Biogeography and Phylogenetics of the Planktonic Foraminifera

Heidi Seears, BSc, MSc

Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

July 2011

Abstract

The planktonic foraminifera are a highly abundant and diverse group of marine pelagic protists that are ubiquitously distributed throughout the worlds' oceans. These unicellular eukaryotes are encased in a calcareous ($CaCO_3$) shell or 'test', the morphology of which is used to identify individual 'morphospecies'. The foraminifera have an exceptional fossil record, spanning over 180 million years, and as microfossils provide a highly successful paleoproxy for dating sedimentary rocks and archiving past climate. Molecular studies, using the small subunit (SSU) ribosomal (r) RNA gene are used here to investigate the biogeographical distributions and phylogenetic relationships of the planktonic foraminifera. Biogeographical surveys of two markedly different areas of the global ocean, the tropical Arabian Sea, and the transitional/sub-polar North Atlantic Ocean, revealed significant genotypic variation within the planktonic foraminifera, with some genetic types being sequenced here for the first time. The foraminiferal genotypes displayed non-random geographical distributions, suggestive of distinct ecologies, giving insight into the possible mechanisms of diversification in these marine organisms. The ecological segregation of genetically divergent but morphologically cryptic genetic types could, however, have serious repercussions on their use as paleoproxies of past climate change. Phylogenetic analyses of the foraminifera based firstly on a partial ~1,000 bp terminal 3′ fragment of the SSU rRNA gene, and secondly on the ~3,000 bp almost complete gene supported the hypothesis of the polyphyletic origins of the planktonic foraminifera, which appear to be derived from up to 5 separate benthic ancestral lineages. The almost complete gene is sequenced here in the planktonic taxa for the first time, though amplification was problematic. In a first step to addressing a pressing need for new genetic markers to support data gained from the SSU rRNA

gene, a culture system was established for the benthic foraminifera, in order to provide a reliable source of DNA for EST library construction or full genome sequencing. Finally, to overcome difficulties associated with the PCR amplification of the foraminifera, a new lysis buffer and DNA extraction procedure was developed. A highly successful buffer was created, allowing high quality DNA to be extracted from foraminiferal specimens, whilst leaving the delicate calcitic shell intact for morphological reference.

Acknowledgements

I would like to thank my supervisor, Chris Wade, for all of his support and guidance during this project, and Kate Darling for her comments and advise. I would also like to thank the BBSRC for funding this work and NERC for providing additional financial support for the collection of specimens.

I am grateful to everyone who participated in the collection of planktonic foraminiferal specimens, in particular Kate Darling and Blair Steel (cruise CD148: Arabian Sea) and Mathew Carroll (cruise CD159: North Atlantic Ocean) and would like to acknowledge the crew and scientists of the RRS Charles Darwin (cruises CD148 & CD159). I am also grateful to Professor John Murray (Southampton) for his assistance in the identification of the British intertidal foraminiferal species collected during this study.

I would like to thank Don McNab and Carole East of the University of Nottingham Academic support unit.

I also thank my lab mates, Ian Fontanilla, Cendrine Hudelot and Mathew Carroll for their friendship and assistance, and also Sheila Keeble for her invaluable technical support. To all of my friends, who have offered great support over the years, I am extremely grateful. In particular I would like to thank Rachel Clark.

Finally, I owe special thanks to my family, without whose support I could never have completed my studies.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

α	Shape parameter
β-ME	Beta-mercaptoethanol
‰	Parts per thousand
1°	Primary
^{16}O	Stable oxygen isotope
^{18}O	Secondary stable oxygen isotope
2°	Secondary
2n	Diploid
3°	Tertiary
3′	Three prime
5′	Five prime
A	Adenine
ANOVA	Analysis of variance
BI	Bayesian inference
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
$CaCO_3$	Calcium carbonate
CE	Cytoplasmic envelope
CTAB	Cetyl trimethylammonium bromide
CTD	Conductivity temperature depth
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DOC	Dissolved organic carbon
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EG	East Greenland Current
EST	Expressed sequence tag
EtBr	Ethidium bromide
FM	Fitch-Margoliash
G	Guanine
GDE	Genetic Data Environment
GITC	Guanidinium isothiocyanate
GTR	General time-reversible
I	Ionic

IOL	Inner organic lining
IR	Irminger Current
K/T	Cretaceous/Tertiary
K-H	Kishino–Hasegawa
LA	Labrador Current
LB	Luria-Bertani
LBA	Long branch attraction
LRT	Likelihood ratio test
LSU (or 28S)	Large subunit
Ma	Million years
MC	Monsoon current
MCMC	Markov Chain Monte Carlo
ME	Minimum evolution
Mg^{2+}	Magnesium 2+ cation
$MgCl_2$	Magnesium chloride
ML	Maximum likelihood
MP	Maximum parsimony
n	Haploid
N	Non-ionic
NA	North Atlantic Current
NaCl	Sodium chloride
NaOAc	Sodium acetate
NJ	Neighbour joining
PCR	Polymerase chain reaction
PO	Portugal Current
POM	Primary organic membrane
PSU	Practical Salinity Units
r	Ribosomal
RELL	Resampling estimated log-likelihoods
RNA	Ribonucleic acid
RPB1	RNA polymerase II largest subunit
rpm	Revolutions per minute
RRS	Royal research ship
RRT	Relative rate test
RRV	Royal research vessel
RV	Research vessel
s.R	Sexual reproduction
Sarkosyl	Sodium N-lauroyl sarcosine
SC	Somali Current

SDS	Sodium dodecyl sulfate
SEC	Southern Equatorial Current
SEM	Scanning electron microscope
sp.	Species
SST	Sea surface temperature
SSU (or 18S)	Small subunit
sub	Substitution
SW	Southwest
T	Thymine
TBE	Tris/Borate/EDTA buffer
TEM	Transmission electron microscope
ti	Transition
Tris	Tris(hydroxymethyl)aminomethane
tv	Transversion
WG	West Greenland Current
X-gal	Bromo-chloro-indolyl-galactopyranoside
Γ	Gamma
χ^2	Chi squared

1 Introduction

1.1 The Foraminifera

The Foraminifera are a diverse group of marine protists that are ubiquitously distributed throughout the world's marine habitats. They are unicellular eukaryote organisms that likely evolved from an amoeba-like ancestor, and comprise of a single cell, usually encased in a protective shell or 'test' that may be organic, agglutinated or calcareous in nature. The foraminifera are distinguished from other rhizopod protoctists by their pseudopodia, which are finely granular and form intricate reticulate networks (reticulopodia) (Lee *et al*., 1990).

The number of extant foraminiferal species has been estimated at approximately 10,000 (Vickerman, 1992). The vast majority of these are benthic taxa, species that inhabit marine sediments. Benthic species first appear in the fossil record during the Cambrian period (Culver, 1991), and since this time have radiated into an enormously diverse group. Distributed globally, the benthic foraminifera form distinct assemblages on the inner and outer continental shelf, upper and lower continental slope, and in deep-sea sediments (reviewed by Sen Gupta, 1999a). Species may live within the sediment (obligate epibenthic), on the sediment (obligate endobenthic), or migrate between the two (Linke & Lutze, 1993).

The planktonic foraminifera have adopted a pelagic mode of life, free-floating in the water column. In contrast to the benthic taxa, the planktonic foraminifera, are represented by far fewer species, estimated at around 40 – 50. Planktonic species are

younger in comparison to their benthic relatives, first appearing in the fossil record during the Jurassic period (Loeblich & Tappan, 1974; Caron & Homewood, 1983).

The planktonic foraminifera show immense diversity and adaptability, both in their morphology and biology and have expanded to fill a wide variety of niches within the global ocean. They are classified taxonomically based on the characteristics of their calcareous shell. Identification is based on general morphology as well as the ultrastructural and microstructural features of the shell (Hemleben *et al*., 1989) obtained by transmission electron microscope (TEM) (Bé *et al*., 1966; Takayanagi *et al*., 1968) and scanning electron microscope (SEM) (Lipps, 1966; Hemleben, 1969a,b; Scott, 1974; Hemleben, 1975; Saito *et al*., 1976; Benjamini & Reiss, 1979; Cifelli, 1982) investigations. The major morphological split is between the spinose planktonic foraminifera (those with spines) and the non-spinose planktonic foraminifera (those without spines) (fig. 1.1) (first recognised by Parker, 1962). The non-spinose taxa can be further divided into the macroperforate, microperforate, and non-spiral groups (fig. 1.1) (summarised by Hemleben *et al*., 1989). Molecular phylogenetic studies have led to an extensive increase in our understanding of the evolutionary relationships of the planktonic foraminifera, refining our views of their taxonomic relationships, as will be discussed later on.

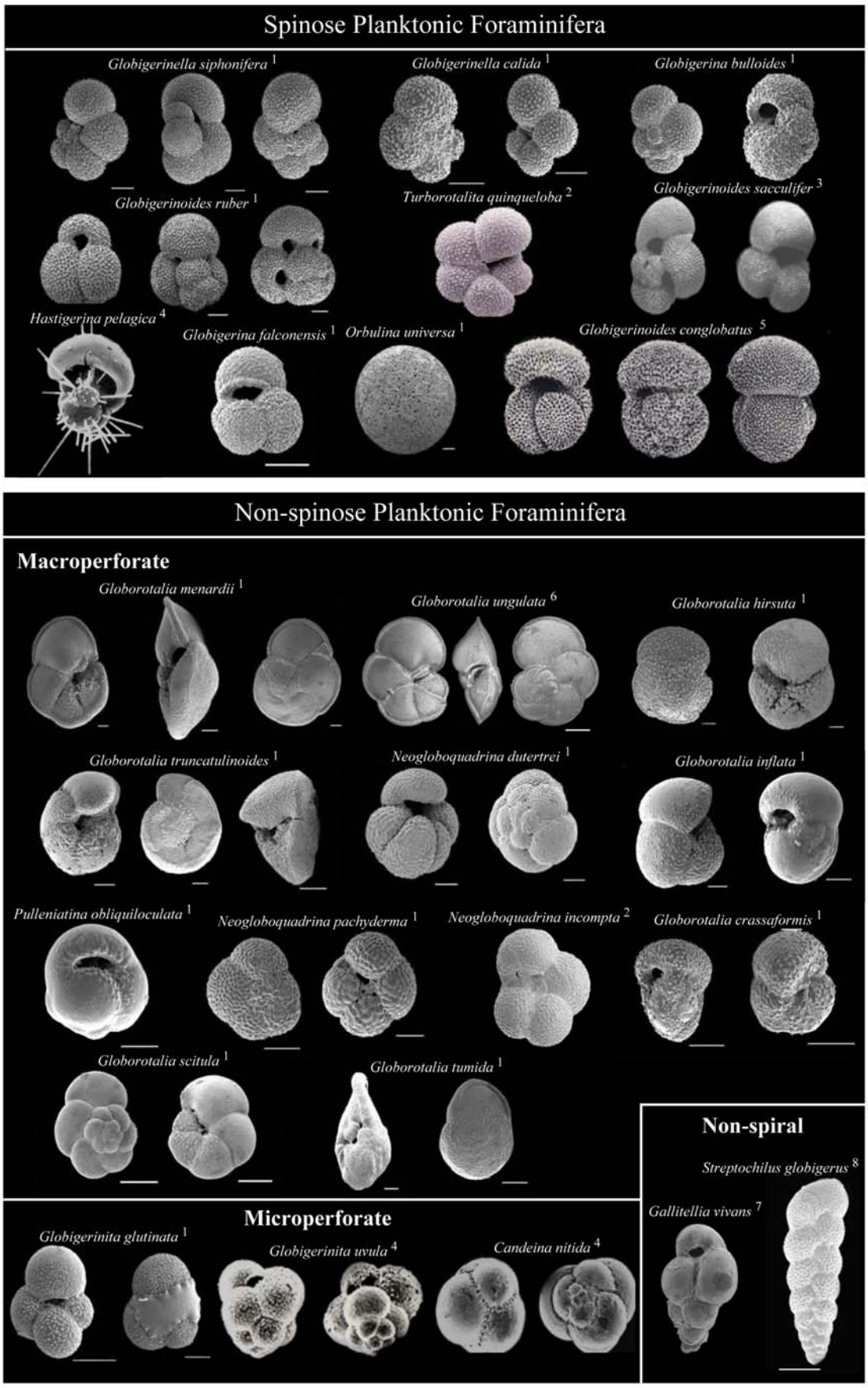


Figure 1.1. Scanning electron microscope (SEM) images of the planktonic foraminifera, showing their major taxonomic groupings. References: (1) Departamento de Paleontologia e Estratigrafia da UFRGS., 2006. (2), Darling & Wade, 2008, (3) Microfossil image recovery and circulation for learning and education (MIRACLE), 2010, (4) Bé, 1977, (5) de Vargas & de Garidel Thoron, 2010 (6) photographs by K. Finger, Berkeley USA (7) Kimoto *et al*., 2009, (8) Darling *et al*., 2009.

1.2 Biological characteristics of the Foraminifera

1.2.1 Cellular structure

The cytoplasm of the foraminifer's single cell is contained within an external test, and fills the internal space to match its shape. Much of the inner space of the test is filled by vacuolated cytoplasm, though the final chamber may be incompletely filled, either as a result of poor health or nutrition, or during chamber construction (Hemleben *et al*., 1989). There are thought to be three zones of intergrading cytoplasm: 1) Compact intrashell cytoplasm, 2) frothy or reticulate cytoplasm, usually observed in the final chamber or at the aperture between the intrashell and extracell spaces, and 3) external cytoplasm comprising of alveolate masses or reticulate to fibrose strands of rhizopodia engulfing the outer surface of the shell. Sticky Rhizopodia (fine cytoplasmic filaments) extend outwards into the surrounding environment forming a radial net that is used for feeding (Hemleben *et al*., 1989). The nucleus is typically located in one of the inner chambers, protected from the external environment. Within the rest of the cellular space, cytoplasmic streaming leads to a fluid movement of material, particularly so towards the aperture and surrounding extrashell cytoplasm. The extrashell cytoplasmic strands are particularly fluid allowing pseudopodia to be extended in all directions, or retracted and flattened to form a covering over the test (Hemleben *et al*, 1989). Three types of pseudopodia may be observed: 1) rhizopods (branching), 2) filopodia (long, thin and straight), and 3) reticulopodia (net-like). In species with spines protruding from the test (spinose foraminifera) rhizopodia may extend along the radially arranged structures, to form a fine web.

1.2.2 Growth and Test Ontogeny

In the more primitive forms of foraminifera (e.g. *Cribrothalammina alba*) the test is agglutinated in nature, held together with organic or calcareous cement. In order for growth to occur, material is added to the inner organic lining of the test at sites distant from the aperture (Goldstein & Barker, 1988). For the majority of foraminifera, however, growth is accomplished by adding a new chamber to the calcareous test at regular intervals, increasing in size each time. For most calcareous benthic foraminifera, (e.g. *Rosalina floridana* and *Ammonia tepida*: Order Rotaliida), the process begins by the formation of a translucent algal cyst that extends over the entire surface of the test. Pseudopodia then coalesce within the cyst forming a template or anlage of the future chamber. The pseudopodia work over the surface of the anlage, forming an inner organic lining (IOL). Once the IOL is complete, cytoplasm from within the test floods the newly formed chamber, forcing the vesicular material of the anlage out through the aperture to form a sheath over the test surface. Calcite deposition then occurs over both the new chamber and the entire test surface. Once construction is complete, the foraminifer breaks free of the cyst (Angell, 1967b, reviewed by Goldstein, 1999).

In the planktonic foraminifera the process is similar, though with some characteristic differences. Feeding rhizopodia are withdrawn and a translucent bulge of cytoplasm emerges from the shell aperture. Fanlike rhizopodia then radiate from the bulge to form the outline of the outer protective envelope. The cytoplasmic bulge gradually extends up to meet the protective envelope. Construction of the anlage then begins. The anlage comprises of a thin cytoplasmic envelope (CE) and the primary organic membrane (POM), a non-living filamentous layer. These ultrastructural elements are

associated with the calcification process. The periphery of the bulge smoothens, forming the outline of the new chamber. Then small plaques of calcite are secreted on both sides of the POM, gradually building up to form a continuous bilamellar wall (unlike in benthic taxa where the wall is unilamellar). In many taxa, pores form in the chamber surface at the sites where cytoplasmic strands passed through sieve-like micropores, leaving a perforated mature chamber. Further thickening continues to occur, and the whole process is complete in approximately 6 hours (Hemleben *et al*., 1989).

Chamber formation is generally similar in spinose and non-spinose taxa, though with spines added in the spinose taxa after the chamber is largely complete (Hemleben *et al*., 1986). Some species such as *Globorotalia menardii* develop a keel, to strengthen the sharply angled rim of the compressed test (fig. 1.1) (Schott, 1973). The keel is formed by the collapse of the chamber wall along the test periphery (Hemleben *et al*., 1977). Most non-spinose species also bear small conical calcitic protuberances called pustules, which serve as anchor points for masses of rhizopodia (Hemleben, 1975; Hemleben *et al*., 1989).

1.2.3 Feeding and Nutrition

The Foraminifera utilize a broad range of feeding mechanisms and nutritional resources. Algae and diatoms form the staple diet of the majority of foraminifera, though bacteria may also form an integral part of the nutritional intake of many species (Lee, 1980). Yeasts, fungi and small animals are also known to be ingested in some cases (Lee *et al*., 1966; Lipps, 1983; Bernhard & Bowser, 1992). Feeding experiments indicate that many species feed selectively (lee *et al*., 1966; Lee, 1980), targeting, for example, a particular species of algae (reviewed by Arnold, 1974;

Anderson *et al*., 1991). Mechanisms employed for feeding include grazing (Jepps, 1942), suspension feeding (Lipps, 1983), deposit feeding (Goldstein & Corliss, 1994), carnivory (Bowser *et al*., 1992), parasitism (Cedhagen, 1994), feeding on phytodetritus (Gooday, 1988, 1993), or even the direct intake of dissolved organic carbon (DOC) (DeLaca *et al*., 1981). Pseudopodia are used in feeding, functioning to gather food (Jepps, 1942) and subdue prey (Bowser *et al*., 1992). In some species they may also function in extrathalamous digestion (Lee *et al*., 1991c; Faber & Lee, 1991).

1.2.4 Symbiosis

Many species of foraminifera display some kind of relationship with other microorganisms. The nature of the relationship varies from true symbiosis, where both individuals benefit, to commensalism, where only one party benefits, but the other is not harmed, to parasitism, where one member is exploited by the other (Hemleben *et al*, 1989). Some species merely sequester chloroplasts on a temporary basis (Lee & Anderson, 1991a). Some 150 extant species of foraminifera harbour algal symbionts, though this represents less than 10 % of the total species number (Lee & Anderson, 1991a). Symbiosis is particularly prevalent in the tropical larger benthic foraminifera (though certain temperate species also carry symbionts) and in the planktonic foraminifera. Symbiotic relationships with algae are thought to benefit the foraminiferal hosts by providing a source of energy from photosynthesis (Falkowski *et al*., 1993; Hallock, 1981a), enhancement of calcification (Duguay, 1983; ter Kuile, 1991), and possibly the removal of host metabolites by the symbiont (reviewed by Hallock, 1999).

Within the benthic foraminifera, 4 milliolid, 3 rotaliid, and 5 globigerinid families are thought to harbour symbionts (Lee & Anderson, 1991a) including diatoms, dinoflagellates, red algae, and chlorophytes (Hemleben *et al*., 1989). Symbiosis appears to have arisen independently in most of these foraminiferal lineages (Hallock, 1999). In contrast, planktonic foraminifera only associate with two types of algal symbiont, dinoflagellates and chrysophytes (Hemleben *et al*., 1989). Within the spinose planktonic foraminifera, *Globigerinoides ruber*, *Globigerinoides conglobatus*, *Globigerinoides sacculifer*, and *Orbulina universa* all harbour dinoflagellate symbionts (Faber *et al*., 1985; Gastrich, 1988; Hemleben *et al*., 1989), possibly of only a single species, *Gymnodinium béii* (Gast & Caron, 1996; Lee & Anderson, 1991a).

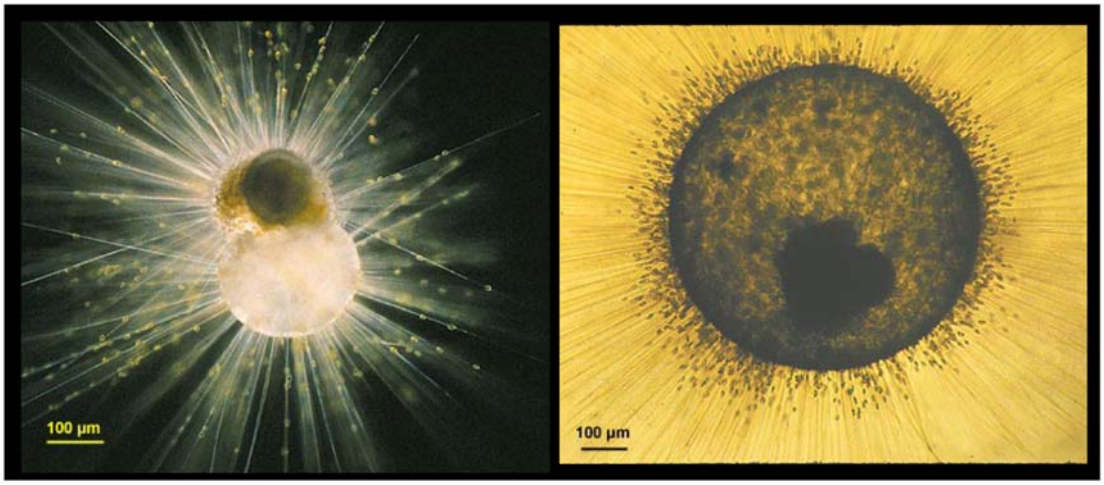


Figure 1.2. Microscope images of the spinose planktonic foraminifers *Globigerinella* sp. (left) and *Orbulina universa* (right), showing algal symbionts enclosed by the foraminiferal cytoplasm along the spines. Pictures taken by O. R. Anderson (2006), licensed to MBL micro*scope.

Turborotalita humilis and *Globigerinella siphonifera* bear chrysophyte symbionts (Faber *et al*., 1985; Gastrich, 1988; Hemleben *et al*., 1989). The symbiotic relationship is thought to be obligative (i.e. survival outside of the relationship would be impossible) in all of these spinose species (Hemleben *et al*., 1989). When observed, the algal symbionts can be clearly seen among the radially arranged spines

(fig. 1.2). *Globigerina bulloides* and *Hastigerina pelagica* are unusual in being symbiont barren, though the latter houses commensals (Spindler & Hemleben, 1980). The non-spinose planktonic foraminifera *Globigerinita glutinata*, *Neogloboquadrina dutertrei, Pulleniatina obliquiloculata, Globorotalia inflata, and Globorotalia menardii* all harbour facultative chrysophytes, whilst the remaining taxa (e.g. *Neogloboquadrina pachyderma*, *Globorotalia truncatulinoides*, *Globorotalia hirsuta*) are symbiont-barren (reviewed in Hemleben *et al*., 1989). Facultative symbionts are housed on a non-permanent basis, photosynthesising within perialgal vacuoles, but may sometimes be digested by the foraminiferan.

1.2.5 Life cycle in the Foraminifera

Benthic foraminifera typically reproduce by a classical dimorphic life cycle, consisting of a regular alternation between sexual and asexual generations (Goldstein, 1999; Lee *et al*., 1991b) (fig. 1.3). The haploid, megalospheric gamont releases gametes (~ 1-4 μm), which are fertilized to produce a zygote, eventually giving rise to a diploid microspheric agamont. The agamont then produces haploid megalospheric young, by multiple fission, and the cycle begins again. A biological dimorphism usually exists between the gamont, which has a single nucleus and a megalospheric test, characterised by a large proloculus (1st chamber) but a relatively small overall diameter, and the agamont, which is multinucleate and has a microspheric test, characterised by a smaller proloculus, but relatively larger overall test diameter (Goldstein, 1999). Exceptions to this rule do exist in some species, where this size relationship may be inverted. This dimorphic life cycle was first recognised by Lister (1895) in a study of the benthic foraminifer, *Elphidium crispum*, and was later confirmed by Schaudinn (1895).

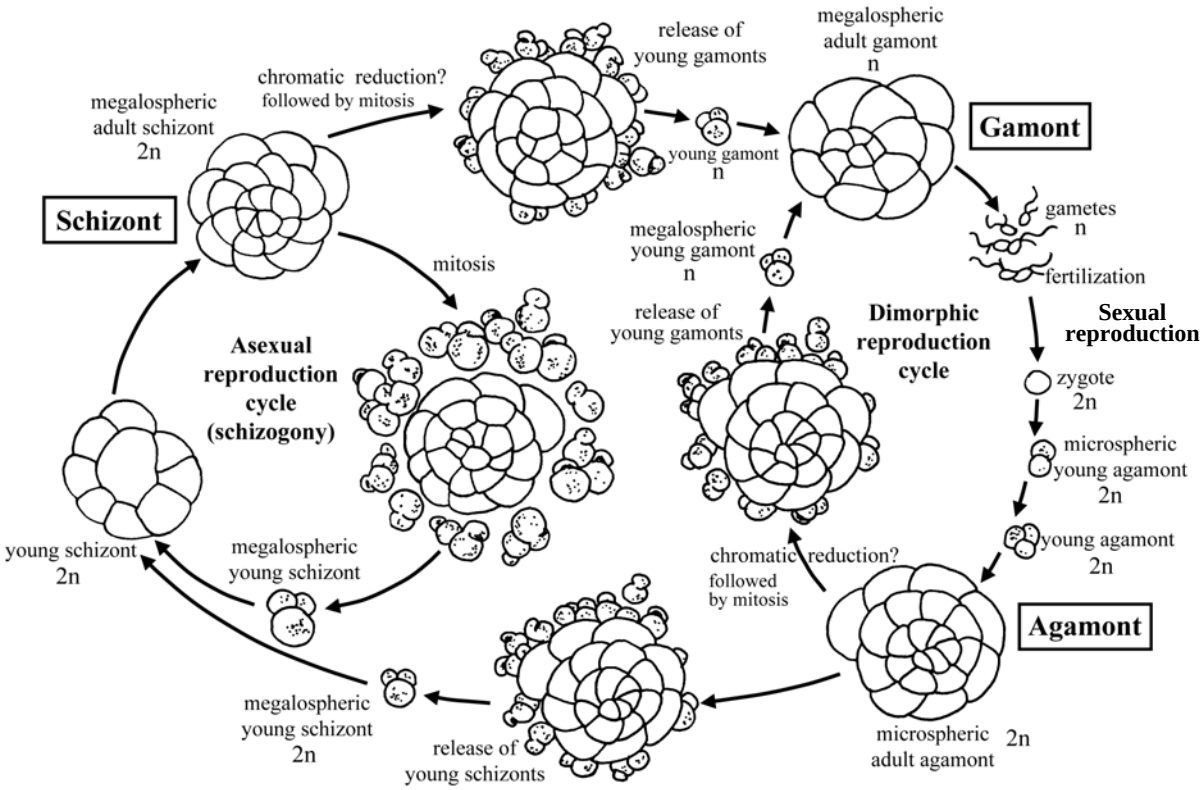


Figure 1.3. Life cycle of the Foraminifera. Most benthic foraminifera display a classic dimorphic life cycle, shown on the right of the diagram, which involves a regular alternation between two generations, the microspheric agamont and the megalospheric gamont. In some benthic species, the life cycle also includes a schizont, which is produced from the agamont, and reproduces asexually. Cyclic asexual schizont formation (schizogony) may occur at this point. Planktonic foraminiferal morphospecies reproduce by sexual reproduction only, seen on the far right of the diagram. Adapted from the life cycle of *Ammonia tepida,* as documented by Stouff *et al*., 1999a.

The alternation of generations may be obligatory (i.e. there is a rigid cycle between sexual and asexual phases) in some foraminifera, for example *Elphidium crispum* (Lister, 1985; Jepps, 1942) and *Glabratella sulcata* (Grell, 1958), or facultative (i.e. the sexual/asexual cycle can be broken under certain conditions or stresses) in others, e.g. *Ammonia tepida* (Bradshaw, 1957; Goldstein & Moodley, 1993) and *Saccammina alba* (Goldstein, 1988). The facultative system is often referred to as a biologically trimorphic life cycle, and involves successive asexual reproduction that inserts a third, biologically distinct form between the agamont and gamont generations, the megalospheric schizont (Fig. 1.3; Stouff *et al*., 1999a). Schizonts reproduce by

multiple fission, to produce either another schizont generation, or megalospheric gamonts, at which point the cycle is closed. Schizogony may become cyclic, with several generations of schizonts following one another (Dettmering *et al.*, 1998). Trimorphism was first suggested as a reproductive strategy in foraminifera by Rhumbler (1909) and was subsequently proposed to be the dominant life cycle in larger foraminifera (Leutenegger 1977; Lee *et al.*, 1991b). Confirmation of this reproductive strategy came when primary successive asexual reproduction was recorded in laboratory cultures of *Heterostegina depressa* (Röttger *et al.*, 1986, 1990), and has since been documented in other species such as the larger foraminifer *Amphistegina gibbosa* (Dettmering *et al.*, 1998; Harney *et al.*, 1998), and in *Ammonia tepida* (Stouff *et al.*, 1999a).

In contrast to the variety of reproductive strategies seen in the benthic foraminifera, only sexual reproduction has ever been recorded in the planktonic taxa (Goldstein, 1999; Hemleben *et al*., 1989). Hemleben *et al*. (1989) suggest that the agamont and multiple fission have been lost, leaving a 'gamic' life cycle (fig. 1.3). The planktonic foraminifera have developed a number of mechanisms for coping with the difficulties of reproduction in an open ocean environment, thus maximising the chances that compatible gametes of the correct species will meet (Hemleben *et al*., 1989). Throughout the year most species will migrate throughout the water column, a strategy that is thought to maximise the use of available food sources. Gametogenic adults usually settle in the water column and accumulate in the thermocline to release their gametes. The deep chlorophyll maximum layer is an optimal zone for reproductive success, providing a stable breeding environment and food source for juveniles. Many species exhibit a lunar or semi-lunar reproductive cycle, allowing

gamete release to be synchronised, though food availability may also play some role in the timing of reproduction. Gametes are released in their hundreds of thousands and though not proven conclusively, evidence suggests that the primary reproductive strategy may be dioceous, with gametes mixing from different parents. The general reproductive strategies and life horizons of some modern planktonic foraminifera, as recorded by Hemleben *et al*. (1989), are shown in fig. 1.4.

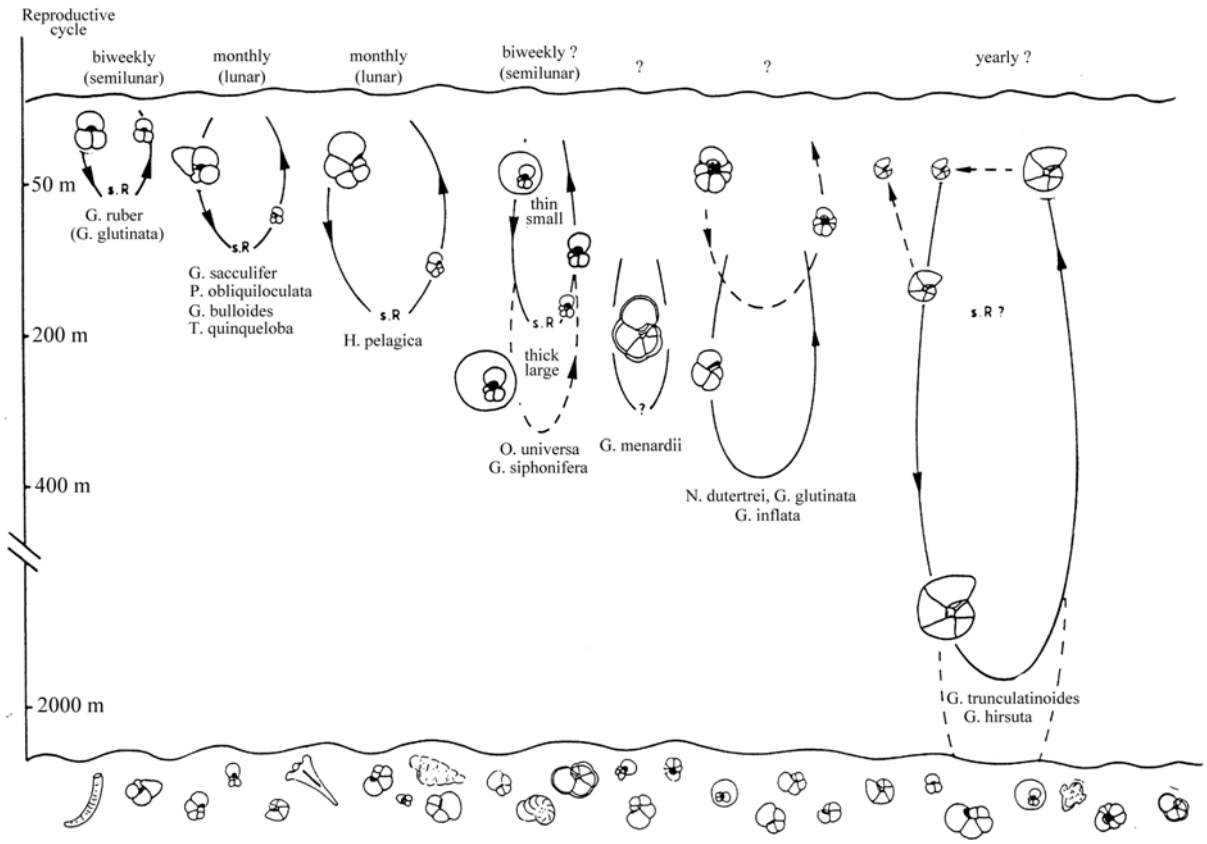


Figure 1.4. Summary of the reproductive strategies and life horizons of some modern planktonic foraminifera from the tropics and subtropics. s.R = sexual reproduction; major patterns span a range from near-surface dwelling forms that reproduce at shorter time intervals to those that reproduce at depth, and rise to the surface. The latter probably have longer reproductive intervals. Taken from Hemleben *et al*. (1989)

1.3 Biogeography and ecology of the Planktonic Foraminifera

Despite an apparent lack of barriers to gene flow in the open ocean, the global ocean is not a uniform environment, but one comprised of regionally distinct ecosystems. From studies of the global distributions of modern planktonic foraminifera, Bé and Tolderlund (1971) divided the global ocean into broad zones, designated as the polar, sub-polar, transitional, sub-tropical and tropical faunal provinces (fig. 1.5), and demonstrated that specific assemblages of morphospecies are associated with each particular region (also see Bé, 1977).

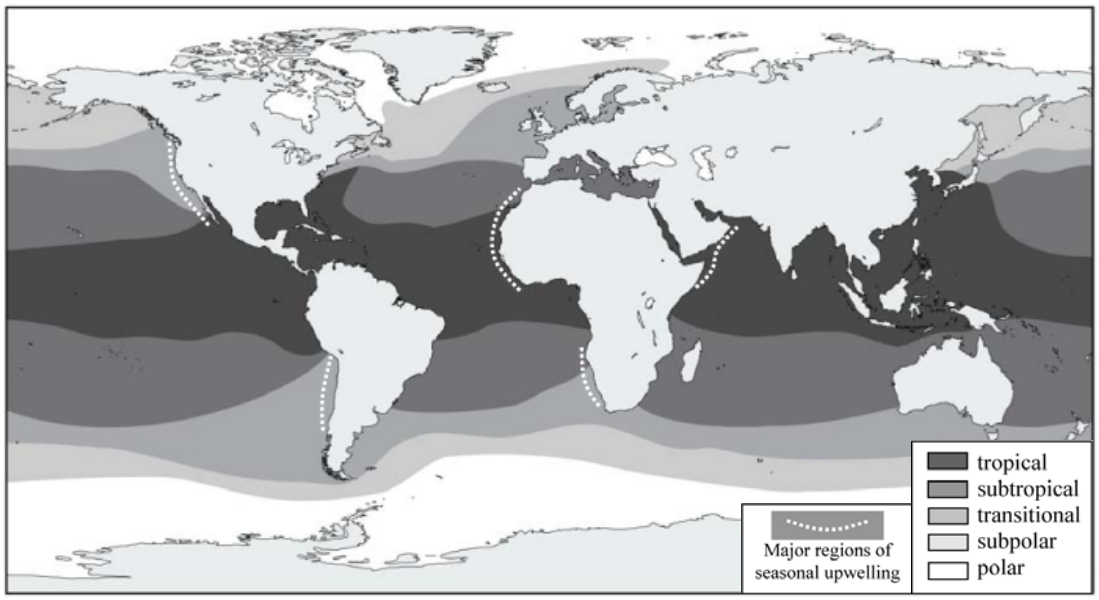


Figure 1.5. World map showing the five major planktonic foraminiferal faunal provinces, devised by Bé & Tolderlund, 1971. Tropical 24-30 °C, subtropical 18-24°C, transitional 10-18 °C, subpolar 5-10 °C, polar 0-5 °C. Areas of seasonal upwelling, considered as the 6th faunal province by Hemleben *et al*. (1989) are also shown (see key).

Discrete assemblages are also found in a transitory province associated with regional upwelling (summarised in Lipps, 1979; Hemleben *et al*, 1989). Individual morphospecies may be found across several zones, but each has a characteristic, usually temperature dependent distribution (fig. 1.6). Within the faunal provinces,

other factors such as salinity, prey abundance, nutrient level, turbidity and illumination may also affect diversity, abundance and distribution locally. The fact that planktonic foraminiferal morphospecies adhere to these faunal provinces, coupled with their widespread global distribution has made them extremely useful in the study of both modern and ancient marine ecosystems (Hemleben *et al*., 1989).

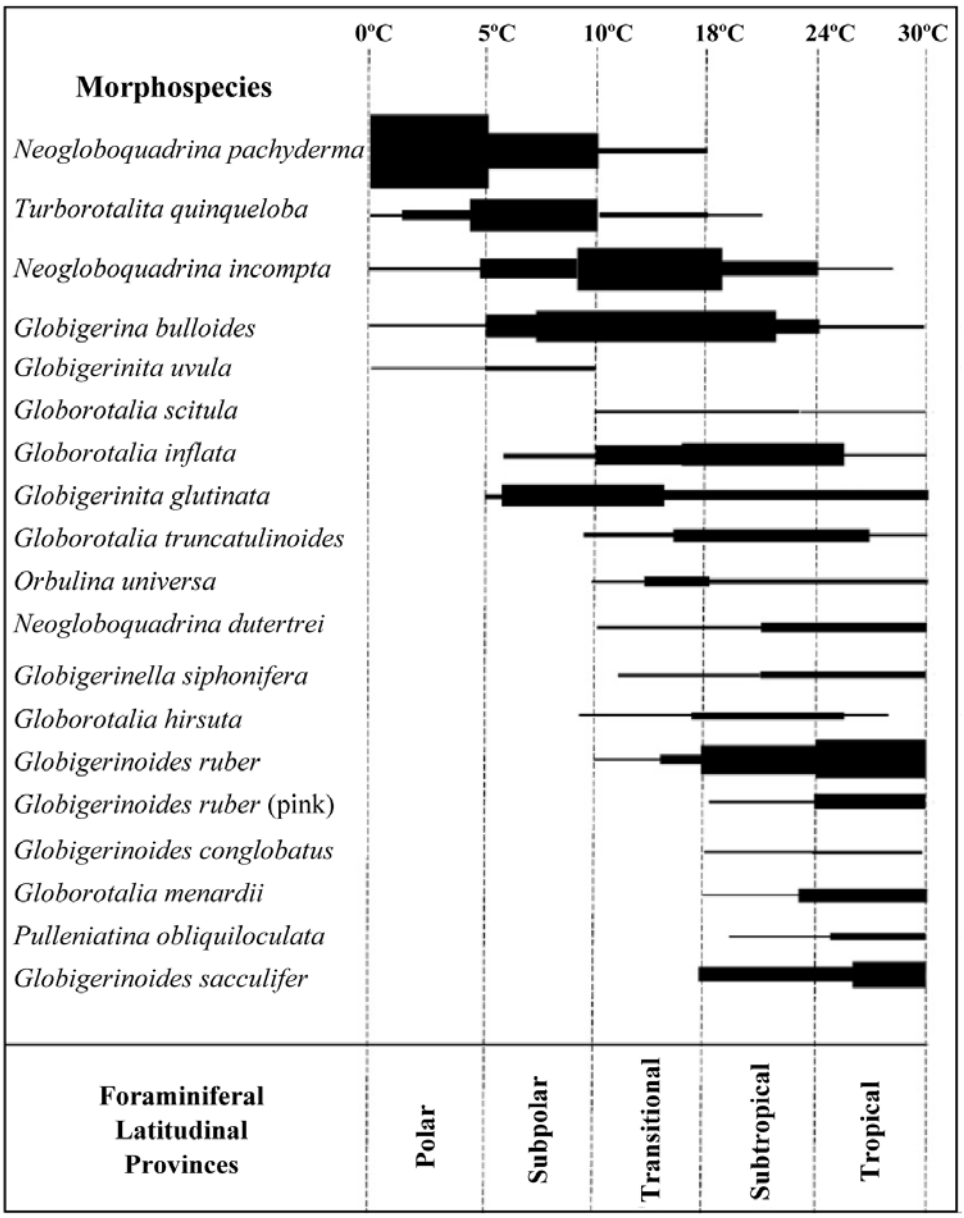


Figure 1.6. Morphospecies assemblages and ranges within the five major planktonic faunal provinces. Varying thickness represents relative abundance within each zone. (Modified from and Bé Tolderlund, 1971 & Darling & Wade, 2008).

1.4 The use of planktonic foraminifera as indicators of past environmental conditions and climatic change

When planktonic foraminifera die their calcitic shells slowly sink in the water column forming a component of "marine snow" (Bishop *et al*., 1977; Silver *et al*., 1978; Wefer *et al*., 1982), which settles on the seabed, forming a layer of sediment in which the shells eventually become fossilised. The planktonic foraminifera have an exceptional fossil record, spanning approximately 180 million years (Ma), and it is this continuous and clearly interpretable fossil record that has afforded planktonic foraminifera great utility in reconstructing past climate, ecological conditions and geological history (e.g. CLIMAP, 1976; Berger, 1979a; Vincent & Berger, 1981; Ruddiman & Sarnthein, 1986; Boersma *et al*., 1987).

Core samples of oceanic sediments provide information on the foraminiferal assemblage (i.e. the species composition/ relative abundances of species) from a given geological time period. Using our knowledge of the ecological habits of extant planktonic foraminiferal morphospecies it is then possible to estimate the past climatic conditions that existed during that time period, based on the species that were present (e.g. Sancetta *et al*., 1972; Imbrie *et al*., 1973; Sachs *et al*., 1977; reviewed by Hemleben *et al*., 1989). Such methods of course works on the assumption that modern planktonic foraminiferal morphospecies respond to their environment in the same way as ancient populations. Only the most abundant morphospecies in the assemblage are considered, removing possible interference from species only present on the periphery of their natural range.

Shell chemical composition, particularly stable isotopes (e.g. the ratio of $^{18}O/^{16}O$), is also widely employed to estimate water temperatures where the planktonic foraminifers grew (reviewed by Anderson & Arthur, 1983; Berger, 1979b; Berger *et al*., 1981; Hemleben *et al*., 1989; Vincent & Berger, 1981). When calcification of foraminiferal shells occurs, the relative amounts of the two isotopes incorporated is dependent on temperature, and thus the ratio of the common isotope ^{16}O to the heavier isotope, ^{18}O, may be used to estimate the water temperature at the time that the calcite of the shell was deposited.

1.5 Classification of the Foraminifera

1.5.1 Criteria for the classification of the Foraminifera

The Foraminifera are numerous and varied in their shell morphology and biology, making the task of compiling a single informative classification extremely difficult. Traditional foraminiferal classification is based almost exclusively on the characteristics of the test, primarily its chemical composition, ultrastructure, mode of formation, and mode of growth (continuous or periodic) (Loeblich & Tappan, 1992).

The Foraminifera can be simply divided into three main groups, according to the chemical composition of the test: 1) organic test, 2) agglutinated test, and 3) calcareous test (Cushman, 1948). Organic tests are composed of a simple membrane comprised of proteins and mucopolysaccharides ("tectin") (Lee, 1990) (e.g. the Allogromiida). Agglutinated tests have an organic lining, coated with a calcareous or ferruginous cement, to which particles of natural materials from the environment are affixed (Lee, 1990) (e.g. the Astrorhizida, Lituolida, Trochamminida & Textulariida). Calcareous tests are comprised of secreted calcium carbonate ($CaCO_3$), usually

calcite, (Lee, 1990) (e.g. the Milliolida, Lagenida, Globigerinida, Buliminida & Rotaliida). The calcite wall may be perforate (e.g. Lagenida, Buliminida, & Globigerinida) or imperforate (e.g. the Milliolida, Caterinida, & Spirillinida) in structure (Carpenter *et al*., 1862; Cushman, 1948; Reuss, 1861; Sen Gupta, 1999b), monolamellar (single layered) (e.g. Lagenida), or bilamellar (double layered) (e.g. Globigerinida, Rotaliida, Buliminida) (Sen Gupta, 1999b), and have either a low magnesium content (many orders, e.g. Carterinida, Globigerinida) or high magnesium content (Milliolida only) (Sen Gupta, 1999b). In two orders (the Robertinida and Involutinida) the secreted $CaCO_3$ test is made from aragonite rather than calcite and in a single order (the Silicoloculinida), the test is formed from opaline silica.

Wall ultrastructure may be used to differentiate between calcitic taxa. Based on crystallographic organisation, three major types of calcareous wall structure are recognised: 1) porcelaneous (e.g. Milliolida), 2) microgranular (e.g. Fusulinida), and 3) hyaline (e.g. Rotaliida) (Lee, 1990). When viewed under reflected light, porcelaneous tests have an opaque, white appearance, compared to microgranular tests, which appear sugary, or hyaline tests which appear glassy (Lee, 1990). Polarised light interference patterns can be further used to differentiate calcitic hyaline orders (Wood, 1949). The pattern produced can be either radial (caused by the perpendicular orientation of the c-axis of calcite crystals in relation to the curvature of the test wall; Wood, 1949) (e.g. Lagenida) or granular (oblique orientation or bundles of several preferred orientations (Towe & Cifelli, 1967; Stapleton, 1973)) (e.g. Milliolida).

The mode of chamber addition or the arrangement of chambers is also a feature used in foraminiferal classification. The foraminiferal test may be comprised of a single

chamber (unilocular), e.g. in certain members of the Astrorhizida, or multiple chambers (multilocular), e.g. in the Lituolida and Trochamminida (agglutinated), the Robertinida (aragonitic), and the Globigerinida, Buliminida, and Rotaliida (calcitic). In unilocular taxa, the chamber may take a simple globular form or a tubular form, which may grow in a flat spiral (planispiral). In multilocular taxa chambers may be added in a number of different ways, for example, in a simple row (uniserial), alternating rows (biserial), a simple spiral (planispiral), in a helicoids spiral (trochospiral), or in a spiral where each chamber forms half a whorl (streptospiral) (planktics) (illustrated by Sen Gupta, 1999b, p24-35, redrawn from Loeblich & Tappan, 1964). Such features were once thought key to foraminiferal classification (d'Orbigny, 1826; Cushman, 1945), however, as similar shell morphologies are present in both agglutinated and calcitic orders, Loeblich & Tappan (1964) argue that 'the same chamber arrangement and form of test may have developed in independent lineages by parallel evolution, without indicating interrelationship of the similarly shaped shells'. Today chamber arrangement is therefore used largely for classification only at the supraordinal level.

Supraordinal classification is usually based on numerous combinations of a diverse range of morphological features including wall pores, wall passages, principal apertural features (separating superfamilies), free or fixed nature of the test, mode of chamber addition, simple or divided nature of the chamber interior and apertural modifications (separating families) (Lee, 1990; Loeblich & Tappan, 1987; Haman, 1988). Other factors such as geological history, and some biological characters may also be taken into account (Loeblich & Tappan, 1987).

In evolutionary terms, a progression is thought to have taken place from the most primitive organic membranous and single chambered forms, through to agglutinated tests, and eventually up to the most advanced calcareous, perforate, trochospiral forms (Cushman, 1948). This progression is usually reflected in modern classifications, which place species within evolutionary lineages, exhibiting gradational morphological transitions, using evidence from the fossil record (Kennett & Srinivasan, 1983; Pearson, 1993). Phylogenies are usually constructed according to shell morphology and geologic occurrence/ biostratigraphy (Tappan & Loeblich, 1988).

1.5.2 Current classification of the Foraminifera

The classification of the Foraminifera shown in fig. 1.7 is a much-simplified adaptation of the classification of Loeblich & Tappan (1992) with morphology added from the large compendium of foraminiferal families also published by Loeblich & Tappan (1987), and from Hemleben *et al*. (1989). Examples of all known orders are shown, with greater detail presented for the Globigerinida (the planktonic foraminifera), the main focus of this thesis.

The Foraminifera are designated as a class, as in Loeblich & Tappan (1992). The classification includes 16 orders, 14 of which are listed by Loeblich & Tappan (1992), with a further two added in accordance with Sen Gupta (1999b). These are the Involutinida (with an aragonite test), which is separated from the Spirillinida (calcite test) and raised to the rank of an order (Sen Gupta, 1999b) and the Silicoloculinida (silica test), which is separated from the Milliolida (calcite test) (Sen Gupta, 1999b; Lee, 1990).

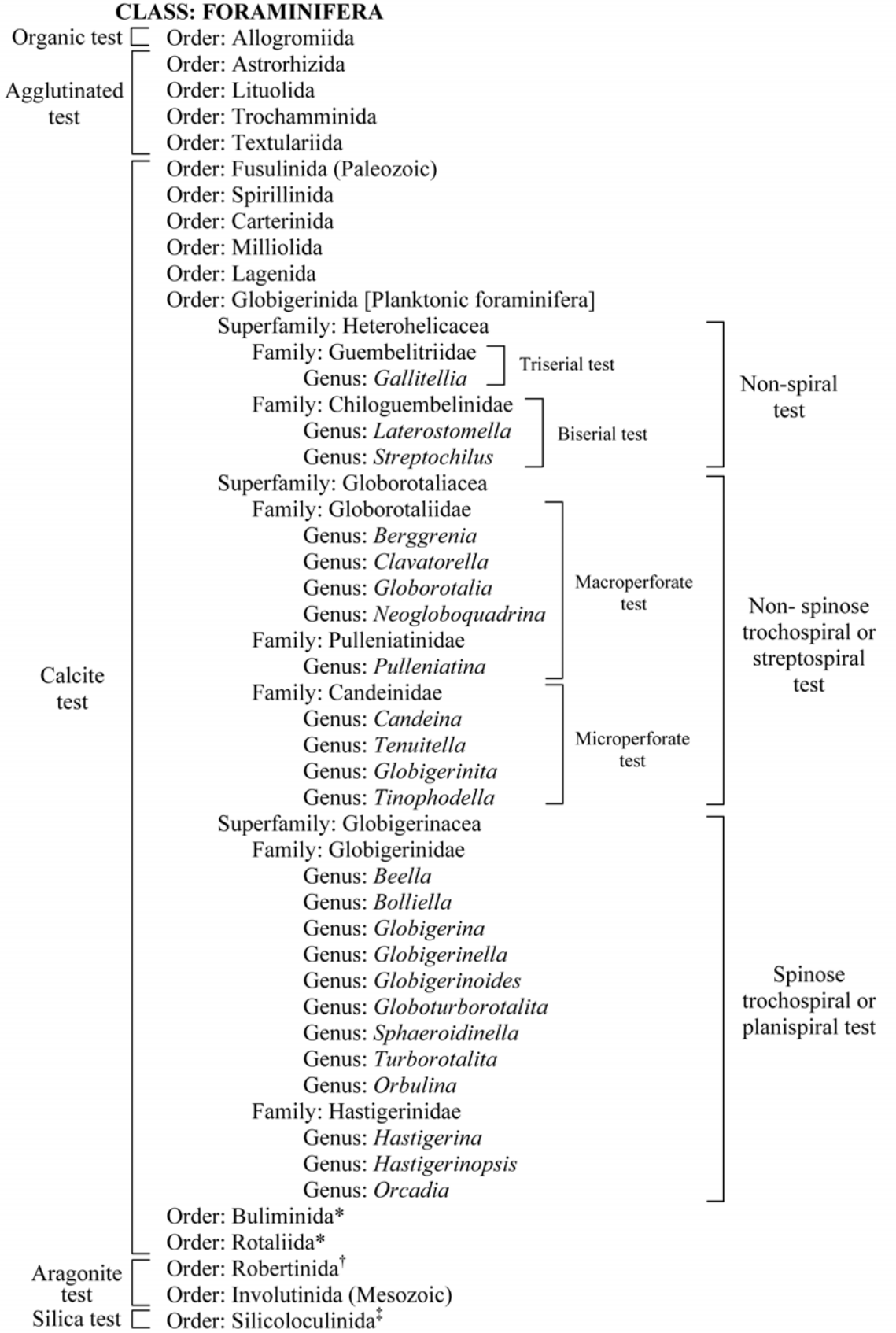


Figure 1.7. Classification of the Foraminifera, based on the morphological characteristic of the test. Adapted from Loeblich & Tappan (1992) and Sen Gupta, 1999b. Morphology is added from Loeblich & Tappan (1987), Hemleben *et al*. (1989) and Simmons *et al*. (1997). The Order Globigerinida, which represents the planktonic foraminifera, is expanded to show all extant superfamilies, with examples of genera. * Split from the Rotaliina of Loeblich & Tappan (1987), in accordance with information from Grigelis (1978), Haynes (1981) and Loeblich & Tappan (1992). † Separated from Spirillinida as in Loeblich & Tappan, 1987. ‡ Separated from Milliolida as in Lee, 1990. Two orders, the Fusulinida and Involutinida are now extinct.

Other points to note are that the Rotaliida and Buliminida, are split from the Rotaliina of Loeblich & Tappan, 1987, following work by Grigelis (1978), Haynes (1981) and Loeblich & Tappan, 1992. Loeblich & Tappan (1987, 1992) also separated the Robertinida from the Spirillinida. Two of the orders within the classification are now extinct (the Fusulinida and Involutinida).

The Order Globigerinida, which represents the planktonic foraminifera, is shown in greater detail (to the genus level) and includes 3 extant superfamilies (the Heteroheilicacea, Globorotaliacea, and Globigerinacea), and 7 extant families (the Guembelitriidae, Chiloguembelinidae, Globorotaliidae, Pulleniatinidae, Candeinidae, Globigerinidae, and Hastigerinidae), as in Loeblich & Tappan (1992). As no details of the suprafamilial structure were provided in the Loeblich & Tappan (1992) classification, generic level hierarchical information within the Globigerinida is taken from Decrouez, 1989 (only extant genera are shown).

1.6 Phylogenetic relationships of the planktonic foraminifera

Methods for inferring relationships among eukaryote taxa are traditionally based on phenotypic characters and the fossil record, as can be seen for the classification of the foraminifers (above). However, genetic sequencing and molecular phylogenies have since challenged the findings of these traditionally used methods (Sogin, 1989), and have added greatly to our understanding of the interrelationships between the foraminiferal taxa, and of the evolutionary mechanisms working on them.

1.6.1 The origins of the Foraminifera: Molecular versus fossil evidence

Much of the early molecular phylogenetic work on the foraminifera focused on their placement in the eukaryote "tree of life". Phylogenetic studies based on partial sequences of the small subunit (SSU) rRNA gene (Darling *et al*., 1996b; Pawlowski *et al*., 1996b; Wade *et al*., 1996) and the large subunit (LSU) rRNA gene (Pawlowski *et al*. (1994b) placed the foraminifera in a monophyletic group, branching outside of the eukaryote "crown" group diversification. The foraminifera branched closely to the plasmodial and cellular slime molds, early in the eukaryotic tree, far earlier than suggested by the fossil record (Pawlowski *et al*., 1994b). One study placed the foraminifera (*Ammonia sp*.) in a highly derived position, clustering with the alveolates (Ampiomplexa, dinoflagellates, & ciliates) within the major eukaryote radiation, or "crown" group (Wray *et al*., 1995), however, these sequences were later determined to be derived from a non-foraminiferal contaminant (Darling *et al*., 1996b; Wade *et al*., 1996).

During these early studies, SSU rRNA sequences were amplified using the "universal" eukaryote primers of White *et al*. (1990), originally designed for use on fungi. Confirming the identity of the early foraminiferal DNA sequences was essential, as the multiple genomes of the various symbionts, commensals and prey items associated with foraminifera could be preferentially amplified over the single genome of the foraminiferan. Early observations indicated that PCR amplification of the SSU rRNA gene from foraminiferal samples produced two bands following gel electrophoresis. Both bands were sequenced and included in phylogenetic analyses, and those sequences clustering together in a monophyletic group, separate from any previously sequenced organisms were taken to be foraminiferal in origin. For both the SSU and

LSU rRNA genes it was found that the eukaryote contaminant sequences were far shorter than those of the foraminifera, allowing for easy identification of the correct band (Darling *et al*. 1996a,b; Wade *et al*., 1996; Pawlowski *et al*., 1994b). To help overcome the potential problem of contamination, gametogenic specimens, with their high ratio of foraminiferal genomes compared to contaminants, were sometimes used to give a higher chance of procuring foraminiferal DNA (Darling *et al*., 1996a,b; Wade *et al*., 1996).

The deep phylogenetic position of the foraminifera, according to analyses of ribosomal DNA genes, contrasts with their Cambrian appearance in the fossil record (Culver, 1991). Early foraminifera were characterised by the presence of a unilocular (single-chambered) agglutinated test (Culver, 1991) and it has been suggested that the group may have evolved from a fragile naked ancestral species, which would naturally be absent from the fossil record. The recent discovery of an extant "naked" foraminiferan, in the form of freshwater protist, *Reticulomyxa filosa*, which clustered amongst the foraminifera in both SSU and Actin phylogenies, seems to confirm this possibility (Pawlowski *et al*., 1999a,b). If true, the origin of the group could be much earlier than suggested by the fossil record, explaining the conflicting molecular and fossil data.

Archibald *et al*. (2003), however, suggest that the early positioning of the foraminifera within the eukaryote tree is an artefact, resulting from the very rapid rates of rDNA evolution seen in the group, and the resulting bias caused by the long-branch attraction phenomenon (Embley & Hurt, 1998; Philippe & Adoutte, 1998; Philippe *et al*., 2000), which pulls the foraminifera into position alongside ancient lineages at the base of the

eukaryote tree. The same effect is likely to be working on other highly derived groups too, for example the Microsporidia (Keeling & Doolittle, 1996; Hirt *et al*., 1999: Keeling *et al*., 2000).

More recent molecular studies have in fact suggested that the foraminifera are more closely related to members of the Cercozoa, in particular Cercomonas and chlorarachniophytes (Archibald *et al*., 2003; Berney & Pawlowski, 2003; Keeling, 2001; Longet *et al*., 2003; Flakowski *et al*., 2005) thus placing their origins once again within the main eukaryote radiation (Berney & Pawlowski, 2003; Keeling, 2001; Longet *et al*., 2003). No members of the Cercozoa had been sequenced at the time of the earlier studies (Darling *et al*., 1996b, Pawlowski *et al*., 1996; Wade *et al*., 1996), and so this relationship was not apparent in the phylogenies produced. In the actin phylogenies of Keeling (2001), the foraminifera consistently fell within the cercozoa and with strong support. The relationship was backed up by evidence of a polyubiquitin amino acid insertion common only to the foraminifera and cercozoa (Archibald *et al*., 2003). On reanalysing the SSU rRNA gene (complete sequences), removing any long-branching lineages from the phylogenetic analyses, Berney & Pawlowski (2003) found the foraminifera to branch consistently with the marine testate filosean *Gromia oviformis*, as a sister group to Cercozoa. The same relationship was recovered in subsequent phylogenetic analyses using the RNA polymerase II gene Longet *et al*. (2003) and Actin gene Flakowski *et al*. (2005). The position of this large clade (Foraminifera, Gromia, Cercozoa) remained poorly resolved, as were the locations of all other major groupings within the eukaryote tree. Though the close affiliations of the Foraminifera are now better understood, further work is needed to pinpoint their exact position in the eukaryote tree.

1.6.2 The origins of the planktonic foraminifera: The move from benthos to plankton

Molecular analyses had shown that the Foraminifera form a monophyletic group within the eukaryotic phylogeny (Archibald *et al*., 2003; Berney & Pawlowski, 2003; Darling *et al*., 1996b; Flakowski *et al*., 2005; Keeling, 2001; Longet *et al*., 2003; Pawlowski *et al*., 1994b, 1996b; Wade *et al*., 1996), however it was also important to determine the evolutionary relationships within the foraminifera, particularly between the benthic and planktonic taxa. Fossil evidence suggested that the earliest planktonic foraminifers evolved from a single benthic lineage, originating in the Mid-Jurassic or earlier (Loeblich & Tappan, 1974; Caron & Homewood, 1983). They may have originated from small benthic foraminifera, the Oberhauserellidae (Tappan & Loeblich, 1988). The movement of foraminifers from the benthos to the plankton thus significantly post-dates the major diversification of benthic foraminifers (Tappan & Loeblich, 1988).

From this point the planktonic foraminifera were subjected to successive extinction and radiation events (Banner & lowry, 1985), and it was assumed that new lineages evolved from the surviving taxa following each extinction event (Tappan & Loeblich, 1988; Norris, 1991; Olsson *et al*., 1992), rather than arising from new and independent adaptations to the planktonic mode of life. Molecular phylogenetic analyses, however, suggest that the planktonic foraminifera are in fact polyphyletic in origin, arising from different benthic ancestors on independent occasions (Darling *et al*., 1997; de Vargas *et al*., 1997). It is unknown how many transitions may have occurred from the benthic to planktonic mode of life during the history of the foraminifera (de Vargas *et al*., 1997), however, repeated phases of adaptation to the planktonic mode of life may be evident in small globular foraminifera during the

Middle and Late Jurassic (Wernli, 1988). Molecular phylogenies suggest that there may be at least 3 extant independent lineages of planktonic foraminifera today (Aurahs *et al*., 2009b; Darling *et al*., 1997, 1999, 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001), consistent with the spinose planktonic foraminifera, non-spinose macroperforates, and non-spinose microperforates (Darling *et al*., 2006; de Vargas *et al*., 1997).

The placement of the spinose Globigerinidae and the non-spinose Globorotaliidae in separate lineages contrasts with the traditional paleontological view of their common origin. The Globorotaliidae were thought to have diverged from a Globigerinid ancestor in the Neogene (Cifelli, 1982; Pearson, 1993), however, the molecular data suggests that the Globorotaliidae are far more closely related to some benthic taxa than to the Globigerinidae (Aurahs *et al*., 2009b; Darling *et al*., 1997, 1999, 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001). There has, in fact, been increasing evidence from molecular phylogenies that the planktonic foraminifera are polyphyletic in origin, evolving independently up to 3 times from separate benthic ancestors (Darling *et al*., 1997, 1999; de Vargas *et al*., 1997, 1998; Stewart *et al*., 2001).

The fossil record shows that the spinose planktonic foraminifera first appeared approximately 65 Ma ago, after the K/T extinctions (Hemleben *et al*., 1991), followed by the first Globorotaliidae (non-spinose), which emerged only 22 Ma ago (Kennett & Srinivasan, 1983). There is compelling evidence to support the separate origins of the spinose and non-spinose planktonic foraminifera. For example, a number of biological similarities exist between the non-spinose planktonic Globorotaliidae and

the benthic foraminifera, suggesting a common ancestry (de Vargas *et al*., 1997; Hilbrecht & Thierstein, 1996; Hemleben *et al*., 1989). The spinose planktonic foraminifera seem highly adapted to the planktonic mode of life, bearing long radial spines, which may aid flotation and certainly allow them to capture prey items e.g. zooplanton and phytoplanton, and house symbionts, thus making them more versatile and able to survive more variable surface water conditions (de Vargas *et al*., 1997). The non-spinose species lack such features, having smooth tests more reminiscent of the benthic taxa. The Globorotaliidae are also herbivorous like benthic species rather than carnivorous like the spinose planktonics (Hemleben *et al*., 1989). Moreover, in laboratory culture, non-spinose globorotaliid specimens have been known to adopt benthic behaviour (Hilbrecht & Thierstein, 1996).

Molecular evidence further indicates that the spinose planktonic foraminifera have evolved separately from the non-spinose planktonic foraminifera, which are in turn far more closely related to the benthic taxa. Extensive SSU rRNA sequence variations have been observed between planktonic foraminiferal species within both the foraminiferal specific insertions and expansion segments (Darling *et al*., 1997). Darling *et al*. (1997) identified a number of substitutional changes that are characteristic of the spinose to non-spinose/ benthic split. In particular a distinctive two base deletion was noted in all planktonic spinose species corresponding to position 2093/4 in *Allogromia* sp. (GenBank accession X86093). In all of the non-spinose planktonic and benthic species, two adenine bases were present in these positions.

Further molecular evidence that the non-spinose foraminifera are more akin to certain benthic taxa than to the spinose planktonic foraminifera came when the first studies of rates of rDNA evolution in the foraminifera were made. Pawlowski *et al*. (1997) reported extreme differences in the rate of rDNA evolution within the foraminifera, the first time such extreme differences had been discovered within a single group of organisms. Corroborating evidence was obtained by de Vargas *et al*. (1997) and Darling *et al*. (1997). Rates of evolution seen in the spinose planktonic foraminifera (globigerinids) are estimated at 50 – 100 times that of most benthic taxa (Pawlowski *et al*., 1997). Most of the globorotaliids (non-spinose macroperforate) show a slower rate of evolution than the spinose taxa, comparable to that of the benthic foraminifera (with the exception of *Globorotalia menardii & truncatulinoides*) (de Vargas *et al*., 1997).

1.6.3 Evolutionary relationships within the planktonic foraminifera

As well as their demonstrated utility in tracing the origins of the foraminifera within the eukaryotes, SSU rDNA sequences contain sufficient evolutionary information to allow the examination of both distant and close relationships within the planktonic foraminifera. In particular, the unprecedented rates of rRNA gene evolution seen in the spinose planktonic taxa (Darling *et al*., 1997; Pawlowski *et al*., 1997) facilitate high-resolution phylogenetic analyses, revealing their interrelationships.

A number of relationships between spinose taxa are consistently recovered in phylogenetic analyses, for example the groupings of *Globigerinoides ruber* with *Globigerinoides conglobatus* (Darling *et al*., 1999, 2000; de Vargas *et al*., 1997; de Vargas & Pawlowski, 1998; Stewart *et al*., 2001; Aurahs *et al*., 2009b), *Globigerinella*

siphonifera with *Globigerinella calida* (de Vargas *et al*., 1997; de Vargas & Pawlowski, 1998), *Globigerina bulloides* with *Globigerina falconensis* and *Turborotalita quinqueloba* (Darling *et al*., 2006; Stewart *et al*., 2001; Aurahs *et al*., 2009b), and *Orbulina universa* with *Globigerinoides sacculifer* (Darling *et al*., 1999, 2000, 2006; de Vargas *et al*., 1997, 2002; de Vargas & Pawlowski, 1998; Stewart *et al*., 2001; Aurahs *et al*., 2009b). *Hastigerina pelagica* has been found to fall at the base of the spinose clade, suggesting an ancestral position (Darling *et al*., 2006; Aurahs *et al*., 2009b).

Within the non-spinose planktonic foraminifera, slower rates of rDNA evolution result in poor resolution and difficulties in ascertaining the placement of taxa. In the majority of past studies only a small number of macroperforate species were included in phylogenies, and though these usually grouped together (Darling *et al*., 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001), such poor taxon sampling made it impossible to say whether the group as a whole would be monophyletic. There is, however, some evidence that *Globorotalia inflata, Pulleniatina obliquiloculata, Neogloboquadrina dutertrei, Neogloboquadrina pachyderma* & *Neogloboquadrina incompta* (previously named *N. pachyderma* right coiling or dextral) form a monophyletic group (Aurahs *et al*., 2009b, Darling *et al*., 2006). In addition, the phylogeny of Aurahs *et al*. (2009b) shows a large, monophyletic group of macroperforate taxa, however, the tree contains only planktonic foraminiferal taxa, and thus cannot confirm the position or monophyly of the macroperforate taxa within the whole of the foraminifera. The microperforate taxa, *Globigerinita glutinata* & *Globigerinita uvula* fall separate from the other non-spinose planktonic taxa, amongst

the benthic foraminifera (Aurahs *et al*., 2009b, Darling *et al*., 2000, 2006; Stewart *et al*., 2001).

The phylogenetic relationships between the planktonic foraminifera together with their origins from benthic ancestors will be explored further during this study (chapter 5).

1.6.4 Cryptic Genetic Diversity Revealed

A degree of morphological plasticity has been observed within the shells of the traditionally recognised morphospecies of planktonic foraminifera, originally thought to be intraspecific variation, or an ecophenotypic effect in response to differing environmental conditions within their adaptive range (Kennett, 1976; Hecht *et al*., 1976; Healy-Williams *et al*., 1985). However, as more foraminiferal SSU rDNA sequences were obtained it became apparent that individual morphospecies were often comprised of complexes of several individual genetic types, revealing that traditional taxonomy had greatly underestimated planktic foraminiferal diversity. Many of these genetic types were found to display distinct ecologies and novel adaptations, often consistent with species-level classification (Darling *et al*., 1997, 1999, 2004, 2006, 2007; de Vargas *et al*., 1997, 1999, 2001, 2002; Huber *et al*., 1997). Examples of planktonic foraminiferal morphospecies for which several SSU rDNA types have been identified are shown in table 1.1. This represents our knowledge to date, though it is likely that more diversity remains to be uncovered.

Table 1.1. Examples of planktonic foraminiferal morphospecies for which multiple SSU rDNA types have been identified

Morphospecies	Number of known genetic types to date	References
Orbulina universa	**3**	2, 3, 11, 14
Globigerinella siphonifera	**>7**	2, 3, 5, 9, 13, 16
Globigerinoides rubber	**5**	1, 2, 3, 5, 9, 10, 15, 16
Globigerina bulloides	**7**	3, 4, 5, 8, 9, 10, 16
Turborotalita quinqueloba	**6**	4, 5, 9, 17
Neogloboquadrina pachyderma	**7**	4, 6, 8, 9
Neogloboquadrina incompta	**2**	5, 7, 9
Globorotalia truncatulinoides	**4**	12, 14

The number of genetic types discovered to date are shown. References: (1) Aurahs *et al*., 2009b; (2) Darling *et al*., 1997; (3) Darling *et al*., 1999; (4) Darling *et al*., 2000; (5) Darling *et al*., 2003; (6) Darling *et al*., 2004; (7) Darling *et al*., 2006; (8) Darling *et al*., 2007; (9) Darling *et al*., 2008; (10) de Vargas *et al*., 1997, (11) de Vargas *et al*., 1999; (12) de Vargas *et al*., 2001 (13) de Vargas *et al*., 2002; (14) de Vargas *et al*., 2004; (15) Pawlowski *et al*., 1997; (16) Stewart *et al*., 2000; (17) Stewart *et al*., 2001.

The discovery of hidden genetic diversity in the planktonic foraminifera adds complexity to our views of their global biogeography, perhaps explaining the apparent occupation of single morphospecies in multiple faunal provincial zones (Bé & Tolderlund, 1971), a pattern that was originally thought to be indicative of a fairly generalist lifestyle. Significantly, the newly discovered genotypes show non-random distributions, suggestive of distinct ecologies (ecotypes) (Darling *et al*., 1997, 1999, 2004, 2006, 2007, 2008; de Vargas *et al*., 1997, 1999, 2001, 2002; Huber *et al*., 1997). Such cryptic diversity particularly impacts on climate change palaeoproxies where the presence of these ecotypes may lead to inaccuracy in analysis. It is therefore vital to gain a better understanding of their global genetic variability and phylogeography to improve quantitative faunal and geochemical palaeoclimate reconstructions.

1.7 Summary of the aims of this thesis

Chapter 1 of this thesis offers a generalised introduction to the Foraminifera and the topics that will be covered throughout the thesis, with details of the general methods used provided in chapter 2.

Chapters 3 and 4 aim to highlight the extensive genetic diversity seen in the planktonic foraminifera, focusing on two contrasting areas of the global ocean, the tropical Arabian Sea (chapter 3), and the transitional/sub-polar North Atlantic Ocean (chapter 4). The genetic types found in each region have been incorporated into a comprehensive phylogeny of the foraminifera, containing a broad range of both planktonic and benthic taxa. The links between genetic variation and the ecological habits of the planktonic foraminiferal genetic types are explored.

In chapter 5, the phylogenetic relationships of the foraminifera are examined, and the origins of the planktonic foraminifera investigated. Traditional classification assumes a monophyletic origin for the planktonic foraminifera, however, molecular work indicates possible polyphyletic origins. A comprehensive phylogeny containing a broad range of planktonic and benthic taxa is presented, constructed from an ~1,000 bp 3′ terminal fragment of the small subunit (SSU) ribosomal (r) RNA gene. The phylogenetic relationships of the foraminifera are explored, and the origins of the planktonic taxa investigated. An additional aim of this chapter was to overcome the difficulties that are frequently encountered during phylogenetic analyses of the foraminifera, including poor resolution of relationships, low bootstrap support of clades, and difficulties in placing certain taxa. It is likely that such problems stem from the use of insufficient data, with only an ~1,000 bp fragment, or roughly a third

of the length of the foraminiferal SSU rRNA gene, traditionally used. Here new sequence data is also presented for approximately 3,000 bp, or almost the complete length of the SSU rRNA gene in the foraminifera. Phylogenetic reconstruction was carried out using a range of methods, and the origins of the planktonic taxa again investigated. For both the shorter ~1,000 bp fragment and the almost full-length ~3,000 bp of the SSU rRNA gene, various phylogenetic hypotheses were tested to assess the rigidity of the optimal phylogenies, and an examination of the relative rates of evolution between the foraminiferal taxa made.

In response to some of the problems experienced during molecular work on the foraminifera, chapter 6 focuses on the development of a method for culturing foraminiferal species (benthic in the first instance) in the laboratory, with the aim of providing a continual source of genetic material for molecular work. This would ultimately allow for better optimisation of PCR amplification methods, for example by allowing extensive experimentation into primer design and PCR conditions. It would also provide a DNA template for genomic sequencing, in order to identify new genetic markers for use in phylogenetic work.

The work described in chapter 7 also aims to remedy some of the problems commonly encountered in molecular studies of the foraminifera (e.g. in chapters 3 –5 of this thesis). The traditionally employed method of extracting and storing DNA from foraminiferal samples can result in poor-quality template DNA, and often leads to high failure rates in PCR amplifications. Here new DNA extraction buffers have been designed and tested, with the aim of developing a technique to effectively remove and

store genetic material from the single cell of the foraminifera, whilst leaving the delicate calcite shell intact, for morphological reference.

The findings of this thesis are summarized and discussed in chapter 8.

1.8 References

Anderson, O. R. 2006. Micro*scope (online). Available at: http://starcentral.mbl.edu /microscope (accessed 13.08.10)

Anderson, O. R., Lee, J. J., & Faber Jr, W. W. 1991. Collection, maintenance and culture methods for the study of living foraminifera. In: *Biology of Foraminifera* (ed. J.J Lee & O. R. Anderson), Academic press (San Diego) pp 335-357

Anderson, T. F., & Arthur, M. A. 1983. Stable isotopes of oxygen and carbon and their application to sedimentologic and paleoenvironmental problems. In: *Stable Isotopes in sedimentary Geology* (eds. Arthur, M. A., Anderson, T. F., Kaplan, I. R., Veizer, J., & Land, L. S.): SEPM Short Course No. 10, Tulsa p., p1-151

Angell, R. W. 1967. The process of chamber formation in the foraminifer Rosalina floridana. *Journal of Proto-zoology*, **14**, 566-574

Archibald, J. M., Longet, D., Pawlowski, J., & Keeling, P. J. 2003. A novel Polyubiquitin structure in Cercozoa and Foraminifera: evidence for a new eukaryotic supergroup. *Molecular Biology and Evolution*, **20** (1), 62-66

Arnold, Z. M. 1974. Field and laboratory techniques for the study of living foraminifera. In: *Foraminifera*, v.1 (eds. Hedley, R. H., & Adams, C. G.), Academic Press, London, p. 153-206

Aurahs, R., Göker, M., Grimm, G. W., Hemleben, V., Hemleben, C., Schiebel, R., & Kucera, M. 2009b. Using the multiple analysis approach to reconstruct phylogenetic relationships among planktonic foraminifera from highly divergent and length-polymorphic SSU rDNA sequences. *Bioinformatics and Biology Insights*, **2009:3**, 155-177

Banner, F. T., & Lowry, F. M. D. 1985. The stratigraphical record of planktonic foraminifera and its evolutionary implications. *Spec. Pap. Paleontol*, **33**, 117-130

Baroin, A., Perasso, R., Qu, L. H., Brugerolle, G., Bachellerie, J., & Adoutte, A. 1988. Partial phylogeny of the unicellular eukaryotes based on rapid sequencing of a portion of 28S ribosomal RNA. *Proceedings National Academy of Sciences of the USA*, **85**, 3474-3478

Bé, A. W. H. 1977. An ecological, zoogeographic and taxonomic review of Recent planktonic foraminifera. In: *Oceanic Micropaleontology,* v.1 (ed Ramsay, A. T. S.) Academic Press, London, pp. 1-100

Bé, A. W. H. and Tolderlund, D.S. 1971. Distribution and ecology of planktonic foraminifera. In: *The Micropaleontology of Oceans* (eds Funnell B.M., Riedel W.R.),

Bé, A. W. H., McIntyre, A., & Breger, D. 1966. The shell microstructure of a planktonic foraminifer, *Globorotalia menardii* (d'Orbigny). *Eclogae geol. Helv*, **59**, 885-896

Bé, A.W.H., 1977. An ecological, zoogeographic and taxonomic review of recent planktonic foraminifera. In: *Oceanic Micropaleontology,* Volume 1(ed. Ramsey A.T.S.). Academic Press, London. pp 1-100.

Benjamini, C & Reiss, Z. 1979. Wall-hispidity and perforation in Eocene planktonic foraminifera. *Micropaleontology*, **25**, 141-150

Berger, W. H. 1979a. Preservation of foraminifera. In: *Foraminiferal ecology and paleoecology* (eds. Lipps, J. H., & Berger, W. H. *et al*.). SEPM Short Course No.6, Houston, Texas, p 105-155

Berger, W. H. 1979b. Stable isotopes in Foraminifera. In: *Foraminiferal Ecology and Paleoecology* (eds. Lipps, J. H., & Berger, W. H.): SEPM Short Course No. 6, Houston, Texas, p156-197

Berger, W. H. 1981. Paleoceanography: The Deep-Sea Record; In: *The Oceanic Lithosphere* (ed. Emiliani, C): The Sea, vol. 7, p. 1437-1519

Berney, C., & Pawlowski, J. 2003. Revised Small Subunit rRNA analysis provides further evidence that foraminifera are related to cercozoa. *Journal of Molecular Evolution*, **57**, S120-S127 DOI: 10.1007/s00239-003-0015-2

Bernhard, J. M., & Bowser, S. S. 1992. Bacterial biofilms as a trophic resource for certain benthic foraminifera. *Marine Ecology Progress Series*, **83**, 263-272

Bishop, J. K. B., Edmond, J. M., Ketten, D. R., Bacon, M. P., & Silker, W. B. 1977. The chemistry, biology and vertical flux of particulate matter from the upper 400 m of the equatorial Atlantic Ocean. *Deep-Sea Research*, **24**, 511-548

Boersma, A., Premoli-Silva, I., & Shackleton, N. J. 1987. Atlantic Eocene planktonic foraminiferal paleohydrographic indicators and stable isotope paleoceanography. *Paleoceanography*, **2**, 287-331

Bowser, S. S., Alexander, S. P., Stockton, W. L., & DeLanca, T. E. 1992. Extracellular matrix augments mechanical properties of pseudopodia in the carnivorous foraminiferan *Astrammina rara*: Role in prey capture. *Journal of Protozoology*, **39**, 724-732

Bradshaw, J. S. 1957. Laboratory studies of the rate of growth of the Foraminifera "*Streblus beccarii* (Linné) var. *tepida* (Cushman)". *Journal of Paleontology* **31** (6), 1138-1147

Bruns, T. D., White, T. J., & Taylor, J. W. 1991. Fungal molecular systematics. *Annual Review of Ecology and Systematics*, **22**, 525-564

Cambridge Univ. Press, London, pp.105-150.

Caron, M., & Homewood, P. 1983. Evolution of early planktic foraminifers. *Marine Micropaleontology*, **7**, 453-462

Caron, M., & Homewood, P. 1983. Evolution of early planktic foraminifers. *Marine Micropaleontology*, **7**, 453-462

Carpenter, W. B., Parker, W. K., & Jones, T. R. 1862. *Introduction to the Study of the Foraminifera*, Ray Society, London

Cedhagen, T. 1994. Taxonomy and biology of *Hyrrokkin sarcophagi* n. gen., et sp. n., a parasitic Foraminiferan (Rosalinidae). *Sarsia*, **79**, 65-82

Cifelli, R. 1982. Early occurrences and some phylogenetic implications of spiny, honeycomb textured planktonic foraminifera. *Journal of Foraminiferal Research*, **12**, 105-115

Cifelli, R. 1982. Textural observations on some living species of planktonic foraminifera. *Smithsonian Contr. To Paleobiol*., Washington, p 1-45

CLIMAP Project members. 1976. The surface of the ice-age Earth. *Science*, **191**, 1131-1137

Culver, S. J. 1991. Early Cambrian foraminifera from West Africa. *Science*, **254**, 689-691

Cushman, J. A. 1945. Parallel evolution in the Foraminifera. *American Journal of Science*, **243-A**, 117-121

Cushman, J. A. 1948. *Foraminifera: Their classification and Economic Use*, 4th Ed., Harvard University Press, Cambridge

Dams, E., Hendriks, L., Van de Peer, Y., Neefs, J. M., Smits, G., Vandenbembt, I., & de Wachter, R. 1988. Compilation of small ribosomal subunit RNA sequences. *Nucleic Acid Research*, **16** (sup), 87-173

Darling, F., & Wade, C. M. 2008. The genetic diversity of planktic foraminifera and the global distribution of ribosomal RNA genotypes. *Marine Micropaleontology* **67**, 216 – 238

Darling, K, F., Thomas, E., Kasemann, S. A., Seears, H. A., Smart, C. W., & Wade, C. M. 2009. Surviving mass extinction by bridging the benthic/planktic divide. *Proceedings of the National Academy of Sciences,* USA, **106**(31), 12629-33

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996a. The isolation and amplification of the 18s ribosomal RNA gene from planktonic foraminifers using gametogenic specimens. *In: Microfossils and oceanic environments* (eds. Moguilevsky, A & Whatley, R.): University of Wales, Aberystwyth Press, 3.1, 249-259

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996b. Molecular phylogeny of the planktonic foraminifera. *Journal of Foraminiferal Research*, **26** (4), 324-330

Darling, K. F., Kucera, M. Wade, C. M. 2007. Global molecular phylogeography reveals persistent Arctic circumpolar isolation in a marine planktonic protist. *Proc. Natl. Acad. Sci.* USA **104**, 5002-5007.

Darling, K. F., Kucera, M., Kroon, D., Wade, C. M. 2006. A resolution for the coiling direction paradox in *Neogloboquadrina pachyderma*. *Paleoceanography* **21**, PA2011, doi:10.1029/2005PA001189.

Darling, K. F., Kucera, M., Pudsey, C. J., Wade, C. M. 2004. Molecular evidence links cryptic diversification in polar plankton to Quaternary climate dynamics. *Proc. Natl. Acad. Sci.* USA. **101**, 7657-7662.

Darling, K. F., Kucera, M., Wade, C. M., von Langen, P., & Pak, D. 2003. Seasonal distribution of genetic types of planktonic foraminifer morphospecies in the Santa Barbara Channel and its paleoceanographic implications. *Paleoceanography,* **18** (2), 1032

Darling, K. F., Wade, C. M., Kroon, D. & Leigh Brown, A. J. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. *Marine Micropaleontoogy,* **30**, 251-266

Darling, K. F., Wade, C. M., Kroon, D., Leigh Brown, A. J. & Bijma,J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulation. *Paleoceanography* **14**, 3-12

Darling, K. F., Wade, C. M., Steward, I. A., Kroon, D., Dingle, R., Leigh Brown, A. J. 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature*, **405**, 43-47.

de Vargas, C., & Pawlowski, J. 1998. Molecular versus taxonomic rates of evolution in planktonic foraminifera. *Molecular Phylogenetics and Evolution*, **9** (3), 463-469

de Vargas, C., Bonzon, M., Rees, N. W., Pawlowski, J., Zaninetti, L. 2002. A molecular approach to diversity and biogeography in the planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Marine Micropaleontology* **870**, 1-16

de Vargas, C. & de Garidel Thoron, T. 2010. CalcOBIS, The Institute of Marine & Coastal Sciences of Rutgers University (online). Available at: http://marine.rutgers.edu/MEEOOP/CalcOBIS/ (accessed 13.08.10)

de Vargas, C., Norris, R., Zaninetti, L., Gibb, S. W., & Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proceedings of the National Academy of Science* USA **96**, 2864-2868

de Vargas, C., Renaud, S., Hilbrecht, H., Pawlowski, J. 2001. Pleistocene adaptive radiation in *Globorotalia truncatulinoides*: genetic, morphologic, and environmental evidence. *Paleobiology* **27**(1), 104-125.

de Vargas, C., Sáez, A. G., Medlin, L. K., Thierstein, H. R. 2004. Super-species in the calcareous plankton. In: *Coccolithophores-from molecular processes to global impact* (eds. Thierstein H. R., Young, J. R.). Springer. pp 271-298.

de Vargas, C., Zaninetti, L., Hilbrecht, H. and Pawlowski, J. 1997. Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record. *J. Mol. Evol.* **45** (3), 285-294

Decrouez, D. 1989. Generic ranges of Foraminiferida. *Revue de Paléobiologie*, **8**, 263-321

DeLaca, T. E., Karl, D. M., & Lipps, J. H. 1981. Direct use of dissolved organic carbon by agglutinated Foraminifera. *Nature*, **289**, 287-289

Departamento de Paleontologia e Estratigrafia da UFRGS. 2006. Foraminiferos (online) Available at: http://www6.ufrgs.br/microfosseis/dados _foraminiferos.htm (accessed 13.08.10)

Dettmering, C., Rottger, R., Hohenegger, J., & Schmalijohann, R. 1998. The trimorphic life cycle in foraminifera: Observations from cultures allow new evaluation. *European Journal of Protistology* **34** (4), 363-368

d'Orbigny, A. 1826. Tableau Méthodique de la Classe des Céphalopodes. *Annales des Sciences Naturelles, Paris (Série 1)*, **7**, 245-314

Duguay, L. E., 1983. Comparative laboratory and field studies on calcification and carbon fixation in foraminiferal-algal associations. *Journal of Foraminiferal Research*, **13**, 252-261

Embley, T. M., & Hirt, R. P. 1998. Early branching Eukaryotes**?** *Current Opinion in Genetics & Development*, **8**, 624-629

Faber, W. W., & Lee, J. J. 1991. Histochemical evidence for digestion in *Heterostegina depressa* and *Operculina ammonoides* (Foraminifera). *Endocytobiosis and Cell Research*, **8**, 53-59

Faber, W. W., Anderson, O. R., Lindsey, J. L., & Caron, D. A. 1985. Algal-foraminiferal symbiosis in the planktonic foraminifer *Globigerinella aequilateralis*, I, Occurrence and stability of two mutually exclusive chrysophytes endosymbionts and their ultrastructure. *Journal of Foraminiferal Research*, **18**, 334-343

Falkowski, P. G., Dubinsky, Z., Muscatine, L., & McCloskey, L. 1993. Population control in symbiotic corals. *BioScience*, **43**, 606-611

Field, K. G., Olsen, G. J., Giovannoni, S. J., Ghiselin, M. T., Raff, E. C., Pace, N. R., & Raff, R. A. 1988. Molecular phylogeny of the animal kingdom. *Science*, **239**, 748-753

Flakowski, J., Bolivar, I., Fahrni, J., & Pawlowski, J. 2005. Actin phylogeny of foraminifera. *Journal of Foraminiferal Research*, **35** (2), 93-102

Gast, R. J., & Caron, D. A. 1996. Molecular phylogeny of symbiotic dinoflagellates from planktonic foraminifera and radiolarians. *Molecular Biology and Evolution*, **13**, 1192-1197

Gastrich, M. D. 1988. Ultrastructure of a new intracellular symbiotic alga found within planktonic foraminifera. *Journal of Phycology*, **23**, 623-632

Goldstein, S. T. 1988. On the life cycle of *Saccammina alba* Hedley, 1962. *Journal of Foraminiferal Research*, **18**, 311-325

Goldstein, S. T. 1999. Foraminifera: A biological Overview. In *Modern Foraminifera* (Ed. Gupta, B. K. S), Kluwer Academic Publishers, p37-55

Goldstein, S. T. 1999. Foraminifera: A biological overview. In: *Modern Foraminifera* (ed. Sen Gupta, B. K.), Kluwer Academic Publishers, p 37-55

Goldstein, S. T. & Moodley, L. 1993. Gametogenesis and life cycle of the foraminifer Ammonia beccarii (Linné) forma tepida (Cushman). *Journal of Foraminiferal Research* **23** (4), 213-220

Goldstein, S. T., & Barker, W. W. 1988. Test ultrastructure and taphonomy of the monothalamous agglutinated foraminifer *Cribrothalammina* n. gen. *Alba* (Heron-Allen and Earland). *Journal of Foraminiferal Research*, **18**, 130-136

Goldstein, S. T., & Corliss, B. H. 1994. Deposit feeding in selected deep-sea and shallow-water benthic foraminifera. *Deep-Sea Research*, **41**, 229-241

Gooday, A. J. 1988. A response by benthic foraminifera to the deposition of phytodetritus in the deep sea. *Nature*, **332**, 70-73

Gooday, A. J. 1993. Deep-sea benthic foraminiferal species which exploit phytodetritus: Characteristic features and controls on distribution. *Marine Micropaleontology*, **22**, 187-205

Grell, K. G. 1954. Der Generationswechsel der Polythalmen Foraminifera Rotaliella heterocaryotica. *Archiv der Protistk* **100**, 211-235

Grigelis, A. A. 1978. Higer foraminiferal taxa. *Paleontological journal*, **12**, 1-9

Hallock, P. 1981. Algal symbiosis: a mathematical analysis. *Marine Biology*, *62*, 249-255

Hallock, P. 1999. Symbiont-Bearing Foraminifera. In: *Modern Foraminifera* (ed Sen Gupta, B. K.), Kluwer Academic Publishers, p. 123-139

Harney, J. N., Hallock, P., & Talge, H. K. 1998. Observations on a trimorphic life cycle in *Amphistegina gibbosa* populations from the Florida Keys. *Journal of Foraminiferal Research* **28** (2), 141-147

Haynes, J. R. 1981. *Foraminifera*. Macmillan, London

Healy-Williams, N., Ehrlich, R., Williams, D.F. 1985. Morphometric and stable isotopic evidence for subpopulations of *Globorotalia truncatulinoides*. *Journal of Foraminiferal Research* **15**, 242–253

Hecht, A.D., Bé, A.W.H., Lott, L. 1976. Ecologic and paleoclimatic implications of morphologic variation of *Orbulina universa* in the Indian Ocean. *Science*, **194**, 422–424

Hemleben, C., Mühlen, D., Olsson, R. K., & Berggren, W. A. 1991. Surface texture and the first occurrence of spines in planktonic foraminifera from the early Tertiary. *Geol Jahrbuch*, **128**, 117-146

Hemleben, Ch. 1969a. Ultramicroscopic shell and spine structure of some spinose planktonic foraminifera. In: *Proc. 1st Int. Conf. Plankt. Microfoss. 2,* (eds. Brönnimann, P., & Renz, H. H.), Leiden, p. 254-256

Hemleben, Ch. 1969b. Zur Morphogenese planktonischer Foraminiferen. *Zitteliana*, **1**, 91-133

Hemleben, Ch. 1975. Spine and pustule relationships in some Recent planktonic foraminifera. *Micropaleontology*, **21**, 334-341

Hemleben, Ch., Anderson, O. R., Berthold, W., & Spindler, M. 1986. Calcification and chamber formation in Foraminifera – a brief overview. In: Leadbeater, B. S. C., & Riding, R. (eds.), *Biomineralization in Lower Plants and Animals*, Systematics Association, Special Volume, **30**, 237-49

Hemleben, Ch., Bé, A. W. H., Anderson, O. R., & Tuntivate-Choy, S. 1977. Test morphology, organic layers and chamber formation of the planktonic foraminifer *Globorotalia menardii* (d'Orbigny). *Journal of Foraminiferal Research*, **7**, 1-25

Hemleben, Ch., Spindler M., Anderson, O. R. 1989. Modern Planktonic Foraminifera. Springer Verlag, New York, 363pp

Hilbrecht, H & Thierstein, H. R. 1996. Benthic behaviour of planktic foraminifera. *Geology*, **24**, 200-202

Hillis, D. M., & Moritz, C. (Eds). 1990. Molecular systematics. Sinauer Ass. Inc, pp1-588

Hirt, R. P., Logsdon jr, J. M., Healy, B., Dorey, M. W., Doolittle, W. F., & Embley, T. M. 1999. Microsporidia are related to Fungi: Evidence from the largest subunit of RNA polymerase II and other proteins. *Proceedings of the National Academy of Science of the USA*, **96**, 580-585

Huber, B. T., Bijma, J., & Darling, K. 1997. Cryptic speciation in the living planktonic foraminifer Globigerinella siphonifera (d'Orbigny). *Paleobiology*, **23** (1), 33-62

Imbrie, J., van Donk, J., & Kipp, N. G. 1973. Paleoclimatic investigation of a late Pleistocene Caribbean deep-sea core: comparison of isotopic and faunal methods. *Quaternary Research*, **3**, 10-38

Jepps, M. W. 1942. Studies on *Polystomella* Lamarck (Foraminifera). *Journal of the Marine Biological Association of the United Kingdom*, **25**, 607-666

Jepps, M. W. 1942. Studies on *Polystomella Lamarck* (Foraminifera). *Journal of the Marine Biological Association of the United Kingdom*, **25**, 607-666

Keeling, P. J. 2001. Foraminifera and Cercozoa are related in Actin phylogeny: two orphans find a home? *Molecular Biology and Evolution*, **18** (8), 1551-1557

Keeling, P. J., & Doolittle, W. F. 1996. Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. *Molecular Biology and Evolution*, **18**, 1551-1557

Keeling, P. J., Luker, M. A., & Palmer, J. D. 2000. Evidence from beta-tubulin phylogeny that Microsporidia evolved from within the Fungi. *Molecular Biology and Evolution*, **17**, 23-31

Kennett, J. P., & Srinivasan, M. S. (eds). 1983. *Neogene planktonic foraminifera, a phylogenetic atlas*. Hutchinson Ross Publishing Company, Stroudsburg, Pennsylvania

Kennett, J.P., 1976. Phenotypic variation in some Recent and late Cenozoic planktonic foraminifera. In: *Foraminifera, vol. 2.* (eds Hedley, R.H., Adams, C.G.), Academy Press, New York, pp. 111–170

Kimoto, K., Ishimura, T., Tsunogai, U., Itaki, T., & Ujiié, Y. 2009. The living triserial planktic foraminifer Gallitellia vivans (Cushman): Distribution, stable isotopes, and paleoecological implications. *Marine Micropaleontology*, **71**, 71-79

Langer, M. R., Lipps, J. H., & Werner, E. P. 1993. Molecular paleobiology of protists: amplification and direct sequencing of foraminiferal DNA. *Micropaleontology*, **39** (1), 63-68

Lee, J. J. 1980. Nutrition and physiology of the foraminifera. In: *Biochemistry and Physiology of Protozoa*, v. 3 (eds. Levandowsky, M., & Hutner, S. H.), Academic Press, New York, p. 43-66

Lee, J. J. 1990. Phylum Granuloreticulosa (Foramininifera). In: *Handbook of Protoctista* (eds. Margulis, L., Corliss, J. O., Melkonian, M., & Chapman, D. J.), Jones and Bartlett, Boston, pp. 524-548

Lee, J. J., & Anderson, O. R. 1991a. Symbiosis in Foraminifera. In: *Biology of Foraminifera* (eds. Lee, J. J., & Anderson, O. R.), Academic Press, London, p. 157-220

Lee, J. J., Faber, W. W. Jr., Anderson, O. R., & Pawlowski, J. 1991b. Life cycles of foraminifera. In *Biology of Foraminifera* (ed. J. J. Lee, & O. R. Anderson), Academic Press, New York, pp. 285-334

Lee, J. J., Faber, W. W., & Lee, R. E. 1991c. Granular reticular digestion – a possible preadaption to benthic foraminiferal symbiosis? *Symbiosis*, **10**, 47-61

Lee, J. J., McEnery, M., Pierce, S., *et al*. 1966. Tracer experiments in feeding littoral foraminifera. *Journal of Protozoology,* **13**, 659-70

Lenaers, G., Scholin, C., Bhaud, Y., Saint-Hilaire, D., & Herzog, M. 1991. A molecular phylogeny of dinoflagellate protists (Pyrrhophyta) inferred from the sequence of 24S rRNA divergent domains D1 and D8. *Journal of Molecular Evolution*, **32**, 53-63

Leutenegger, S. 1977. Reproduction cycles of larger foraminifera and depth distribution of generations: *Utrecht Micropaleontological Bulletin* **15**, 26-34

Linke, P., & Lutze, G. F. 1993. Microhabitat preferences of benthic foraminifera – a static concept or a dynamic adaptation to optimise food acquisition? *Marine Micropaleontology*, **20**, 215-234

Lipps, J. H. 1966. Wall structure, systematics, and phylogeny studies of Cenozoic planktonic Foraminifera. *Journal of Paleontology*, **40**, 1257-1275

Lipps, J. H. 1983. Biotic interactions in benthic foraminifera. In: *Biotic Interactions in Recent and Fossil Benthic Communities* (eds. Tevez, M. J. J., & McCall, P. L.), Plenum, New York, p. 331-376

Lipps, J.H., 1979. The Ecology and Paleoecology of planktic Foraminifera. In: *Foraminiferal Ecology and Paleoecology* (eds. Lipps, J.H., Berger, W.H., Buzas, M.A., Douglas, R.G., Ross, C.A.), SEPM Short Course #6, Houston, Texas, pp. 62-104

Lister, J. J. 1895. Contributions to the life history of the Foraminifera. *Philosophical Transactions of the Royal Society of London, Series B*, **186**, 401-453

Lister, J. J. 1895. Contributions to the life history of the Foraminifera. *Philosophical Transactions of the Royal Society of London, Series B*, **186**, 401-453

Loeblich, A. R., & Tappan, H. 1987. *Foraminiferal Genera and Their Classification*, v. 1-2, Van Nostrand Reinhold, New York

Loeblich, A. R., & Tappan, H. 1992. Present Status of foraminiferal classification. In: *Studies in Benthic Foraminifera* (eds. Takayangi, Y & Saito, T), Proceedings of the Fourth International Symposium on Benthic Foraminifera, Sendai, 1990 (Benthos '90), Tokai University Press, Tokyo, Japan, pp. 93-102

Loeblich, Jr., A. R., & Tappan, H. 1964. Sarcodina, Chiefly 'Thecamoebians' and Foraminiferida. In: *Treatise on Invertebrate Paleontology* (ed. R. C. Moore), Geological Society of America, Boulder, Part C, v. 1-2, 900 pp

Loeblich, Jr., A. R., & Tappan, H. 1974. Recent advances in the classification of the Foraminiferida. In: *Foraminifera*, 1 (eds. Hedley, R. H., & Adams, C. G.). Academic Press, London, p1-53

Longet, D., Archibald, J. M., Keeling, P. J., & Pawlowski, J. 2003. Foraminifera and Cercozoa share a common origin according to RNA polymerase II phylogenies.

International Journal of Systematic and Evolutionary Microbiology, **53**, 1735-1739

Microfossil image recovery and circulation for learning and education (MIRACLE). 2010. Foraminifera (online). Available at: http://www.ucl.ac.uk/GeolSci/micropal/foram.html

Norris, R. D., 1991. Biased extinction and evolutionary trends. *Paleobiology*, **17** (4), 388-399

Olsson, R. K., Hemleben, C., Berggren, W. A., & Liu, C. 1992. Wall texture classification of planktonic foraminifera genera in the lower Danian. *Journal of Foraminiferal Research,* **22** (3), 195-213

Parker, F. L. 1962. Planktonic foraminiferal species in Pacific sediments. *Micropaleontology*, **8**, 219-254

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C., & Bowser, S. S. 1999a. Molecular evidence that *Reticulomyxa filosa* is a freshwater naked foraminifer. *Journal of Eukaryotic Microbiology*, **46** (6), 612-617

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C., & Bowser, S. S. 1999b. Naked foraminiferans revealed. *Nature*, **399**, 27

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C., Gouy, M. and Zaninetti, L. 1997. Extreme differences in rates of molecular evolution of foraminifera revealed by comparison of ribosomal DNA sequences and the fossil record. *Mol. Biol. Evol.* 14 (5), 498-505

Pawlowski, J., Bolivar, I., Fahrni, J., & Zaninetti, L. 1994a. Taxonomic identification of foraminifera using ribosomal DNA sequences. *Micropaleontology*, **40** (4), 373-377

Pawlowski, J., Bolivar, I., Guiard-Maffia, J., & Gouy, M. 1994b. Phylogenetic position of foraminifera inferred from LSU rRNA gene sequences. *Molecular Biology and Evolution*, **11** (6), 929-938

Pearson, P. N. 1993. A lineage phylogeny for the Paleogene planktonic foraminifera. *Micropaleontology*, **39**, 193-232

Perasso, R., Baroin, A., Qu, L. H., & Bachellerie, J. P. 1989. Origin of the algae. *Nature*, **399**, 142-144

Philippe, H., & Adoutte, A. 1998. The molecular phylogeny of Eukaryota: Solid facts and uncertainties. In: *Evolutionary relationships among Protozoa* (eds Coombs, G., Vickermann, K., Sleigh, M., & Warren, A.). Chapman & Hall, London, pp25-26

Philippe, H., Lopez, P., Brinkmann, H., Budin, K., Germot, A., Laurent, J., Moreira, D., Müller, M., & Le Guyader, H. 2000. Early branching or fast evolving Eukaryotes? An answer based on slowly evolving positions. *Proceedings of the Royal Society of London, series B,* **267**, 1213-1222

Reuss, A. E. 1861. Entwurf einer Systematischen Zummenstellung der Foraminiferen. *Sitzungsberichte der K. Akademie der Wissenschaften, Wien*, **44**, 354-396

Rhumbler, L. 1909. Die Foraminiferen (Thalamorphoren) der plankton-Expedition, Teil. In *Ergebnisse der Plankton-Expedition der Humboldt-Stiftung, v. 3,* (ed. V. Hensen), Lipsius und Tischer, Kiel, p131

Röttger, R., Fladung, M., Schmaljohann, R., Spindler, M., & Zacharias, H. 1986. A new hypothesis: The so-called megalospheric schizont of the larger foraminifer, *Heterostegina depressa* D'Orbigny, 1862, is a separate species. *Journal of Foraminiferal Research* **16**, 141-149

Röttger, R., Kruger, R., & De Ruk, S. 1990. Trimorphism in foraminifera (Protazoa): Verification of an old hypothesis. *European Journal of Protistology* **25**, 226-228

Ruddiman, W. F., & Sarnthein, M., *et al*. 1986. Paleoclimatic linkage between high and low latitudes. *Nature*, **322**, 211-212

Sachs, H. M., Webb, T., & Clark, D. R. 1977. Paleoecological transfer functions. *Ann. Rev. Earth Planet. Sci*, **5**, 159-178

Saito, T., Thompson, P. R., & Breger, D. 1976. Skeletal ultra-microstructure of some elongate-chambered planktonic foraminifera and related species. In: *Selected Papers in honour of Prof. Kiyoshi Asano: Progress in Micropaleontology, Spec. Pub*. (eds. Takayanagi, Y., & Saito, T.), New York, p 278-304

Sancetta, C., Imbrie, J., Kipp, N. G., McIntyre, A., & Ruddiman, W. F. 1972. Climatic record in North Atlantic deep-sea core V23-82: comparison of the last and present interglacials based on quantitative time series. *Quaternary Research*, **2**, 363-367

Schaudinn, P. 1895. Uber den Dimorphisms bei Foraminiferen. *Sitzungsberichte der Gesellschaft naturforschender Freunde zu Berlin*, **5**, 87-97

Schlegel, M. 1991. Protist evolution and phylogeny as discerned from small subunit ribosomal RNA sequence comparisons. *European Journal of Protistology*, **27**, 207-219

Schott, G. H. 1973. Ontogeny and shape in *Globorotalia menardii*. *Journal of Foraminiferal Research*, **3**, 142-146

Scott, G. H. 1974. Pustulose and honeycomb topography in *Globigerinoides trilobus*. *Micropaleontology*, **20**, 466-472

Sen Gupta, B. K. 1999a. Introduction to Modern Foraminifera. In: *Modern Foraminifera* (ed. Sen Gupta, B. K.). Kluwer Academic Publishers, p 3-6

Sen Gupta, B. K. 1999b. Systematics of modern Foraminifera. In: *Modern Foraminifera* (ed. Sen Gupta, B. K.). Kluwer Academic Publishers, p 7-36

Silver, M. W., Shanks, A. L., & Trent, J. D. 1978. Marine snow: micro-plankton habitat and source of small-scale patchiness in pelagic populations. *Science*, **201**, 371-373

Sogin, M. L. 1989. Evolution of Eukaryotic Microorganisms and Their Small Subunit Ribosomal RNAs. *American Zoologist*, **29**, 487-499

Sogin, M. L. 1991. Early evolution and the evolution of eukaryotes. *Current Opinions in Genetics and Development*, **1**, 457-463

Sogin, M. L. 1997. History assignment: when was the mitochondrion founded? *Current Opinions in Genetics and Development*, **7**, 792-799

Spindler, M., & Hemleben, C. 1980. Symbionts in planktonic foraminifera (Protozoa). *Endocytobiology, Endosymbiosis and Cell Biology*, **1**, 133-140

Stapleton, R. P. 1973. Ultrastructure of some tests of some Recent Foraminifera. *Palaeontographica,* pt. A, **142**, 16-49

Stewart, I. A. 2000. The molecular evolution of planktonic foraminifera and its implications for the fossil record. Ph.D. thesis, University of Edinburgh

Stewart, I. A., Darling, K. F., Kroon, D., Wade, C. M., & Troelstra, S. R. 2001. Genotypic variability on subarctic Atlantic planktic foraminifera. *Marine Micropaleontology*, **43**, 143-153

Stouff, V., Lesourd, M., & Debenay, J. P. 1999a. Laboratory observations on asexual reproduction (schizogony) and ontogeny of *Ammonia tepida* with comments on the life cycle. *Journal of Foraminiferal Research* **29** (1), 75-84

Takayanagi, Y., Niitsuma, N., & Sakai, T. 1968. Wall microstructure of *Globorotalia truncatulinoides* (d'Orbigny). *Tohoku Univ. Sci. Repts., Ser. 2 (Geol)*, **40** (2), 141-170

Tappan, H. & Loeblich, A. R. Jr. 1988. Foraminiferal evolution, diversification, and extinction. *Journal of Paleontology,* **62** (5), 695-714

ter Kuile, B. 1991. Mechanisms for calcification and carbon cycling in algal symbiont-bearing foraminifera. In: *Biology of Foraminifera* (eds Lee, J. J., & Anderson, O. R.), Academic Press, New York, pp. 73-89

Towe, K. M., & Cifelli, R. 1967. Wall ultrastructure in the calcareous Foraminifera: Crystallographic aspects and a model for calcification. *Journal of Paleontology*, **41**, 742-762

Vickerman, K. 1992. The diversity and ecological significance of Protozoa. *Biodiversity and Conservation*, **1**, 334-341

Vincent, E., & Berger, W. H. 1981. Planktonic foraminifera and their use in paleoceanography. In: *The Oceanic Lithosphere* (ed. Emiliani, C.), *The Sea*, vol 7, chap. 25, New York, p 1025-1119

Wade, C. M., Darling, K. F., Kroon, D., & Leigh Brown, A. J. 1996. Early evolutionary origin of the planktic foraminifera inferred from small subunit rDNA sequence comparisons. *Journal of Molecular Evolution*, **43** (6), 672-677.

Wefer, G., Suess, E., Balzer, W., Liebezeit., G., Mueller, P. J., Ungerer, C. A., & Zenk, W. 1982. Fluxes of biogenic components from sediment trap deployment in circumpolar waters of the Drake Passage: *Nature*, **299**, 145-147

Wernli, R. 1988. Les protoglobigérines (foraminifères) du Toarcien et de l'Aalénien du Domuz Dag (Taurus Occidental, Turquie). *Ecologae geol. Helv.*, **81**, 661-668

White, T.J., Burns, T., Lee, S., & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (eds Innis MA, Gelfland DH, Sninsky JJ, White TJ), Harcourt Brace Jovanovich, San Diego, pp 315 – 322

Woese, C. R., & Olson, G. J. 1986. Archaebacterial phylogeny: perspectives on the urkingdoms. *Systematic and Applied Microbiology*, **7**, 161-177

Wood, A. 1949. The structure of the wall of the test in the Foraminifera: its value in classification. *Quarterly journal of the Geological Society of London*, **104**, 229-255

Wray, C. G., Langer, M. R., DeSalle, R., Lee, J. J., & Lipps, J. H. 1995. Origin of the foraminifera. *Proc. Natl. Acad. Sci. USA*, **92**, 141-145

2 Materials and Methods

2.1 Collection and preparation of foraminiferal specimens

2.1.1 Planktonic foraminiferal samples

All planktonic foraminiferal samples were collected by ship, on the following cruises: RV Professor Logachev, Denmark Strait, North Atlantic Ocean, Aug/Sept 1997 (collected by I. Stewart); Discovery, Cruise 262, North Atlantic Ocean, April 2002 (collected by K.F. Darling); RRS Charles Darwin, cruise CD148, NERC: Arabian Sea, June/July 2003 (Collected by K. F. Darling & B. Steel); RRS Charles Darwin, cruise CD159, NERC: North Atlantic Ocean, July 2004 (collected by M. Carroll). Samples were collected by pumping (5-6 m depth) from the ship's non-toxic water supply through a plankton screen (83, 150 or 200 μm mesh), or by vertical net tow (0-200 m depth, 83 μm mesh) in waters of a depth ranging from approximately 1,000 – 3, 500 m, dependent on location. For genetic analysis, a representative sample of specimens was collected at each station. Individual specimens were identified using a stereomicroscope, and morphotype and cytoplasmic colouration were recorded by digital video imaging. Only adult specimens containing cytoplasm were selected for genetic analysis.

In the ship's laboratory, the live planktonic foraminiferal samples were individually crushed in a lysis buffer containing 50 mM Tris buffer (pH 8.6), 2 mM EDTA, 0.1 % Triton X-100 and 0.5 % Na deoxycholate (Holzmann & Pawlowski, 1996), and incubated for 1 hour at 60 °C. They were then transported at room temperature to the

lab, where they were stored at –80 °C. Material from this lysis buffer solution was subsequently used directly as the DNA template for PCR amplifications.

For transect assemblage assessment, bulk samples were taken at each station with the specimens either dried on slides directly or collected as bulk samples in ethanol. The preserved assemblages were then individually picked and placed onto micropalaeontological slides.

2.1.2 Benthic foraminiferal samples

Live, benthic foraminifera were collected from Brancaster, Norfolk in May 2007 and May 2009. Sediment was collected at low tide from tidal mudflats, from the green, algal-rich surface layer, where the foraminifera are abundant. The sediment was sieved (212 μm) and washed through with seawater, before being transported to the lab.

In the lab, thin layers (~50 mm) of the sieved sediment, containing the live foraminifera, were placed in plastic tubs, covered with a 5 cm deep layer of fresh seawater (collected at high tide on the day of the foraminifera collection), with a loose lid to prevent evaporation. The sediment was washed twice with fresh seawater, allowing it to settle in between, and any large organisms, such as nematode worms, removed to avoid decay and contamination of the water. After 24 hours, live forams, with a healthy orange cytoplasm (seen on the surface of the sediment), were removed by pipette to a 19 cm diameter petri dish containing fresh seawater (salinity ~ 27 – 30 ppt). Individuals of particular target species were then selected for use in a) culture

experiments and b) experiments into the development of a new DNA extraction method.

2.2 DNA amplification by the polymerase chain reaction (PCR)

2.2.1 The small subunit ribosomal RNA gene

Ribosomal genes have proven to be of great utility in reconstructing phylogenies. Sequences of the large subunit (LSU) (or 28S) and small subunit (SSU) (or 18S) ribosomal (r) RNA genes are characterised by the presence of both conserved regions, which provide valuable information regarding the origins and phylogenetic relationships between distant taxonomic groups (Baroin *et al*., 1988; Perasso *et al*., 1989; Sogin, 1991; Schlegel, 1991), and divergent regions, which may be used for the phylogenetic study of closely related taxa (Lenaers *et al*., 1991). The SSU rRNA gene has proven to be an enormously useful marker for investigating foraminiferal evolutionary relationships (Darling *et al*., 1997, 1999, 2000, 2004; Wade *et al*., 1996; de Vargas *et al.,* 1997, 1999, 2001, 2002; Pawlowski *et al*., 1997), with unusually high rDNA sequence divergence within the foraminifera even allowing for its use in inferring within-morphospecies relationships (Darling *et al*., 2003, 2006, 2007; de Vargas *et al*., 2002).

For phylogenetic studies of the foraminifera, a partial, approximately 1,000 bp fragment of the 3′ terminal region of the SSU rRNA gene is typically amplified and sequenced, corresponding to the 30-48 region of the eukaryotic SSU rRNA secondary-structure model (Neefs *et al*., 1990) (see schematic diagram, fig. 2.1). The region contains 4 expansion segments (V7a, V7b, V8, & V9) that are present in most eukaryotes, and 3 foraminiferal specific insertions (F1, F2, & F3), which show

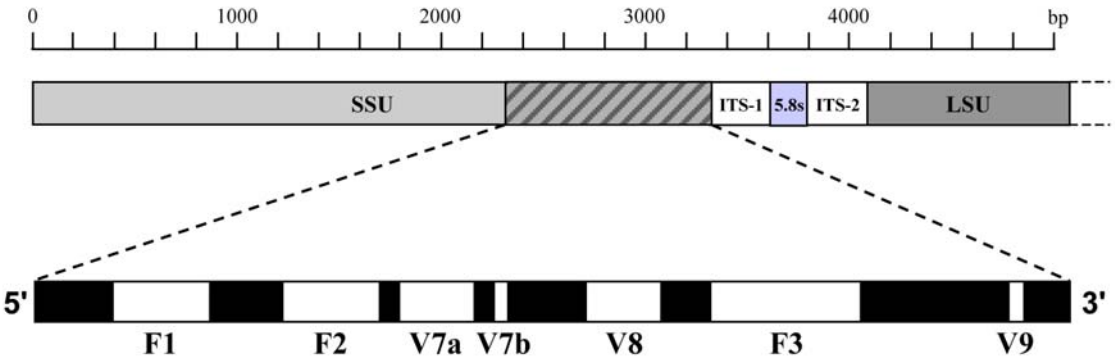


Figure 2.1. Schematic diagram of the SSU rRNA gene, showing the ~1,000 bp 3′ terminal region commonly used in molecular studies of the foraminifera. V7 – V9 represent variable length expansion segments present in most eukaryotes and F1 – F3 represent three insertions that are unique to the foraminifera. F1 may represent the V6 variable region observed only in prokaryote sequences (Neefs *et al*., 1990). Scale is given according to the complete sequence of *Trochammina* sp. (accession number X86095). Adapted from de Vargas *et al*., 1997 and Darling *et al*., 1997.

considerable length and sequence variability, even within the foraminifera (Darling *et al*., 1996b; Wade *et al*., 1996). This ~1000 bp region of the SSU rRNA gene is utilised in most phylogenetic studies of the foraminifera (Darling *et al*., 1996a,b, 1997, 2000, 2003, 2006, 2007; de Vargas *et al*., 1999, 2002; Stewart *et al*., 2001; Wade *et al*., 1996), while a smaller ~500 bp fragment at the start of the same region is used to confirm the identity of genotypes within morphospecies (Darling *et al*., 2003, 2006, 2007; de Vargas *et al*., 2002).

2.2.2 PCR primers

Foraminifera are unicellular organisms, carrying only small amounts of DNA, particularly the planktonic foraminifera, which bear only a single copy of the genome. Two nested rounds of PCR are therefore necessary to produce sufficient yields of PCR product. Initially, pre-existing "universal" eukaryote PCR primers (White *et al*., 1990) were successfully applied to both the benthic and planktonic foraminifera, and

many foraminifera-specific primers have been designed subsequently (Darling *et al*., 1997; de Vargas *et al*., 1997, 2002; Pawlowski *et al*., 1996).

In this study, the traditionally used partial ~1,000 bp terminal fragment of the SSU rRNA gene was utilised to reconstruct phylogenies of the foraminifera. The fragment was amplified in two rounds, by a nested PCR approach. In the first round (1° PCR) primer C5 was coupled with either primer 138 or NS8, and in the second round (2° PCR), primer 2082F was coupled with primer 3014R or primer FS3 with 138, dependent on success (for primer positions and sequences see figure 2.2 and table 2.1).

For the identification of the genetic types within morphospecies (used in the biogeographical surveys; chapters 3 & 4) an ~500 bp fragment of the SSU rRNA gene was utilised, using the 1° PCR described above, plus a secondary PCR using primers 2082F and 2514R (see figure 2.2 and table 2.1 for primer positions and sequences).

In addition, a new method was developed for the PCR amplification of almost the complete SSU rRNA gene (~3,000 bp), for use in phylogenetic reconstruction of the planktonic foraminifera. This is the first time that an extensive fragment of the SSU rRNA gene has been sequenced in the planktonic foraminifera, as previous attempts to amplify and visualise the complete planktonic foraminiferal SSU rRNA gene on an agarose gel have been unsuccessful (Darling *et al*., 1996a). Three rounds of nested PCR were necessary before strong bands could be achieved on the agarose gels. A large number of new foraminiferal-specific primers were designed for the purpose, some of which are in equivalent positions to the 'universal eukaryote primers' of

White *et al*. (1990). Full details of the methodology (including primers used) are given in chapter 5. A schematic diagram of the SSU rRNA gene, indicating primer positions and the coverage of the nested PCR fragments are shown in figure 2.2 (primer sequences shown in table 2.1).

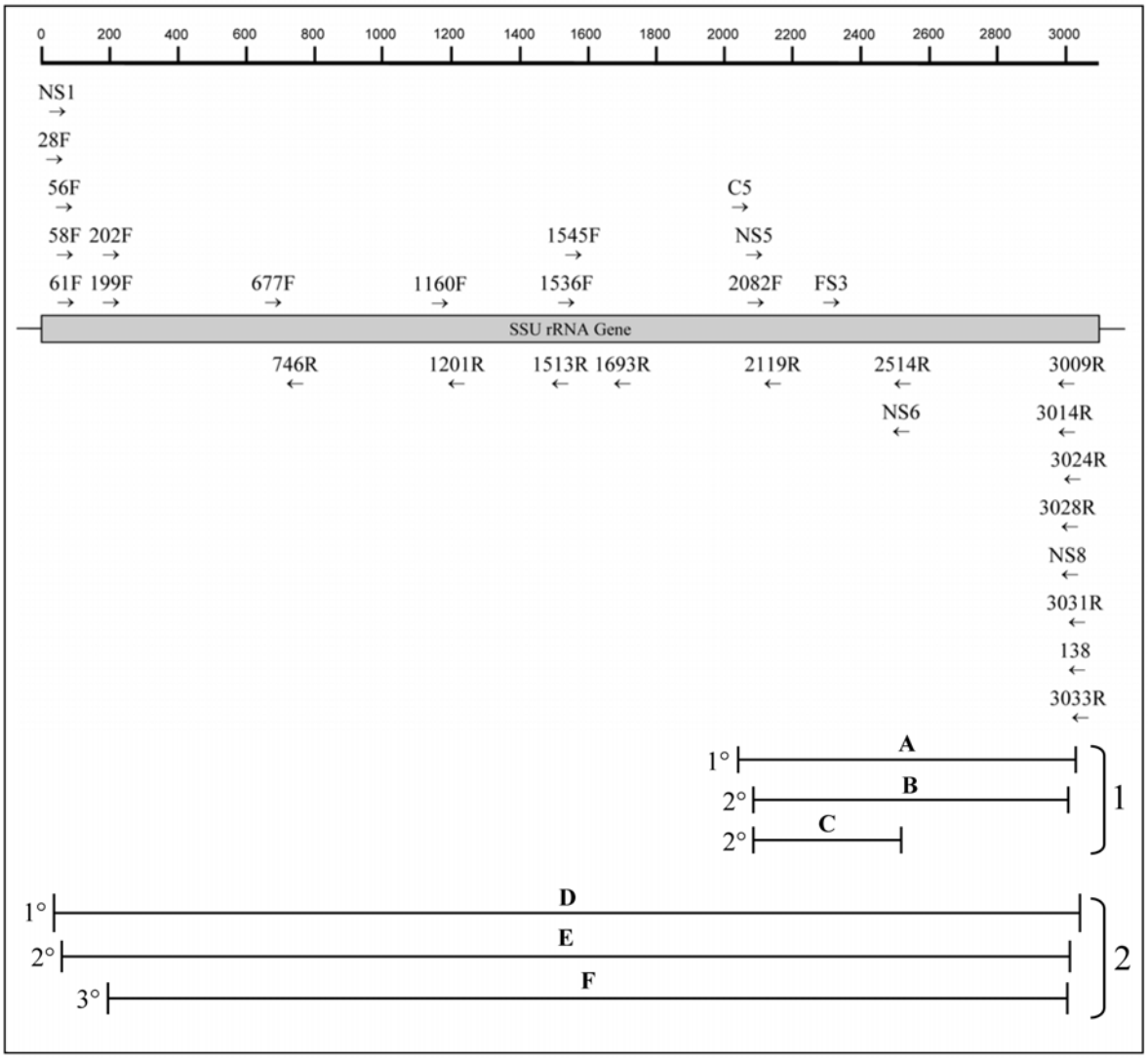


Figure 2.2. Schematic diagram of the SSU rRNA gene showing the positions of primers used for both PCR and sequencing in this study. Positions are based on the complete sequence of *Allogromia* sp. (GenBank X86093). The gene fragments produced using a nested PCR approach are shown. 1 = transect studies (see chapters 3 & 4), A = primary PCR, B = secondary PCR (1000 bp fragment for phylogenetic reconstruction), C = secondary PCR (500 bp fragment for genotype identification.), 2 = ~3000 bp amplification (see chapter 5), D = primary PCR, E = secondary PCR, F = tertiary PCR. The exact primers used varied according to success, as described in chapters 3, 4, & 5.

Table 2.1. Primers used for the PCR amplification and DNA sequencing of the foraminifera

SSU Primer	Sequence	Direction	Reference
28Fa	5′ - GATTCTGCCAGCTGTTCATACGC - 3′	Forward	This study
NS1	5′ - GTAGTCATATGCTTGTCTC - 3′	Forward	White *et al*. 1990
56F	5′ - TTGAGCTCAAAGATTAAGCCATGCA - 3′	Forward	This study
58F	5′ - GAGCTCAAAGATTAAGCCATGCAAG - 3′	Forward	This study
61F	5′ - CTCAAAGATTAAGCCATGCAAGTGG - 3′	Forward	This study
199F	5′ - CAACTGCGGATAGCTGTTTAATACA - 3′	Forward	This study
202F	5′ - CTGCGGATAGCTGTTTAATACAGTC - 3′	Forward	This study
677F	5′ - ATTCGGAGGAGTAGTTTCTGATCC - 3′	Forward	This study
746R	5′ - GGCAAGTTACGCGCCTGCTGC - 3′	Reverse	This study
1160F	5′ - GGCAAGTCTGGTGCCAGCAGC - 3′	Forward	This study
1201R	5′ - CCAACTACGAACCTCTTAACCGC - 3′	Reverse	This study
1513R	5′ - GGTCAACACATTTCACCGCTCGC - 3′	Reverse	This study
1536F	5′ - CGAGCGGTGAAATGTGTTGACCC - 3′	Forward	This study
1545F	5′ - GTGAAATGTGTTGACCCTATTAAGAC - 3′	Forward	This study
1693R	5′ - ACGACGAGGGTATCTGATCCTC - 3′	Reverse	This study
C5	5′ - GTAGTATGCACGCAAGTGTGA - 3′	Forward	Designed by C.M. Wade
NS5	5′ - AACTTAAAGGAATTGACGGAAG - 3′	Forward	White *et al*., 1990
2082F	5′ - TGAAACTTGAAGGAATTGACGGAAG - 3′	Forward	Modified from NS5, White *et al*., 1990
2119R	5′ - GGTAAGATTTCCCGCGTTGAGTC - 3′	Reverse	This study
FS3	5′ - GTGATCTGTCTGCTTAATTGC - 3′	Forward	Designed by C.M. Wade
NS6	5′ - GCATCACAGACCTGTTATTGCCTC - 3′	Reverse	White *et al*.,1990
2514R	5 ′- GGCATCACAGACCTGTTATTGCC - 3′	Reverse	Modified from NS6, White *et al*., 1990
3009R	5′ - CCGATGCCTTGTTACGACTTCTC - 3′	Reverse	This study
3014R	5′ - GTCGTAACAAGGCATCGGTAG - 3′	Reverse	This study
3024R	5′ - TGCAGGTTCACCTACCGATGCC - 3′	Reverse	This study
3028R	5′ - CCTTCTGCAGGTTCACCTACCGA - 3′	Reverse	This study
3031R	5′ - GATCCTTCTGCAGGTTCACCTAC - 3′	Reverse	This study
NS8	5′ - TCCGCAGGTTCACCTACGGA - 3′	Reverse	White *et al*., 1990
138	5′ - TGATCCTGCAGGTTCACCTAC - 3′	Reverse	Medlin *et al*., 1988
3033R	5′ - AATGATCCTTCTGCAGGTTCACCT - 3′	Reverse	This study
M13F	5′ - GTAAAACGACGGCCAG - 3′	Forward	Provided with the TOPO® TA Cloning® kit (Invitrogen™)
M13R	5′ - CAGGAAACAGCTATGAC - 3′	Reverse	

With each of the methods described above, if PCR amplification failed, several combinations of primers were tried until a product was amplified. Conditions of the PCR amplifications were varied until an optimal method was attained (see below).

2.2.3 PCR components

Listed in table 2.2 are the PCR components used, and their concentrations in a 50 µl reaction volume. For the amplification of the ~ 500 bp and ~1,000 bp terminal 3´fragments of the SSU rRNA gene (chapters 3, 4, & 7), 3 µl of template DNA was used in the 1° PCR. For the transect studies (chapters 3 & 4), the lysis buffer in which the samples had been crushed (see section 2.1.1) was used directly as the template. For the newly developed DNA extraction procedures discussed in chapter 7, purified DNA was used as the template. 1 µl of 1° PCR product was then used as the template for the 2° PCR. For amplification of the ~3,000 bp, almost complete SSU rRNA gene, 5 µl of the lysis buffer in which samples had been crushed was used as the template in the 1° PCR. In both the 2° and 3° PCR, 1 µl of PCR product from the previous round was used as the template. For all rounds of PCR, negative controls (template replaced by distilled water) and positive controls (using a stock of benthic foraminiferal DNA stored in lysis buffer at –20 °C) were also included.

Table 2.2. Concentrations of components used in PCR amplifications

Component		Stock concentration	Volume used (µl)	Final concentration
dNTPs*	(500–1000 bp amplification)	1.25 mM	8.0	200 µM
	(>1000 bp amplification)	1.25 mM	12.0	300 µM
PCR buffer (Qiagen or neb)†		10X	5.0	1X
Q solution (Qiagen)‡		5X	10.0	1X
$MgCl_2$		2.5 mM	3.0	1.5 mM
Primer 1		10 µM	1.0	0.2 µM
Primer 2		10 µM	1.0	0.2µM
DNA polymerase (Taq or $Vent_R^{®}$)§	Taq (Qiagen)	5 units/µl	0.2	1 unit
	$Vent_R^{®}$ (neb)	2 units/µl	0.5	1 unit
DNA template		-	1-5 µl¶	-
Sterile distilled water		-	To make final volume of 50 µl	-

* dNTP concentration dependent on length of target fragment. 500-1000 bp corresponds to fragments A, B, C (fig. 2.1), > 1000 bp corresponds to fragments D, E, F (fig. 2.1). † Includes 15 mM $MgCl_2$. ‡ Q solution added when using Taq to increase the yield from weak samples, facilitating the amplification of difficult templates by modifying the melting behaviour of DNA. § Taq polymerase used primarily. $Vent_R$ used for repeats of failed samples. ¶ Template volume varied according to PCR round and length of fragment.

2.2.4 PCR running conditions

Thermal cycling was carried out using a Perkin Elmer cycler. The PCR running conditions (tables 2.3 & 2.4) were designed primarily to suit the amplification of the approximately 3000 bp full-length SSU rRNA gene (chapter 5), however, they worked equally well for the amplification of the shorter fragments (500 – 1000 bp) used in the transect studies (chapters 3 & 4), and were therefore adopted as a standard method for all. The temperature of the annealing phase was kept high (55 °C) for the 1°PCR reactions in order to discourage random annealing of primers to poorly matched contaminant eukaryote templates (table 2.3). In the 2° and 3° PCR reactions, where a greater proportion of foraminiferal templates would be present, this temperature was lowered to 50 °C (table 2.4) to promote annealing. Alternative PCR running conditions were also developed for the amplification of the ~500 bp and ~1,000 bp fragments of the SSU rRNA gene, following poor success rates in PCR. Two extra

cycles were added following the hot start, incorporating a lower annealing temperature of 45 °C to encourage annealing in the early stages of the PCR.

Table 2.3. 1° PCR running conditions

Phase	Temp (°C)	Duration	No. of Cycles
Hot Start	96	2 mins	1
Denaturation	96	30 Secs	35
Annealing	55	30 Secs	
Extension	72	2 - 4 mins*	

* 2 mins for ~1,000 bp fragment, 4 mins for ~3,000 bp almost complete gene

Table 2.4. 2° and 3° PCR running conditions

Phase	Temp (°C)	Duration	No. of Cycles
Hot Start	96	2 mins	1
Denaturation	96	30 Secs	35†
Annealing	50	30 Secs	
Extension	72	2 - 4 mins*	

* 2 mins for ~1,000 bp fragment, 4 mins for ~3,000 bp almost complete gene. † for samples producing very weak bands the no. of cycles were increased to 40

Table 2.5. Alternative PCR running conditions

Phase	Temp (°C)	Duration	No. of Cycles
Hot Start	96	2 mins	1
Denaturation	96	1 min	2
Annealing	45	2 mins	
Extension	72	2 mins	
Denaturation	96	30 Secs	35
Annealing	55	30 Secs	
Extension	72	2 mins	

2.3 Gel electrophoresis

PCR products were separated and visualized through gel electrophoresis using the following protocol:

1. PCR products (50 µl volumes) were loaded into wells on a 1.5 % agarose gel (1.5g agarose in 100 ml TBE buffer (54 g Tris, 27.5 g boric acid and 20 ml 0.5 EDTA in 1000 ml distilled water to make 5X TBE buffer, then diluted to 1X before use), with 0.5 µg/ml ethidium bromide (EtBr) added), submerged in a tank containing 1X TBE.
2. A current of 100 –120 V was applied through the gel using a portable power supply, and run until the negatively charged DNA fragments of the PCR product had migrated approximately 2 thirds of the distance towards the next set of wells, in the direction of the positive electrode (as indicated by the progression of a blue loading buffer). The DNA fragments become separated according to size, larger fragments moving more slowly through the gel matrix than smaller fragments.
3. The agarose gel was then removed from the tank to be visualised and photographed using a Bio-Rad gel doc system (ethidium bromide in the agarose gel intercalates between the nitrogen bases of the DNA and fluoresces under UV light (Reece, 2004).
4. The target band (visualised on a UV transilluminator), identified according to size by comparison to a molecular size marker, was then excised from the gel using a scalpel.

2.4 Gel extraction

Extraction of DNA bands from agarose gels was undertaken using the Eppendorf Perfectprep® Gel Extraction Kit. The protocol follows the manufacturers' instructions.

1. The PCR product was excised from the agarose gel with a sterile, sharp scalpel and placed in a 1.5 ml microcentrifuge tube.
2. The gel slice was weighed. Three volumes of Binding Buffer were added to every volume of the excised gel (100 mg ~ 100 µl).
3. The gel slices were incubated at 50 °C for 10 minutes (or until the gel slice had completely dissolved). To help dissolve the gel, the tubes were inverted every 2-3 minutes during the incubation.
4. One gel volume of ice-cold isopropanol was added and mixed to precipitate the PCR product.
5. A spin column with a membrane filter was placed in a 2 ml collection tube. To bind the PCR product, the sample was applied to the spin column provided and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the column was placed back into the same collection tube. Maximum volume capacity of spin column was 800 µl. For sample volumes of more than 800 µl, the remaining samples were also loaded and the collection tube centrifuged again until all the samples were used up.
6. 750 µl of the wash buffer were added to the column and centrifuged at 13,000 rpm for 1 minute. After discarding the flow-through the column was replaced in the collection tube and centrifuged at 13,000 rpm for another minute to completely remove traces of the wash buffer.

7. The spin column was placed into a clean 2 ml collection tube. 30 µl of the elution buffer (10 mM Tris-Cl, pH 8.5) was added to the centre of the membrane filter and the collection tube centrifuged at 13,000 rpm for 1 minute to elute the PCR product.
8. The spin column was discarded, and the eluted product was stored at –20 °C until further use.

2.5 DNA quantification

Eluted PCR products were quantified using a NanoDrop® ND-1000 spectrophotometer prior to DNA sequencing.

2.6 Cloning

Cloning of the PCR product prior to sequencing was necessary for those foraminiferal morphospecies that carry several different copies of the SSU rRNA gene (certain non-spinose planktonic taxa). If such multi-template genomes are sequenced directly, variable sites among the different copies often manifest as ambiguous sites in the resulting gene sequence, and small insertions or deletions may mean that several offset traces are overlaid, rendering them unreadable. To avoid such problems, three clones were made and sequenced for any multi-template taxa in this study. To circumvent the potential problem of Taq errors, which were observed in some of the cloned sequences, each clone was sequences three times and a majority rule consensus sequence constructed.

Cloning was undertaken using the Invitrogen™ TOPO TA Cloning® Kit. In the cloning method used, DNA is incorporated into a *pUC* plasmid vector, and utilises the incorporation of an ampicillin-resistance gene to allow the transformed *E. coli* bacteria to grow on a media inhospitable to most bacteria. Insertion of the target DNA disrupts the *lacZ* gene, which codes for a subunit of the β-galactosidase enzyme that breaks down galactose. Colonies of successfully transformed bacteria appear white when compared to bacterial colonies retaining a functional *lacZ* gene in the plasmid, which appear blue.

The protocol below follows the manufacturers' instructions for the Invitrogen™ TOPO TA Cloning® Kit. The method was modified to use half volumes of competent cells and cloning reaction reagents.

2.6.1 Selective agar plate preparation

1. 15-20 ml of LB (Luria-Bertani) agar containing 50 mg/ml ampicillin was poured into each Petri dish and set aside at room temperature to solidify. The agar plates were left slightly open in a drying cabinet for 30 minutes to remove condensation from the lid.
2. The agar plates were then warmed at 37 °C for 30 minutes.
3. 80 µl of 20 mg/ml X-gal was spread on each agar plate, after which they were incubated at 37 °C until use.

2.6.2 Setting up the TOPO TA Cloning® reaction (Invitrogen™)

1. The following reagents (table 2.6) were mixed gently and incubated for five minutes at room temperature (22 – 23 °C):

Table 2.6. Reagents used in the TOPO TA cloning® reaction

Reagent	Volume (µl)
PCR product	0.5 to 2
Salt solution	0.5
Water	Add to total volume of 2.5
TOPO® vector	0.5
Final Volume	3

The method was modified to use half the volumes of those suggested by the manufacturer

2. The reaction was placed on ice until needed

2.6.3 Transforming One Shot® TOP10 competent cells

1. 1 µl of the TOPO® cloning reaction was added to 25 µl of One Shot® chemically competent *E. coli* cells, mixed gently, and incubated on ice for 15 minutes.
2. The cells were heat-shocked for 30 seconds at 42 °C without shaking. This allowed the cells to take in the plasmids. The tubes were immediately transferred in ice.
3. 125 µl of room temperature S.O.C medium was added, and the tube then capped tightly and shaken horizontally (200 rpm) at 37 °C for one hour.
4. 20 µl and 50 µl from each transformation were spread on pre-warmed (37 °C) selective agar plates containing X-gal and incubated overnight at 37 °C. Two

different volumes were plated to ensure that at least one had well-spaced colonies.

5. 10 white colonies were picked and cultured overnight in 5 ml LB medium containing 50 ug/ml ampicillin (dark blue colonies were not used).

2.6.4 Plasmid isolation

Plasmid DNA was isolated from the overnight LB liquid culture using a QIAprep® Spin Miniprep kit (QIAgen®) and a microcentrifuge. The following protocol follows the manufacturers' instructions (excluding the preparation stages in steps 1-3).

1. A small amount of each culture (~500 µl) was set aside in a separate tube where ~125 µl of 80% sterile glycerol was added. These tubes were then stored at –80 °C so that the culture could be re-grown in the near future should the need arise.
2. The remaining culture was transferred to a microcentrifuge tube, and centrifuged for five minutes at 13,000 rpm to concentrate the bacterial cells into a pellet. The supernatant was then discarded.
3. The process was repeated until most of the culture was used up.
4. The pelleted bacterial cells were re-suspended in 250 µl of Buffer P1 and transferred to a microcentrifuge tube.
5. 250 µl of Buffer P2 was added and the suspension mixed thoroughly by inverting the tube 4-6 times.
6. 350 µl of Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4-6 times.

7. The sample was centrifuged for 10 mins at 13,000 rpm in a tabletop microcentrifuge, to form a compact white pellet.
8. The supernatant from step 7 was applied to a QIAprep spin column and centrifuged for 30-60 s.
9. The QIAprep spin column was washed by adding 0.5 ml of Buffer PB and centrifuging for 30-60 s, with the flow-through discarded.
10. The QIAprep spin column was washed again by adding 0.75 ml of Buffer PE and centrifuging for 30-60 s, with the flow-through discarded.
11. The column was centrifuged for an additional 1 min to remove residual wash buffer.
12. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. The DNA was eluted by adding 50 μl of Buffer EB (10 mM Tris-Cl, pH 8.5) to the centre of the column, and allowing it to stand for 1 min before centrifuging for 1 min.

2.6.5 Cycle sequencing

Cycle sequencing was undertaken using the Applied Biosystems™ PRISM™ BigDye® Version 3.1 sequencing chemistry. The protocol below is modified from the manufacturers' instructions. Volumes were reduced by half for most components, and less than 20 % of the standard amount of BigDye® was used.

Listed below are the components used for the cycle sequencing reaction (table 2.7), and the running parameters used (table 2.8). Sequencing of the sense and antisense strands was carried out in separate tubes. The primers used are listed in the methods

section of each chapter. See above for primer positions and sequences (fig. 2.2, table 2.1).

Table 2.7. Components of the cycle sequencing reaction

Component		Stock concentration	Volume used (μl)	Final concentration
BigDye® Ready Reaction Mix* (for 500-1000 bp PCR product)		2.5X	1.0	0.25X
Sequencing Primer		1 μM	1.6	0.16 μM
Sequencing buffer*		5X	2.0	1X
Template (purified PCR product)	Direct sequencing	-	Add 5-20 ng per 500-1000 bp to be sequenced	
	Sequencing from a clone	-	Add 25-100 ng per 500-1000 bp to be sequenced†	
Sterile distilled water		-	To make a final volume of 10 μl	-

* Supplied by Applied Biosystems™. † Amount of template multiplied by 5 when using a cloned product to allow for the presence of 4/5 plasmid DNA

Table 2.8. Cycle sequencing running parameters

Temperature (°C)	Duration	No. of cycles
96	1 min	1
96	10 sec	25
50	5 sec	
60	4 min	

Once the sequencing reaction was completed, samples were sent to the University of Edinburgh or to the Natural History Museum, London to be sequenced directly on an Applied Biosystems 377 DNA sequencer.

2.7 DNA sequence analysis

2.7.1 Assembly of the SSU rDNA sequences

Sense and anti-sense SSU rDNA sequences were assembled to form consensus contigs within Gap4 of the Staden package (version 1.5.3) (Staden *et al*., 2000). Each sequence was carefully checked for errors, and the primer sites removed. Any unreliable sequences were discarded, and the sequencing reaction repeated. For cloned specimens each consensus contig was constructed from three replicates of the sequences, to eliminate Taq errors.

Consensus sequences output from Gap4 were aligned manually within version 2.2 of the Genetic Data Environment (GDE) package (Smith *et al*., 1994). Each new sequence was subjected to a BLAST search, to confirm that it was foraminiferal in origin, and crosschecked against other published sequences already present in the GDE alignment (aligned by C. Wade) to confirm its genotype identity or to highlight it as a novel genetic type. Within the alignment, sequences were again checked by eye for unusual bases, and the sequencing trace data consulted to confirm either errors or genuine sequence differences.

2.7.2 Selecting nucleotide sites for phylogenetic analysis

Due to the extreme genetic distances observed within the foraminifera, only a limited proportion of nucleotide sites can be reliably aligned across all taxa. Stringent criterions were observed for the selection of sites, and any nucleotides that could not be unambiguously aligned were excluded from the subsequent phylogenetic analyses. This conservative approach was observed to eliminate positions that could be subject to errors in positional homology.

2.7.3 Evaluating sequences for evidence of substitution saturation

When measuring the distance between two strands of aligned DNA we count the number of sites at which they differ. The proportion of homologous sites is known as the observed distance (or p-distance), and is expressed as the number of nucleotide differences per site. However, across evolutionary time multiple substitutions (or 'hits') will accumulate per site, until eventually the sequences become random or 'saturated' (Strimmer & von Haeseler, 2009). In phylogenetic analyses, sequence saturation can lead to underestimates of the amount of evolutionary change that has taken place, and descendant sequences can appear similar even if the similarity is not brought about by descent from a common ancestor (homoplasy) (Graur & Li, 2000). If the sequence data is saturated to a severe degree, the optimal model of DNA sequence evolution may no longer be able to correct for multiple hits (Xia *et al*., 2003). Before being subjected to phylogenetic estimation, the sequences were therefore checked for saturation, using plots based on the number of substitutions; a standard procedure used to check for evidence of saturation in sequence datasets (Morisson, 2006; Xia *et al.*, 2009). For this study plots were made firstly of uncorrected pairwise transition (ti) and transversion (tv) distances against pairwise total uncorrected distances, and secondly of uncorrected pairwise transition (ti) distances against transversion (tv) distances. As highly divergent sequences are more prone to substitutions than closely related sequences, saturation (which generally occurs in transitions before transversions) would be observed as a curve and eventual plateau in the line of best fit (Salemi, 2009). No evidence of saturation was evident in the datasets used in this study, with transitions and transversions increasing linearly (discussed in chapter 5).

2.8 Phylogenetic Analysis

2.8.1 Choosing a model of sequence evolution using the likelihood ratio test (LRT)

The model of sequence evolution used in a phylogenetic analysis may be relatively simplistic, or more complex (parameter-rich). Ideally the chosen model should be complex enough to explain the observed data, but not so complex as to be subject to impractically long computations, or require overly large datasets (Swafford *et al*., 1996). For this study, the simplest models of evolution, including JC69 (Jukes & Cantor, 1969), F81 (Felsenstein, 1981), and K2P (Kimura, 1980) were not considered for use since more parameter-rich models have been determined to be optimal for 80% of 208 published datasets, by Kelchner & Thomas (2007). Two parameter-rich models were considered for use; the general time-reversible (GTR) model (Lanave *et al*., 1984), plus the slightly less sophisticated HKY85 model (Hasegawa, *et al*. 1985). The HKY85 model allows for different rates of substitution for transitions and transversions as well as allowing for unequal base frequencies (Hasegawa *et al.*, 1985). The GTR model allows all six pairs of substitution to have different substitution rates as well as allowing for unequal base frequencies (Rodriguez *et al.*, 1990). Both models can be used with or without the addition of a Gamma (Γ) correction, which accounts for rate heterogeneity between sites (Yang 1993).

For pairs of evolutionary models that are nested (i.e. where the simpler model is nested within the more complex model), their fit to a particular dataset can be compared using the likelihood ratio test (LRT) (Goldman, 1993; Schmidt, 2009; Swofford *et al*., 1996), utilising maximum likelihood scores calculated in PAUP* (version 4.0d65; Swofford, 1998). The test statistic is given as $LR = 2(\ln L_1 - \ln L_0)$

where L_1 is the log likelihood under the more complex (parameter-rich) model and L_0 is the log likelihood under the simpler model. The likelihood under the more complex model will always be equal to or higher than that of the simpler model, and the significance can be determined by deriving the probability or p-value of the obtained difference. With nested models of evolution, twice the difference in the likelihood scores between models is approximately Chi squared (χ^2) distributed. The p-value is determined by applying the difference in log likelihood scores, together with the degrees of freedom (the difference in number of parameters between the models), to a Chi squared table. The simpler model is rejected in favour of the more complicated one, if the difference in log likelihood scores is significant.

The LRT test was used to compare the fit of the GTR model (Lanave *et al*., 1984), and the slightly less sophisticated HKY85 model to the datasets in this study, both with and without a Gamma (Γ) correction (Yang, 1993). For all datasets the best model proved to be GTR + Γ.

2.8.2 Phylogenetic tree reconstruction

Molecular phylogenies can be constructed using either the raw character state data directly (ie. where each homologous position in a sequence alignment is considered directly without conversion to a distance) or by converting the character state data to a matrix of pairwise distances from which a tree is then built. Discrete character-state methods include maximum parsimony, maximum likelihood and Bayesian inference. Distance methods include neighbour-joining, minimum evolution and Fitch-Margoliash.

Phylogenetic tree reconstruction methods can also be classified according to the manner in which they search for the best tree. Algorithmic methods (e.g. neighbour joining) construct trees by clustering sequences following an algorithm, and typically generate a single best estimate phylogeny for the data, with the phylogenetic criterion defined by the tree-building algorithm. Optimality criterion methods (eg. minimum evolution, Fitch-Margoliash, maximum likelihood, Bayesian inference, and maximum parsimony) define a criterion (an objective function), by which alternative trees may be compared. The criterion is used to give each alternative tree a score, which allows the optimum tree (under the criterion) to be selected as the best explanation of the data (an algorithm is used merely to compute the value of the objective function and for searching for trees that optimise this value).

Here phylogenetic trees were constructed using a range of methods including Bayesian inference (BI; Ronquist & Huelsenbeck, 2003; Larget & Simon, 1999) (character state & optimality criterion), maximum likelihood (ML; Felsenstein, 1981) (character state & optimality criterion), neighbour joining (NJ; Saitou & Nei, 1987) (distance & algorithmic), Fitch-Margoliash (FM; Fitch & Margoliash, 1967) (distance & optimality criterion), minimum evolution (ME; Rzhetsky & Nei, 1992) (distance & optimality criterion), and maximum parsimony (MP; Fitch, 1971) (character state & optimality criterion).

BI was performed using the MrBayes (version 3.1.2) package (Ronquist & Huelsenbeck, 2003) using a GTR+ Γ model (Lanave *et al*., 1984; Yang 1993) and with the tree space explored using four chains of a Markov Chain Monte Carlo (MCMC) algorithm for between 1 & 5 million generations, sampling every 100

generations. In order to ensure adequate chain swapping, a range of heating parameters were tested with the final tree constructed using the optimal temperature. The run was terminated only after the Bayesian MCMC searches had reached a stationary phase (plateau), indicating convergence of the chain onto the target distribution, and a consensus tree built using the last 1000 trees. Bayesian posterior probabilities were obtained within MrBayes from these last 1000 trees.

ML analysis was undertaken within the Phyml package (Guindon & Gascuel, 2003) using a GTR+ Γ model (Lanave *et al*., 1984; Yang 1993), with parameters estimated within Phyml. NJ, FM, ME and MP analyses were performed using PAUP* (version 4.0d65; Swofford, 1998). For NJ, FM and ME methods distances were corrected for multiple hits using a GTR+ Γ model (Lanave *et al*., 1984; Yang, 1993). The rate matrix, base frequencies, and shape parameter (α) of the gamma distribution (based on 16 rate categories) were estimated using likelihood by iteration from an initial NJ tree. The parameters estimated from the initial tree were then used to build a new NJ tree and the parameters re-estimated, with this process repeated until there was no further improvement in likelihood. For FM, ME and MP methods, tree searching used a heuristic procedure with tree-bisection-reconnection branch swapping. Bootstrap re-sampling (Felsenstein, 1985b) was undertaken with 1000 bootstrap replicates in order to assign support to particular branches within the tree.

2.8.3 Relative rate tests (RRT)

The degree of substitutional rate divergence between pairs of taxa or taxon groups, within the foraminiferal phylogeny, was assessed by means of the relative rate test (RRT) (Sarich & Wilson, 1967). The RRT allows for the comparison of rates of

evolution between two taxa (or groups of taxa), without any knowledge of divergence time. This is achieved simply by comparing the substitutional rates in the two closely related taxa, with a third more distantly related outgroup, in the case of this study the agglutinated benthic foraminifer, *Allogromia* sp. The test was carried out using the GRate package (Müller, K, unpublished) (see appendix 2), using Maximum Likelihood estimates of substitutions per site (with a GTR + Γ model). Within the package, standard errors were estimated via bootstrapping (Efron, 1982, Felsenstein, 1985a) and the significance of differences between groups tested using a two-tailed z-test.

2.8.4 Hypothesis testing

The topology of an optimal phylogenetic tree may not necessarily appear as expected. For example, the relationships shown may not agree with traditional taxonomical classifications. The Kishino–Hasegawa (KH) test (Kishino & Hasegawa, 1989) can be used to test the likelihood of alternative phylogenetic hypotheses. The KH test utilised here was based on the resampling estimated log-likelihoods (RELL) method (Kishino *et al*., 1990) (as implemented in PAUP*) a variant of the non-parametric bootstrap that is computationally less demanding (Schmidt, 2009). In the test, the likelihood score of the optimal tree produced from the original phylogenetic analysis is compared to that of a tree in which the topology has been constrained to fit an alternative hypothesis. For example, certain groups of taxa may fall in independent groups in the optimal phylogenetic tree, but in a large monophyly in taxonomical classifications (see chapter 5). In this case the likelihood scores of the optimal tree, and a tree in which the taxa had been constrained to form a monophyly would be compared. If the likelihood score for the optimal tree is significantly better than for

the alternative topology, the alternative hypothesis can be rejected (see appendix 9.5 for details).

2.9 References

Baroin, A., Perasso, R., Qu, L. H., Brugerolle, G., Bachellerie, J., & Adoutte, A. 1988. Partial phylogeny of the unicellular eukaryotes based on rapid sequencing of a portion of 28S ribosomal RNA. *Proc. Natl. Acad. Sci. USA*, **85**, 3474-3478

Darling K. F., Kucera M., Pudsey C. J., & Wade C. M. 2004. Molecular evidence links cryptic diversification in polar plankton to Quaternary climate dynamics. *Proc. Natl. Acad. Sci. USA*, **101**, 7657-7662

Darling K.F., Kroon D., Wade C.M., Leigh Brown A.J. 1996b. Molecular phylogeny of the planktic foraminifera. J*ournal of Foraminiferal Research,* **26,** (4), 324-330

Darling, K. F., Kroon, D., Wade, C.M., Leigh Brown, A.J., 1996a. The isolation and amplification of the 18S ribosomal RNA gene from planktonic foraminifers using gametogenic specimens. In: *Microfossils and Oceanic Environments* (eds Moguilevsky A, Whatley RC), University of Wales, Aberystwyth Press, Chapter 3.1, pp 249-259

Darling, K. F., Kucera, M., Wade, C. M., von Langen, P., and Pak, D. 2003. Seasonal distribution of genetic types of planktonic foraminifer morphospecies in the Santa Barbara Channel and its paleoceanographic implications. *Paleoceanography,* **18** (2), 1032

Darling, K. F., Wade, C. M., Kroon, D., & Leigh Brown, A. J. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. *Marine Micropaleontoogy,* **30**, 251-266

Darling, K.F., Kucera, M. Wade, C.M., 2007. Global molecular phylogeography reveals persistent Arctic circumpolar isolation in a marine planktonic protist. *Proc. Natl. Acad. Sci.* USA **104**, 5002-5007

Darling, K.F., Kucera, M., Kroon, D., Wade, C.M., 2006. A resolution for the coiling direction paradox in *Neogloboquadrina pachyderma*. *Paleoceanography* **21**, PA2011, doi:10.1029/2005PA001189

Darling, K.F., Wade, C.M., Steward, I.A., Kroon, D., Dingle, R., Leigh Brown, A.J., 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature*, **405**, 43-47

Darling,K.F., Wade,C.M., Kroon,D., Leigh Brown,A.J. and Bijma,J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulation. *Paleoceanography* **14**, 3-12

de Vargas C., Renaud S., Hilbrecht H., Pawlowski J. 2001. Pleistocene adaptive radiation in *Globorotalia truncatulinoides*: genetic, morphologic, and environmental evidence. *Paleobiology,* **27,** (1), 104-125

de Vargas C., Zaninetti L., Hilbrecht H., Pawlowski J. 1997. Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record. *Journal of Molecular Evolution,* **45**, (3), 285-294

de Vargas, C., Bonzon, M., Rees, N. W., Pawlowski, J., Zaninetti, L. 2002. A molecular approach to diversity and biogeography in the planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Marine Micropaleontology* **870**, 1-16

de Vargas, C., Norris, R., Zaninetti, L., Gibb, S. W., Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proc. Natl. Acad. Sci. USA* **96**, 2864-2868

Efron, B. 1982. The Jackknife, Bootstrap, and other resampling plans. *CBMS-NSF Regional conference series in applied mathematics*, Society for industrial and applied mathematics, Philadelphia

Felsenstein, J. 1985a. Phylogenies and the comparative method. *American Naturalist* **125**, 1-15

Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution,* **17**, 368-376

Felsenstein, J., 1985b. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783-79

Fitch, W. H. 1971. Toward defining the course of evolution: minimum change for a special tree topology. *Systematic Zoology*, 20: 406-416

Fitch, W. M., Margoliash, E. 1967. Construction of phylogenetic trees. A method based on mutation distances as estimated from cytochrome *c* sequences is of general applicability. *Science,* 155**:** 279-284

Goldman, N. 1993. Statistical tests of models of DNA substitution. *Journal of Molecular Evolution*, **36**, 182-198

Graur, D. and Li, W. H. 2000. *Fundamentals of Molecular Evolution*, 2nd ed. Sinauer Associates, Inc., U.S.A., pp. 481

Guindon S., & Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, **52,** (5), 696-704

Hasegawa, M., Kishino, H. and Yano, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **21**, 160-174

Holzmann, M., & Pawlowski, J. 1996. Preservation of foraminifera for DNA extraction and PCR amplification. *Journal of Foraminiferal Research*, **26** (3), 264-267

Jukes, T. H., & Cantor C. R. 1969. Evolution of Protein Molecules. In: *Mammalian Protein Metabolism* (ed Munro, H.N.). Academic Press, New York, pp. 21-132

Kelchner, S.A. and Thomas, M.A. 2007. Model use in phylogenetics: some key questions. *Trends in Ecology and Evolution*, **22** (2), 87-94

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111-120

Kishino, H., & Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution*, **29**, 170-179

Kishino, H., Miyata, T., & Hasegawa, M. 1990. Maximum likelihood inference of protein phylogeny and the origin of chloroplasts. *Journal of Molecular Evolution*, **31**, 151-160

Lanave, C, Preparata G, Saccone C, Serio G. 1984. A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution,* **20**, 86-93

Larget B., Simon D.L. 1999. Markov chain monte carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution*, **16,** 750-759

Lenaers, G., Scholin, C., Bhaud, Y., Saint-Hilaire, D., & Herzog, M. 1991. A molecular phylogeny of dinoflagellates protists (Pyrrhophyta) inferred from the sequence of 24S rRNA divergent domains D1 and D8. *Journal of Molecular Evolution*, **32**, 53-63

Medlin, L., Elwood H. J., Stickel S., and Sogin M. L. 1988. Sequence analysis of enzymatically amplified genomic small subunit rRNA genes from the diatom, *Skeletonema pustulata. Gene*, **71**, 491-499

Morrison, D.A. 2006. Phylogenetic analyses of parasites in the new millennium. *Advances in Parasitology*, **63**, 1-124

Neefs, J., Van de Peer, Y., Hendriks, L., De Wachter, R. 1990. Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research*, **18**, 2237-2242

Pawlowski J., Bolivar I., Fahrni J.F., de Vargas C., Gouy M., Zaninetti L. 1997. Extreme differences in rates of molecular evolution of foraminifera revealed by comparison of ribosomal DNA sequences and the fossil record. *Molecular Biology and Evolution*, **14,** (5), 498-505

Pawlowski, J., Bolivar, I., Fahrni, J. F., Cavalier-Smith, T., & Gouy, M. 1996. Early Origin of Foraminifera Suggested by SSU rRNA Gene Sequences. *Molecular Biology and Evolution*, **13** (3), 445-450

Perasso, R., Baroin, A., Qu, L. H., Bachellerie, J., & Adoutte, A. 1989. Origin of the algae. *Nature*, **339**, 142-144

Rodriguez, F., Oliver, J.L., Marin, A. and Medina, J.R. 1990. The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology*, **142**, 485-501

Ronquist, F., Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19: 1572-1574

Rzhetsky, A., Nei, M. 1992. A simple method for estimating and testing minimum-evolution trees. Mol. Biol. Evol. 9, 945-967

Saitou, N., and Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425

Salemi, M. 2009. Genetic distances and nucleotide substitution models: practice. In: *The phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing* (eds. Lemey, P., Salemi, M., & Vandamme, A-M). Cambridge University Press, Cambridge UK

Sarich, V. M. & Wilson, A. C. 1967. Immunological time scale for hominid evolution. *Science,* **158**, 1200-1203

Schlegel, M., 1991. Protist evolution and Phylogeny as Discerned from Small Subunit Risosomal RNA sequence comparisons. *European Journal of Protistology*, **27**, 207-219

Schmidt, H. A. 2009. Testing tree topologies, theory. In: *The Phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing*, 2nd ed. (eds. Lemey, P., Salemi, M. and Vandamme, A-M.). Cambridge University Press, New York, pp. 381-404

Smith, S.W., Overbeek, R., Woese, R., Gilbert, W. and Gillevet, P.M., 1994. The genetic data environment and expandable GUI for multiple sequence analysis. *Comput. Appl. Biosci*., **10**, 671-675

Sogin, M. L., 1991. Early evolution and the origin of eukaryotes. *Current Opinions in Genetics and Development*, **1**, 457-463

Staden, R. Beal, K.F. and Bonfield, J.K. 2000. The Staden package, 1998. Methods in Molecular Biology **132**: 115-130

Stewart, I. A., Darling, K. F., Kroon, D., Wade, C.M., Troelstra, S.R. 2001. Genotypic variability on subarctic Atlantic planktic foraminifera. *Marine Micropaleontology*, **43**, 143-153

Strimmer, K & von Haeseler, A. 2009. Genetic distances and nucleotide substitution models. In: *The phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing* (eds. Lemey, P., Salemi, M., & Vandamme, A-M). Cambridge University Press, Cambridge UK

Swofford D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Swofford, D. L., Olsen, G.J., Waddell, P.J. and Hillis, D.M. 1996. Phylogenetic Inference. In: *Molecular Systematics*, 2nd ed. (eds. Hillis, D.M., Moritz, C. and Mable, B.K.). Sinauer Associates, Inc., USA, pp. 495-496

Wade, C. M., Darling, K. F., Kroon, D., & Leigh Brown, A. J. 1996. Early evolutionary origin of the planktic foraminifera inferred from small subunit rDNA sequence comparisons. *Journal of Molecular Evolution*, **43** (6), 672-677

White TJ, Burns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (eds Innis MA, Gelfland DH, Sninsky JJ, White TJ), Harcourt Brace Jovanovich, San Diego, pp 315 – 322

Xia, X. 2009. Assessing Substitution Saturation With DAMBE. In: *The Phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing*, 2nd ed. (eds. Lemey, P., Salemi, M. and Vandamme, A-M.). Cambridge University Press, New York, pp. 615-630

Xia, X., Xie, Z., Salemi, M., Chen, L. and Wang, Y. 2003. An index of substitution saturation and its application. *Molecular Phylogenetics and Evolution*, **26**, 1-7

Yang, Z. 1993. Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates vary over sites. *Molecular Biology and Evolution,* **10**, 1396-1401

3 Genotypic variability in the planktonic foraminifera of the central Arabian Sea

3.1 Introduction

The planktonic foraminifera are a highly abundant, diverse and ubiquitous group of marine pelagic protists that can be found throughout the worlds' oceans. They have an exceptional fossil record, spanning over 180 million years (Ma) and as microfossils, their shells provide a highly successful tool for dating rocks and ocean sediments, studying evolutionary processes and archiving past climate. Their usefulness as indicators of past climate stems from the fact that individual species of planktonic foraminifera have characteristic environmental preferences that are reflected in their spatial and temporal distribution in the oceans and the chemistry of their calcite shells. The accurate identification of planktonic foraminiferal species from the morphological characteristics of their shells (morphospecies) is essential to the success of such methods. However, this is now questioned by evidence from the small subunit (SSU) ribosomal (r) RNA gene, which is widely used in studies of their evolutionary relationships. High levels of previously unrecognized diversity have been discovered within the traditionally known morphospecies, with individual morphospecies often comprised of several genetic types (genotypes), some of which may be species in their own right (Darling *et al*., 1997, 1999, 2004, 2006, 2007; Darling & Wade, 2008; de Vargas *et al*., 1997, 1999, 2001, 2002; Huber *et al*, 1997). Perhaps more significantly, the newly discovered genotypes show non-random distributions, suggestive of distinct ecologies (ecotypes) (Darling *et al*., 1997, 1999, 2004, 2006, 2007, 2008; de Vargas *et al*., 1997, 1999, 2001, 2002; Huber *et al*., 1997). Such cryptic diversity particularly impacts on climate change palaeoproxies where the

presence of these ecotypes may lead to inaccuracy in analysis. It is therefore vital to gain a better understanding of their global genetic variability and phylogeography to improve quantitative faunal and geochemical palaeoclimate reconstructions.

Extensive sampling effort is required throughout the different water masses of the global ocean to address this issue. Increased taxon sampling will provide valuable information about the ecological habits of individual genotypes, the phylogenetic relationships within and between sibling clusters and their position within the foraminifera. In addition, surveying genotype distribution across the oceans can reveal geographical connectivity between different oceanic regions, providing clues to present and past ocean circulation, evolutionary drivers and the evolutionary history of foraminiferal species (Darling *et al*, 2000, 2004, 2006 and 2007; de Vargas *et al*., 1999, 2001, 2002). Although genetic surveys of the planktonic foraminifera have now been undertaken over a wide range of oceanic water masses (Aurahs, *et al.*, 2009; Darling *et al*., 1996a, b, 1997, 1999, 2000, 2004, 2007; de Vargas *et al*., 1997, 1999, 2002, 2004; Stewart, 2001; Ujiié & Lipps, 2009), gradually adding to the global picture of genotype distribution, these studies have generally ranged more towards the mid to higher latitudes with the tropics remaining relatively under-sampled by comparison. In fact, many more morphospecies occur in the transitional to lower latitudes than the higher latitudes (Rutherford *et al*., 1999) and these tropical and subtropical regions together with their wind driven upwelling regions, play an equally vital role in the Earth's climate system.

The Arabian Sea was chosen as a tropical region of high priority as it is one of the richest marine biological areas of the world and is also a major contributor to global

ocean productivity and biogenic carbonate burial. It is therefore currently under intensive study to understand its role in both the modern and palaeoenvironmental global ocean/climate system. The Arabian Sea is known to harbour a wide range of planktonic foraminiferal morphospecies (Brummer & Kroon, 1988), providing an excellent opportunity to obtain an understanding of the foraminiferal ecosystem in a tropical water column. Moreover, the Arabian Sea has been the focus of a number of studies linking physical oceanographic conditions and the distribution of planktonic foraminiferal morphospecies (Prell *et al*., 1981; Cullen & Prell, 1984; Kroon, 1991; Schiebel *et al*, 2004). These studies were however based entirely on the identification of morphospecies from their morphological characteristics alone and evidence from other subtropical/tropical regions strongly indicate that the morphospecies of the Arabian Sea are highly likely to harbour cryptic genotypes/ecotypes in such a dynamic ecosystem.

The Arabian Sea is a unique marine environment since its circulation is completely reversed biannually by seasonally reversing monsoon winds (Schott, 1983; Swallow, 1984). It is therefore subject to greater seasonal variability than any other ocean basin on the globe (Schott & McCreary, 2001; Clemens *et al*., 1991). During the summer months (June – September), low pressure over Asia and high pressure over the Indian Ocean drives strong winds in a southwesterly direction (the southwest monsoon). Conversely, in winter (November – February), high pressure over the Asian continent and low pressure over the Indian Ocean causes the monsoon winds to reverse and weaken (the northeast monsoon). In summer, the southwest monsoon leads to the formation of a major low-level air current, the Findlater jet (Findlater, 1996), which in turn causes the formation of upwelling zones in the coastal regions of Somalia,

Yemen, and Oman (Lee *et al*., 2000). The seasonal effects of the monsoon on mixed-layer dynamics and upwelling in the Arabian Sea are pronounced and have a major effect on the primary productivity (phytoplankton growth) and biogeochemistry of the region (Wiggert *et al*., 2002; Banse, 1987; Banse & English, 2000). Nutrients brought into the euphotic zone by coastal upwelling and offshore Ekman pumping are transported into the open ocean by wind-driven mixing (McCreary *et al*., 1996; Lee *et al*., 2000) and lateral advection (Young & Kindle, 1994; Prasanna Kumar *et al*., 2001). This leads to an enormous increase in primary productivity in the region (Banse & English, 1994, Bauer *et al*., 1991) and the normally oligotrophic waters of the Arabian Sea transform into one of the most productive marine environments on Earth.

This study describes the SW monsoon phase of the seasonal cycle and represents the first genetic study on the planktonic foraminifers of the Arabian Sea. Wind speeds over the Arabian Sea are at their strongest during the SW monsoon limiting the deployment of multinet systems for foraminiferal sampling and making CTD (conductivity temperature depth) profiling challenging. Nevertheless, it was possible to sample the mixed layer at this time and this study specifically focuses on the genetic variation in the SSU rRNA gene of mixed layer planktonic foraminiferal morphospecies of the central Arabian Sea during the summer SW monsoon of 2003. Environmental effects are most pronounced during this period with a distinct disparity between adjacent water masses. The north/south cruise transect crossed this divide, providing an opportunity to compare the adaptations of planktonic foraminiferal morphospecies and individual genetic types to the different ecological settings. Since the Arabian Sea is subject to such extreme seasonality, it will be important to sample

during the inter-monsoon period in the future to obtain perspective on the planktonic foraminiferal genotypes that overlay one another in the sediment throughout the seasonal cycle.

3.2 Aims and Objectives

The aim of this study was to conduct a survey of the planktonic foraminiferal morphospecies, and their component SSU rRNA genetic types, within the unique marine environment of the central Arabian Sea mixed layer, during the summer SW monsoon. At this time a distinct disparity exists between the more eutrophic, high salinity hydrographic conditions of the northern water mass and the oligotrophic, low salinity conditions of the southern water mass. This offers an excellent opportunity to investigate the potential effects of divergent ecological adaptations on the biogeographical distributions and diversification of the planktonic foraminiferal morphospecies and their component genetic types. Phylogenetic analyses were employed to elucidate the positions of the Arabian Sea morphospecies/ genetic types within a comprehensive foraminiferal phylogeny, and their biogeographical distributions across the region examined. The data gained here was also compared to current knowledge of the global distributions of tropical/ sub-tropical planktonic foraminifera, with the view to examining the processes of dispersal and diversification in the global ocean.

3.3 Materials and Methods

3.3.1 Cruise track and oceanographic setting

Specimens of planktic foraminifera were collected at nine stations along a north/south cruise transect in the central Arabian Sea (20°22.81N/64°29.36E–02°36.03S/56°54.75E) during the summer monsoon of late June/July 2003 (fig. 3.1A; cruise Charles Darwin CD148, NERC) (collection by Kate Darling & Blair Steel). The oceanography of the Arabian Sea during the SW monsoon is shown in figures 3.1B-E to demonstrate the environmental conditions prevailing along the cruise transect. A cyclonic surface circulation appears during the SW monsoon that drives an eastward flowing monsoon current (MC), north of 10°S across the equatorial region (fig. 3.1B). A temperature gradient forms from west to east, starting where cooler water upwells off the Arabian coast increasing gradually to reach temperatures up to 29 °C in the west (fig. 3.1C). There is a clear north/south differentiation in salinity, with high salinity water forming in the upper Arabian Sea due to excess evaporation, extending to between stations 4 and 5 on the transect while the southern half of the region has a lower salinity, as water is transported in from the Bay of Bengal, where excess precipitation and abundant runoff predominate (fig. 3.1D). Elevated levels of primary productivity are typical of the northern Arabian Sea during the summer months (fig. 3.1E), as nutrient rich water is transported offshore from regions of upwelling along the coasts of Somalia and Oman (Wyrtki, 1973). The southern Arabian Sea conversely, is highly oligotrophic with a water mass interface around stations 4 -5 of the cruise transect (fig. 3.1E). Conductivity, temperature, depth (CTD) profiles from station 3 (15°01.11 N/65°00.02E) indicate that the mixed layer was 75m deep at this position with a temperature of 28.5 °C and salinity of 36.7 psu, consistent with fig. 3.1C and 3.1D maps.

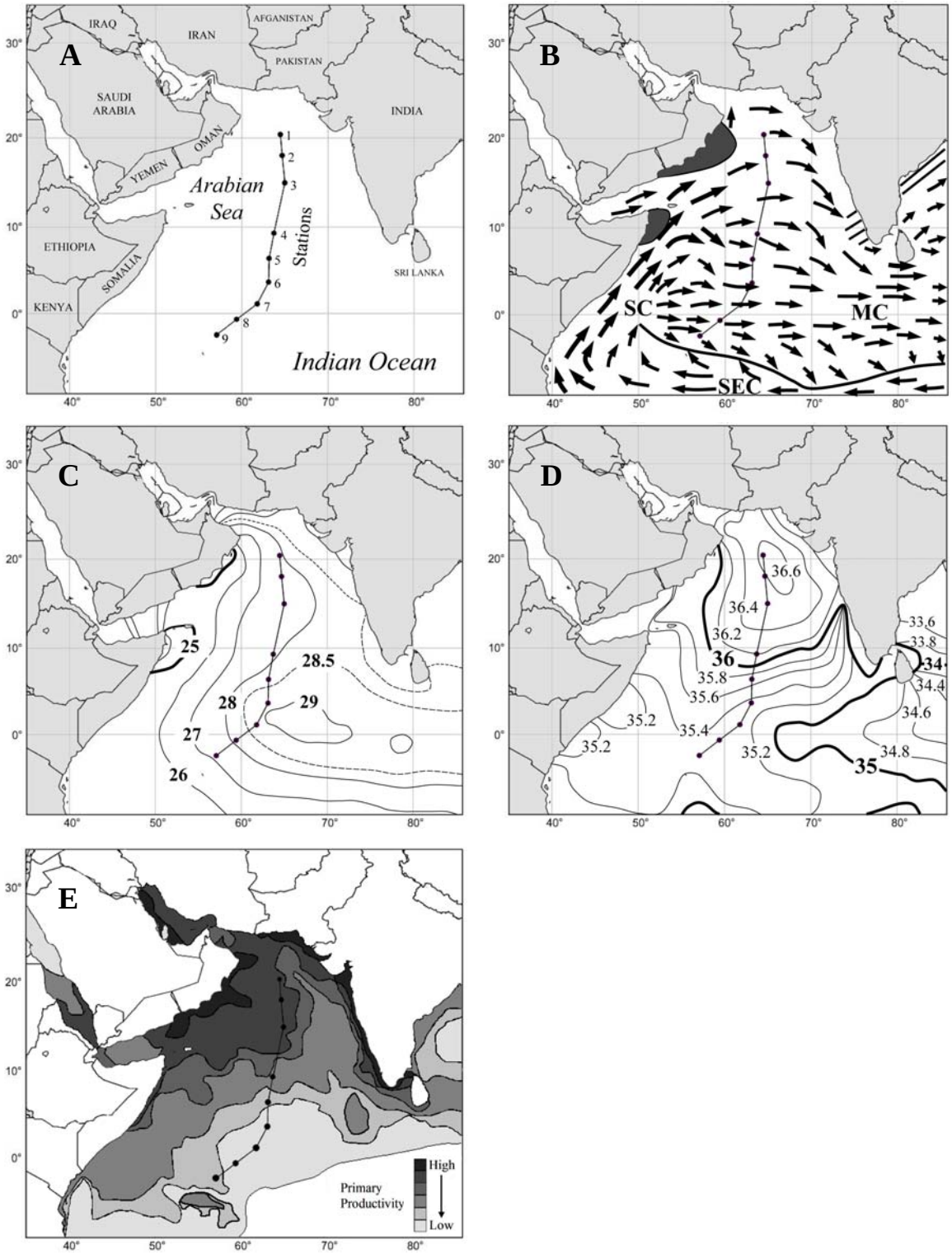


Figure 3.1. Maps of the Arabian Sea showing: (A) CD148 cruise transect and stations, (B) Surface currents during August at the SW monsoon peak. Regions of intense seasonal upwelling (dark grey), weak sporadic upwelling (hatched) SC = Somali Current, MC = Monsoon Current, SEC = Southern Equatorial Current (modified from Cullen and Prell, 1984), (C) Average sea-surface temperature (SST) (°C) for the SW Monsoon in July 2005 (adapted from Locarnini *et al*., 2006), (D) Average sea-surface salinity (PSU) for the SW Monsoon in July 2005 (adapted from Antonov *et al*., 2006), (E) Average primary productivity during the SW monsoon in July – September 1979 (adapted from Coastal Zone Colour Scanner composite images of the region, NASA Earth-Sun System Division, Earth Sciences (GES) Data and Information Services Center (DISC) Distributed Active Archive Center (DAAC)).

At this position, the thermocline dipped steeply between 75 and 150m (19 °C) and then reduced its steepness coincident with a salinity minimum of 35.7psu. Projections of mixed layer depth in July from Prasanna Kumar & Narvekar (2005) indicate a mixed layer depth of ~50 m north of station 3, shoaling to a 40 m mixed layer depth south of station 5.

3.3.2 Planktic foraminiferal sampling

Samples were collected either by pumping (5 m depth) from the ships' non-toxic water supply through a plankton screen (83 μm mesh) or by vertical net tow (0-100 and 0-200 m depth, 83 μm mesh) in waters with an average depth of 3,500 m. For genetic analysis, a representative sample of specimens was collected at each station. Individual specimens were identified using a stereomicroscope, and morphotype and cytoplasmic colouration were recorded by digital video imaging. Only adult specimens containing cytoplasm were selected for genetic analysis. These were crushed in a lysis buffer (Holzmann & Pawlowski, 1996) and incubated for 1 hour at 60 °C, before being transported to the lab where they were stored at –80 °C. For assemblage assessment, bulk samples were taken at each station with the specimens either dried on slides directly or collected as bulk samples in ethanol. The preserved assemblages were then individually picked and placed onto micropalaeontological slides (carried out by K. Darling). The high incidence of small juveniles compared to the low incidence of mature specimens made identification too uncertain to carry out relative abundance counts along the transect however visual assessment of the bulk assemblages was undertaken.

3.3.3 DNA amplification and sequencing

The PCR amplification of an approximately 1000 bp region of the terminal 3′ end of the foraminiferal SSU rRNA gene was carried out using a nested PCR approach. 3 μl of template were used in the first round of PCR (1° PCR), using primer C5 coupled with either primer 138 or NS8 (see chapter 2, section 2.2.2 for primer sequences and positions). Two secondary (2°) PCR reactions were performed, using 1 μl of product from the first round as the template. The first of these utilised primers 2082F and 2514R to amplify ~500 bp, for use in the identification of within-morphospecies genotypes. The second involved the amplification of the ~1,000 bp fragment in every genotype found in the Arabian Sea, for use in phylogenetic analysis. Two primer combinations were used: primers 2082F and 3014R and primers FS3 and 138, dependent on success (see chapter 2, section 2.2.2 for primer sequences and positions). Following poor success rates, an additional pass was made through the failed samples using primers designed for the amplification of the full-length SSU rRNA gene (see chapter 5) (1° PCR = 56F and 3033R (5 μl template), 2° PCR = 61F and 3024R (1 μl template), 3° PCR = 2082F and 2514R (for ~500 bp) or 2082F and 3014R (for ~1,000 bp) (1μl template), (see chapter 2, section 2.2.2 for primer sequences and positions). Reactions were performed using Taq polymerase in the first instance, and again using $Vent_R$ polymerase, following poor success rates. PCR reaction conditions were as described in chapter 2, sections 2.2.3 and 2.2.4. Amplification products were separated by gel electrophoresis and purified using an Eppendorf Perfectprep® Gel Extraction Kit (see chapter 2, sections 2.3 & 2.4). For taxa where direct sequencing was impossible due to the presence of multiple templates, cloning of the 1000bp fragment was carried out prior to sequencing using the TOPO TA cloning® method (Invitrogen™) (see chapter 2, section 2.6). Both

sense and antisense strands were sequenced directly on an Applied Biosystems™ 377 DNA sequencer using Applied Biosystems™ BigDye® v3.1 terminator cycle sequencing (primers 2082F, 2514R (500 bp fragment), primers 2082F, 3014R (1000 bp fragment), also primers M13R, M13F for clones) (see chapter 2, section 2.6.5 for details).

3.3.4 Sequence analysis and phylogenetic reconstruction

SSU rDNA sequences were assembled using Gap4 in the Staden package (Staden *et al*., 2000) and aligned manually within the Genetic Data Environment (GDE) package (version 2.2) (Smith *et al*., 1994). Arabian Sea genotypes were identified by BLAST search and comparison to existing sequences in a foraminiferal alignment, revealing those sequences that were novel to this locality. 90 foraminiferal taxa were selected for use in the main phylogenetic analysis based on 407 unambiguously aligned bp sites, including all species/genotypes obtained from the Arabian Sea, together with examples of every species and genotype of planktic foraminifera currently available in GenBank, plus a representative group of benthic foraminiferal taxa (1 per family in GenBank) (for taxa list see appendix 9.1, and for alignment see appendix 9.7.1).

To improve resolution, additional phylogenies were constructed for 4 of the most common Arabian Sea morphospecies, thus allowing a greater number of unambiguously aligned sites to be recruited into the analyses (*Globigerinella siphonifera/ Globigerinella calida* (668 bp), *Globigerinoides ruber/ Globigerinoides conglobatus* (589 bp), *Globigerina bulloides* (669 bp), *Turborotalita quinqueloba* (748 bp)) (for alignments see appendices 9.7.2, 9.7.3, & 9.7.5).

Phylogenetic trees were constructed using Bayesian inference (BI; Ronquist & Huelsenbeck, 2003, Larget & Simon, 1999), maximum likelihood (ML; Felsenstein, 1981), neighbour joining (NJ; Saitou & Nei, 1987), Fitch-Margoliash (FM; Fitch & Margoliash, 1967), minimum evolution (ME; Rzhetsky & Nei, 1992), and maximum parsimony (MP; Fitch, 1971) (FM, ME, MP sub-set trees only). In all methods multiple hits were accounted for using a general time-reversible (GTR) model with a gamma (Γ) correction (Lanave *et al*., 1984; Yang, 1993) (see main methods, chapter 2, section 2.8.2 for details).

3.4 Results

363 specimens of planktonic foraminifera were collected from 8 stations along a cruise transect in the Arabian Sea during the summer monsoon of 2003 (fig. 3.1). Small subunit rRNA gene sequences were successfully amplified for 213 individual specimens. Twenty different genotypes were recognised from 13 different mixed layer morphospecies (spinose: *Globoturborotalita rubescens* (pink), *Globigerinoides ruber, Globigerinoides sacculifer*, *Globigerinella siphonifera, Globigerina bulloides*, *Orbulina universa*, *Turborotalita quinqueloba*; non-spinose macroperforate: *Pulleniatina obliquiloculata*, *Neogloboquadrina dutertrei*, *Globorotalia menardii*, *Globorotalia ungulata*; non-spinose microperforate: *Globigerinita glutinata*; bi-serial: *Streptochilus globigerus*). Of these, two morphospecies; *G. rubescens* (pink) and *G. ungulata* were sequenced for the first time and four new genotypes of *G. ruber*, *G. siphonifera*, *T. quinqueloba* and *G. glutinata* were identified. In the case of *G. ungulata*, the three specimens sequenced exhibited the discriminating morphological features of this morphospecies, which is described as having a keel structure on the umbilical shoulder of the test (Hemleben *et al*, 1989). However, we do not rule out the possibility that they are *G. tumida,* since some workers believe *G. ungulata* to be an immature form of *G. tumida.* Here we continue to call the morphospecies we sequenced *G. ungulata*. Visual assessments of the bulk samples confirmed that all morphospecies found in the central Arabian Sea mixed layer were genotyped.

3.4.1 Foraminiferal phylogeny

A comprehensive phylogeny of the foraminifera based on the analysis of 407 bp of the SSU rRNA gene is shown in figure 3.2. It includes examples of all planktonic foraminiferal morphospecies and genotypes sequenced to date plus representatives of the major groups of benthic taxa (see appendix 9.1 for taxa list). The phylogenetic positions of the Arabian Sea sequences are highlighted on the tree. All of the methods of phylogeny reconstruction employed in this study were largely consistent in their inferred trees.

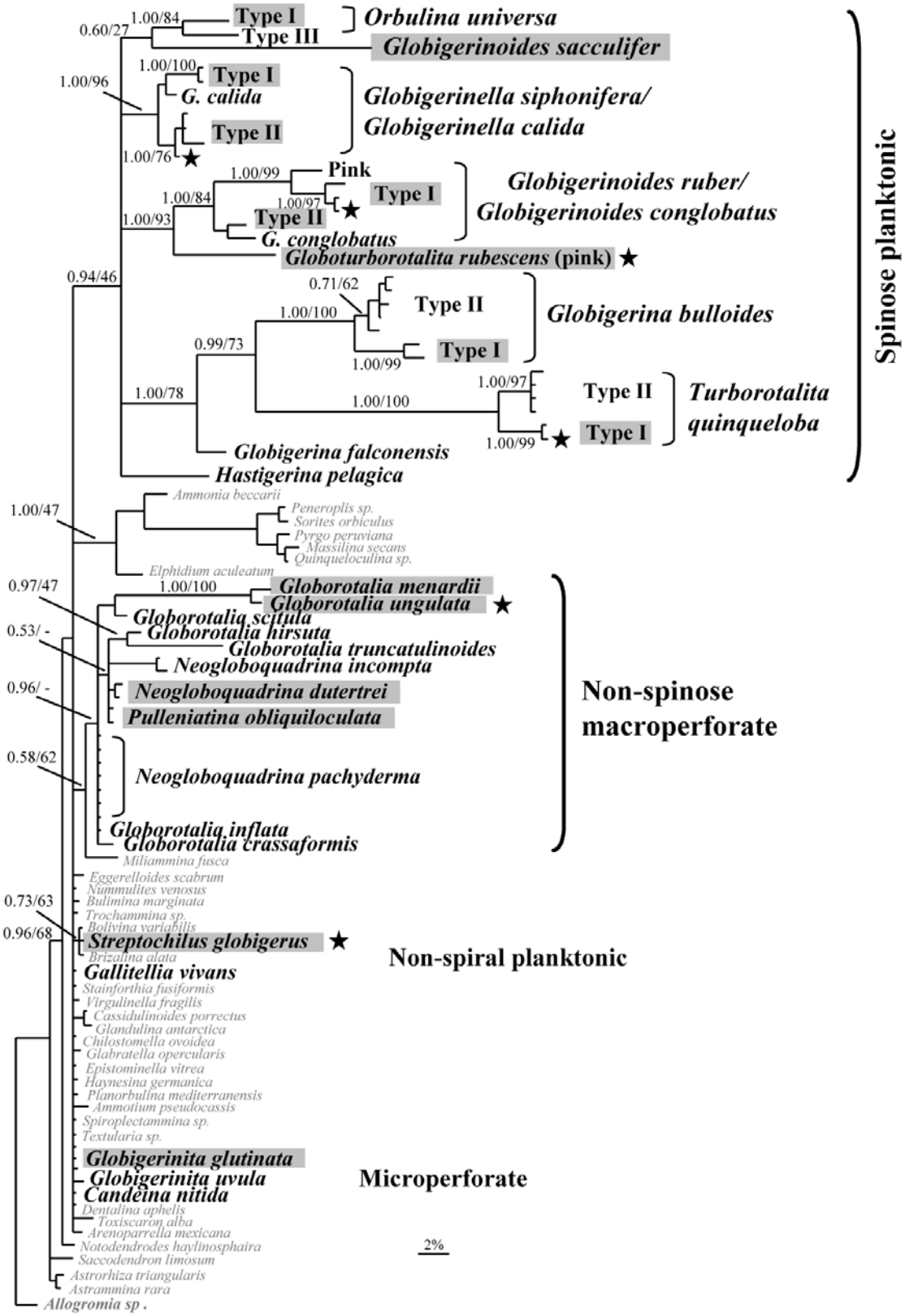


Figure 3.2. Bayesian inference SSU rDNA phylogenetic tree showing the position of the Arabian Sea morphospecies and genotypes within the foraminifera. The phylogeny is based on 407 unambiguously aligned nucleotide sites and is rooted on the benthic foraminifer *Allogromia sp.* Bayesian posterior probabilities (from last 1000 trees, obtained within MrBayes) and ML bootstraps (expressed as a percentage, 1000 replicates) are shown on the tree (BI posterior probabilities/ ML bootstraps). The scale bar corresponds to a genetic distance of 2 %. Benthic foraminiferal taxa are shown in grey text, and planktonic foraminifera are shown in black. Morphospecies and genotypes found in the Arabian Sea are shown on a grey background. ★ indicates novel sequences obtained from the Arabian Sea cruise (CD148). The sequence for *S. globigerus* is also presented in Darling *et al.* (2009). For taxa list and GenBank accession numbers see appendix 9.1.

3.4.1.1 The spinose planktonic morphospecies grouping

The spinose planktonic foraminifera (*G. siphonifera*, *G. calida*, *O. universa*, *G. sacculifer*, *G. rubescens* (pink), *G. ruber*, *G. conglobatus*, *G. bulloides*, *T. quinqueloba*, *G. falconensis* and *H. pelagica*) form a monophyletic group within the SSU phylogeny (fig. 3.2). This group is recovered with all methods of tree construction, though only weakly supported in bootstrap analysis (p= 0.94 BI, 46 % ML). Although there is relatively little structure at the base of the spinose clade, the spinose taxa appear partitioned into four principal clusters, consistent with previous phylogenetic studies (Darling *et al*., 1999, 2000, 2006; Stewart *et al*., 2001).

Globigerinella cluster: *Globigerinella siphonifera* and *G. calida* cluster together in a well-supported clade within the spinose planktonics (p=1 BI, 96 % ML; fig. 3.2). A phylogeny constructed from 668 bp (fig. 3.3a) shows the relationships among the genetic types within this clade more clearly. Three primary divisions are observed within *Globigerinella* (figs. 3.2 and 3.3a); *G. siphonifera* Type I (p=1 BI, 100 % ML, 100 % NJ, 100 % MP; fig. 3.3a), *G. siphonifera* Type II (p=0.99 BI, 97 % ML, 97 % NJ, 96% MP; fig. 3.3a) and *G. calida* as described by Stewart *et al.* (2000) and Darling & Wade (2008). *Globigerinella siphonifera* Type Ia sub-divides into two subtle subtypes; $Ia_{(1)}$ and $Ia_{(2)}$. The Type II clade sub-divides into IIa and IIb and IIa again sub-divides into a number of subtle sub-types, previously shown as the IIa complex in Darling & Wade (2008). Three subtypes are apparent, $IIa_{(1)}$ (Accession U80788), $IIa_{(2)}$ (Accessions AF102227, AJ3905674, Z83960), and $IIa_{(3)}$ (Arabian Sea). Much of the genetic variability within these sub-types occurs in the variable regions of the SSU rRNA gene leading to little support for branches within the IIa cluster (fig. 3.3a).

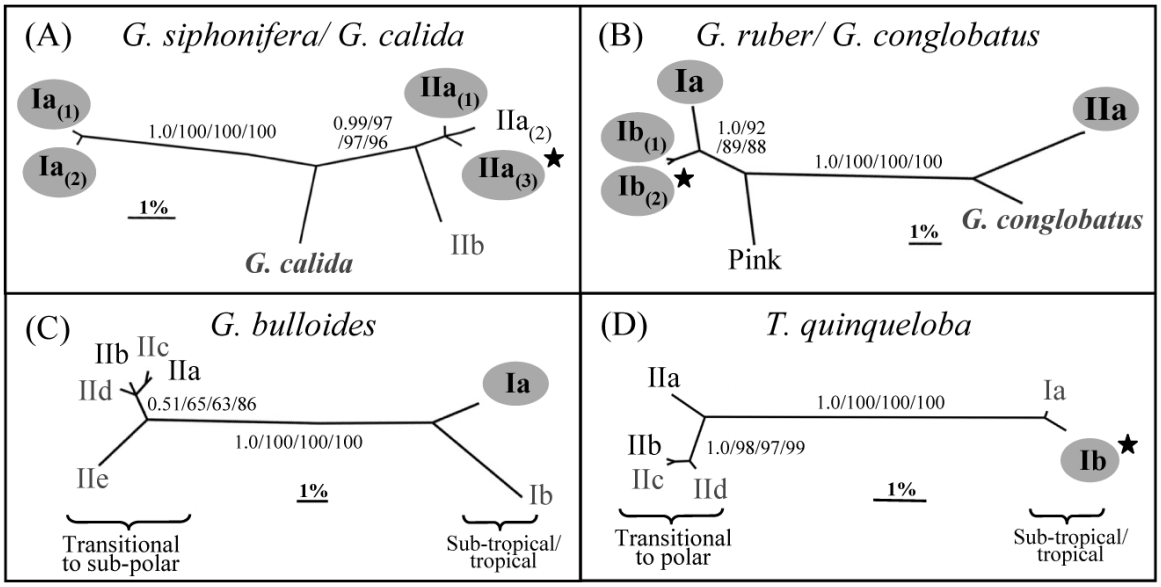


Figure 3.3. SSU rDNA phylogenetic trees of (A) *Globigerinella siphonifera/ Globigerinella calida* (668 unambiguously aligned nucleotide sites), (B) *Globigerinoides ruber/ Globigerinoides conglobatus* (589 bp), (C) *Globigerina bulloides* (669 bp), (D) *Turborotalita quinqueloba* (748 bp). The phylogenies were constructed using Bayesian Inference and are unrooted. Bayesian posterior probabilities and ML, NJ, and MP bootstraps (expressed as a percentage) are shown on the trees (BI/ML/NJ/MP bootstraps). The scale bar corresponds to a genetic distance of 1 %. Morphospecies and genotypes found in the Arabian Sea are shown on a grey background. ★ indicates novel sequences obtained from the Arabian Sea cruise (CD148).

In total, four distinct genotypes of *G. siphonifera* were found in the Arabian Sea mixed layer; Types $Ia_{(1)}$, $Ia_{(2)}$, $IIa_{(1)}$ and the novel $IIa_{(3)}$.

***Globigerinoides sacculifer* and *Orbulina universa* cluster:** The next grouping within the spinose planktonic region of the main foraminiferal tree (fig. 3.2) shows *G. sacculifer* clustering with *O. universa* as demonstrated by Darling *et al* (1999), with similarly little support (p= 0.60 BI, 27 % ML; fig. 3.2). No intra-specific genotypic variation has been found to date in *G. sacculifer*, and indeed the sequences from the Arabian Sea are identical to those already in GenBank. *Orbulina universa* was very rare in the mixed layer. Only a single individual of *O. universa* Type I was genotyped.

***Globoturborotalita rubescens* (pink), *Globigerinoides ruber,* and *Globigerinoides conglobatus* cluster:** *Globoturborotalita rubescens* (pink) is included in a foraminiferal phylogeny for the first time and falls as the sister taxon to *G. ruber/G. conglobatus* in the main spinose planktonic region of the tree (p= 1.0 BI, 93 % ML; fig. 3.2). A phylogeny constructed from 589 bp shows the relationships among the *G. ruber/G. conglobatus* genotypes (fig. 3.3b). The overall topology remains the same as in previous studies (Darling *et al*., 1999, 2008), with two primary subdivisions: *Globigerinoides ruber* (pink)/*G. ruber* (white) Type I and *G. conglobatus*/ *G. ruber* (white) Type IIa (p=1 BI, 100 % ML, 100 % NJ, 100 % MP; fig. 3.3b). As in Darling *et al*. (1999, 2000), the *G. ruber* (white) Type I lineage sub-divides into Ia and Ib (p=1 BI, 92 % ML, 89 % NJ, 88 % MP; fig. 3.3b). However, a subtly different variant of Ib was discovered in the Arabian Sea, splitting Ib into subtypes $Ib_{(1)}$ and the new $Ib_{(2)}$. In total, four distinct genotypes of *G. ruber* (white) were found in the Arabian Sea mixed layer; Types Ia, $Ib_{(1)}$, the novel $Ib_{(2)}$ and IIa. *Globigerinoides conglobatus* was not found in the Arabian Sea mixed layer assemblage.

***Globigerina bulloides, Turborotalita quinqueloba,* and *Globigerina falconensis* cluster:** These morphospecies form a distinct clade within the spinose planktonic group (p= 1 BI, 78 % ML; fig. 3.2) with three principal divisions (Stewart *et al* 2001). *Globigerina falconensis* diverges first (p= 1 BI, 78 % ML; fig. 3.2.) followed by the divergence of the *T. quinqueloba* and *G. bulloides* lineages. *Globigerina bulloides* (p=1 BI, 100 % ML; fig. 3.2) and *T. quinqueloba* (p=1 BI, 100 % ML; fig. 3.2) are both well supported in the phylogeny with both morphospecies falling on relatively long branches in the tree. A phylogeny of the *G. bulloides* cluster based on 669 sites (fig. 3.3c), shows the complex of genotypes found within this morphospecies to date.

The 7 distinct genotypes split principally into Types I and II (p=1 BI, 100 % ML, 100 % NJ, 100 % MP; fig. 3.3c). Type I is associated with warmer waters and subdivides into Ia (found in the Arabian Sea mixed layer) and Ib (fig. 3.3c). Type II is associated with cooler waters and comprises 5 individual sub-types that group together strongly in the tree (p=1 BI, 100 % ML, 100 % NJ, 100 % MP; fig. 3.3c). Within this group, the topology is consistent with previous studies (Darling *et al*., 2007, 2008). No specimens of *G. bulloides* Type II were found in the central Arabian Sea mixed layer. A phylogeny of the *T. quinqueloba* cluster based on 748 sites (fig. 3.3d) again shows a principal split between Type I and Type II (p=1 BI, 100 % ML, 100 % NJ, 100 % MP; fig. 3.3d). Again, this split divides the warm (Type I) from cool (Type II) types. Only one specimen of *T. quinqueloba* was successfully sequenced and was found to be a new genotype of Type I. This has been labelled Type Ib, and falls together with Type Ia in the 748 bp tree (p=1 BI, 100 % ML, 100 % NJ, 100 % MP; fig. 3.3d). Type II currently comprises 4 individual sub-types that group together strongly in the tree (p=1 BI, 100 % ML, 100 % NJ, 100 % MP; fig. 3.3d). Within this group, the topology is consistent with previous studies (Darling & Wade 2008).

Hastigerina pelagica: This spinose morphospecies falls at the base of the spinose group in the 407 bp phylogeny (fig. 3.2). Although relationships are poorly supported in this part of the tree, it appears to be positioned separately from the other spinose lineages, falling as a sister taxon to the other spinose groups. *Hastigerina pelagica* is unique among the planktonic foraminifera, both in its morphology and biology, and its ancestry is still strongly debated by micropalaeontologists (Schiebel & Hemleben 2005). *Hastigerina pelagica* was not found in the central Arabian Sea mixed layer.

3.4.1.2 The non-spinose planktonic foraminifera

The non-spinose planktonic foraminifera fall within three separate regions of the tree (fig. 3.2), consistent with the macroperforate, microperforate and non-spiral planktonic groupings (Hemleben *et al*, 1989). Rates of evolution among the non-spinose planktonics are generally magnitudes lower than the rates observed in the planktonic spinose group and are more akin to those observed in benthic species (Pawlowski *et al*, 1997). There is therefore little resolution among the non-spinose planktonics with only a few exceptions.

Non-spinose macroperforates: The macroperforates form a monophyletic group within the phylogeny comprising *Globorotalia* (*menardii, ungulata, truncatulinoides, crassaformis, hirsuta, scitula and inflata*), *Neogloboquadrina* (*incompta, dutertrei and pachyderma*) and *Pulleniatina* (*obliquiloculata*) (fig. 3.2). This group is recovered with all methods of phylogeny reconstruction, though is only weakly supported in bootstrap analysis (p= 0.96 BI, - ML). Of the macroperforates, only *G. menardii, G. ungulata, N. dutertrei* and *P. obliquiloculata* were found in the central Arabian Sea mixed layer. Due to its very high evolution rate compared with other macroperforate taxa, the placement of *G. menardii* has often proved problematic. In the current phylogeny, the position of *G. menardii* is consistent with taxonomic classification, placing it with the other macroperforates, however, its placement was inconsistent across methods of tree construction (data not shown). Although some very minor sequence variation was detected in *G. menardii*, it cannot be considered sufficient to warrant sub-type status. A closely related morphospecies, *G. ungulata,* has been sequenced in this study for the first time. *Globorotalia menardii* and *G. ungulata* fall together on a relatively long branch (with all phylogenetic methods) (p= 1 BI, 100 %

ML, fig. 3.2). Three specimens were sequenced, with no variation detected. Some *neogloboquadrinid* morphospecies exhibit a degree of intra-individual variation in the most variable regions of their SSU gene repeats not used for phylogenetic analysis (Darling & Wade, 2008). The sequences of *N. dutertrei* obtained in this study did show some degree of variation, but without extensive cloning, it is impossible to determine whether they reflect the presence of more than one genotype in the water column. All *P. obliquiloculata* sequences obtained during this study were identical to each other, however they differed subtly from those currently available in GenBank. As yet, it is unknown whether this is due to sequencing errors in those submitted to GenBank, intra-individual variation or whether they are a different genotype. Though not present in the Arabian Sea, a new sequence for *Globorotalia scitula* was added during this study. *Globorotalia scitula* consistently fell within the macro-perforate clade, though it's positioning with *G. menardii* and *G. ungulata* was not recovered with all methods of tree construction (data not shown) and has little support (fig. 3.2).

Non-spinose microperforates: There are possibly 7 morphospecies of non-spinose microperforate planktonic foraminifera (Hemleben *et al*, 1989) of which only three have been sequenced to date; *G. uvula* (Stewart *et al*., 2001), *G. glutinata* (Darling *et al*., 2000) and *Candeina nitida* (Ujiié & Lipps, 2009). They fall outside of the main non-spinose 'macroperforate' group falling separately among the benthics (fig. 3.2). Only *G. glutinata* was found along the cruise transect and displayed distinct sequence variation between specimens. Comparing the Arabian Sea sequences to those currently in GenBank, it appears that there are three subtly different genotypes, which are named here as Type $1a_{(1)}$, $1a_{(2)}$ (accession Z83974.1, de Vargas, 1997), and $1a_{(3)}$. Only Types $1a_{(1)}$ and $1a_{(3)}$ were found in the central Arabian Sea mixed layer. Recent

cloning of *G. glutinata* from North-West Pacific assemblages (Ujiié & Lipps, 2009) indicates that these are most likely to be genuine subtype differences and not simply intra-individual variation in the SSU gene repeats.

3.4.1.3 Non-spiral planktonics (biserial and triserial)

There are two planktonic morphospecies of the biserial genus *Streptochilus* (Hemleben *et al*, 1989) and one morphospecies of the triserial genus *Gallitellia* (Kroon and Nederbragt, 1990). Only *Streptochilus globigerus* was found in the mixed layer of the central Arabian Sea. This morphospecies also exhibits a degree of intra-specific variation in the SSU sequences similar to that observed in some benthic foraminiferal species (Darling & Wade, 2008). The sequence gained here for *S. globigerus* is also presented in Darling *et al*. (2009), where the species was surprisingly found to cluster with the infaunal benthic biserial species *Bolivina variabilis* from the Kenyan coastal region (sequence from Ertan *et al*, 2004). The tri-serial morphospecies, *Gallitellia vivans,* has been sequenced for the first time only recently (Ujiié *et al*., 2008), and falls in an unresolved position within the benthic taxa (fig. 3.2).

3.4.2 Biogeography

Analysis of the morphospecies genotype distribution data (fig. 3.4) combined with a visual assessment of the bulk assemblage data showed some distinct ecological segregation related to the physical oceanography of the Arabian Sea.

3.4.2.1 Globigerinoides ruber (fig. 3.4A; n=75)

Assessment of the bulk assemblage samples revealed that *G. ruber* was the dominant morphospecies in the Arabian Sea during the SW monsoon. It was found in high numbers in the more eutrophic, high salinity water of the north and occurred in significantly lower numbers in the more oligotrophic lower salinity water mass to the south. There are four genotypes of *G. ruber* in the Arabian Sea assemblage, which have distinctive biogeographies. Only Type IIa (n=24) and Type $Ib_{(2)}$ (n=46) were found in the eutrophic high salinity water mass of the northern Arabian Sea. The other *G. ruber* genotypes Ia (n=4) and $Ib_{(1)}$ (n=1) were not found in the northern water mass following extensive genotyping of the water column. These genotypes were found in low numbers within the southern water mass, with only a single specimen of *G. ruber* Type Ia identified at station 9.

3.4.2.2 Globigerinella siphonifera (fig. 3.4B; n=24)

Globigerinella siphonifera was distributed throughout the transect and four genotypes were identified. The newly recognised Type $IIa_{(3)}$ (n=19) was distributed throughout the cruise transect, thriving equally in both the northern and southern water masses. The other genotypes appeared more rare. Type $Ia_{(2)}$ (n=1) was found only in the northern water mass and Types $Ia_{(1)}$ (n=1) and $IIa_{(1)}$ (n=3) were found in low numbers in the southern water mass.

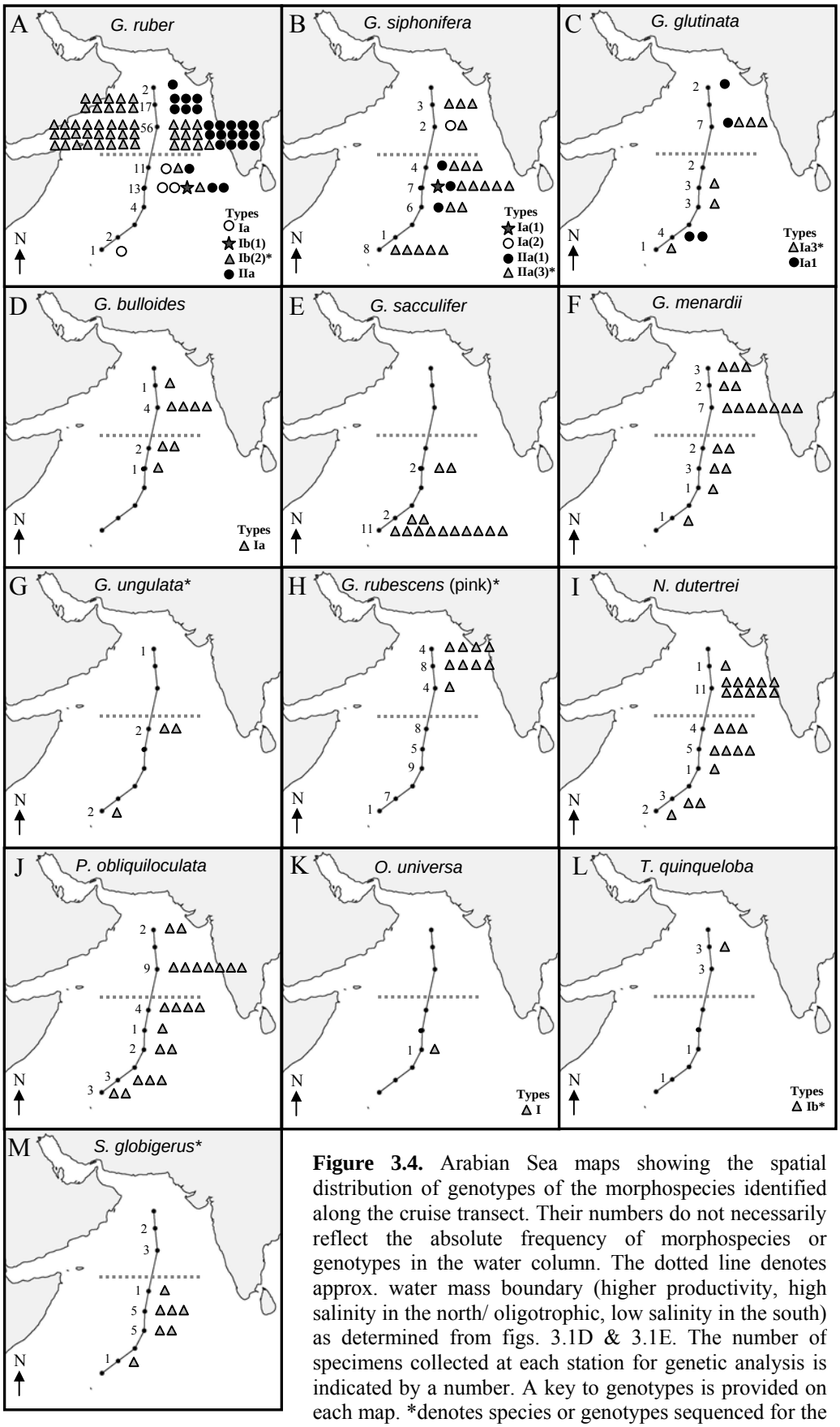


Figure 3.4. Arabian Sea maps showing the spatial distribution of genotypes of the morphospecies identified along the cruise transect. Their numbers do not necessarily reflect the absolute frequency of morphospecies or genotypes in the water column. The dotted line denotes approx. water mass boundary (higher productivity, high salinity in the north/ oligotrophic, low salinity in the south) as determined from figs. 3.1D & 3.1E. The number of specimens collected at each station for genetic analysis is indicated by a number. A key to genotypes is provided on each map. *denotes species or genotypes sequenced for the first time.

3.4.2.3 Globigerinita glutinata (fig. 3.4C; n=10)

Globigerinita glutinata was found throughout the transect. Two potentially distinct subtypes of Type Ia were identified in the central Arabian Sea mixed layer; Types $1a_{(1)}$ and $1a_{(3),}$ each distributed along the length of the cruise transect.

3.4.2.4 Globigerina bulloides (fig. 3.4D; n=8)

Globigerina bulloides was present in very low numbers in the bulk samples and was distributed mainly in the more eutrophic, high salinity water mass of the northern region. Genotyping revealed only one genetic type, Type Ia, confined to the northern water mass.

3.4.2.5 Globigerinoides sacculifer (fig. 3.4E; n=14)

Globigerinoides sacculifer was found only in the southern waters, south of station 4. Only a single genotype was found, which was identical to all other *G. sacculifer* sequenced to date.

3.4.2.6 Globorotalia menardii (fig. 3.4F; n=18)

The bulk assemblage data clearly showed that *G. menardii* increased in numbers towards the southern part of the cruise transect though this pattern was not reflected in the number of specimens collected for genotyping (fig. 3.4F). Despite the distribution difference between the water masses, only a single genotype was found in the mixed layer along the cruise transect.

3.4.2.7 Globorotalia ungulata (fig. 3.4G; n=3)

Assessment of the bulk assemblage showed that *Globorotalia ungulata* was more common in the southern part of the cruise transect. Only three specimens were sequenced and a single genotype found.

3.4.2.8 Globoturborotalita rubescens **(pink) (fig. 3.4H; n=9)**

Globoturborotalita rubescens (pink) was present throughout the transect, though only nine specimens were successfully amplified. This newly sequenced morphospecies showed no sequence variation in the specimens collected between stations 1-3.

3.4.2.9 Neogloboquadrina dutertrei **(fig. 3.4I; n=22)**

Neogloboquadrina dutertrei was distributed along the length of the cruise transect, and is most likely represented by a single genotype in the Arabian Sea. However, as in most *Neogloboquadrina*, *N. dutertrei* specimens exhibit intra-individual variation in their SSU gene repeats and the presence of more than one genotype cannot be ruled out without extensive cloning.

3.4.2.10 Pulleniatina obliquiloculata **(fig. 3.4J; n=21)**

Pulleniatina obliquiloculata was distributed along the length of the cruise transect. Only a single genotype was found.

3.4.2.11 Orbulina universa (fig. 3.4K; n=1)

Orbulina universa was very rare in the water column. Only a single specimen of Type I was identified in the southern water mass at station 6.

3.4.2.12 Turborotalita quinqueloba (fig. 3.4L; n=1)

It is difficult to differentiate *T. quinqueloba* from tiny juveniles of other morphospecies, but mature specimens were rare. Only a single specimen of Type Ia was amplified at station 2.

3.4.2.13 Streptochilus globigerus (fig. 3.4M; n=7)

The biserial morphospecies, *S. globigerus*, was found in substantial numbers along the length of the cruise transect (bulk sample assessment). Only a single genotype was identified.

3.5 Discussion

3.5.1 The newly sequenced morphospecies and their phylogenetic placement within the all foram phylogeny

The overall structure of the foraminiferal SSU rDNA phylogeny is largely unchanged from previous studies (Darling *et al.,* 1997, 1999, 2000, 2006; de Vargas *et al.,* 1997; Stewart *et al.,* 2001). The planktonic foraminifera do not form a single monophyletic unit in the tree but instead, the planktonic spinose species fall together in a monophyletic group separate from the non-spinose species. Amongst the non-spinose foraminifera, the macroperforates fall together in the molecular phylogeny consistent with their taxonomic grouping (Hemleben *et al*, 1989). The non-spinose micro-perforate taxa and the non-spiral biserial planktonic taxa fall separately from the macroperforates, though their placement is ambiguous. Three morphospecies of planktonic foraminifera are included within the molecular phylogeny for the first time.

***Globoturborotalita rubescens* (pink):** This spinose morphospecies is highly conspicuous in the sediment assemblage as it is one of only two morphospecies with red colouration in its shell. It is possibly closely related to *Globoturborotalita tenella* (Parker), which has yet to be sequenced. It is a shallow-dwelling morphospecies that is most common in the tropics but is also found into the sub-tropical province (Bé, 1977). It can be locally common and has its highest relative frequency in the Arabian Sea (12 %) in the western region (Kroon, 1991). In the main foraminiferal phylogeny (fig. 3.2), it diverges first within a well-supported cluster from the *G. ruber*/*G. conglobatus* group. Fossil record studies show that it first appeared in the Middle Pliocene, around 3.6 million years ago (Kennett and Srinivasan, 1983). *Globoturborotalita rubescens* (pink) is very difficult to distinguish from *Globigerina*

woodi (Chaisson and Pearson, 1997), from which it may have evolved via the morphospecies *Globigerina decoraperta* (Kennett and Srinivasan, 1983). *Globigerina woodi* is also the possible ancestral lineage of *G. ruber,* which would be highly consistent with the molecular phylogeny.

***Globorotalia ungulata*:** This morphospecies is said to occur rather sporadically in tropical waters (Hemleben *et al*, 1989). It appeared in the Late Pliocene around 2.5 million years ago and is thought to have evolved from *G. tumida* (Kennett and Srinivasan, 1983). *Globorotalia ungulata* and *G. menardii* fall together at the end of a relatively long branch in the main phylogeny (fig. 3.2). It will not be possible to determine globorotaliid relationships and ancestry until all the other extant morphospecies have been sequenced.

***Streptochilus globigerus*:** This is one of the two known morphospecies of extant biserially coiled planktonic foraminifera. Once thought to be extinct, *Streptochilus* was discovered in plankton tows south of India (*S. globulosus*) (de Klasz *et al*., 1989) and in the North Atlantic (*S. globigerus*) (Hemleben *et al*., 1989). It is a small-sized morphospecies that frequents tropical to transitional waters and lives in highly productive deep water, appearing abundantly in coastal surface water in areas of upwelling, but can also be found in the open ocean (Hemleben *et al*, 1989). This is the first time that any biserial planktonic morphospecies has been included in a comprehensive phylogeny of the foraminifera. *Streptochilus globigerus* not only falls among the benthic, rather than planktonic foraminifera (fig. 3.2), but also has an extremely high sequence identity to the benthic species *Bolivina variabilis,* sufficient to suggest that *S. globigerus* and *B. variabilis* are one and the same morphospecies

(Darling *et al*., 2009). Indeed, subsequent phylogenetic analyses of the SSU rDNA sequences of *B. variabilis* and *S. globigerus* have revealed that *S. globigerus* falls well within the *B. variabilis* radiation (Darling *et al* 2009).

3.5.2 Biogeographical distribution and ecology of the morphospecies and genotypes of the central Arabian Sea mixed layer

The cruise transect was conducted during the SW monsoon, when environmental conditions were most pronounced and a strong disparity existed between the northern and southern water masses. This provided the opportunity to determine the ecological adaptations of planktonic foraminiferal morphospecies and their individual genetic types in the mixed layer of two different water masses. The high salinity northern water mass (stations 1-3 on the cruise transect) has elevated levels of primary productivity (fig. 3.1B-E). The southern water mass, conversely, has a lower salinity and is highly oligotrophic, with a boundary separating the two water masses between Station 3 and 4 on the cruise transect.

3.5.2.1 Geographical distribution of morphospecies: bulk assemblage data

Visual assessment of the bulk assemblages (provided by K. Darling) showed that the more eutrophic region of the northern Arabian Sea mixed layer in late June/July was dominated by *G. ruber* with lower numbers of *N. dutertrei*, *P. obliquiloculata*, *G. menardii* and *G. glutinata* also present. This is consistent with assemblages found in the surface sediments of the region (*G ruber* 20-40 %, *G. glutinata* < 10 %; *G. menardii* 10 %; *N. dutertrei* 5-10 %; *P. obliquiloculata* >10 %; *G. bulloides* <5 %; *G. siphonifera* 3-5 %; Cullen & Prell, 1984) with two notable exceptions. Firstly, *G. sacculifer* constitutes over 10 % of the core top assemblage of the northern region (Cullen & Prell, 1984), but was entirely absent in the SW monsoon water column of

the northern eutrophic water mass in July, even in the deep 200 m net. *Globigerinoides sacculifer* did not appear in the assemblage until reaching the frontal zone at station 4. High numbers of *G. sacculifer* were also reported in this frontal zone during the SW monsoon in June 1984 (Kroon, 1991), consistent with this study. The difference between water column and core top abundance in *G. sacculifer* during the SW monsoon indicates that it must be present in higher numbers in the northern region of the Arabian Sea during the more oligotrophic inter-monsoon period. Guptha *et al*, (1994), certainly found a greater proportion of *G. sacculifer* in the nutrient depleted northern water mass in late September/October, though Schiebel *et al* (2004) found considerable numbers of *G. sacculifer* in the water column at a station equivalent to our station 3 in July/August. The second exception to the core top assemblage below the northern water mass is that small species such as *G. rubescens* (pink) may have been underestimated when sieved at 150µm, since *G. rubescens* (pink) was present in the water column in relatively high numbers in this study yet is only recorded as constituting less than <1 % in the sediment assemblage (Cullen & Prell, 1984).

Bulk samples indicate that the dominant morphospecies in the southern more oligotrophic water mass was *G. sacculifer* during the SW monsoon, with proportionately lower numbers of *G. ruber*. This indicates that there may be a strong element of seasonality in the sediment assemblage in both the northern and southern regions, since these differences are not reflected in the sediment assemblage counts where both are present in high numbers throughout (Cullen & Prell, 1984). The other major morphospecies that was more prevalent within the southern region was

G. menardii, which increased towards the later stations. This is most likely related to the increased mixing and nutrification at the interface with the Southern Equatorial Current, since *G. menardii* is often associated with productive, upwelling regions. *Globigerina bulloides* was very rare in the southern water mass during the SW monsoon.

The bulk samples show that *S. globigerus* was present in high numbers along much of the cruise transect. The distribution of *S. globigerus* is poorly known because of its sporadic appearance and small test size. They most likely have a tychopelagic life style, occupying a niche in both the benthos and plankton. This is extensively discussed in Darling *et al* (2009).

3.5.2.2 Geographical distribution and ecology of within-morphospecies genotypes

Within the Arabian Sea mixed layer, two morphospecies; *Globigerinoides ruber* (fig. 3.4A) and *Globigerinella siphonifera* (fig. 3.4B) were each represented by four individual genetic types that exhibited apparent ecologically distinct distribution patterns during the SW monsoon. The *G. ruber* morphospecies is represented by two highly divergent lineages, Type I and Type II (see phylogeny: fig. 3.3B), which likely represent separate species (Darling & Wade, 2008). Types Ia, Ib and II were all present in the Arabian Sea mixed layer, represented by the genotypes Ia, $Ib_{(1)}$, $Ib_{(2)}$ (new subtype) and IIa. As discussed above, there was an apparent decreasing trend from north to south in the proportion of *G. ruber* present in the mixed layer along the cruise track, however, there was also a clear difference in genotype distribution between the eutrophic, high salinity northern water mass and the oligotrophic, lower salinity southern water mass (fig. 3.4A). The northern water mass contained only two

of the *G. ruber* genotypes ($Ib_{(2)}$ and IIa), both of which were present in equally high numbers. The other two genotypes (Ia and $Ib_{(1)}$) were found only in the southern water mass. The absence of these two genotypes from the northern water column perhaps suggests that they are not adapted to more eutrophic high salinity conditions. The more numerous genotype from the southern region, Type Ia, likely dominates during the more oligotrophic periods of the seasonal cycle. A clear ecological distinction could be seen between the *G. ruber* Type Ia and Type II lineages.

Interestingly, *G. ruber* Type $Ib_{(1)}$ and the newly recognised Type $Ib_{(2)}$ also appeared ecologically distinct in their distribution patterns, despite these subtypes being only subtly different at the genetic level. Type $Ib_{(2)}$ had a dominant presence in the Northern Arabian Sea, whilst only a single individual of Type $Ib_{(1)}$ was found, in the southern water mass. As Type $Ib_{(1)}$ was not identified in a population of 70 individual specimens in the northern water mass, it could indicate a different ecological optimum to that of Type $Ib_{(2)}$, and a possible cause of their recent divergence.

Both major lineages of *Globigerinella siphonifera* (Types I & II) were present in the Arabian Sea mixed layer (fig. 3.4B), represented by subtypes $Ia_{(1)}$, $Ia_{(2)}$, $IIa_{(1)}$ and $IIa_{(3)}$. Although their numbers were relatively low, it was clear that *G. siphonifera* Type II (n=22) was the dominant *G. siphonifera* lineage along the whole cruise transect, thriving within the varying hydrographic conditions of both the northern and southern water masses. The two representatives of the *G. siphonifera* Type I lineage (Types $Ia_{(1)}$ & $Ia_{(2)}$), conversely, were represented by only single individuals. The newly recognised $IIa_{(3)}$ genotype was distributed throughout both water masses in large numbers (n=19), whereas Type $IIa_{(1)}$ was found only in small numbers in the southern

water mass (n=3). This suggests that even at this low level of genetic distinction (fig. 3.3a), Types $IIa_{(1)}$ and $IIa_{(3)}$ may have distinctly different ecologies, possibly driving their genetic separation.

The genetic types of *G. ruber* and *G. siphonifera,* unarguably have distinct geographical distributions within the Arabian Sea mixed layer, a likely indicator of ecological differentiation. Partitioning by the frontal systems between the two quite disparate environments is one of the most likely drivers of divergence and speciation in the open ocean (Schluter, 2001; Darling *et al*, 2004). Unravelling these relationships would be a considerable step forward to improve quantitative faunal and geochemical palaeoclimate reconstructions. However, there is an additional possibility that such distributions could arise as a result of competitive exclusion, whereby, a well-established genotype, by sheer weight of numbers, may prevent another from occupying a region, as proposed by Aurahs *et al.* (2009). This has particularly been noted to take place between closely related types, and could explain, for example the mutually exclusive distributions of closely related *G. ruber* types $Ib_{(1)}$ and $Ib_{(2)}$ here. In addition, *G. siphonifera* Type $IIa_{(3),}$ the dominant type here, may be competitively excluding other *G. siphonifera* genotypes. Sympatric occurrence of more divergent types is thought to be possible due to pronounced ecological niche separation (Aurahs *et al*. 2009), and indeed here we see distantly related *G. ruber* types $Ib_{(2)}$ and IIa co-existing in the North of the Arabian Sea in great numbers.

The remaining planktonic foraminiferal morphospecies of the Arabian Sea mixed layer were largely represented by only single genotypes. *Globigerina bulloides* and *Globigerinoides sacculifer*, in particular, displayed non-random biogeographical

distributions between the water masses (figs. 3.4D and 3.4E), again pointing to selective ecological requirements.

Globigerina bulloides is more typical of sub-polar regions (Bé and Tolderlund, 1971), but also characterises upwelling zones in lower latitudes (Naidu and Malmgren, 1996). It is a minor background morphospecies in the central Arabian Sea (Cullen and Prell, 1984), occurring in low numbers, and mostly confined to the northern water mass (bulk sample assessment and fig.3.4D). The two major *G. bulloides* lineages follow a temperature-dependent distribution globally, with Type I occurring in warm waters, and Type II occurring in cold waters (fig. 3.3c; Darling & Wade, 2008). Of the two known tropical/subtropical genotypes (Type Ia and Ib) (Darling & Wade, 2008), only Type 1a was present in the Arabian Sea mixed layer. It occurred predominately towards the north of the region (fig. 3.4D), being absent from the most southerly reaches of the transect (stations 6 – 9). This absence from the most oligotrophic, lower salinity waters perhaps indicates an adaptation to slightly more eutrophic, higher salinity conditions. Interestingly, *Globigerina bulloides* dominates the planktonic foraminiferal assemblages in the upwelling coastal waters of the Arabian Sea (Schiebel *et al*. 2004). Here temperatures are considerably cooler than those found in the central Arabian Sea, and the question therefore remains whether this warm water genotype (1a) is ecologically distinct from those found in high numbers in the upwelling regions of the Arabian Sea.

The single genotype of *G. sacculifer* was confined to the southern oligotrophic water mass in this study (fig. 3.4E) reflecting its possible adaptation to more oligotrophic waters (Halicz and Reiss, 1981; Reiss, 1980). It has been postulated that other factors

such as the chlorophyll maximum or thermocline development may affect its distribution (Weikert, 1987), and its status in the Arabian Sea water column has been shown to vary with temperature, salinity, nutrients and thermocline depth (Schiebel *et al*, 2004). Salinity is an unlikely limiting factor as *G. sacculifer* is a euryhaline species, capable of tolerating salinities in a range of 24 ‰ – 47 ‰ (Hemleben *et al*, 1989).

Although *G. menardii* (fig. 3.4F) was present throughout the transect, the bulk samples indicate that its numbers tended to increase in the assemblage towards the most southern part of the cruise transect, indicating a possible ecological preference. This is in contrast to the other globorotaliid in the assemblage, *G. ungulata* (fig. 3.4G), which occurred at relatively low levels throughout.

The remaining prominent morphospecies within the assemblage *G. rubescens* (pink), *N. dutertrei* and *P. obliquiloculata* (figs. 3.4H, 3.4I and 3.4J) were represented by only single genotypes, exhibiting wide distributions along the whole transect. Although different genotypes of *N. dutertrei* (Darling *et al*, 2003) and *P. obliquiloculata* have been recognised (unpublished observation), extensive sampling and cloning will be required before their biogeographical distribution can be determined. Microperforate morphospecies *Globigerinita glutinata*, may have been represented by two distinct genotypes, however, these too showed broad distributions across the transect area.

Orbulina universa (fig. 3.4K) and *T. quinqueloba* (fig. 3.4L) were each represented by a single genotype and were found in very low numbers in the central Arabian Sea mixed layer in June/July. Insufficient data prevents comment on their ecology, and it

may be that these individuals simply arrived in the region by expatriation, a phenomenon commonly observed in planktonic foraminifera, whereby individuals are carried, by currents, outside of their ecologically optimal range.

The final morphospecies, *Streptochilus globigerus,* was found throughout the cruise transect and is of particular interest. *Streptochilus globigerus* (fig. 3.4M) is an unusual, sporadically occurring, biserial planktonic foraminifer (de Klasz *et al*., 1989; Schiebel and Hemleben, 2005). As discussed above, the SSU rDNA sequences for *S. globigerus* showed a surprisingly high level of sequence identity to the benthic species *Bolivina variabilis* from the Kenyan coastal region (Ertan *et al*, 2004), located south west of our central Arabian Sea sampling stations, indicating that they are the same species (Darling *et al*, 2009). In the benthos, *B. variabilis/S. globigerus* lives as a shallow to intermediate infaunal dweller in the continental shelf sediments. During the SW monsoon (fig. 3.1b), its populations become expatriated by the winds and currents far offshore, where they continue to live and grow as plankton in the open ocean (Darling *et al*, 2009). *Streptochilus globigerus* is therefore tychopelagic in nature (McQuoid, and Nordberg, 2003), exploiting both benthic and planktonic habitats (Darling *et al*, 2009). The high degree of genetic identity within the Arabian Sea benthic and planktonic biserial foraminifers indicates that they must represent a single genetically connected population. Whether there is two-way gene flow between the benthos and plankton or whether their population identity is maintained by continual re-seeding from the benthos is as yet unknown.

3.5.3 Global phylogeography of the Arabian Sea genotypes

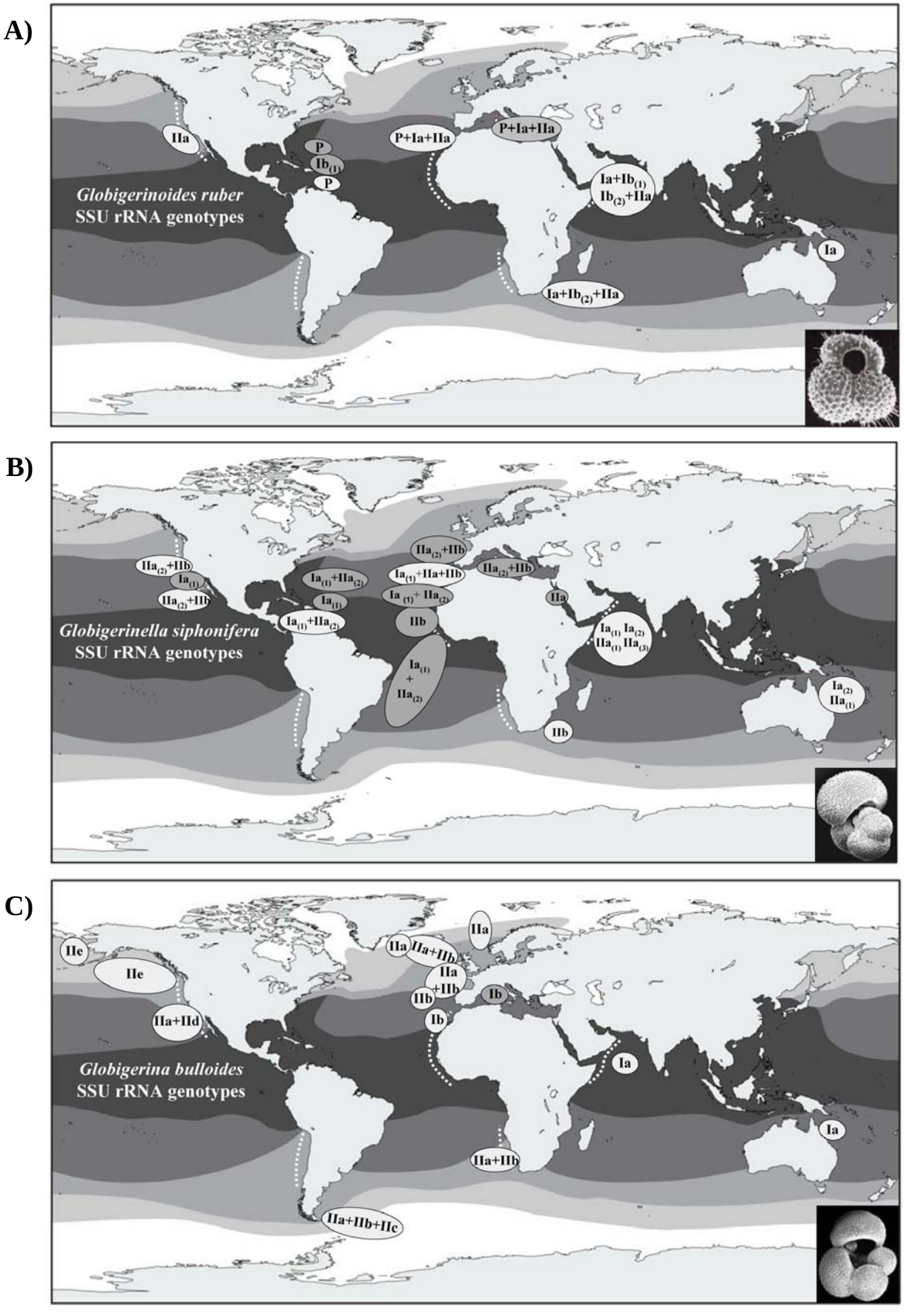
The apparent lack of barriers to gene flow in the open ocean in combination with the high dispersal potential of pelagic taxa clearly impacts on their rate of diversification and speciation. Indeed, planktonic foraminiferal diversity appears very low at around 50 morphospecies when compared with their benthic counterparts of several thousand. Nevertheless, specific assemblages are found associated with regionally distinct ecosystems, which are traditionally divided into polar, sub-polar, transitional, sub-tropical and tropical faunal provinces (Bé & Tolderlund, 1971; Bé, 1977). Discrete assemblages are also found in a transitory province associated with regional upwelling (summarised in Lipps, 1979; Hemleben *et al*, 1989). Within all these provinces, other factors such as salinity, prey abundance, nutrient level, turbidity and illumination also affect their diversity, abundance and distribution locally. Planktonic foraminiferal diversity peaks in the subtropics (Rutherford *et al*, 1999) where there is vertical niche partitioning and decreases steeply towards the poles where there is little permanent thermal structure. The level of genetic variation observed within planktonic foraminiferal morphospecies imposes a further tier of complexity as individual genetic types within morphospecies clades may also have distinct ecologies and biogeographical distributions (summarised in Darling & Wade, 2008).

Genotyping reveals geographical connectivity to other regions of the oceans, providing clues to present and past ocean circulation, evolutionary drivers and the evolutionary history of foraminiferal species (Darling *et al*, 2000, 2004, 2006, 2007; de Vargas *et al*., 1999, 2001, 2002). As yet, sampling in the tropics and subtropics is sparse and considerable caution must be taken in interpreting global distribution of tropical and subtropical morphospecies and genotypes. However, some ecological

perspective can be gained in comparing central Arabian Sea mixed layer genotypes with those in other regions of the tropical and subtropical global ocean. Twenty distinct genotypes of 13 morphospecies were collected from the Arabian Sea mixed layer (figs. 3.2 and 3.3). Global connectivity is discussed below for *G. ruber*, *G. bulloides*, *G. sacculifer*, *O. universa*, *G. siphonifera* and *T. quinqueloba*, for which sufficient global biogeographical evidence is available (see fig. 3.5). The remaining morphospecies *G. menardii* (fig. 3.4F), *G. rubescens* (pink) (fig. 3.4H), *G. ungulata* (fig. 3.4G), *N dutertrei* (fig.4I), *P. obliquiloculata* (fig. 3.4J), *G. glutinata* (fig. 3.4C), *S. globigerus* (fig. 3.4M) are either currently represented by single genotypes globally or the evidence for the presence of multiple genotypes is unclear.

3.5.3.1 Globigerinoides ruber

G*lobigerinoides ruber* (figs. 3.4A, 3.5A) is a warm-water specialist, being distributed throughout the tropical to transitional provinces globally, but having no true cold-water representatives (Bé, 1977; Darling *et al*., 2008). This Arabian Sea study is the first to sample a population of *G. ruber* in truly tropical waters and sample numbers are sufficient to provide clues to their ecological adaptations. Despite the great distance separating them genetically (fig. 3.3b), Type I and Type II do not appear to represent the major ecological divisions between *G. ruber* genotypes in the Arabian Sea. Type Ib_2 and IIa both frequent the more eutrophic, high salinity northern water mass in great numbers. Subtypes of both Type I and Type II lineages appear globally widespread within the tropical to transitional provinces (Darling & Wade, 2008). To date they have been found in three subtropical localities: off the Canary Islands (Stewart, 2000), off the Crozet Islands (Darling & Wade, 2008) and in the Mediterranean (Aurahs *et al*., 2009) (fig. 3.5A).


A)
IIa
P
Ib(1)
P
P+Ia+IIa
P+Ia+IIa
Ia+Ib(1)
Ib(2)+IIa
Ia
Ia+Ib(2)+IIa
Globigerinoides ruber
SSU rRNA genotypes
B)
IIa(2)+IIb
Ia(1)
IIa(2)+IIb
Ia(1)+IIa(2)
Ia(1)
Ia(1)+IIa(2)
IIa(2)+IIb
Ia(1)+IIa+IIb
Ia(1)+ IIa(2)
IIb
IIa(2)+IIb
IIa
Ia(1) Ia(2)
IIa(1) IIa(3)
Ia(1)
+
IIa(2)
Ia(2)
IIa(1)
IIb
Globigerinella siphonifera
SSU rRNA genotypes
C)
IIe
IIe
IIa+IId
IIa
IIa
IIa+IIb
IIa
+IIb
IIb
Ib
Ib
Ia
Ia
IIa+IIb
IIa+IIb+IIc
Globigerina bulloides
SSU rRNA genotypes

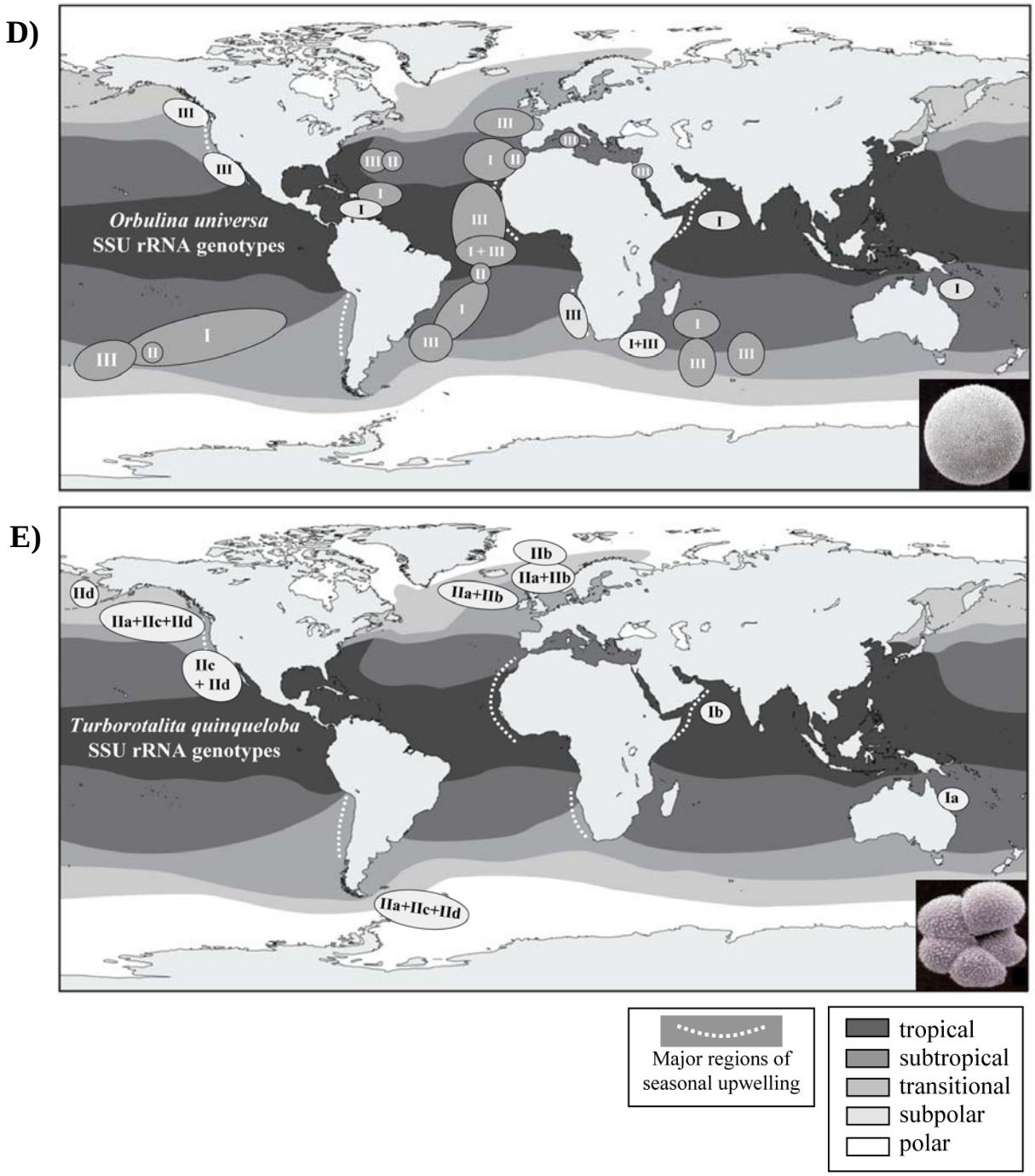


Figure 3.5. The global biogeographical distribution of SSU rRNA genotypes isolated to date for some of the morphospecies found in the Arabian Sea (those for which sufficient data exists). **A)** *Globigerinoides ruber* (Aurahs *et al*., 2009; Darling *et al*., 1997, 1999, 2003; de Vargas *et al*., 1997; Pawlowski *et al*., 1997; Stewart *et al*., 2000), **B)** *Globigerinella siphonifera* (Darling *et al*., 1997, 1999, 2003, 2008; de Vargas *et al*., 2002; Stewart *et al*., 2000), **C)** *Globigerina bulloides* (Darling *et al*., 1999, 2000, 2003, 2007, 2008; de Vargas *et al*., 2007; Stewart *et al*., 2000), **D)** *Orbulina universa* (Darling *et al*., 1997, 1999, 2008; de Vargas *et al*., 1999, 2004), **E)** *Turborotalita quinqueloba* (Darling *et al*., 2000, 2003, 2008; Stewart *et al*., 2001). Genotypes isolated by the Darling *et al*. group are shown in light grey. Those isolated by the de Vargas *et al*., Pawlowski *et al*., or Aurahs *et al*. groups are shown in dark grey. For a full list of collection sites and references see appendix 9.6. The five major planktonic foraminiferal faunal provinces (modified from Bé and Tolderlund, 1971) are shown, together with areas of seasonal upwelling (see key). Modified from Darling *et al*. (2008).

Arabian Sea types Ia, $1b_{(1)}$ and IIa are cosmopolitan, found in both the Indo-Pacific and Atlantic Oceans, whereas Type $1b_{(2)}$ has so far been found only in the Indo-Pacific and is potentially endemic to the region. Considerable evidence suggests that *G. ruber* Types I and II represent a species level divergence, which occurred many millions of years ago (Darling *et al*, 1999). The lack of clear ecological difference between *G. ruber* Types I and II in the Arabian Sea (fig. 3.4A) suggests that this may have been an ancient vicariant divergence, though living in sympatry does not necessarily exclude ecological divergence in planktonic foraminifers (Huber *et al*, 1997). Much more work will have to be carried out to determine whether subtle ecological differences divide the Type I from the Type II lineages within the global population. Ecological divisions were, however, clearly observed at the subtype level between Types Ia and Ib in the Arabian Sea. *Globigerinoides ruber* Type Ia occurred only in the southern, more oligotrophic and low salinity water mass in the Arabian Sea, though their numbers were low (fig. 3.4A). Further, ecological divisions were also observed at the third subtle level between $Ib_{(1)}$ and $Ib_{(2)}$. In both these cases ecological partitioning by the frontal systems are likely drivers of divergence (Schluter, 2001; Darling *et al*, 2004).

3.5.3.2 Globigerinella siphonifera

Globigerinella siphonifera (figs. 3.4B, 3.5B) is a common sub-tropical to tropical morphospecies that diminishes in abundance towards the cooler limits of the transitional province, and is commonly found in boundary currents, upwelling areas and near continental margins (Bé and Tolderlund, 1971). The *G. siphonifera* lineages fall into two main groups: Type I and Type II (fig. 3.3a), both of which are represented in the Arabian Sea. Both the degree of genetic divergence and their

extensive biological differences indicate that these major divisions represent distinct biological species (Huber *et al*, 1997). The Type II lineage again splits into two (Type IIa and IIb), which de Vargas *et al.* (2002) also considered different species due to their distinct biogeographical and ecological differences. Two subtle subtypes of *G. siphonifera* Type I have been sequenced to date ($Ia_{(1)}$ and $Ia_{(2)}$) and both occur in the Arabian Sea. Type $Ia_{(1)}$ appears globally cosmopolitan but Type $Ia_{(2)}$ has yet to be found in the Atlantic (Darling & Wade, 2008) and may be confined within the Indo-Pacific. It is possible that like some other tropical specialists (Darling *et al*, 1999, 2004), $Ia_{(2)}$ may be prevented by its particular specialisation from transiting into the Atlantic Ocean by the cool African Cape corridor. The Type I subtypes occupy the same water column as Type IIa in both the Atlantic and Indo-Pacific, appearing associated with more oligotrophic to mesotrophic conditions with a deep chlorophyll maximum. The Type IIa genotypes harbour a great deal of low-level genetic variation (fig. 3.3a), which has previously been represented as a complex (Darling & Wade, 2008). An examination of all Type IIa sequences to date reveals 3 distinct subtypes (fig. 3.3a). Two of these sub-types were found in the Arabian Sea (IIa $_{(1)}$ and IIa $_{(3)}$). Type $IIa_{(1)}$, found previously at the Great Barrier Reef (Darling *et al*., 1997), shows an Indo-Pacific restricted distribution much like that of Type $Ia_{(2)}$. Type IIa $_{(3)}$, a new subtype found only in the Arabian Sea thus far, is either highly restricted in its distribution or has yet to be found elsewhere. Subtype $IIa_{(2)}$ was not found in the Arabian Sea but is widespread throughout the Atlantic Ocean (determined from GenBank sequences from Darling *et al*., 1997 and de Vargas *et al*., 2002). Type IIb is found widely in the Atlantic (de Vargas *et al*., 2002; Darling & Wade, 2008), but was not present in the northern more eutrophic mixed layer of the Arabian Sea during the SW monsoon. Although normally associated with areas of high primary productivity

and upwelling (de Vargas *et al*., 2002), this genotype appeared absent throughout the cruise transect, though the number genotyped was low.

3.5.3.3 Globigerina bulloides

Globigerina bulloides (fig. 3.4D, 3.5C) is most common in sub-polar regions (Bé and Tolderlund, 1971). It also characterises upwelling zones in lower latitudes (Naidu and Malmgren, 1996), and has been found in lower numbers in association with non-upwelling tropical waters (Darling *et al*., 2000). *Globigerina bulloides* is represented by two main lineages, Type I and Type II, which display distinctly different ecologies. Type I is represented by two known genetic types, which occur in warm tropical to sub-tropical waters, while Type II is represented by a complex of five cool water genotypes (fig. 3.3c), found from polar to transitional waters (Darling & Wade, 2008).

In the Arabian Sea mixed layer, the warm water *G. bulloides* Type Ia was present, and was restricted to the northern eutrophic water mass (fig. 3.4D). This genotype has also been found in the Coral Sea but not as yet in the Atlantic where a different warm water Type, Ib has been identified off the Canary Islands (Stewart, 2000) and in the Mediterranean Sea (de Vargas *et al*., 1997). Type Ib is highly divergent from Type Ia (fig. 3.3c), indicating that they must have been genetically isolated for some considerable time. Type Ia may possibly be confined within the Indo-Pacific, where, like some other tropical specialists (Darling *et al*, 1999, 2004), it may be prevented by its particular specialisation from transiting into the Atlantic Ocean through the cool African Cape corridor.

3.5.3.4 Globigerinoides sacculifer

Globigerinoides sacculifer (fig. 3.4E) forms a major component of the tropical/subtropical assemblage with its peak abundance occurring in a global belt between 20°N and 20°S (Bé and Tolderlund, 1971; Bé, 1977), the highest numbers being observed in the tropics (Darling & Wade, 2008). Globally, it has been collected for genetic study from the Caribbean (Darling *et al*., 1996; de Vargas *et al*., 1997, Pawlowski *et al*., 1997), the Mediterranean Sea (de Vargas *et al*., 1997), the Great Barrier Reef (Darling *et al*., 1997) and the Northwest Pacific (Ujiié & Lipps, 2009). Interestingly, even though this morphospecies exhibits high levels of morphological variation (Bijma & Hemleben, 1994), no intra-specific variation has been detected in its SSU rRNA gene sequences, indicating that gene flow between the global ocean populations must be sufficient to maintain its genetic homogeneity. This is supported by its presence today in the warm Agulhas Current Eddies (Lončarić, 2006), which transports them from the Indian Ocean into the South Atlantic around the African Cape.

3.5.3.5 Orbulina universa

Orbulina universa (figs. 3.4K, 3.5D) is a globally ubiquitous species, but is restricted to water temperatures of between 10 °C and 30 °C (Bé & Tolderlund, 1971). The localised distribution of *O. universa* genotypes is strongly correlated to the level of primary productivity in the water column (de Vargas *et al*., 1999, Darling & Wade, 2008). Considering the global ubiquity and varied adaptations of *O. universa* genotypes, it is interesting that all three genotypes of *O. universa* (Types I-III; de Vargas *et al*., 1999) were virtually absent in the central Arabian Sea in both the

northern and southern water masses. Clearly, the physical conditions prevailing in the central Arabian Sea are not favourable to any genotype of *O. universa*.

3.5.3.6 Turborotalita quinqueloba

The biogeography and evolution of the genotypes of *T. quinqueloba* (figs. 3.4L, 3.5E) has become a point of interest in recent years. *Turborotalita quinqueloba* is primarily known as a sub-polar morphospecies (Bé and Tolderlund, 1971), though it had been recognised in the tropical assemblage (Kroon, 1991). Small numbers of *T. quinqueloba* were found in the Arabian Sea mixed layer during the SW monsoon assemblage along the length of the cruise transect. As in *G. bulloides*, there are two main lineages in *T. quinqueloba,* which exhibit distinctly different ecologies. Type I genotypes are associated with warm waters whereas Type II genotypes are associated with transitional and cool waters (Darling & Wade, 2008). Type Ib, found in the Arabian Sea mixed layer, is new and clusters closely with the warm water Type Ia from the Coral Sea (fig. 3.3D). Together, they are separated by a great distance from the Type II grouping. Whether Type Ib is endemic to the Arabian Sea remains to be determined.

3.6 Conclusions

During the SW monsoon, pronounced environmental conditions lead to a strong disparity between the northern and southern water masses of the Arabian Sea. We find a distinct difference in the distribution and ecology of the planktonic foraminifera of the Arabian Sea mixed layer at this time, segregating morphospecies and genotypes between the high salinity, more eutrophic north and the lower salinity, oligotrophic south. In the north, *G. ruber* dominated, followed by *N. dutertrei, P. obliquiloculata, G. menardii,* and *G. glutinata.* In the south *G. sacculifer* dominated, followed by *G. ruber* and *G. menardii.* For those morphospecies represented by complexes of several discrete genetic types, within the Arabian Sea mixed layer, individual genotypes were found to have distinct ecologies and novel adaptations to differing physical oceanographic conditions. *Globigerinoides ruber* showed a clear ecological distinction between its Type Ia and Types II lineages, supporting past opinions that they represent independent species (Darling & Wade, 2008). Within both *G. ruber* and *G. siphonifera*, subtle sub-types were found to display differing geographical distributions, indicating that they may have individual ecological habits, though it is possible that competitive exclusion may also play some role in their distribution (Aurahs *et al*., 2009). The ability of the genetic types to become specialised and adapted to life in regionally distinct ecosystems is a likely driver of divergence and speciation in the open ocean, running counter to the apparent lack of barriers to gene flow. Differences between core top assemblage data (Cullen and Prell, 1984) and morphospecies counts from the mixed layer during the SW monsoon indicate a level of seasonal variation in morphospecies distribution across the Arabian Sea. It will therefore be important in future work to sample during both the monsoon and inter-monsoon periods for direct comparison.

Comparing the Arabian Sea mixed layer genotypes to those in other regions of the tropical and subtropical global ocean reveals geographical connectivity to other ocean regions, providing clues to ocean circulation, evolutionary drivers and evolutionary history in the planktonic foraminifera. For example, representatives of the major lineages of *G. ruber*, Type I and Type II, are found to coexist globally suggesting ancient vicariant divergence rather than ecological partitioning. However, this study particularly shows that ecological divisions also exist, as demonstrated between the *G. ruber* Types Ia and Ib, and $Ib_{(1)}$ and $Ib_{(2)}$ within the Arabian Sea mixed layer. Another notable pattern indicates an apparent segregation of *G. bulloides* warm water genotypes between the Atlantic Ocean and the Indo-Eastern Pacific Oceans. The *G. bulloides* Type Ib (Atlantic Ocean) and Type Ia (Indo-Eastern Pacific) are highly divergent, suggesting that they have been genetically isolated for some considerable time. In *G. siphonifera*, Type $Ia_{(1)}$ is cosmopolitan whilst Type $Ia_{(2)}$ has been found only in the Indo-Pacific to date. It is likely that the African landmass forms a barrier to the dispersal of tropical/sub-tropical specialists, with the cool and inhospitable waters around the South African Cape impeding their transit between the major oceans (Darling & Wade, 2008). Some more widespread genetic types have clearly overcome this barrier, allowing gene flow to occur on a global scale. *G. sacculifer* has a global distribution and genetic homogeneity indicating that sufficient gene flow between its global populations must exist.

It is clear that in using morphospecies concepts, the true diversity of the planktonic foraminifera has been greatly underestimated, and the individual ecological habits of the genetic types overlooked. The latitudinal provincial definition of the biogeography of the planktonic foraminifera (Bé and Tolderlund, 1971; Bé, 1977) is

simplistic and fails to recognize that multiple genetic types of a morphospecies may co-exist within the same province, whilst actually inhabiting their own ecologically defined niches. The resulting repercussions on the use of planktonic foraminiferal morphospecies as palaeoproxies for past climate change is potentially substantial and demonstrates the value of diversity studies on this scale. Additionally, wide-scale genotyping of the planktonic foraminifera is proving immensely valuable for our understanding of the short and long term global processes involved in marine protist evolution and speciation through time.

3.7 References

Antonov, J. I., Locarnini, R. A., Boyer, T. P., Mishonov, A. V., & Garcia, H. E. 2006. *World Ocean Atlas 2005, Volume 2: Salinity* (ed Levitus, S). NOAA Atlas NESDIS 62, U.S. Government Printing Office, Washington, D.C., 182 pp.

Aurahs, R, Grimm, G. W., Hemleben, V., Hemleben, C., & Kucera M. 2009. Geographical distribution of cryptic genetic types in the planktonic foraminifer *Globigerinoides ruber. Molecular Ecology,* **18**, 1692 - 1706

Banse, K. (1987) Seasonality of phytoplankton chlorophyll in the central and southern Arabian Sea. *Deep Sea Research*, **34**, 713-723

Banse, K., & English D. 2000. Geographical differences in seasonality of CZCS-derived phytoplankton pigment in the Arabian Sea for 1978–1986. *Deep-Sea Research II* **47**, 1623-1677

Banse, K., & English D. C. 1994. Seasonality of coastal zone colour scanner phytoplankton pigment in the offshore oceans. *Journal of Geophysical Research,* **99**, 7323-7345

Bauer, S., Hitchcock, G.,& Olson, D. 1991. Influence of monsoonally-forced Ekman dynamics upon the surface layer depth and plankton biomass distribution in the Arabian Sea. *Deep-Sea Research,* **38**, 531-553

Bé, A. W. H. 1977. An ecological, zoogeographic and taxonomic review of recent planktonic foraminifera. In: *Oceanic Micropaleontology, Volume 1*. (ed Ramsey ATS), Academic Press, London. pp 1-100

Bé, A.W.H., & Tolderlund, D. S. 1971. Distribution and ecology of planktonic foraminifera. In: *The Micropaleontology of Oceans* (eds Funnell BM, Riedel WR), Cambridge University Press, London, pp.105-150

Bijma, J., & Hemleben, C. 1994. Population dynamics of the planktic foraminifer *Globigerinoides sacculifer* (Brady) from the central Red Sea. *Deep-Sea Research*, **41** (3) 485 – 510

Brummer, G. J. A., & Kroon, D. 1988. *Planktonic foraminifers as tracers of ocean climate history*. Free University Press, Amsterdam, p. 6-346

Chaisson, W.P., & Pearson P. N. 1997. Planktonic foraminifer biostratigraphy at Site 925: Middle Miocene-Pleistocene. In: *Proceedings of the Ocean Drilling Program, Scientific Results*, (eds Shackleton NJ, Curry WB, Richter C, Bralower TJ), College Station, TX, 154, 3-32

Clemens, S., Prell, W. D., Murray, D., Shimmield, G., & Weedon, G. 1991. Forcing mechanisms of the Indian Ocean monsoon. *Nature* **353**, 720-725

Cullen, J. L., & Prell, W. L. 1984. Planktonic Foraminifera of the Northern Indian Ocean: Distribution and preservation in surface sediments. *Marine Micropaleontology*, **9**, 1-52

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996a. Molecular phylogeny of the planktic foraminifera. J*ournal of Foraminiferal Research,* **26**, (4), 324-330

Darling, K. F., Kucera, M., Kroon, D., & Wade, C. M. 2006. A resolution for the coiling direction paradox in *Neogloboquadrina pachyderma*. *Paleoceanography,* **21**, PA2011, doi:10.1029/2005PA001189

Darling, K. F., Kucera, M., Pudsey, C. J., & Wade C. M. 2004. Molecular evidence links cryptic diversification in polar plankton to Quaternary climate dynamics. *Proc. Natl. Acad. Sci. USA*, **101**, 7657-7662

Darling, K. F., Kucera, M., & Wade, C. M. 2007. Global molecular phylogeography reveals persistent Arctic circumpolar isolation in a marine planktonic protist. *Proc. Natl. Acad. Sci. USA,* **104**, 5002-5007

Darling, K. F., Kucera, M., Wade, C. M., von Langen, P., & Pak, D. 2003. Seasonal distribution of genetic types of planktonic foraminifer morphospecies in the Santa Barbara Channel and its paleoceanographic implications. *Paleoceanography,* **18**, (2) 1032-1042

Darling, K. F., & Wade, C. M. 2008. The genetic diversity of planktic foraminifera and the global distribution of ribosomal RNA genotypes. *Marine Micropaleontology,* **67**, 216 - 238

Darling, K. F., Wade, C. M., Kroon, D., & Leigh Brown, A. J. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. *Marine Micropaleontol*ogy, **30**, 251-266

Darling, K. F., Wade, C. M., Kroon, D., Leigh Brown, A. J., & Bijma, J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulation. *Paleoceanography,* **14**, 3-12

Darling, K. F., Wade, C. M., Steward, I. A., Kroon, D., Dingle, R., & Leigh Brown, A. J. 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature,* **405**, 43-47

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996b. The isolation and amplification of the 18S ribosomal RNA gene from planktonic foraminifers using gametogenic specimens. In: *Microfossils and Oceanic Environments* (eds Moguilevsky A, Whatley RC), University of Wales, Aberystwyth Press, Chapter 3.1, pp 249-259

de Klasz, I., Kroon, D., & Van Hinte, J. E. 1989. Notes on the foraminiferal genera *Laterostomella* de Klasz and Rerat and *Streptochilus* Brönnimann and Resig. *Journal of Micropaleontology,* **8**, 215-226

de Vargas, C., Bonzon, M., Rees, N. W., Pawlowski, J., & Zaninetti, L. 2002. A molecular approach to diversity and biogeography in the planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Marine Micropaleontology,* **870**, 1-16

de Vargas, C., Norris, R., Zaninetti, L., Gibb, S. W., & Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proc. Natl. Acad. Sci. USA,* 96, 2864-2868

de Vargas, C., Renaud, S., Hilbrecht, H., & Pawlowski, J. 2001. Pleistocene adaptive radiation in *Globorotalia truncatulinoides*: genetic, morphologic, and environmental evidence. *Paleobiology,* **27**, (1), 104-125

de Vargas, C., Sáez, A.G., Medlin, L. K., & Thierstein, H. R. 2004. Super-species in the calcareous plankton. In: Coccolithophores-from molecular processes to global impact (eds Thierstein HR, Young JR), Springer. pp 271-298

de Vargas, C., Zaninetti, L., Hilbrecht, H., & Pawlowski, J. 1997. Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record. *Journal of Molecular Evolution,* **45**, (3), 285-294

Ertan, K. T., Hemleben, V., & Hemleben, Ch. 2004. Molecular evolution of some selected benthic foraminifera as inferred from sequences of the small subunit ribosomal DNA. *Marine Micropaleontology,* **53**, 367-388

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution,* **17**, 368-376.

Findlater, J. 1996. A Major Low-level Air Current Near the Indian Ocean during the Northern Summer. *Quarterly Journal of the Royal Meteorological Society*, **95**, 362-380

Guindon, S., & Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, (5), 696-704

Halicz, E., & Reiss, Z. 1981. Paleoecological relations of Foraminifera in a desert-enclosed sea – The Gilf of Aqaba (Elat), Red Sea. *Marine Ecology*, **2**, (1), 15-34

Hemleben, Ch., Spindler, M., & Anderson, O. R. 1989. Modern Planktonic Foraminifera. Springer Verlag, New York, 363pp

Holzmann, M., & Pawlowski, J. 1996. Preservation of foraminifera for DNA extraction and PCR amplification. *Journal of Foraminiferal Research*, **26**, 264-267

Huber, B.T., Bijma, J., & Darling, K. F. 1997. Cryptic speciation in the living planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Paleobiology*, **23**, 33-62.

Kennett, J. P., & Srinivasan, M. S. 1983. *Neogene Planktonic Foraminifera, a Phylogenetic Atlas*, Hutchinson and Ross, Stroudsburg, Pennsylvania

Kroon, D. 1991. Distribution of extant planktic foraminiferal assemblages in Red Sea and Northern Indian Ocean surface waters. *Revista Española de Micropaleontologia*, **23**, (1), 37-74

Kroon, D., & Nederbragt, A. 1990. Ecology and paleoecology of triserial planktic Foraminifera. *Marine Micropaleontology*, **16**, 25-38

Lanave, C., Preparata, G., Saccone, C., & Serio, G. 1984. A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution,* 20, 86-93.

Larget, B., & Simon, D. L. 1999. Markov chain monte carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution*, **16**, 750-759

Lee, C., Jones, B., Brink, K., & Fischer, A. 2000. The upper-ocean response to monsoonal forcing in the Arabian Sea: seasonal and spatial variability. *Deep-Sea Research II*, **47**, 1177-1226

Lipps, J. H. 1979. The Ecology and Paleoecology of planktic Foraminifera. In: *Foraminiferal Ecology and Paleoecology* (eds Lipps JH, Berger WH, Buzas MA, Douglas RG, Ross CA), SEPM Short Course #6, Houston, Texas, pp. 62-104

Locarnini, R. A., Mishonov, A. V., Antonov, J. I., Boyer, T. P., & Garcia, H. E. 2006. *World Ocean Atlas 2005, Volume 1: Temperature (*ed Levitus S) NOAA Atlas NESDIS 61, U.S. Government Printing Office, Washington, D.C., 182 pp.

Lončarić, N. 2006. Planktic foraminiferal content in a mature Agulhas eddy from the SE Atlantic: Any influence on foraminiferal export fluxes? *Geologia Croatica*, **59**, (1), 41–50

McQuoid, M. R., & Nordberg, K. 2003. The diatom *Paralia sulcata* as an environmental indicator species in coastal sediments. *Estuarine, Coastal and Shelf Sciences,* **56**, 339

Naidu, P. D., & Malmgren, B. A. 1996. A high resolution record of late Quaternary upwelling along the Oman Margin, Arabian Sea based on planktonic foraminifera. *Paleoceanography*, **11**, (1), 129-140

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C., Gouy, M., & Zaninetti, L. 1997. Extreme differences in rates of molecular evolution of foraminifera

revealed by comparison of ribosomal DNA sequences and the fossil record. *Molecular Biology and Evolution*,**14**, (5), 498-505

Prasanna Kumar, S., Madhupratap, M., Dileep Kumar, M., Muraleedharan, P. M., de Souza, S. N., Gauns, M., & Sarma, V. V. S. S. 2001. High biological productivity in the central Arabian Sea during summer monsoon driven by Ekman pumping and lateral advection. *Current Science*, **81**, 1633-1638

Prasanna Kumar, S., & Narvekar, J. 2005. Seasonal variability of the mixed layer in the central Arabian Sea and its implication on nutrients and primary productivity. *Deep-Sea Research II*, **52**, 1848–1861

Prell, W. L., Curry, W. B. 1981. Faunal and isotopic indices of monsoonal upwelling: western Arabian Sea. *Oceanologica Acta*, **4**, 91-98

Reiss, Z., Luz, B., Almogi-Labin, A., Halicz E., Winter A., Wolf M., & Ross D. A. 1980. Later Quaternary Paleoceanography of the Gulf of Aqaba (Elat), Red Sea. *Quaternary Research*, **14**, 294-308

Ronquist, F., & Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572-1574

Rutherford, S., D'Hondt, S., & Prell, W. 1999. Environmental controls on the geographic distribution of zooplankton diversity. *Nature*, **400**, 749-753

Saitou, N., & Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406-425

Schiebel, R., & Hemleben, C. 2005. Modern Planktic Foraminifera. *Paläontologische Zeitschrift,* **79** (1), 135-148

Schiebel, R., Zeltner, A., Treppke, U. F., Waniek J. J., Bollmann, J., Rixen, T., & Hemleben C. 2004. Distribution of diatoms, coccolithophores and planktic foraminifers along a trophic gradient during SW monsoon in the Arabian Sea. *Marine Micropaleontology,* **51** (3-4) 345-371

Schluter, D. 2001. Ecology and the origin of species. *Trends in Ecology and Evolution*, **16**, 372-380

Schott, F. 1983. Monsoon response of the Somali Current and associated upwelling. *Progress in Oceanography,* **12**, 357-381

Schott, F. A., & McCreary Jr., J. P. 2001. The Monsoon Circulation of the Indian Ocean. *Progress in Oceanography,* **51**, 1-123

Smith, S. W., Overbeek, R., Woese, R., Gilbert, W., & Gillevet, P. M. 1994. The genetic data environment and expandable GUI for multiple sequence analysis. *Computer Applications in the Biosciences*, **10**, 671-675

Staden, R. 1996. The Staden Sequence Analysis Package. *Molecular Biotechnology,* **5**, 233-241

Steinke, S., Chiu, H.Y., Yu, P. S., Shen, C. C., Löwemark, L., Mii, H. S., & Chen, M. T. 2005. Mg/Ca ratios of two *Globigerinoides ruber* (white) morphotypes: Implications for reconstructing tropical/subtropical surface water conditions. *Geochemistry, Geophysics, Geosystems,* **6**, doi: 10.1029/2005GC000926

Stewart, I. A. 2000. The molecular evolution of planktonic foraminifera and its implications for the fossil record. Ph.D. thesis, University of Edinburgh.

Stewart, I. A., Darling K. F., Kroon D., Wade C. M., & Troelstra S. R. 2001. Genotypic variability on subarctic Atlantic planktic foraminifera. *Marine Micropaleontology*, **43**, 143-153

Swallow, J. C. 1984. Some Aspects of the Physical Oceanography of the Indian Ocean. *Deep-Sea Research*, **31**, 639-650

Swofford, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Ujiié, Y., & Lipps, J. H. 2009. Cryptic diversity in planktic foraminifera in the northwest Pacific Ocean. *Journal of Foraminiferal Research*, **39**, 145-154

Wang, L. 2000. Isotopic signals in two morphotypes of *Globigerinoides ruber* (white) from the South China Sea: Implications for monsoon climate change during the last glacial cycle. *Palaeogeogr. Palaeoclimatol. Palaeoecol*., **161**, 381-394.

Weikert, H. 1987. Plankton and the pelagic environment. In: *Key environments Red Sea* (eds Edwards AJ, Head SM) Pergamon Press, Oxford, pp. 90-111

White, T. J., Burns, T., Lee S., & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (eds Innis MA, Gelfland DH, Sninsky JJ, White TJ), Harcourt Brace Jovanovich, San Diego, pp 315 – 322

Wiggert, J. D., Murtugudde, R. G., & McClain, C. R. 2002. Processes controlling interannual variations in wintertime (Northeast Monsoon) primary productivity in the central Arabian Sea. *Deep-Sea Research II*, **49**, 2319-2343

Wiggert, J. P., Kohler, K. E., Hood, R. R., & Olson, D. B. 1996. A four-component ecosystem model of biological activity in the Arabian Sea. *Progress in Oceanography*, **37**, 193-240

Williams, D. F., Be, W. H., & Fairbanks, R. G. 1981. Seasonal stable isotopic variations in living planktonic foraminifera from Bermuda plankton tows. *Palaeogeography, Palaeoclimatology, Palaeoecology,* **33**, 71-102.

Wyrtki, K. 1973. An equatorial jet in the Indian Ocean. *Science*, 181, 262-264

Yang, Z. 1993. Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates vary over sites. *Molecular Biology and Evolution*, **10**,1396-1401

Young, D. K., & Kindle, J. C. 1994. Physical processes affecting availability of dissolved silicate for diatom production in the Arabian Sea. *Journal of Geophysical Research-Oceans,* **99**, (C11), 22619-22632

4 Genotypic variability in the planktonic foraminifera of the North Atlantic Ocean

4.1 Introduction

Planktonic foraminiferal sampling of the North Atlantic Ocean allows the exploration of a typical high latitude morphospecies assemblage, and provides a complete contrast to the sampling of the tropical Arabian Sea. The morphospecies and genetic types found here are highly adapted to colder conditions and have thrived in this setting.

At these high latitudes foraminiferal diversity is far lower than that of the tropics or sub-tropics (Bé & Tolderlund, 1971; Rutherford *et al*., 1999). The vertical stratification and niche partitioning, which lead to high diversity in the tropics/sub-tropics, are far less pronounced at higher latitudes. Additionally, high seasonality and instability in the hydrographic conditions mean less permanently stable niche spaces in which species can differentiate (Darling *et al*., 2008). Nevertheless, the high latitude planktonic foraminifera have been studied a great deal, primarily due to their great utility as a paleoproxy for climate change, but also for the information they can provide for investigating patterns of vicariance and gene flow in the global oceans (Darling *et al*., 2000, 2003, 2004, 2006, 2007; de Vargas *et al*., 1999; Stewart *et al*., 2001).

High latitude morphospecies of planktonic foraminifera are highly adapted to cold-water polar and subpolar conditions and most display a clear bipolar, or anti-tropical distribution (Bé & Tolderlund, 1971; Darling & Wade, 2008). Identical genetic types have been found in the polar/ subpolar waters of both the northern and southern hemispheres in several morphospecies, (Darling *et al*., 2000, 2006, 2008), suggesting

a connectivity between the populations. A continual gene flow would be needed to maintain homogeneous bipolar populations and may indicate that individuals are continually being transported across the tropics, which should be highly inhospitable to these cold-water adapted types (Darling *et al*., 2000, 2008).

Some genetic types, however, show highly restricted geographical distributions, indicating localised endemism and a lack of widespread dispersal. This is perhaps surprising in light of the fact that, like many pelagic organisms, the planktonic foraminifera are highly cosmopolitan, and ubiquitously spread throughout the global ocean. They are clearly capable of long-distance transit and dispersal, possessing a high potential for gene flow on a global scale (Darling *et al*., 2000). The existence of rare or isolated genetic types then indicates that in certain cases differentiation is occurring either in allopatry, through vicariance, or in sympatry by ecological partitioning. The data examined in this study indicate that probable examples of both mechanisms may even be operating within a single morphospecies.

This study focuses on the genetic variation in the SSU rRNA gene of mixed layer planktonic foraminiferal morphospecies of the North Atlantic Ocean, collected along a transect from Scotland to Newfoundland in July 2004. The transect traverses both the subpolar (5-10 °C) and transitional (10-18 °C) provinces, offering an excellent opportunity to study the adaptations of within-morphospecies genetic types to localised hydrographical conditions, and thus the process of genetic divergence due to ecological partitioning. 7 morphospecies and their genetic types were identified, including *Globigerina bulloides*, *Orbulina universa*, *Turborotalita quinqueloba* (spinose planktonic), *Neogloboquadrina pachyderma* (left-coiling),

Neogloboquadrina incompta (also known as *N. pachyderma* (right-coiling): see Darling *et al*., 2006; Ottens, 1992), *Neogloboquadrina inflata* (non-spinose planktonic) and *Globigerinita uvula* (non-spinose microperforate planktonic). Phylogenetic analyses were conducted to ascertain the positioning of the North Atlantic genotypes in an all-foraminifera SSU rRNA phylogeny. The localised biogeography of individual genetic types within each North Atlantic foraminiferal morphospecies was investigated, highlighting the process of diversification by ecological partitioning. In addition, the global distributions of the North Atlantic genetic types were examined in order to investigate the processes of global gene flow and vicariance in this marine pelagic group.

The manner in which diversification is occurring within the planktonic foraminifera is certainly complex, involving a combination of the effects of current oceanic circulation, historical global temperature change, and localised ecological conditions. The possible mechanisms by which reproductive isolation, and thereby divergence, is occurring are discussed.

4.2 Aims and Objectives

The aim of this study was to conduct a survey of the planktonic foraminiferal morphospecies, and their component SSU rRNA genetic types, within the typical high latitude setting of the North Atlantic Ocean. Phylogenetic analyses would be employed to elucidate the positions of the North Atlantic morphospecies/ genetic types within the Foraminifera, and their biogeographical distributions across the region examined. An additional study of the global biogeography of these high latitude foraminiferal genotypes would provide insight into the role of both geographical isolation and long-distance dispersal in the diversification of these marine organisms.

4.3 Materials and Methods

4.3.1 Cruise track and oceanographic setting

Specimens of planktic foraminifera were collected at 27 stations along an east/west cruise transect in the North Atlantic Ocean (57˚07'N: 11˚09'W-50˚53'N: 49˚54'W), between Scotland and Newfoundland in July 2004 (fig. 4.1A; cruise Charles Darwin CD159, NERC, collected by M. Carroll). Water depth ranged from approximately 1,000 – 3, 500 m. Figures 4.1B - 4.1E demonstrate the environmental conditions prevailing along the cruise transect. The most notable currents in the area are the North Atlantic (NA) current, which transports warmer water up from the south, and the East Greenland (EG) current, which transports cold water down from the polar Arctic in the north (fig. 4.1B). A temperature and salinity gradient exists between the southeast of the region and the northwest (figs. 4.1C, 4.1D). In terms of foraminiferal provinces, as defined by Bé & Tolderlund (1971), 10 °C represents the approximate boundary at which the transitional waters of the east give way to the colder sub-polar waters of the west, and 5 °C indicates the start of the true polar province. The North Atlantic is a highly productive region, with primary productivity decreasing slightly from north to south (fig. 4.1E).

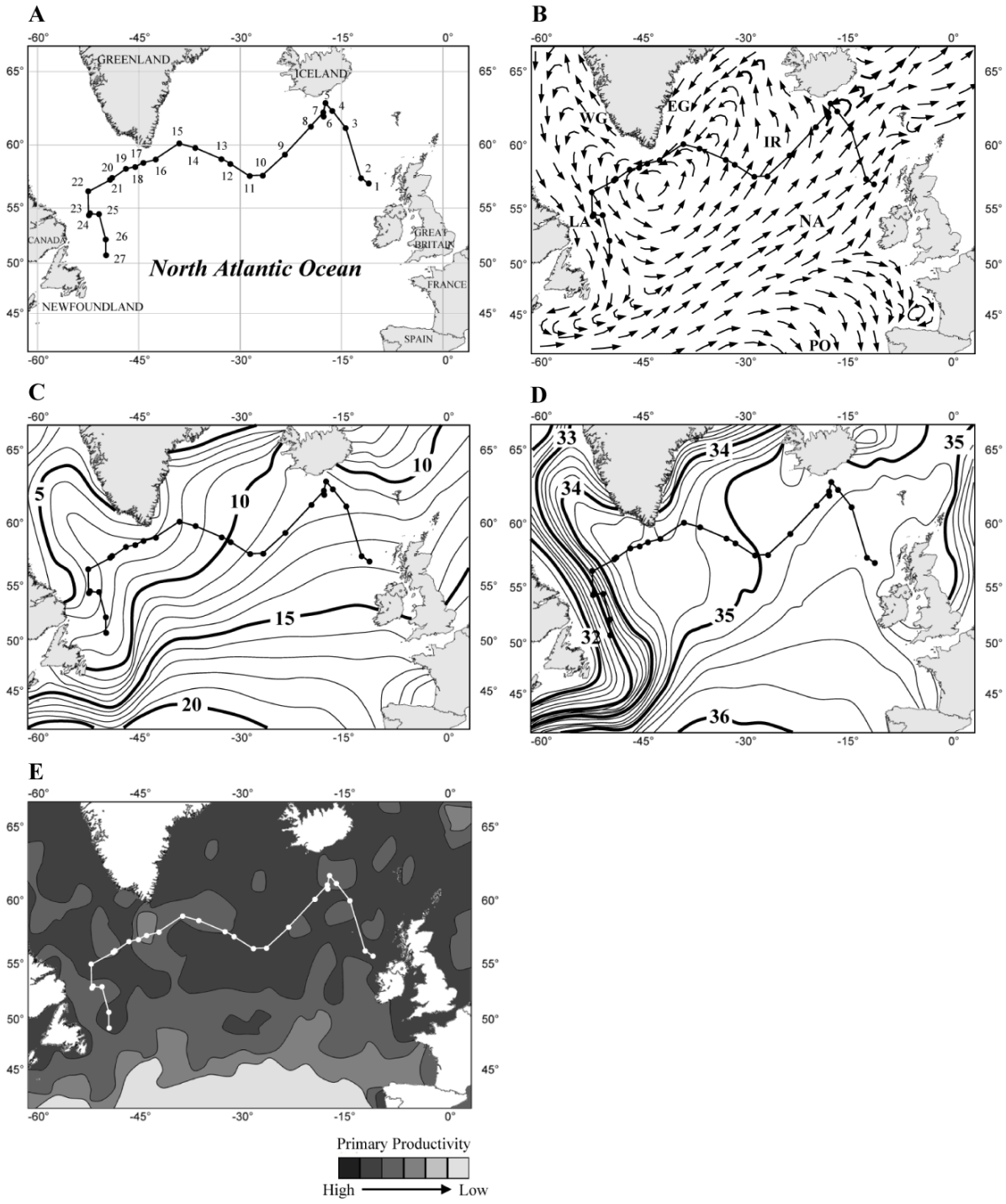


Figure 4.1. Maps of the North Atlantic Ocean showing: (A) CD159 cruise transect and stations, (B) Surface currents during July, modified from U.S. Navy, 1976, Marine Climatic Atlas of the World, Volumes 1 – 5. NA: North Atlantic Current, IR; Irminger Current, EG; East Greenland Current, WG; West Greenland Current, LA; Labrador Current, PO; Portugal Current, (C) Average sea-surface temperature (SST) (°C) for July 2005 (adapted from Locarnini *et al*., 2006), (D) Average sea-surface salinity (PSU) for July 2005 (adapted from Antonov *et al*., 2006), (E) Average primary productivity during July – September 2004 (adapted from Coastal Zone Colour Scanner composite images of the region, NASA Earth-Sun System Division, Earth Sciences (GES) Data and Information Services Center (DISC) Distributed Active Archive Center (DAAC)).

4.3.2 Planktic foraminiferal sampling

Samples of foraminifera were collected by passing water from the ship's 'non-toxic' water supply, pumped from a depth of ~ 6m, through a plankton screen (200 μm mesh) on deck. During unfavourable weather conditions the ship's indoor 'non-toxic' water supply was sieved (150 μm mesh). Vertical tows to 200 m (with a 150 μm mesh) were performed twice, however, no foraminifera were recovered and the method was abandoned. For genetic analysis, a representative sample of specimens was collected at each station. Individual specimens were identified using a stereomicroscope, and morphotype and cytoplasmic colouration were recorded by digital video imaging. Only adult specimens containing cytoplasm were selected for genetic analysis. These were crushed in a lysis buffer (Holzmann *et al*., 1996) and incubated for 1 hour at 60 °C, before being transported to the lab where they were stored at –80 °C. Planktonic foraminiferal sampling was carried out by M. Carroll.

4.3.3 DNA amplification and sequencing

For the identification of the genetic types of planktonic foraminifera found in the North Atlantic Ocean, a nested PCR approach was used to amplify an approximately 500 bp region of the terminal 3′ end of the foraminiferal SSU rRNA gene. A 1° PCR reaction using 3μl template and primer C5 with either primer 138 or NS8 was followed by a 2° PCR using 1μl PCR product from the first round as a template and either primers 2082F and 2514R or primers FS3 and 138. Following poor success rates, an additional pass was made through the failed samples using primers designed for the amplification of the full-length SSU rRNA gene (1° PCR = 56F and 3033R (5 μl template), 2° PCR = 61F and 3024R (1 μl template), 3° PCR = 2082F and 2514R (1

µl template) (see chapter 2, section 2.2.2 for primer sequences and positions). Reactions were performed using Taq polymerase in the first instance, and again using $Vent_R$ polymerase, following limited success. PCR reaction conditions were as described in chapter 2, sections 2.2.3 and 2.2.4. Amplification products were separated by gel electrophoresis and purified using an Eppendorf Perfectprep® Gel Extraction Kit (see chapter 2, sections 2.3 & 2.4). Both sense and antisense strands were sequenced directly on an Applied Biosystems 377 DNA sequencer using Applied Biosystems™ BigDye® v3.1 terminator cycle sequencing (primers 2082F, 2514R) (see chapter 2, section 2.6.5 for details).

4.3.4 DNA sequence analysis and phylogenetic reconstruction

SSU rDNA sequences were assembled using Gap4 in the Staden package (Staden *et al*., 2000) and aligned manually within the Genetic Data Environment (GDE) package (version 2.2) (Smith *et al*., 1994). North Atlantic genotypes were identified by BLAST search and comparison to existing sequences in the alignment. Sequences for the North Atlantic taxa were incorporated into a 407 bp all-foraminifera phylogeny, as previously described in chapters 3, section 3.3.4 (taxa list and ~1,000 bp alignment shown in appendices 9.1 and 9.7.1). To improve resolution, additional phylogenies were constructed for 4 of the North Atlantic morphospecies/groupings, thus allowing a greater number of unambiguously aligned sites to be recruited into the analyses (*Globigerina bulloides* (669 bp), *Turborotalita quinqueloba* (748 bp), the neogloboquadriniids (666 bp), *Neogloboquadrina pachyderma* (811 bp)) (for alignments see appendices 9.7.4, 9.7.5, 9.7.6, & 9.7.7).

Phylogenetic trees were constructed using Bayesian inference (BI; Ronquist & Huelsenbeck, 2003, Larget & Simon, 1999), maximum likelihood (ML; Felsenstein, 1981), neighbour joining (NJ; Saitou & Nei, 1987), Fitch-Margoliash (FM; Fitch & Margoliash, 1967), minimum evolution (ME; Rzhetsky & Nei, 1992), and maximum parsimony (MP; Fitch, 1971) (FM, ME, MP sub- set trees only). In all methods multiple hits were accounted for using a general time-reversible (GTR) model with a gamma (Γ) correction (Lanave *et al*., 1984; Yang 1993) (see chapter 2, section 2.8.2, for details).

4.4 Results

799 specimens of planktonic foraminifera were collected from 27 stations along a cruise transect in the North Atlantic Ocean during July 2004 (Fig.1a). Small subunit rRNA gene sequences were successfully amplified for 164 individual specimens. Eight different genotypes were recognised from 7 mixed layer morphospecies (spinose: *Globigerina bulloides*, *Orbulina universa*, *Turborotalita quinqueloba*; non-spinose: *Neogloboquadrina pachyderma* (left-coiling), *Neogloboquadrina incompta, Neogloboquadrina inflata*; microperforate: *Globigerinita uvula)*. No novel genotypes were discovered in the North Atlantic samples.

4.4.1 Placement of the North Atlantic taxa in the foraminiferal SSU rRNA phylogeny

The placement of the North Atlantic foraminiferal sequences in a comprehensive phylogeny of the foraminifera based on the analysis of 407 bp of the SSU rRNA gene is shown in fig. 4.2. Examples of all planktonic foraminiferal morphospecies and genotypes sequenced to date plus representatives of the major groups of benthic taxa are included. All of the methods of phylogeny reconstruction employed in this study were largely consistent in their inferred trees.

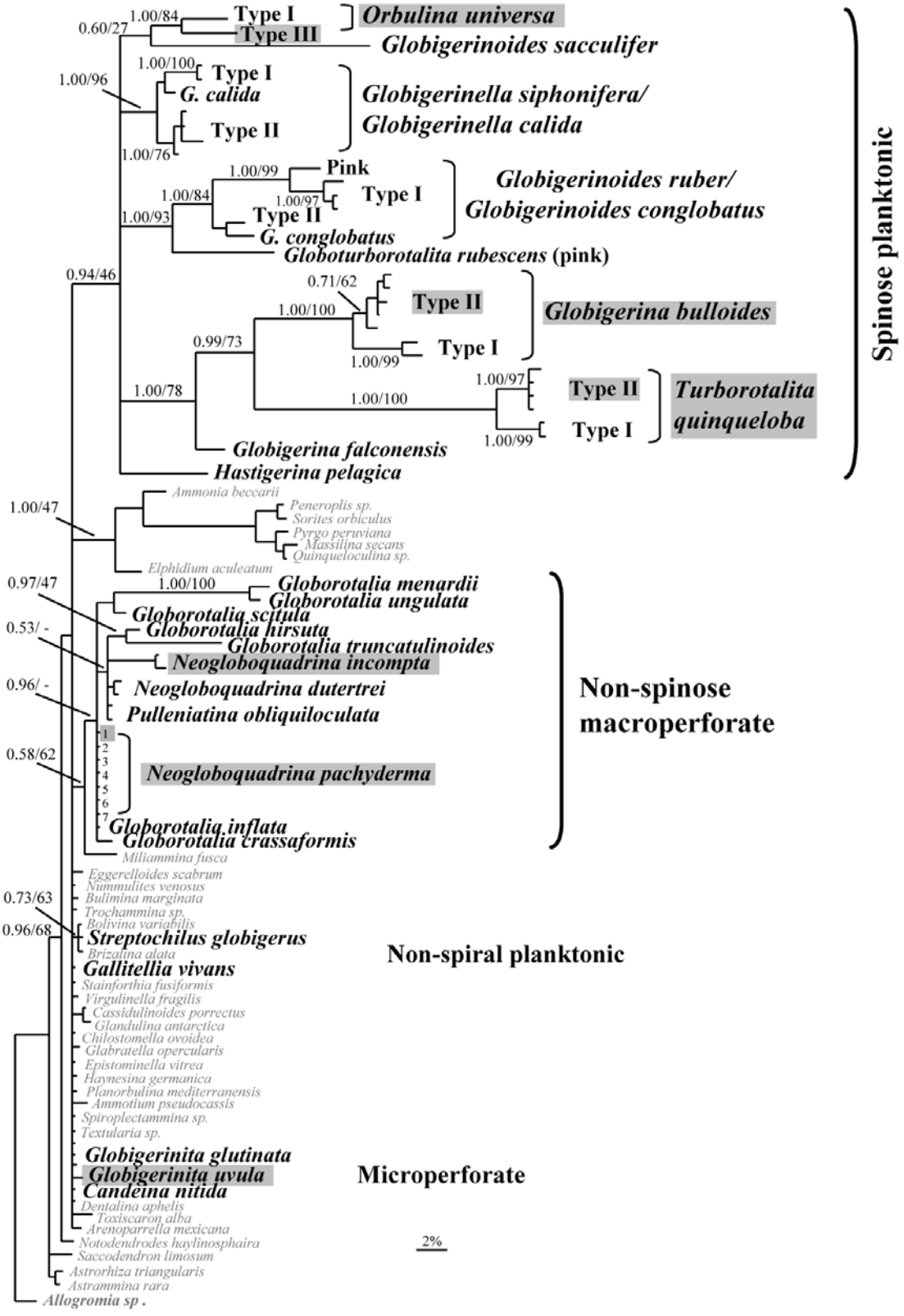


Figure 4.2. Bayesian inference SSU rDNA phylogenetic tree showing the position of the North Atlantic morphospecies and genotypes within the foraminifera. The phylogeny is based on 407 unambiguously aligned nucleotide sites and is rooted on the benthic foraminifer *Allogromia sp.* Bayesian posterior probabilities (from last 1000 trees, obtained within MrBayes) and ML bootstraps (expressed as a percentage, 1000 replicates) are shown on the tree (BI posterior probabilities/ ML bootstraps). The scale bar corresponds to a genetic distance of 2 %. Benthic foraminiferal taxa are shown in grey text, and planktonic foraminifera are shown in black. Morphospecies and genotypes found in the North Atlantic Ocean are shown on a grey background. For taxa list and GenBank accession numbers see appendix 9.1.

4.4.1.1 Spinose planktonic morphospecies

Three of the North Atlantic morphospecies, *Orbulina universa, Globigerina bulloides,* and *Turborotalita quinqueloba* fall into the spinose planktonic region of the foraminiferal phylogeny (p= 0.94 BI, 46 % ML; Fig. 4.2). Only one of the three known genetic types of *O. universa* was identified in the North Atlantic, type III. A phylogeny re-drawn from de Vargas *et al*. (1999), shows the relationships between the three *O. universa* types, with Type III falling as a sister taxa to Type II (fig 4.3a). *Globigerina bulloides* and *Turborotalita quinqueloba* fall as sister taxa in the tree (p= 0.99 BI, 73 % ML; Fig. 4.2), clustering together with *Globigerina falconensis* to form a well-supported group (p= 1.00 BI, 78 % ML). Seven genotypes have been recognised within the *Globigerina bulloides* morphospecies to date and can be separated into two main groups, Type I (warm water adapted) (p= 1.00 BI, 100 % ML, 100 % NJ, 100 % MP), and Type II (cold water adapted) (p= 1.00 BI, 100% ML, 100 % NJ, 100 % MP) (fig. 4.3b) (see also fig. 4.2). The two genotypes of *G. bulloides* collected in the North Atlantic belong to the Type II grouping and were identified as types IIa and IIb. Both cluster together with Types IIc and IId, though with only weak support (p= 0.51 BI, 41 % ML, 63 % NJ, 86 % MP). Type IIe lies separate within the Type II group and may represent an earlier divergence. The 6 genotypes of *Turborotalita quinqueloba* can also be split into two main groups, Type I (warm water) (p= 1.00 BI, 100 % ML, 100 % NJ, 100 % MP) and Type II (cold water) (p= 1.00 BI, 100 % ML, 100 % NJ, 100 % MP) (fig. 4.3c) (see also fig. 4.2). In the North Atlantic, types IIa and IIb were both present. Type IIb falls together with Types IIc and IId in the phylogeny (p= 1.00 BI, 98 % ML, 97 % NJ, 99 % MP). Type IIa falls separate and may represent an earlier divergence.

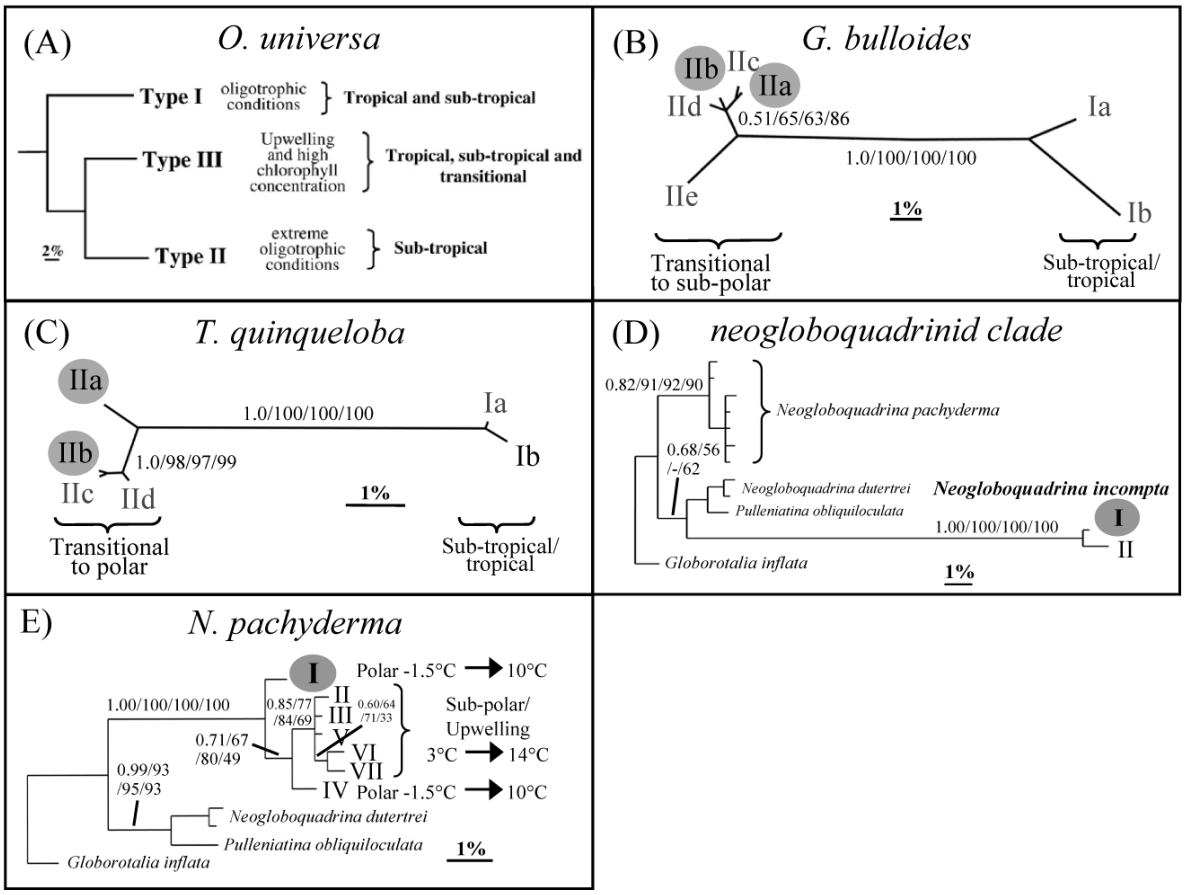


Figure 4.3. SSU rDNA phylogenetic trees of (A) *Orbulina universa* (re-drawn from de Vargas *et al*., 1999), (B) *Globigerina bulloides* (669 unambiguously aligned nucleotide sites) (unrooted), (C) *Turborotalita quinqueloba* (748 bp) (unrooted), (D) neogloboquadrinid clade showing the position of *Neogloboquadrina incompta* (666 bp) (rooted on *G. inflata*), (E) *Neogloboquadrina pachyderma* (811 bp) (rooted on *G. inflata*). The phylogenies were constructed using Bayesian Inference. Bayesian posterior probabilities and ML, NJ, and MP bootstraps (expressed as a percentage) are shown on the trees (BI/ML/NJ/MP bootstraps). The scale bar corresponds to a genetic distance of 1 %. Morphospecies and genotypes found in the North Atlantic Ocean are shown on a grey background.

4.4.1.2 Non-spinose macroperforate morphospecies

Two non-spinose macroperforate morphospecies, *Neogloboquadrina pachyderma* (left-coiling) and *Neogloboquadrina incompta,* were collected in the North Atlantic. Both fall within the monophyletic macroperforate clade, together with *Globorotalia* (*menardii, ungulata, truncatulinoides, crassaformis, hirsuta, scitula and inflata*), *Neogloboquadrina dutertrei,* and *Pulleniatina obliquiloculata* (p= 0.96 BI, - ML) (fig. 4.2) (Clade recovered with all methods of tree construction).

Neogloboquadrina pachyderma and *Neogloboquadrina incompta* are morphologically similar, so much so that *N. incompta* was originally considered to be a right-coiling variety of *N. pachyderma* (Ottens, 1992). Genetic data coupled with geographical distribution and ecology has since led to them being re-defined as separate species (Darling *et al*., 2000, 2004, 2006).

Two genetic types of *N. incompta* have been identified to date and it is Type I that is found in the North Atlantic. A phylogeny of the neogloboquadrinids constructed from 666 bp (fig. 4.3d) shows Types I and II falling together on a long branch (p= 1.00 BI, - ML). The higher resolution of this phylogeny shows *N. incompta* clustering with *N. dutertrei* and *P. obliquiloculata,* though with only low support (p= 0.68 BI, 56 % ML, - NJ, 62 % MP). This relationship was recovered with all methods of tree construction (BI, ML, FM, ME, MP) with the exception of neighbour-joining, which grouped *N. incompta* and *N. pachyderma* together (50 % bootstrap support), as found by Darling *et al*. (2006).

Seven separate genotypes have been recognised within *N. pachyderma* so far (figs. 4.3e), only one of which, Type I, was found in the North Atlantic. In the phylogeny in fig. 4.3e, based on 811 nucleotide sites, the *N. pachyderma* types fall together in a strongly supported clade (p= 1.00 BI, 100 % ML, 100 % NJ, 100 % MP) (fig. 4.3e). Within this clade, Type I appears to represent the earliest divergence, with Type IV then falling as a sister to the remaining *N. pachyderma* types (p= 0.71 BI, 67 % ML, 80 NJ, 49 % MP). Types II, III, V, VI, & VII fall together (p= 0.85 BI, 77 % ML, 84 NJ, 69 % MP), probably representing a more recent diversification. Types VI and VII

cluster together within this group (p= 0.60 BI, 64 % ML, 71 NJ, 33 % MP) and seem to represent the most recent divergence.

4.4.1.3 Non-spinose microperforate morphospecies

Only one microperforate morphospecies, *Globigerinita uvula*, was found in the North Atlantic Ocean. Little molecular work has been carried out on *G. uvula* to date and until recently only a single sequence was available on GenBank (Stewart *et al*., 2001), also derived from a North Atlantic specimen. The sequences collected during the current study showed minor differences to this sequence in the variable regions of the SSU rRNA gene, however, they are unlikely to be significant enough to represent a new genetic type. A possible second genetic type of *G. uvula* has been identified by Aurahs *et al*. (2009), though further work is needed to confirm this identification. Of the 7 microperforate morphospecies thought to exist, only 3 have been sequenced to date (*G. uvula*, *Globigerinita glutinata,* and *Candeina nitida*), and these fall separately among the benthic foraminiferal taxa in the tree (fig. 4.2).

4.4.2 Biogeography

Analysis of the morphospecies genotype distribution data (fig. 4.4) reveals geographical segregation within the North Atlantic Ocean, likely resulting from a difference in ecological requirements of these genetically different types and morphospecies.

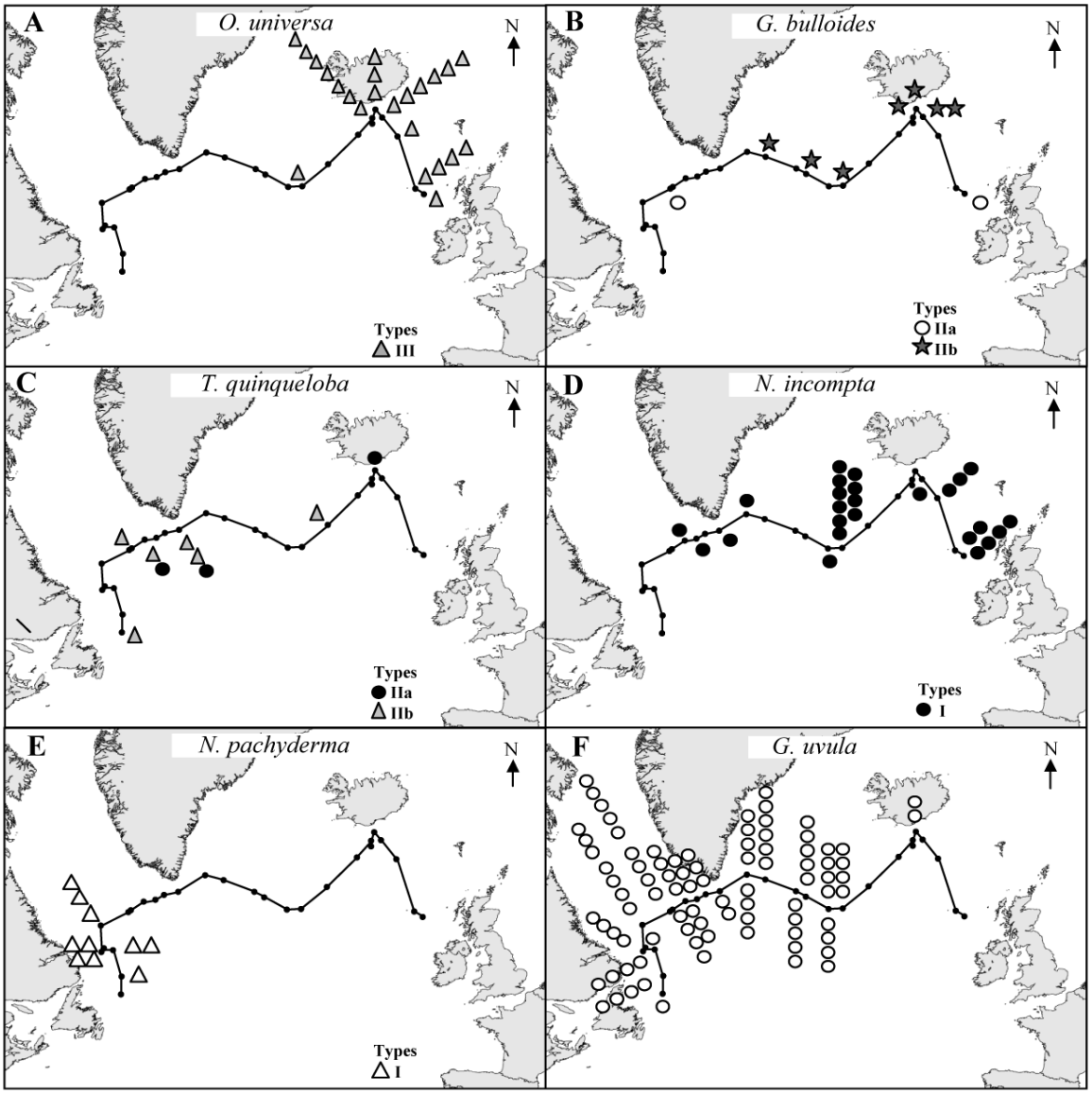


Figure 4.4. North Atlantic Ocean maps showing the spatial distribution of genotypes of the planktonic foraminiferal morphospecies identified along the cruise transect. Their numbers do not necessarily reflect the absolute frequency of morphospecies or genotypes in the water column. A key to genotypes is provided on each map.

*4.4.2.1 **Orbulina universa*** (spinose) (Fig. 4.4A; n=23).

The single genetic type of *Orbulina universa* found in the North Atlantic, type III, was almost entirely restricted in its distribution to the first 7 stations on the transect, in the northeast of the region. It was completely absent in the west, past station 10.

4.4.2.2 Globigerina bulloides (spinose) (Fig. 4.4B; n=9).

Two genetic types of *G. bulloides* were found in the North Atlantic, types IIa and IIb. The majority of samples sequenced belonged to *G. bulloides* Type IIb, which was distributed between stations 4 & 14 on the transect. Only two specimens of *G. bulloides* Type IIa were sequenced successfully, occurring at either end of the transect (stations 1 & 21).

4.4.2.3 Turborotalita quinqueloba (spinose) (Fig. 4.4C; n=9).

Two genetic types of *Turborotalita quinqueloba,* types IIa and IIb, were found in the North Atlantic. Neither were particularly numerous and both occurred along the length of the transect. The apparent increase in frequency towards the west of the region is an artefact resulting from high numbers of PCR failures in this morphospecies.

4.4.2.4 Neogloboquadrina incompta (non-spinose) (Fig. 4.4D; n=24).

Neogloboquadrina incompta Type I was numerous in the North Atlantic from stations 1-19, but was completely absent beyond this point (stations 20-27).

4.4.2.5 Neogloboquadrina pachyderma (non-spinose) (Fig. 4.4E; n=12).

Neogloboquadrina pachyderma Type I was less numerous in the North Atlantic than its sister species *Neogloboquadrina incompta.* It was restricted in its distribution to the south west of the region, from stations 22-27.

*4.4.2.6 **Globigerinita uvula*** (Macroperforate) (Fig. 4.4F; n=87).

Globigerinita uvula was by far the most numerous morphospecies found in the North Atlantic. At the time of this transect study its distribution appeared skewed towards the west of the region (stations 10-27), being largely absent from the east (stations 1-9).

4.5 Discussion

4.5.1 Placement of the North Atlantic foraminiferal morphospecies & genetic types within the SSU rRNA phylogeny

The overall topology of the foraminiferal SSU rDNA phylogeny is consistent with previous studies (Darling *et al*., 1997, 1999, 2000, 2006; de Vargas *et al.,* 1997; Stewart *et al*., 2001). The planktonic spinose species fall together in a monophyletic group separate from the non-spinose species. Amongst the non-spinose foraminifera, the macroperforates fall together in the molecular phylogeny consistent with their taxonomic grouping (Hemleben *et al*, 1989). The non-spinose micro-perforate taxa and the non-spiral biserial planktonic taxa fall separately from the macroperforates, though their placement is ambiguous.

4.5.1.1 Spinose planktonic morphospecies

The placement of the North Atlantic taxa is largely consistent with previous studies. *Orbulina universa* falls together with *Globigerinoides sacculifer* in the spinose planktonic region of the tree, as in Darling *et al*. (1999, 2000, 2006) and Stewart *et al*. (2001), though as in these studies the support is only weak (p= 0.60 BI, 27 % ML) (fig. 4.2). *Orbulina universa* Type III (North Atlantic) fell as would be expected with *O. universa* Type I (Darling *et al*., 1999, 2000, 2006; de Vargas *et al*., 1997, 1999; Stewart *et al*., 2001). The relationship between the 3 known genotypes of *Orbulina universa*, differentiated by their adaptations to differing levels of primary productivity in the oceans (Darling *et al*., 2008; de Vargas *et al*., 1999), is better demonstrated in fig. 4.3a (redrawn from de Vargas *et al*., 1999).

Globigerina bulloides and *Turborotalita quinqueloba* fall together in the spinose region of the tree (p= 0.99 BI, 73 % ML) (fig. 4.2), as is consistent with the phylogenies of Darling *et al*. (2000, 2006) and Stewart *et al*. (2001). Within the complex of genotypes making up the *G. bulloides* morphospecies, the principal split is between Type I (warm-water) and Type II (cold-water) (figs. 4.2 & 4.3b), as was found by Darling *et al*. (2007, 2008). The North Atlantic types, IIa and IIb both fall within the cold-water Type II group (p= 1.0 BI, 100 % ML, 100 % NJ, 100 % MP; Fig. 4.3b). Types IIa, IIb, IIc, & IId fall closely together in the phylogeny, with Type IIe diverging slightly earlier. Within the genetic types of *T. quinqueloba,* the principal division is again between Type I (warm-water) and Type II (cold-water) (Figs. 4.2 & 4.3c). Type II comprises 4 individual sub-types that group together strongly in the tree (p= 1.0 BI, 100 % ML, 100 % NJ, 100 % MP; Fig. 4.3c). Of the types found in the North Atlantic, Type IIa may represent an early divergence in the group, whereas Type IIb clusters closely together with Types IIc & IId (p= 1 BI, 98 % ML 97 % NJ, 99 % MP; Fig. 4.3c). The topology is consistent with previous studies (Darling & Wade, 2008).

4.5.1.2 Non-spinose macroperforate morphospecies

In the non-spinose region of the tree, poor resolution often leads to problems defining firm groupings, however the two morphospecies found in the North Atlantic, *Neogloboquadrina incompta* and *N. pachyderma*, fall as part of a loosely-supported monophyletic macroperforate group (p=0.96 BI, - ML) (fig. 4.2). *Neogloboquadrina incompta* and *N. pachyderma* were once thought to be variants of the same species, but have since been re-designated as separate species based on genetic, morphological, and biogeographical data (Darling *et al*., 2000, 2004, 2006). The two

morphospecies are highly divergent genetically and do not cluster together within the main foraminiferal phylogeny (fig. 4.2). Of the two known genetic types of *N. incompta* (Types I & II), only Type I was found in the North Atlantic. In a phylogeny of the neogloboquadrinid taxa, based on 666 bp (fig. 4.3d), the two types fall together on a long branch (p= 1.00 BI, 100 % ML, 100 NJ, 100 % MP; fig. 4.3d), grouping with *N. dutertrei* and *P. obliquiloculata*, though with only low support (p= 0.68 BI, 56 % ML, - NJ, 62 % MP). The topology was recovered with 5 methods of tree reconstruction (BI, ML, FM, ME, & MP), contradicting the NJ phylogeny of Darling *et al*. (2006), where *N. incompta* diverged from *N. pachyderma*. The current NJ analysis differs from the other methods used, supporting the Darling *et al*. (2006) topology.

The relationships between the 7 genotypes of *N. pachyderma* are poorly resolved within the all-foraminifera phylogeny (fig. 4.2). In the phylogeny based on 811 bp (fig. 4.3e), however, the *N. pachyderma* genotypes fall together in a strongly supported clade (p= 1.00 BI, 100 % ML, 100 % NJ, 100 % MP). Within this clade, Type I, the only *N. pachyderma* type found in the North Atlantic, appears to represent the earliest divergence, with type IV then falling as a sister to the remaining *N. pachyderma* types (p= 0.71 BI, 67 % ML, 80 NJ, 49 % MP). Types II, III, V, VI, & VII fall together (p= 0.85 BI, 77 % ML, 84 NJ, 69 % MP), probably representing more recent diversifications. Types VI and VII cluster together within this group (p= 0.60 BI, 64 % ML, 71 NJ, 33 % MP) and seem to represent the most recent divergence. The topology is consistent with that reported by Darling *et al*. (2004, 2007).

4.5.1.3 Non-spinose microperforate morphospecies

The three microperforate planktonic foraminiferal morphospecies included in the phylogeny, *Globigerinita glutinata*, *Globigerinita uvula* and *Candeina nitida* fall separately among the benthic foraminifera (fig. 4.2). As in previous studies, resolution in this region of the tree is poor (Darling *et al*., 1999, 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001), and although the taxa are closely related (uncorrected p distance (PAUP*) between *G. uvula* & *G. glutinata* = 0.00737; *G. uvula* & *C. nitida* = 0.00737; *G. glutinata* & *C. nitida* = 0.00246), they quite surprisingly do not form a monophyletic group. Only *G. uvula* was found in the North Atlantic.

4.5.2 Biogeography of the North Atlantic planktonic foraminiferal genotypes

4.5.2.1 Orbulina universa

Only a single genetic type of the spinose morphospecies, *Orbulina universa*, Type III, was identified in the North Atlantic. This type showed a pronounced distribution, being almost entirely restricted to the northeast of the region (stations 1-7; Fig. 4.4A, maps). Both SST and salinity are lower here than in the west (fig. 4.1C, 4.1D), however, temperature is likely to be the most important factor in restricting its distribution. *Orbulina universa* is a ubiquitous species, but is restricted to water temperatures of between 10 °C and 30 °C (Bé & Tolderlund, 1971). In the North Atlantic it occurs only in the transitional waters (~ 10 – 18 °C) of the NA current, at a minimum of 11°C (figs. 4.1B, 4.1C, 4.4A), and is absent from the sub-polar waters of the west (~ 10 °C – 6 °C). In addition, localised distribution in *O. universa* is strongly correlated to the level of primary productivity in the water column (de Vargas *et al*., 1999, Darling & Wade, 2008). Type III appears in areas of upwelling and high

chlorophyll concentration, as is found in the most northerly reaches of the North Atlantic. *Orbulina universa* has not been studied in the North Atlantic previously, however, it has been sampled further south, in the transitional waters off the west coast of France (de Vargas *et al*., 1999).

4.5.2.2 Globigerina bulloides

Two genotypes of the spinose morphospecies *G. bulloides*, types IIa and IIb, were identified among the samples collected in the North Atlantic. Both belong to the cool-water type II grouping. The most numerous genotype, Type IIb, was abundant from stations 4 – 14, but absent from the western leg of the transect (stations 15 – 27) (fig. 4.4b). Poor success of PCR amplifications unfortunately meant that only two specimens of *G. bulloides* Type IIa were amplified, making it difficult to determine its geographical distribution in the region. The two Type IIa samples amplified were found at either end of the transect (east & west) (fig. 4.4B), suggesting a broad distribution across the region. *Globigerina bulloides* has been extensively sampled in the North Atlantic (Stewart *et al*., 2001) and though types IIa and IIb co-habit the region, the extent of their distributions is different, reflecting independent ecological adaptations. Both in this study and that of Stewart *et al*., (2001), *G. bulloides* Type IIb was found to be restricted to the east of the North Atlantic, the western limit of its distribution terminating between 30° - 35° W. Stewart *et al*., 2001 recorded high numbers of Type IIa in the northwest of the region, and only small numbers to the east. Sea surface temperature is the primary factor shaping the distributions of these types. Type IIa predominates in the colder subpolar waters (~ 5 – 10 °C) of the East Greenland (EG) Current (figs. 4.1B, 4.1C), whereas Type IIb predominates in the warmer, transitional waters (~ 10 – 18 °C) of the Irminger (IR) Current to the east

(figs. 4.1B, 4.1C) (Stewart *et al*., 2001). Type IIa tolerates colder conditions than Type IIb, extending further north in the Atlantic (Darling & Wade, 2008; Stewart *et al*., 2001), whereas Type IIb has a greater abundance in slightly warmer waters and extends further south (Stewart, 2000). Darling & Wade (2008) report that Type IIa advances north ahead of Type IIb during the spring plankton blooms of the North Atlantic, and is the only genotype present in the most northerly subpolar latitudes.

Globigerina bulloides is a typical eutrophic morphospecies, occurring primarily in high-nutrient environments and during phytoplankton blooms (Deuser *et al*., 1981; Ganssen & Kroon, 2000; Hemleben *et al*., 1989; Kroon, 1988; Ottens, 1991). It has been shown, through carbon isotope analysis, to reflect the northward migrating spring bloom in the North Atlantic Ocean (Ganssen & Kroon, 2000). With greater sampling of the Type IIa and IIb genetic types in the North Atlantic it may be possible to distinguish different nutrient requirements on an intraspecific level, and thus enhance the utility of *G. bulloides* as a proxy for paleonutrients and productivity.

4.5.2.3 Turborotalita quinqueloba

Two genetic types of the spinose morphospecies *Turborotalita quinqueloba* were found in the North Atlantic, types IIa and IIb, both belonging to the cold-water group II genotypes. Neither occurred in very high numbers across the transect (fig. 4C), though it has been noted by Darling *et al*. (2008) that the small size of this morphospecies can mean that individuals pass through the net or sieve used for sampling, leading to their abundance being underestimated. Stewart *et al*. (2001), found types IIa and IIb to co-exist in the east of the North Atlantic, but found only Type IIa in the colder waters of the EG Current in the northwest. We find both types

occurring along the whole transect from east to west, in both transitional (~ 10 – 18 °C) and subpolar (5 – 10 °C) waters (figs. 4.4C & 4.1C). Although the western limit of the current transect passes further south than that of Stewart *et al*. (2001), the Sea surface temperature (SST) at the stations harbouring type IIb (16 & 19; Figs. 4.1A) is actually the same as the EG Current (~ 8 °C; Fig 4.1C). This suggests that low sample numbers were responsible for Type IIb failing to be found in the western part of the Stewart *et al*. (2001) transect, rather than a superior adaptation of type IIa to cooler temperatures. Additionally, in the Norwegian Sea (65°N) only 2 *T. quinqueloba* Type IIa samples were identified compared to 14 of Type IIb, and further north only Type IIb was found (Darling *et al*., 2008). Although sample numbers were low, this points towards an adaptation of Type IIb to colder temperatures than Type IIa, the converse of the findings of Stewart *et al*. (2001). Clearly further sampling is needed to resolve this matter. It can also be noted that the results of this transect put the range of *T. quinqueloba* into the edge of the Labrador Sea (west of the region), an area reported to be devoid of this morphospecies by Bé & Tolderlund (1971).

4.5.2.4 Neogloboquadrina incompta

Until recently *N. incompta* was considered to be the right-coiling variety of *N. pachyderma*. However, they have since been re-designated as separate species based on biogeography, ecology, and degree of genetic distinction (Darling *et al*., 2000, 2004, 2006, 2008). Only a single genetic type was found in the North Atlantic, Type I. *Neogloboquadrina incompta* Type I occurs from station 1-19 and is most numerous in the east but is completely absent from the far west (stations 20-27) (fig. 4.4D). Sea surface temperature is most likely determining the distribution of this genetic type. *Neogloboquadrina incompta* Type I has previously been recorded only in subpolar

and transitional waters (Darling *et al*., 2004, 2006; Stewart *et al*., 2001), and is found restricted to the warmer (subpolar ~ 5 – 10 °C & transitional ~ 10 – 18 °C) waters of the North Atlantic in this study (figs. 4.1C, 4.4D).

4.5.2.5 Neogloboquadrina pachyderma

Neogloboquadrina pachyderma Type I, conversely, only begins its range at station 22 and extends to station 27 (fig. 4.4E). It appears to be cold-water adapted and has only been found in polar waters to date (Darling *et al*., 2004, 2007). Here in the North Atlantic, *N. pachyderma* Type I is most likely being transported down from the polar waters in the north via the East Greenland (EG) Current. Stewart *et al*. (2001) found no *N. pachyderma* Type I in the North Atlantic, only *N. Incompta* (named right-coiling *N. pachyderma* at the time). The transect extended only from the UK to Greenland, passing through the relatively warm North Atlantic (NA) and Irminger (IR) currents (Fig 4.1B). It did not extend to the southwest, into the colder region (stations 16-27, current transect), where the Labrador (LA) current flows. There is a striking pattern in the geographical distribution of *N. incompta* and *N. pachyderma* along the North Atlantic transect. Each morphospecies occupies a geographical range that is completely uninhabited by the other (figs. 4.4D, 4.4E), offering an excellent example of separation due to differing ecological requirements.

4.5.2.6 Globigerinita uvula

The microperforate species *Globigerinita uvula* was the most abundant morphospecies collected along the North Atlantic transect. All samples belonged to a single genetic type, the same as that identified in the same locality by Stewart *et al*. (2001) (note: 5 bp different in variable regions). Stewart *et al*. (2001), however, found only 4

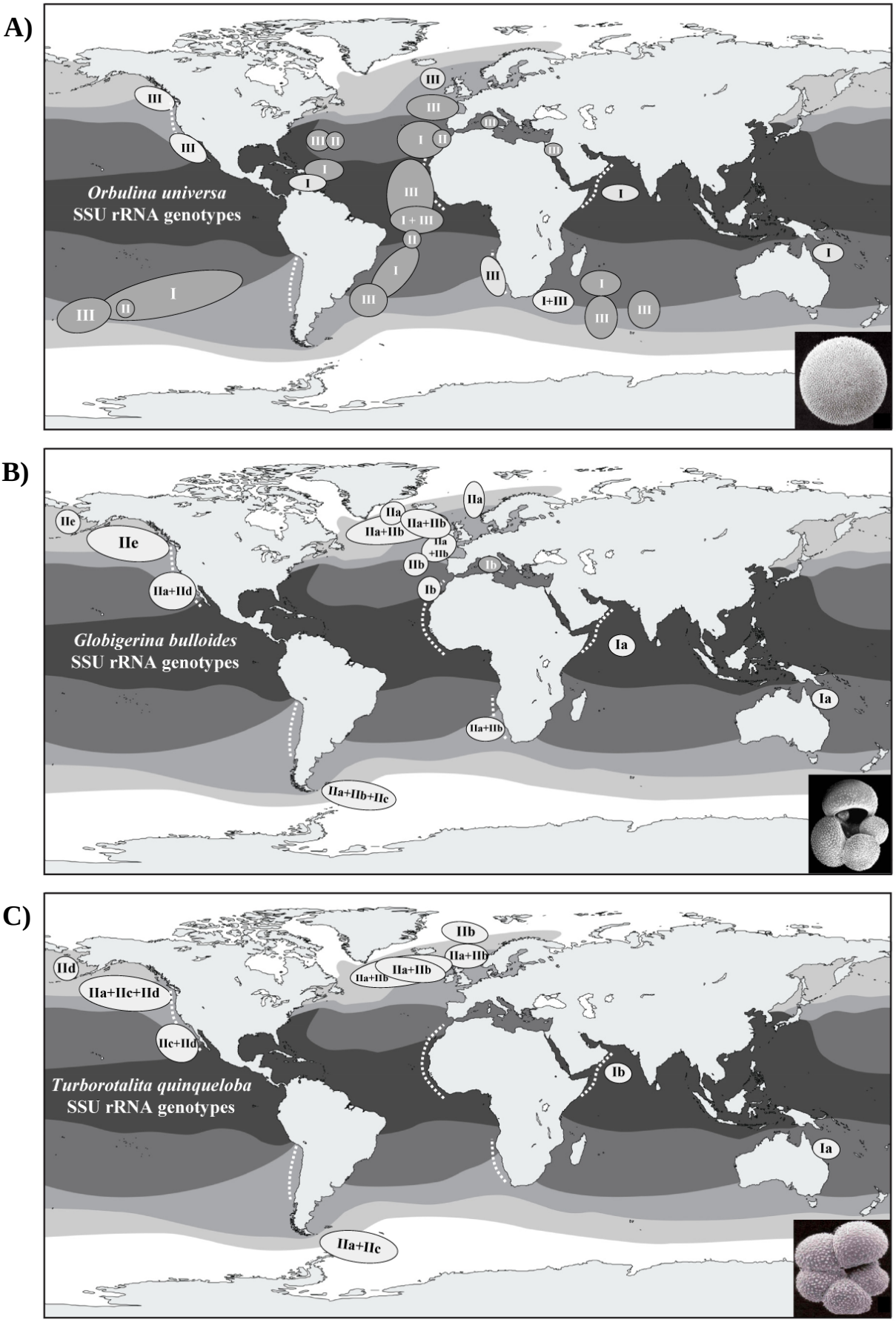
specimens of *G. uvula* in the North Atlantic, 2 to the east of the region, and 2 in the EG current to the northwest. The distribution of *G. uvula* specimens found on the current transect appears heavily skewed towards the west of the region (stations 10-27) (fig. 4.4E), an area not covered by the Stewart *et al*. (2001) transect. Again, the distribution of this morphospecies is likely to be determined by localised differences in SST. Bé (1977) recorded that *G. uvula* (*G. bradyi*) only occurs in subpolar waters, at temperatures of between 5 °C and 10 °C. This finding is supported here where the majority of specimens were found in an area with a SST of 7 °C to 12 °C (figs. 4.1C, 4.4E), consistent with the subpolar province (Bé & Tolderlund, 1971). Some of the samples were found just inside the warmer transitional waters, but only 2 in 87 samples occurred in the transitional waters further to the east (station 5), and are likely to have been transported here passively by the IR current. It is probable that there is an aspect of seasonality in the distribution of planktonic foraminifera, such as *G. uvula*, in the North Atlantic Ocean. The transect of Ottens (1992), bisects the current transct at approximately station 10. In the relatively cool month of April (1988), they recorded low numbers of *G. uvula* at this locality and in the warmer months of August/September (1986), they recorded none.

4.5.3 Global biogeography of the North Atlantic planktonic foraminiferal genotypes

4.5.3.1 Orbulina universa

Orbulina universa (fig. 4.5A) is widely distributed from the tropics to subpolar regions (Hemleben *et al*. 1989), though its abundance peaks in the subtropical and transitional zones (Bé & Tolderlund, 1971). Three genotypes of *Orbulina universa* have been identified, Types I, II, and III (Darling *et al*., 1997, 1999; de Vargas *et al*., 1997, 1999). These have been extensively sampled by de Vargas *et al*. (1999, 2004),

and were originally named as Caribbean, Sargasso, and Mediterranean types respectively. The geographical distribution of the types is strongly correlated with stratification of the water column and primary productivity (Darling & Wade, 2008; de Vargas *et al*., 1999, 2004). Type III, the only type found in the North Atlantic, is found globally in tropical-transitional waters, usually in areas of upwelling and high chlorophyll concentration (reviewed in Darling & Wade, 2008; de Vargas *et al*., 1999, 2004) (fig. 4.5A). The North Atlantic is one such area, boasting high levels of productivity (fig. 4.1E). Type III has the broadest temperature tolerance of the three types, occurring in tropical, subtropical, and transitional waters. Type I occurs only in tropical and subtropical waters (Darling & Wade, 2008; de Vargas *et al*., 1999, 2004) and the rare type II has been found only in the subtropics (de Vargas *et al*., 1997, 1999, 2004). In contrast to *O. universa* Type III, both types I and II are adapted to oligotrophic conditions (Darling & Wade, 2008; de Vargas *et al*., 1999, 2004) and would not be suited to life in the highly eutrophic North Atlantic.


A)
Orbulina universa
SSU rRNA genotypes
B)
Globigerina bulloides
SSU rRNA genotypes
C)
Turborotalita quinqueloba
SSU rRNA genotypes

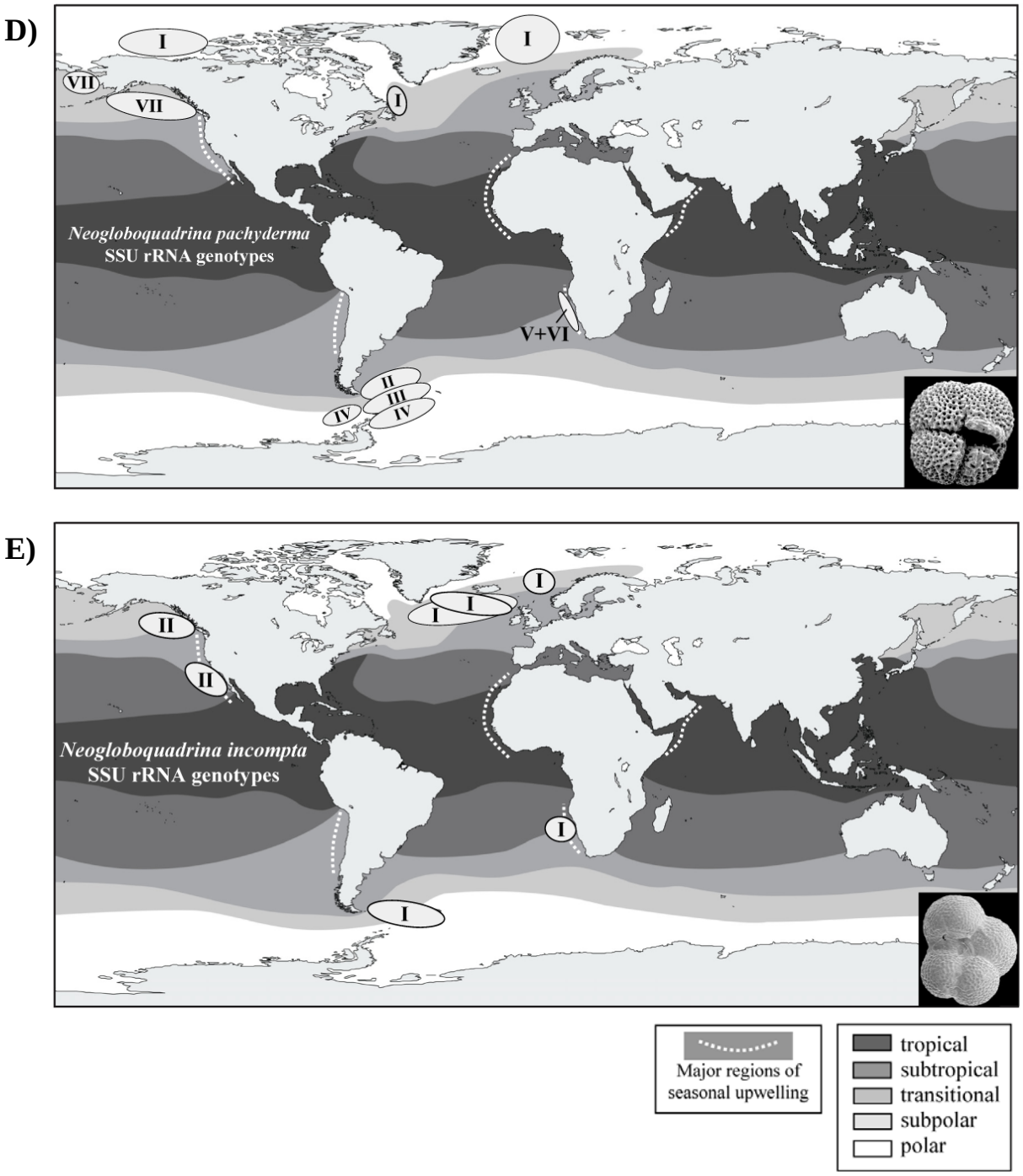


Figure 4.5. The global biogeographical distribution of SSU rRNA genotypes isolated to date for some of the morphospecies found in the Arabian Sea (those for which sufficient data exists). **A)** *Neogloboquadrina pachyderma* (Darling *et al*., 2000, 2004, 2007), **B)** *Neogloboquadrina incompta* (Darling *et al*., 2003, 2006), **C)** *Globigerina bulloides* (Darling *et al*., 1999, 2000, 2003, 2007, 2008; de Vargas *et al*., 2007; Stewart *et al*., 2000), **D)** *Turborotalita quinqueloba* (Darling *et al*., 2000, 2003, 2008; Stewart *et al*., 2001), **E)** *Orbulina universa* (Darling *et al*., 1997, 1999, 2008; de Vargas *et al*., 1999, 2004). Genotypes isolated by the Darling *et al*. group are shown in light grey. Those isolated by the de Vargas *et al*. group are shown in dark grey. For a full list of collection sites and references see appendix 9.6. The five major planktonic foraminiferal faunal provinces (modified from Bé and Tolderlund, 1971) are shown, together with areas of seasonal upwelling (see key). Modified from Darling *et al*. (2008).

4.5.3.2 Globigerina bulloides

Globigerina bulloides (fig. 4.5B) is the most abundantly found planktonic foraminiferal morphospecies in the subpolar province (Bé & Tolderlund, 1971). The genetic types of *G. bulloides* can be split into two phylogenetically divergent groups, Type I and Type II (fig. 4.3B). The Type I genotypes (Ia & Ib) are restricted to the warm-water tropics and sub-tropics (Darling *et al*., 1997, 2008; de Vargas *et al*., 1997; Stewart, 2000), and will not be discussed further. The type II genotypes, of which two were found in the North Atlantic (Types IIa & IIb), occur only in colder waters and are found globally within the transitional to subpolar provinces (Darling *et al*., 2000, 2008; Stewart 2000; Stewart *et al*., 2001). Both Types IIa & IIb show a bipolar distribution in the Atlantic Ocean (Darling *et al*., 2000). In the Pacific Ocean, Type IIb is completely absent and only a single specimen of IIa has been identified, in the transitional waters of the Santa Barbara Channel (Darling *et al*., 2003).

The type II group includes 3 further genotypes that are absent from the North Atlantic. In the Antarctic, Type IIc is found alongside IIa and IIb (Darling *et al*., 2000). In the Pacific, two further types are found, Type IIe, which extends far to the north into cold subpolar waters (Darling *et al*., 2007), and Type IId, which has been found only in the transitional waters of the Santa Barbara Channel (Darling *et al*., 2003). Neither IIe nor IId is present in the Atlantic Ocean.

Within *G. bulloides* some genetic types are bipolar in their global distribution (Types IIa & IIb), pointing to the continual transit of individuals between the poles and a substantial amount of gene flow, whilst others are not bipolar (Types IIc, IId, & IIe) and may have diverged in allopatry as a result of vicariance. Taking into account

phylogenetics and biogeography, it seems likely that an ancestral type of the Type II grouping initially split into two; firstly, the ancestor/precursor to Type IIe, which has remained restricted to the North Pacific (Type IIe) and secondly a precursor type to the rest of the Type II genotypes (IIa, IIb, IIc, IId), which was ubiquitous in its distribution and probably bipolar in both the Atlantic and Pacific Oceans. From this ubiquitous type, the remaining Type II genotypes then diverged. It is possible that Type IIe was restricted from dispersing across the warm tropics due to a more rigid adaptation to cold subpolar waters (Darling *et al*., 2007). Type IIa, which is one of the most recently diverged types, has remained ubiquitous, indicating that it is perhaps capable of transiting the tropics thus maintaining gene flow and a homogeneous global cold-water population. Type IIb is bipolar only in the Atlantic Ocean (fig. 4.5B, Darling *et al*., 2000, 2007, 2008), suggesting that it is currently unable to transit the tropical Pacific. Genetic types that show a more restricted distribution are Types IIc and IId, and these may be more adapted to localised ecological conditions. Type IIc has only been identified in the subpolar Antarctic (fig. 4.5B) (Darling *et al*., 2000), where it likely diverged from the more ubiquitous Type IIa (fig. 4.3B). Type IId has been found to tolerate a wide range of hydrologic conditions, including upwelling and non-upwelling regimes, and the presence or absence of a well-developed thermocline. Yet despite this flexibility, it is only found in the transitional waters of the North Pacific (Santa Barbara Channel; Darling *et al*., 2003, 2008), and may be endemic to this location. It is completely absent from the neighbouring subpolar waters of the North Pacific.

4.5.3.3 Turborotalita quinqueloba

Turborotalita quinqueloba (fig. 4.5C) is one of the five dominant morphospecies within the polar/subpolar assemblage (Bé & Tolderlund, 1971). As in *G. bulloides*, the genetic types of *T. quinqueloba* can be split into two major groups; Type I (warm-water) and Type II (cold-water) (fig. 4.3C). The two Type I genotypes, Types Ia & Ib occur only in the tropics and sub-tropics (Darling *et al*., 1997, 2008) and will not be discussed further. Of the 4 known Type II genotypes, only two, Types IIa & IIb are found in the North Atlantic. Type IIa has also been sampled in the Antarctic (Darling *et al*., 2000), and in the North Pacific (Darling *et al*., 2007), giving it a bipolar distribution in both major oceans, and indicating that transit of individuals may be taking place across the inhospitable tropics, leading to constant gene flow between the poles. Type IIb, conversely, has only been found in the North Atlantic (Darling *et al*., 2008; Stewart *et al*., 2001), possibly indicating an endemism to this region. In the Antarctic Ocean, and the Pacific Ocean (North & South), close relative, Type IIc (fig. 4.3c) is found instead. It seems reasonable to assume that either a precursor of Type IIc, or a ubiquitous ancestor of both types was once present in the North Atlantic, but that trans-tropical transit has since ceased, allowing the new Type IIb to differentiate in allopatry. Type IIb being adapted to very cold waters (as was seen in the Norwegian Sea; Darling *et al*., 2008), may have been prevented from transiting the tropics and mixing with the Southern hemisphere populations due to warmer global temperatures in the recent Quaternary. Type IId is restricted in its distribution to the North Pacific Ocean, being found only in the Santa Barbara channel (Darling *et al*., 2003) and the subpolar North Pacific gyre (Darling & Wade 2008). Again this may reflect an endemism to a single location.

4.5.3.4 Neogloboquadrina pachyderma

Neogloboquadrina pachyderma (fig. 4.5D) is a subpolar to polar specialist that can survive within sea ice (certain genetic types), and withstand salinities of up to 82 ‰ (Hemleben, 1989). It is the dominant species of the Arctic and Antarctic Oceans (Bé & Tolderlund, 1971), and has an anti-tropical distribution. 7 separate genetic types of *N. pachyderma* have been identified to date (Darling *et al*., 2004, 2007, 2008). All are cold-water adapted with. They do, however, have somewhat divergent ecologies, within the subpolar and polar provinces (Darling *et al*, 2008). *Neogloboquadrina pachyderma* Type I is a true polar type, occurring in waters of –1.5 °C to 10 °C (Darling *et al*., 2008). In addition to the North Atlantic, Type I has only been found in the polar waters of the Arctic Ocean (Darling *et al*., 2004, 2007). It appears to be restricted to the Northern hemisphere. Type IV is the only other true polar type, however this is found only in the Antarctic (Darling *et al*., 2004). The remaining 5 genotypes of *N. pachyderma* (Types II, III, V, VI, & VII) are all closely related and are restricted to subpolar waters, of temperatures 3 °C – 14 °C and upwelling systems (Darling *et al*., 2004, 2007, 2008). Type VII is the only type in addition to Type I to be found in the northern hemisphere (North Pacific Subpolar Gyre; Darling *et al*., 2007). Like Type I, it appears to be absent from the southern hemisphere. In fact, none of the genotypes of *N. pachyderma* show a homogeneous bipolar distribution (fig. 5D) (Darling *et al*., 2004, 2008), indicating that the polar populations are currently isolated from one-another, thus preventing gene flow.

The genetic types of *N. pachyderma* provide excellent examples of diversification occurring in both allopatry due to vicariance, and in sympatry due to ecological partitioning (Darling *et al*., 2004, 2007, 2008). Reviewing the phylogenetic and

biogeographical evidence (figs. 4.3E, 4.5D), it seems likely that the modern *N. pachyderma* types originated from a cosmopolitan polar/ subpolar ancestral type, which had a global or global anti-tropical distribution during the last major cooling event of the Tertiary period, 2.5-3.5 Ma (Driscoll & Haug, 1998) (Darling *et al*., 2004). Subsequently, gene flow between the poles has ceased, possibly due to increasing global temperatures transforming the tropics into a barrier, geographically isolating populations at the different poles. The ancestral Type I prevailed in the Arctic, and is found there today (fig. 4.5D). In the Antarctic several genetic types diverged in allopatry, forming two groups in the phylogeny (fig. 4.3E), firstly Type IV which like Type I is a true polar specialist (- 1.6 °C to 10 °C), and secondly a cluster of the remaining *N. pachyderma* types (Types II, III, V, & VI), which are adapted to sub-polar or upwelling conditions (3 °C to 14 °C). Molecular data suggests that the divergence between Type I and the remaining types of *N. pachyderma* occurred ~1.8-1.5 Ma, during the early Quaternary (Darling *et al*., 2004). Observed morphological differences between Arctic and Antarctic populations of *N. pachyderma* (Kennett, 1970) may reflect their divergence (Darling *et al*., 2004).

The divergence between Type IV and the remaining *N. pachyderma* types is thought to have occurred during the mid-Pleistocene ~ 1.1-0.5 Ma (Darling *et al*., 2004). Types II, III, V, VI, & VII cluster together in the phylogeny (Fig. 4.3E), and probably represent a later divergence. Most of these types have likely emerged through adaptation to differing ecological conditions in the subpolar waters of the southern hemisphere (Darling *et al*., 2004), with the exception of Type VII, which is found only in the Northern hemisphere (fig. 4.5D). Darling *et al*. (2004) found Type II to be distributed within the warmer waters of the Subantarctic Front, whereas Type III had a

more widespread distribution. They proposed that the 3 genetic types of *N. pachyderma* found in the Antarctic (II, III, IV) could also reflect the different morphological types of this morphospecies recorded in the Southern Ocean by Kennett (1968). Types V & VI are particularly restricted in their distribution, being found only in the Benguela Upwelling System (Darling *et al*., 2004, 2008), and may be adapted to the specific hydrological conditions of this location. Such types could have been seeded from the Southern Ocean during a glacial period, from subpolar waters advected into the Benguela current system (Darling *et al*., 2004). Type VII is likewise restricted in its distribution, being only found in the North Pacific Subpolar Gyre (Darling *et al*., 2007).

It is interesting to note that the two genetic types found in the northern hemisphere (Types I & VII) are separated by a larger distance phylogenetically than types VI & VII, which are isolated in different hemispheres (fig. 4.3E). Despite their geographical separation, types VI and VII cluster closely in the phylogeny (fig. 4.3E). It could be deduced that a common, bipolar ancestor of Types VI and VII once existed in the Pacific, with a loss of transit across the tropical pacific subsequently leading to Type VII diverging allopatrically in the North Pacific, and Type VI in the Benguela upwelling. The *N. pachyderma* phylogeny (fig. 4.3E) suggests that such an event occurred much later than the original diversification which left Types I and the ancestor of the remaining genetic types isolated in different hemispheres.

4.5.3.5 Neogloboquadrina incompta

Neogloboquadrina incompta (fig. 4.5E) is a true cold-water specialist, being found only in transitional and subpolar waters globally (Darling *et al*., 2006, 2008; Ottens, 1992). Only 2 genetic types of *N. incompta* have been identified to date, Types I & II

(Darling *et al*., 2003, 2006, 2008; Stewart *et al*., 2001). In addition to being present in the North Atlantic, Type I has been found in subpolar Antarctic waters and the Benguela Upwelling System (Darling *et al*., 2006), making it bipolar in the Atlantic. Darling *et al*. (2008) propose that this type is likely to occur in subpolar and transitional waters throughout the whole of the Southern Ocean, being transported by the Antarctic Circumpolar Current. *Neogloboquadrina incompta* Type I has not been identified in the Pacific Ocean as yet; here instead we find the second type of *N. incompta*, Type II. Type II has been found only in the subpolar waters of the Northeast Pacific and is potentially endemic to this area (Darling *et al*., 2006, 2008).

The bipolar anti-tropical distribution of *N. incompta* Type I, in the Atlantic Ocean, suggests that gene flow must be taking place between the polar populations of the Northern and Southern Hemispheres, despite the apparent barrier of the tropics to this cold-water adapted type. However, in the North Pacific Type I is absent, and instead the potentially endemic Type II is found in its place. It seems likely that at one time there was a global, bipolar ancestral type of *N. incompta* (possibly Type I), and that though this type has maintained a bipolar distribution in the Atlantic, through regular gene flow, in the Pacific such gene flow is not occurring, allowing Type II to diverge in allopatry. Certainly a recent divergence of Types I & II is suggested in the phylogeny (fig 4.3D).

4.5.3.6 Globigerinita uvula

Globigerinita uvula is a very small microperforate morphospecies, which is easily distinguished from its close relative *Globigerinita glutinata* by the high spire of its test (Kennett and Srinivasan, 1983). Its peak abundance occurs in the subpolar region,

though it may also be found in polar waters (Bé & Tolderlund, 1971). *Globigerinita uvula* has not been well studied from a molecular viewpoint. Until recently only a single sequence had been published (Stewart *et al*., 2001), representing the same genetic type that was found in the North Atlantic during this current study. Subsequently 5 new sequences have been released, which cluster with the close relative of *G. uvula*, *Globigerinita glutinata* based on analysis of the SSU rRNA gene (Aurahs *et al*., 2009), identifying them as a possible new genotype of *G. uvula*. The findings were not conclusive, however, and further work is needed on the subject. The lack of data makes it impossible to assess the global distribution of genetic types within *G. uvula*, however, as a morphospecies it is said to be typically found in high latitude assemblages (Hemleben *et al*., 1989). Along with *T. quinqueloba* and *N. pachyderma*, it is one of the 3 dominant morphospecies in the polar/sub-polar oceans (Schiebel & Hemleben, 2005). *Globigerinita uvula* primarily inhabits subpolar waters between 5 °C to 10 °C, for example it predominates in the subpolar waters of the North Atlantic and North Pacific (Bé, 1977). It has a bipolar distribution, and in the southern hemisphere has been recorded in the slightly colder waters of the South of Antarctic Polar Front (Bé, 1977). It may also occur in upwelling systems (Benguela current; Oberhänsli *et al*., 1992). The discovery of the potentially new genetic type of *G. uvula* by Aurahs *et al*. (2009) in subtropical waters off the Azores (35.0014 N, 21.0028 W) points to the existence of a possible warm-water type of the morphospecies. In the southwestern Atlantic Ocean, samples of *G. uvula* have also been recorded within cold intrusions in the sub-tropical province (Boltovskoy *et al*., 2000).

4.5.4 Gene flow, vicariance, and speciation in the high latitude planktonic foraminifera

4.5.4.1 Gene flow between polar populations

Studying the geographical distribution of SSU rRNA genotypes in high latitude planktic foraminiferal morphospecies gives great insight into the patterns of gene flow or vicariance across the global ocean (Darling *et al*., 2000, 2004, 2006, 2007). The planktonic foraminifera found at high latitudes, for example the North Atlantic, all display cold-water adaptation, and therefore an anti-tropical distribution globally. Because of the potential barrier of the warmer, inhospitable tropics, the polar populations should exist in complete isolation from one another. The establishment of the polar provinces is thought to have occurred approximately 16 – 8 Ma (Kennett *et al*., 1985; Darling *et al*., 2000), and if these populations had existed in geographical isolation since this time a substantial degree of genetic divergence would be expected. However, as we have seen, 3 separate morphospecies contain homogenous bipolar genetic types (*G. bulloides* Types IIa, IIb, *T. quinqueloba* Types IIa, IIc, IId, and *N. incompta* Type I) (Darling *et al*., 2000, 2006, 2008).

For genetic homogeneity to exist between polar populations in the different hemispheres, genetic exchange must be occurring. The mechanisms by which trans-tropical mixing of foraminiferal populations could occur is unknown, however, there are a number of possibilities. Genetic homogeneity could be sustained by a continual exchange of individuals between the two polar regions. For genetic exchange to be continual, these cold-water adapted genotypes would have to cross the inhospitable waters of the tropics. Darling *et al*. (2000) proposed that cool boundary currents in the east of the subtropical Atlantic Ocean (West African coast) could act as corridors for the introduction of cool-water types into cool seasonal upwelling zones, such as

the Benguela Current. From here, foraminifera could pass passively into the permanent equatorial upwelling zone (2 – 9 °C cooler than surrounding surface water), where a genetic exchange could take place between the northern & Southern hemisphere populations meeting in these waters. However, current circulations in the Atlantic dictate that these populations would have no cool-water corridor back to their respective polar origins, and would need to survive warmer tropical waters in the west of the Atlantic as they were passively transported back to the poles. This is therefore unlikely to be the mechanism operating. A second theory also put forward by Darling *et al*. (2000) was that transit could occur by tropical submergence into the cooler levels of the thermocline, however, without sampling foraminiferal assemblages in these deep waters this cannot be confirmed. It has even been suggested that foraminifera could be the accidental passengers of the Arctic tern (*Sterna paradisaea*) during its annual migration from the Antarctic to its Arctic breeding grounds (Von Hippel, 2001). However, for genetic homogeneity to be reached between foraminiferal populations in the two polar regions, this would have to be occurring on a massive scale.

Another possibility is that genetic exchange between the polar populations is intermittent, occurring only at times when the global climate is cooler. During cooling cycles planktonic foraminiferal subpolar assemblages could expand into the equatorial zone (Darling *et al*. 2000), thereby allowing individuals to pass between the two hemispheres uninterrupted (e.g. during the last glacial period of the quaternary, 1.8 Ma.). Sedimentary records of the lower latitudes show the frequent occurrence of subpolar foraminiferal assemblages within the equatorial zone during these cooling periods (McIntyre *et al*., 1989), indicating that this scenario is quite likely.

The question has been raised as to whether equal levels of genetic exchange take place between the polar populations in the Atlantic and the Pacific. From a study of *N. pachyderma* and *G. bulloides*, Darling *et al*. (2007), concluded that far fewer genetic types displayed a bipolar distribution in the Pacific Ocean than in the Atlantic, suggesting the existence of a more formidable barrier to trans-tropical transport in the Pacific Ocean. However, reviewing the data presented by Darling *et al*. (2008) on the global distributions of the genetic types within 3 morphospecies, *T. quinqueloba, N. incompta* and again *G. bulloides* (figs. 4.5B, 4.5C, 4.5E), it appears that transport between hemispheres may be equally common in both major oceans. To date, 4 bipolar genetic types have been identified in the Atlantic (*N. incompta* Type I, *T. quinqueloba* Type IIa, and *G. bulloides* Types IIa & IIb) and 4 bipolar types in the Pacific Ocean (*T. quinqueloba* Type IIa, IIc, IId, and *G. bulloides* Types IIa) (Darling *et al*., 2008).

4.5.4.2 Allopatric divergence of genetic types

Whilst homogeneous genetic types of planktonic foraminifera have been found separated by huge geographical distances (i.e. at separate poles), suggesting long-distance gene flow, other genetic types represent populations that have diverged in allopatry due to vicariance. Molecular evidence shows that vicariant differentiation and allopatric processes prevail in the polar, more isolated regions (Darling *et al*., 2004), with some morphospecies showing distinct genetic types at each pole. For example, a number of genetic types of planktonic foraminifera are found only in the Southern Hemisphere (Antarctic & Southern Oceans), including *Globigerina bulloides* Type IIc (subpolar), and *N. pachyderma* Types II, III (subpolar), IV (polar), V & VI (Benguela upwelling) (Darling *et al*., 2003, 2004, 2007, 2008). Other genetic

types are harboured only in the Northern Hemisphere, including *N. pachyderma* Type I (polar), *T. quinqueloba* Type IIb (Subpolar/polar) (Arctic & North Atlantic Oceans), and *N. pachyderma* Type VII, *N. incompta* type II, *T. quinqueloba* Type IId, and *G. bulloides* Types IIe & IId (North Pacific Ocean) (Darling *et al*., 2003, 2007, 2008; Stewart *et al*., 2001). *Globigerina bulloides* Type IId is particularly restricted, being found exclusively in the Santa Barbara Channel. The North Pacific has a high species diversity in comparison with the North Atlantic indicating that endemism may be prevalent here. It has been postulated that the region may be the point of origin of many genetic types (Darling *et al*., 2007).

Genetic differentiation has also occurred between populations in the North Pacific and North Atlantic Oceans in certain morphospecies. For example, *G. bulloides* Types IIe and IId, *T. quinqueloba* IId, *N. pachyderma* Type VII, and *N. incompta* Type II occur in the North Pacific Ocean, whereas *G. bulloides* Type IIb, *T. quinqueloba* IIb and *N. incompta* Type I occur in the North Atlantic. The North American land mass presents a formidable barrier to transit between the North Pacific and North Atlantic Oceans, with most types being unable to pass through the extremely cold waters of the Arctic Ocean. However, other factors also affect the transit of planktonic foraminifera across the region.

In *N pachyderma,* Type I is widespread throughout the North Atlantic and Arctic Oceans but is absent from the North Pacific, its transit between the two being prevented by currents flowing predominately from the North Pacific into the Arctic Ocean through the shallow Bering Straight (Darling *et al*., 2007). Likewise, the transit of genetic types from the North Pacific (*G. bulloides* Types IIe and IId, *T.*

quinqueloba IId, *N. pachyderma* Type VII, and *N. incompta* Type II) into the Arctic Ocean and, from there, the North Atlantic is prevented by the inhospitable nature of the Bering Straight. Darling *et al*. (1997) observed that "although there is a considerable flow of water from the North Pacific into the Arctic Ocean that would be expected to carry passively floating plankton north (Woodgate *et al*., 2005), no living planktonic foraminifers were found in the shallow region of the Bering Strait and Chukchi Sea over a distance of ~1,000 km".

4.5.4.3 Sympatric divergence of genetic types

In addition to genetic types developing in allopatry, several genetic types may be found co-habiting in a single region, indicating that divergence is occurring in sympatry. For such localised sympatric divergence to occur either ecological segregation must occur or reproductive isolation (i.e. gametes released on a different time scale). Adaptation to differing sea surface temperatures is one of the most common causes of ecological segregation in the planktonic foraminifera. In the North Atlantic *T. quinqueloba* Types IIa & IIb occur within the same geographical region, however, the range of Type IIb (a potentially endemic type) extends further north (Darling *et al*., 2008), suggesting an adaptation to colder conditions. In the Southern Ocean, *T. quinqueloba* Type IIa also seems adapted to colder waters than Type IIc, occurring south of the Subantarctic Front (Darling *et al*., 2000). In the Southern Ocean, *N. pachyderma* Types II, III, & IV are all found in close geographical proximity, however Type II is restricted to the warmer subpolar waters, Type IV is restricted to the very cold polar waters, and Type III is found in both provinces (Darling *et al*., 2000, 2004). In *G. bulloides*, Type IIa is found in the coldest subpolar waters in both hemispheres (Darling *et al*., 2000, 2008; Stewart *et al*., 2001), whereas

Type IIb is restricted to slightly warmer transitional waters (Stewart *et al*., 2001), as is confirmed in this study. In the North Pacific, *G. bulloides* Types IIa & IId inhabit the warm transitional waters of the Santa Barbara Channel, while Type IIe may be endemic to the cold subpolar waters of the North Pacific Gyre (Darling *et al*., 2007). Type IIc is found only in the Southern Ocean, and is limited to the warmer waters north of the Subantarctic Front (Darling *et al*., 2000).

Temperature is not always the primary factor determining the distribution and segregation of genetic types. The three genetic types of *Orbulina universa*, I, II, III, for example, can be found co-habiting in waters of the same temperature, across multiple provinces globally (de Vargas *et al*., 1999). Here it is the degree of stratification of the water column and therefore levels of primary productivity that determines distribution with Types I & II showing an adaptation to oligotrophic waters, and Type III an adaptation to high productivity areas (de Vargas *et al*., 1999). Indeed, though it has not been specifically investigated, there may be genetic types of other morphospecies that are distributed according to productivity levels. There are a number of types found specifically in upwelling areas, which are known for their nutrient-rich waters. For example, *N. pachyderma* Types V and VI are very specifically adapted to the conditions of the Benguela Upwelling System (Darling *et al*., 2004) and *G. bulloides* Type IId is found exclusively in the Santa Barbara Channel (Darling *et al*., 2007).

4.5.5 Methodological problems

A particularly high rate of failure was observed in the PCR amplifications undertaken during this study, with sequences being gained for only 164 out of 799 specimens. Multiple primer pairs were tested but with limited success. Low success rates are not uncommon in PCR amplifications of foraminiferal samples, however, it is clearly a problem that needs further attention. It may be possible some samples were dead on collection, though the primary cause of the observed PCR failures is likely to rest with the method used to store the samples prior to PCR amplification. In foraminiferal studies the approach almost exclusively used involves the incubation and storage of samples in the lysis buffer of Holzmann *et al*. (1996). Material is then used directly from the buffer for PCR, without a phenol/chloroform or alcohol precipitation stage. It is likely that the samples not only deteriorate over time but that unwanted contaminants from the buffers themselves are carried over into the PCR, inhibiting the reaction. There is a clear need for the development of a new storage and DNA extraction method for use on the foraminifera, a matter that will be covered further in chapter 7. In addition, it may be necessary to re-design the primers used for the PCR amplification of the SSU rRNA gene in the foraminifera and to further optimise the PCR method used. There would also be a great benefit to employing additional molecular markers, to corroborate the results found. A continual source of foraminiferal DNA would be required in both cases, and could be produced in the lab through culturing, as will be discussed in chapter 6.

4.6 Conclusions

The phylogenetic placement of the North Atlantic taxa within the foraminifera was found to be consistent with previous studies. Within the North Atlantic Ocean, patterns in geographical distribution of planktonic foraminiferal SSU rDNA genetic types supports previous evidence of ecological partitioning (Darling *et al*., 2003 2006, 2008; de Vargas *et al*., 1999; Stewart *et al*., 2001), a likely mechanism of diversification in these marine pelagic organisms. Sea surface temperature appears to be the dominant factor governing the geographical distributions of most morphospecies and genetic types within the North Atlantic, though nutrient availability/ productivity has also been shown to affect *G. bulloides* distribution in the region (Ganssen & Kroon, 2000). With further investigation, it may be possible to distinguish different nutrient requirements between the genetic types of *G. bulloides*, greatly enhancing their utility as a proxy for paleonutrients and productivity. The existence of genetically homogeneous populations within some morphospecies at the northern and southern hemisphere poles (Darling *et al*., 2000, 2006, 2008) points to the continual transit of individuals across the inhospitable tropics, though the mechanism by which gene flow is occurring remains unknown. Despite the high dispersal potential of the planktonic foraminifera, the global biogeography of some genetic types, coupled with phylogenetic evidence indicates that allopatric diversification has taken place, possibly as a result of the geographical isolation of these cold-water types during interglacial periods. Finally, is has become clear that the development of superior laboratory methods is needed to improve the yield of PCR amplification in molecular studies of the Foraminifera.

4.7 References

Aurahs, R., Grimm, G. W., Hemleben, V., Hemleben C., & Kucera, M. 2009. Geographical distribution of cryptic genetic types in the planktonic foraminifer *Globigerinoides ruber. Molecular Ecology,* **18**, 1692 – 1706

Bé, A. W. H. & Tolderlund D. S. 1971. Distribution and ecology of planktonic foraminifera. In: *The Micropaleontology of Oceans* (eds Funnell BM, Riedel WR), Cambridge University Press, London, pp.105-150

Bé, A. W. H. 1977. An ecological, zoogeographic and taxonomic review of recent planktonic foraminifera. In: *Oceanic Micropaleontology, Volume 1*. (ed Ramsey ATS), Academic Press, London. pp 1-100

Boltovskoy, E., Boltovskoy, D., & Brandini, F. 2000. Planktonic Foraminifera from south-western Atlantic epipelagic waters: abundance, distribution and year-to-year variations. *J. Mar. Biol. Ass. U.K.*, **79**, 203-213

Darling, K. F, Kucera, M., & Wade, C. M. 2007. Global molecular phylogeography reveals persistent Arctic circumpolar isolation in a marine planktonic protist. *Proc. Natl. Acad. Sci. USA,* **104**, 5002-5007

Darling, K. F., & Wade, C. M. 2008. The genetic diversity of planktic foraminifera and the global distribution of ribosomal RNA genotypes. *Marine Micropaleontology,* **67**, 216 – 238

Darling, K. F., Kucera, M., Kroon, D., & Wade, C. M. 2006. A resolution for the coiling direction paradox in *Neogloboquadrina pachyderma*. *Paleoceanography,* **21**, PA2011, doi:10.1029/2005PA001189

Darling, K. F., Kucera, M., Pudsey, C. J., & Wade, C. M. 2004. Molecular evidence links cryptic diversification in polar plankton to Quaternary climate dynamics. *Proc. Natl. Acad. Sci. USA*, **101**, 7657-7662

Darling, K. F., Kucera, M., Wade, C. M., von Langen, P., & Pak, D. 2003. Seasonal distribution of genetic types of planktonic foraminifer morphospecies in the Santa Barbara Channel and its paleoceanographic implications. *Paleoceanography,* **18**, (2) 1032-1042

Darling, K. F., Wade, C. M., Kroon, D., & Leigh Brown, A. J. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. *Marine Micropaleonto*logy, **30**, 251-266

Darling, K. F., Wade, C. M., Kroon, D., Leigh Brown, A. J., & Bijma, J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulation. *Paleoceanography,* **14**, 3-12

Darling, K. F., Wade, C. M., Steward, I. A., Kroon, D, Dingle, R, & Leigh Brown, A. J. 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature,* **405**, 43-47

Deuser, W. G., Ross, E. H., Hemleben, C., & Spindler, M. 1981. Seasonal changes in species composition, number, mass, size, and isotopic composition of planktonic foraminifera settling to the deep Sargasso Sea. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **33**, 103-127

de Vargas, C, Norris R, Zaninetti, L, Gibb, S. W., & Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proc. Natl. Acad. Sci. USA,* **96**, 2864-2868

de Vargas, C., Zaninetti, L., Hilbrecht, H., & Pawlowski, J. 1997. Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record. *Journal of Molecular Evolution,* **45**, (3), 285-294

Driscoll, N. W., & Haug, G. H. 1998. A Short Circuit in Thermohaline Circulation: A Cause for Northern Hemisphere Glaciation? *Science*, **282**, 436-438

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution,* **17**, 368-376

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783-791

Fitch, W. H. 1971. Toward defining the course of evolution: minimum change for a special tree topology. Systematic Zoology, **20**, 407-416

Fitch, W. M., & Margoliash, E. 1967. Construction of phylogenetic trees. A method based on mutation distances as estimated from cytochrome *c* sequences is of general applicability. *Science,* **155,** 279-284

Ganssen, G. M., & Kroon, D. 2000. The isotopic signature of planktonic foraminifera from NE Atlantic surface sediments: implications for the reconstruction of past oceanic conditions. *Journal of the Geological Society*, **157** (3), 693-699

Guindon, S., & Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, **52**, (5), 696-704

Hemleben, Ch., Spindler, M., & Anderson, O. R. 1989. Modern Planktonic Foraminifera. Springer Verlag, New York, 363pp

Holzmann, M., & Pawlowski, J. 1996. Preservation of foraminifera for DNA extraction and PCR amplification. *Journal of Foraminiferal Research*, **26**, 264-267

Kennett, J. P. 1968. Latitudinal variation in Globigerina pachyderma (Ehrenberg) in surface sediments of the southwest Pacific Ocean. *Micropaleontology* **14**, 305-318

Kennett, J. P. 1970. Comparison of *Globigerina pachyderma* (Ehrenberg) in Arctic and Antarctic. Contrib. Cushman Found. Foram. Res., **21**, 247–50

Kennett, J. P., Keller, G. & Srinivasan, M. S. 1985. Miocene planktonic foraminiferal biogeography and paleoceanographic development of the Indo-Pacific region. *Geol. Soc. Am*. **163**, 197-236

Kennett, J. P., & Srinivasan, M. S. 1983. *Neogene Planktonic Foraminifera, a Phylogenetic Atlas*, Hutchinson and Ross, Stroudsburg, Pennsylvania

Kroon, D. 1988. Distribution of extant planktic foraminiferal assemblages in Red Sea and northern Indian Ocean surface waters. In: *Planktonic foraminifers as tracers of ocean-climate history* (eds. Brummer, G. J. A., & Kroon, D.), Free University Press, Amsterdam, 229-267

Lanave, C., Preparata, G., Saccone, C., & Serio, G. 1984. A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution,* **20**, 86-93

Larget, B., & Simon, D. L. 1999. Markov chain monte carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution*, **16**, 750-759

McIntyre, A., Ruddiman, W. F., Karlin, K., & Mix, A. C., 1989. Surface water response of the equatorial Atlantic Ocean to orbital forcing. Paleoceanography, **4**, 19-55

Oberhänsli, H., Bénier, C., Meinecke, G., Schmidt, H., Schneider, R., & Wefer, G. 1992. Planktic foraminifers as tracers of ocean currents in the eastern South Atlantic. *Paleoceanography*, **7**, 607–632

Ottens, J. J. 1992. April and August Northeast Atlantic surface water masses reflected in planktonic foraminifera. *Netherlands Journal of Sea Research*, **28** (4), 261-283

Ronquist, F., & Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572-1574

Rutherford, S., D'Hondt, S., & Prell, W. 1999. Environmental controls on the geographic distribution of zooplankton diversity. Nature, **400**, 749-753

Rzhetsky, A., & Nei, M. 1992. A simple method for estimating and testing minimum-evolution trees. Mol. Biol. Evol. **9**, 945-967

Saitou, N., & Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 407-425

Schiebel, R., & Hemleben, C. 2005. Modern Planktic Foraminifera. *Paläontologische Zeitschrift,* **79** (1), 135-148

Smith, S.W., Overbeek, R., Woese, R., Gilbert, W., & Gillevet, P.M. 1994. The genetic data environment and expandable GUI for multiple sequence analysis. *Computer Applications in the Biosciences*, **10**, 671-675

Staden, R., Beal, K. F., & Bonfield, J. K. 2000. The Staden package, 1998. Methods in *Molecular Biology*, **132**: 115-130

Stewart, I. A. 2000. The molecular evolution of planktonic foraminifera and its implications for the fossil record. Ph.D. thesis, University of Edinburgh

Stewart, I. A., Darling, K.F., Kroon, D., Wade, C. M., & Troelstra, S. R. 2001. Genotypic variability on subarctic Atlantic planktic foraminifera. *Marine Micropaleontology*, **43**, 143-153

Swofford, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Von Hippel, F. A. 2001. Evolution not all at sea. *Journal of Biogeography*, **28**, 673

Woodgate R., Aagaard, A. K., & Weingartner, T. J. 2005. *Deep Sea Res* Part II, **52**, 3116–3149

Yang, Z. 1993. Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates vary over sites. *Molecular Biology and Evolution*, **10**,1396-1401

5 Phylogenetic Relationships of the Planktonic Foraminifera

5.1 Introduction

5.1.1 Adopting the planktonic mode of life

Of the estimated 10, 000 extant species of foraminifera (Vickerman, 1992), nearly all inhabit the benthic environment, dwelling in marine and fresh-water sediments. A relatively small number of foraminiferal species, by comparison, totalling approximately 40 – 50 and belonging to 15 genera, have left the benthos and adopted a planktonic mode of life, free-floating in the water column (Kennett & Srinivasan, 1983; Saito *et al*., 1981; Hemleben *et al*., 1989).

The planktonic oceanic environment affords numerous advantages, including an extensive geographic area, the possibility for rapid dispersal by currents, and the availability of resources, including, light, carbon dioxide, oxygen, dissolved nutrients, and for zooplankton like the foraminifera, a rapidly regenerating source of food (e.g. phytoplankton prey) (Tappan & Loeblich, 1973).

Life in the planktonic environment requires considerable morphological adaptation, which is reflected in the convergent evolution of such adaptations in the many groups of organisms that have taken this path (Tappan & Loeblich, 1973). An explosive phase of adaptive radiation usually follows when an organism first enters the plankton, with selective pressures tending to modify those structures that are particularly concerned with life in the new environment (Mayr, 1960; Tappan & Loeblich, 1973).

Some distinctive morphologies have developed in the planktonic foraminifera that aid passive suspension in the water column, an essential feature for planktonic life. In the spinose taxa, elaborate radial spines and a network of pseudopodia provide a high surface-to-volume ratio, aiding both suspension and feeding, and may afford some protection against predation. Similar forms are evident in the Radiolaria (De Weaver *et al*., 2001; Petrushevskaya *et al*., 1976). Other taxa, such as the non-spinose foraminifera, *G. menardii* and *G. ungulata* possess a flattened keel structure, which may be positioned to maintain the largest possible area at right angles to the direction of sinking. Large or numerous perforations in the calcareous test of most planktonic foraminifera reduce the weight of the test, allowing the ornate morphologies to develop (Bolli *et al*., 1957; Douglas & Savin, 1972; Lipps, 1966; Tappan & Loeblich, 1973). Such features aid organisms whose method of 'flotation' could be more accurately described as a controlled descent (Ruttner, 1963), however, other adaptations are employed to aid buoyancy.

In certain planktonic organisms, position in the water column may be maintained by the use of fat globules within the cytoplasm (e.g. in the green alga, Botryococcus; Fogg, 1965), or gas vacuoles (e.g. in marine blue-green algae such as *Trichodesmium*, bacteria, and certain protozoans; Tappan & Loeblich, 1973). It is not clear if such structures exist in the planktonic foraminifera, though certain fibrillar bodies found in both spinose and non-spinose taxa may be linked to this function (Anderson & Bé, 1976).

The buoyancy necessary for planktonic life may have originally evolved as a temporary mechanism in the benthic foraminifera, which like other sessile organisms

are negatively buoyant, allowing them to stay fixed in the sediment. Temporary buoyancy mechanisms exist in a number of benthic organisms, for example in the extant benthic foraminifer, *Rosalina globularis*. Here megalospheric individuals, produced asexually, may develop a large globular float chamber in order to release flagellated gametes into the surface waters, aiding dispersal (Tappan & Loeblich, 1973). Other organisms, such as the blue-green alga, *Lyngbya*, form gas "pseudovacuoles" in order to avoid unfavourable conditions in lake sediments by rising to the surface (Hutchinson, 1967). Similar temporary adaptations have been noted in the rhizopod thecamoebian, *Arcella*, which forms gas bubbles in its cytoplasm to self-right it, and in *Difflugia,* which uses oil globules and a gas vacuole to become planktonic for ~ 4 months a year (Schönborn, 1962).

Whatever the mechanism, the planktonic foraminifera have become an extremely successful and ubiquitous part of the marine plankton. The question to be asked now is did the extant planktonic foraminifera originate from a single benthic ancestor, which made a chance transition, or has the event occurred numerous times, giving rise to separate modern lineages?

5.1.2 Paleontological evidence for the origins of the planktonic Foraminifera

Due to their excellent preservation as microfossils, the evolution and phylogenetics of the Foraminifera has been well studied and their classification, based on morphological characteristics of the test, thoroughly catalogued (Cushman, 1948; Decrouez, 1989; d'Orbigny, 1826; Kennett & Srinivasan, 1983; Loeblich and Tappan, 1987, 1992; Pearson, 1993). The earliest benthic foraminifera, identified as simple agglutinated forms, appear in the fossil record, during the Early Cambrian (~ 540 Ma

ago) (Culver, 1991). The planktonic foraminifera appear far later, during the Early Jurassic period (~ 180 - 200 Ma ago) (Caron & Homewood, 1983; Görög, 1994; Loeblich & Tappan, 1974), indicating that the planktonic foraminifera evolved from pre-existing benthic ancestors, as is the case in a number of other groups of organisms (Tappan & Loeblich, 1973).

Though the stratigraphic ranges of the planktonic foraminifera are well known (Kennett & Srinivasan, 1983), their precise origin within the benthic group remains elusive, as the far-reaching morphological changes that accompany movement into the planktonic habitat have obscured their relationships and ancestry (Tappan & Loeblich, 1973). The traditional view, reflected in current classifications, is that the extant planktonic foraminifera represent a single monophyletic lineage, first appearing in "globigerinid" form in the Middle to Early Jurassic (~180 – 200 Ma ago) (Caron & Homewood, 1983; Decrouez, 1989; Görög, 1994; Kennett & Srinivasan, 1983; Loeblich & Tappan, 1974, 1987, 1992). Some evidence suggests that they may have evolved from a single lineage of small benthic foraminifera, the Oberhauserellidae (Tappan & Loeblich, 1988), though other occurrences of small globular forms in the fossil record during the Middle and Late Jurassic may indicate independent adaptations to the planktonic mode of life (Wernli, 1988).

Throughout their history, the planktonic foraminifera have undergone numerous alternating periods of extinctions and radiations (Banner & Lowry, 1985, Tappan & Loeblich, 1973), the most devastating extinction event occurring at the boundary of the Cretaceous and Tertiary (K/T) periods (65.5 Ma ago) (Brinkhuis & Zachariasse, 1988; Liu & Olsson, 1992; Olsson *et al*., 1999), with a second major event at the

Eocene/Oligocene boundary (34 Ma ago) (Bolli, 1986). It is assumed that subsequent planktonic radiations, following major extinctions, evolved from surviving planktonic forms (Norris, 1991; Olsson *et al*., 1992; Tappan & Loeblich, 1988), rather than arising from new adaptations to the planktic habitat from the benthos. Olsson *et al*. (1992) and Culver (1993), for example, propose that all Cenozoic globigerinids derived from two Cretaceous genera (*Guembelitria* and *Hedbergella*), survivors of the K/T extinction.

5.1.3 Molecular evidence for the origins of the planktonic foraminifera

It is, however, possible that the move from benthos to plankton has occurred numerous times throughout the history of the foraminifera. In contrast to their assumed monophyly, according to traditional classifications, molecular phylogenetic analyses of the SSU rRNA gene in fact suggest that the planktonic foraminifera are polyphyletic in origin, arising from more than one benthic ancestor on independent occasions (Darling *et al*., 1997; de Vargas *et al*., 1997). It is not precisely known how many transitions may have occurred from the benthic to planktonic mode of life (de Vargas *et al*., 1997), however, molecular phylogenies, based on an ~1,000 bp partial 3′ terminal fragment of the SSU rRNA gene, suggest that there may be at least 3 independent extant lineages of planktonic foraminifera (Aurahs *et al*., 2009b; Darling *et al*., 1997, 1999, 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001), broadly consistent with the morphological groupings of the spinose, non-spinose macroperforate, and non-spinose microperforate planktonic groups (Hemleben *et al*., 1989).

The placement of the planktonic foraminifera in 3 broad groups is consistently recovered in molecular studies, across many methods of tree reconstruction (Aurahs *et*

al., 2009b; Darling *et al*., 1997, 1999, 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001), however, poor resolution of the deep relationships within the SSU rDNA phylogenies has left the precise benthic origins of the major planktonic groups uncertain. Furthermore, the positions of certain taxa within the phylogeny are often inconsistent between different methods of tree reconstruction (de Vargas *et al*., 1997; Pawlowski *et al*., 1997), and bootstrap support for the major groupings may be low. The monophyletic spinose planktonic clade (Globigerinidae & Hastigerinidae), for example, has gained high bootstrap support in some analyses (Darling *et al*., 1997, 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001), but low bootstrap support in others (Darling *et al*., 1999; de Vargas *et al*., 1997; Pawlowski *et al*., 1997). The non-spinose macroperforate grouping (Globorotaliidae, & Pulleniatinidae) too generally receives low bootstrap support (Darling *et al*., 2006; de Vargas *et al*., 1997). In addition, in areas where only small genetic distances exist between closely related taxa, little phylogenetic structure may be seen (e.g. between *Globigerinita glutinata* and *Globigerinita uvula*; Darling *et al*., 2006; Stewart *et al*., 2001, or between the genetic types of *Neogloboquadrina pachyderma*; Darling *et al*., 2000, 2006).

The unusually variable, and sometimes extreme rates of rDNA evolution seen in the foraminifera (Darling *et al*., 1999; de Vargas *et al*., 1997; de Vargas & Pawlowski, 1998; Pawlowski *et al*., 1997) may in part be responsible for some of the difficulties associated with the phylogenetic analyses, however, a lack of informative data may also be responsible. To date, molecular studies of the planktonic foraminifera have utilised only the last ~1,000 bp of the SSU rRNA gene, approximately a third of its length in the foraminifera, and of these only between ~ 400 and 500 nucleotide sites can be unambiguously aligned across all taxa, for use in phylogenetic analysis

(Darling *et al*., 1997, 1999, 2000, 2006; de Vargas *et al*., 1997; Pawlowski *et al*., 1997; Stewart *et al*., 2001) (see chapters 3 & 4). There is clearly a pressing need to increase the amount of data being used in order to resolve some of the difficulties encountered.

5.2 Aims and Objectives

The primary aim of this study was to determine how many independent extant lineages exist within the planktonic foraminifera and to elucidate their origins within the benthic foraminifera. The approach was two-fold; firstly the phylogenetic relationships of the planktonic foraminifera were re-explored using the traditional ~1000 bp terminal 3´ fragment of the SSU rRNA gene (with a comprehensive list of benthic and planktonic taxa), and secondly the problems of poor resolution and support in previous phylogenies were addressed, using phylogenetic analyses based on the almost complete SSU rRNA gene.

5.3 Methods

5.3.1 Planktic foraminiferal specimens

Planktic foraminiferal specimens were obtained via research vessel cruise (various locations), with collections made via plankton net tow, or pumping of the ship's non-toxic water supply. Details of the specimens successfully amplified in PCR, and their origins are listed in appendix 9.1.

5.3.2 DNA amplification and sequencing

DNA amplification and sequencing of the partial ~1,000 bp terminal 3′ fragment of the SSU rRNA gene was as described in chapters 3 (see also general materials and methods, chapter 2).

For the amplification of the almost complete SSU rRNA gene (approximately 3000 bp; see chapter 2, section 2.2.2 and fig. 2.2), a nested PCR approach was employed, utilising 3 rounds of amplification. For planktonic foraminifera, which unlike the benthic foraminifera do not bear multiple copies of their genome, 3 rounds of amplification were necessary in order to produce strong bands, one more than for the partial ~1,000 bp fragment. Many combinations of both existing and newly designed foraminifera-specific primers were experimented with. Trials were conducted using a range of 5′ primers (NS1, 28F, 56F, 58F, 61F, & 202F), and 3′ primers (3009R, 3014R, 3024R, 3028R, NS8, 3031R, 138, & 3033R) in the 1°, 2° and 3° rounds of PCR (see chapter 2, section 2.2.2 for primer positions and sequences). The greatest success was gained using primers 56F and 3033R in the 1° PCR (5 μl DNA template), followed by a 2° PCR using primers 61F and 3024R (1 μl DNA template), and a 3°

PCR using primers 199F and 3014R (1 μl DNA template) (see chapter 2, section 2.2.2 for primer positions and sequences). Two species, *Globorotalia menardii* and *Globorotalia ungulata* were amplified using the above 1° and 2° PCR, but for the third round, were amplified in three sections using the following primers: 1) 61F & 2119R, 2) 2082F & 2514R, and 3) FS3 & 3024R.

PCR amplification was attempted for multiple specimens of each planktonic foraminiferal morphospecies/genotype available. For specimens that failed to amplify using the standard approach (above), the PCR was repeated using multiple combinations of the listed PCR primers.

PCR optimisation experiments were also undertaken, and a number of factors varied, including the amount of magnesium added, type of DNA polymerase (Taq or $Vent_R$), dNTP concentration, annealing temperature, amount of template DNA (1 μl, 3 μl, 5 μl, 10 μl), dilutions of 1° & 2° PCR products between rounds, and PCR clean up between rounds. The final conditions used are described fully in chapter 2, sections 2.2.3 and 2.2.4.

Amplification products were separated by gel electrophoresis and purified using an Eppendorf Perfectprep® Gel Extraction Kit (chapter 2, section 2.4). The majority of taxa were then cloned using the TOPO TA cloning® method (Invitrogen™) (see chapter 2, section 2.6). Cloning was necessary in those species that bear multiple templates of their genome, which may contain subtle differences that can lead to sequencing errors. For each specimen three clones were isolated and, to circumvent the potential problem of Taq errors (observed in some of the cloned sequences), each

clone was sequences three times and a majority rule consensus sequence constructed. Cloning was also employed for specimens where the yield of PCR product obtained was insufficient to enable direct sequencing.

Both sense and antisense strands were sequenced directly on an Applied Biosystems 377 DNA sequencer using Applied Biosystems™ BigDye® v3.1 terminator cycle sequencing (see chapter 2, section 2.6.5 for details). Sequencing was undertaken in sections of ~ 800 – 1000 bp, using various combinations of the primers shown in the schematic diagram (fig. 5.1) (see chapter 2, section 2.2.2 for sequences). Contigs were assembled using Gap4 in the Staden package version 1.5.3 (Staden *et al*., 2000) and a consensus sequence output.

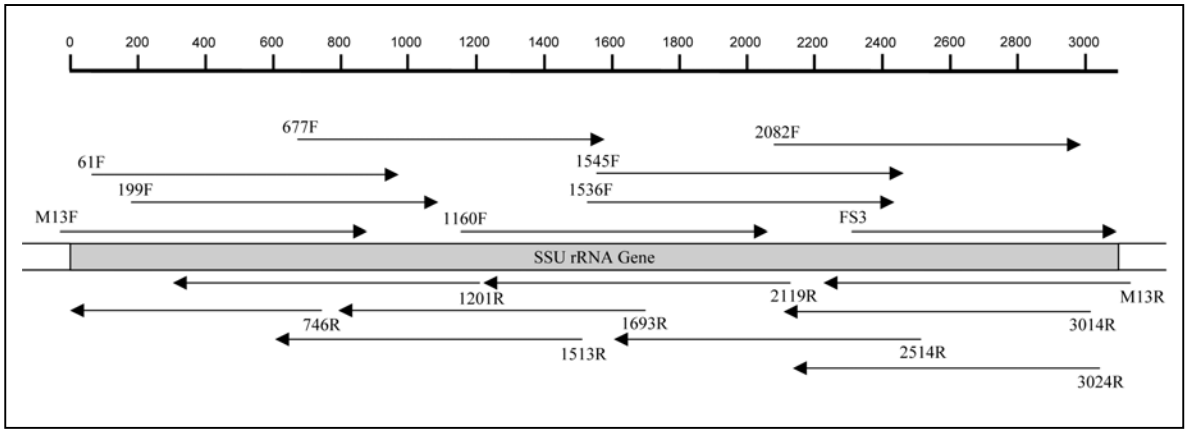


Figure 5.1. Schematic diagram of the SSU rRNA gene showing the positions of the primers used in the DNA sequencing of an ~ 3000 bp fragment. Arrows indicate the direction and length of sequence gained (~ 800 – 1000 bp) with each primer. Primers M13F and M13R (Invitrogen™), were used for cloned sequences only and are located outside the SSU rRNA gene on the TOPO® plasmid vector.

5.3.3 DNA sequence analysis and phylogenetic reconstruction

Sequence analysis and phylogenetic reconstruction of the partial ~1,000 bp terminal 3′ fragment of the SSU rRNA gene have been previously described in chapters 3 & 4. Sequences used are listed in appendix 9.1.

For the ~3,000 bp, almost full-length fragment of the SSU rRNA gene, sequences were aligned manually within the Genetic Data Environment (GDE) package (version 2.2) (Smith *et al*., 1994). In total, 1002 nucleotide sites could be unambiguously aligned across all foraminiferal taxa. Phylogenetic analyses were carried out using the 13 morphospecies of planktonic foraminifera (those for which the ~ 3000 bp fragment could be successfully obtained), plus the 21 benthic foraminiferal morphospecies for which full-length SSU rRNA sequences are currently available on GenBank (see appendix 9.1 for details). Phylogenetic trees were constructed using Bayesian inference (BI; Ronquist & Huelsenbeck, 2003, Larget & Simon, 1999), maximum likelihood (ML; Felsenstein, 1981), and neighbour-joining (NJ; Saitou & Nei, 1987). In all methods multiple hits were accounted for using a general time-reversible (GTR) model with a gamma (Γ) correction (Lanave *et al*., 1984; Yang 1993) (see chapter 2, section 2.8 for details).

5.3.4 Relative rate tests (RRT)

The degree of rate variation between pairs of taxa or taxon groups, within the foraminiferal phylogeny, was assessed by means of the relative rate test (RRT) (Sarich & Wilson, 1967) (chapter 2, section 2.8.3). The RRT allows for the comparison of rates of evolution between two taxa, without any knowledge of divergence time. This is achieved simply by comparing the substitutional rates in the two closely related

taxa, with a third more distantly related outgroup, in the case of this study, agglutinated benthic foraminifer, *Allogromia* sp. The test was carried out using the GRate package (Müller, K) (see appendix 9.3), using maximum likelihood estimates of substitutions per site (with a GTR + Γ model). Within the package, standard errors were estimated via bootstrapping (Efron, 1982, Felsenstein, 1985) and the significance of differences between groups tested using a two-tailed z-test.

5.3.5 Hypothesis testing

The Kishino–Hasegawa (KH) RELL test (Kishino & Hasegawa, 1989), as implemented in PAUP* (see chapter 2, section 2.8.4) was used to test the likelihood of alternative phylogenetic hypotheses. Tree topologies were constrained to fit varying hypotheses regarding the origins of the planktonic foraminifera from benthic ancestors, and compared to the optimal tree derived from BI analysis.

5.4 Results

5.4.1 Molecular data

5.4.1.1 Sequence alignments

Sequences for the ~ 1,000 bp partial fragment of the terminal 3′ region of the SSU rRNA gene were aligned across 61 foraminiferal morphospecies (see appendix 9.1 for taxa list). These included 27 morphospecies of planktonic foraminifera (order Globigerinida), of which 11 were spinose planktonic (32 sequences), 11 were non-spinose macroperforate (20 sequences), 2 were non-spiral planktonic (2 sequences), and 3 were non-spinose microperforate (3 sequences), together with 34 morphospecies of benthic foraminifera (one from every family in GenBank), covering the orders; Rotaliida (14 morphospecies), Milliolida (5 morphospecies), Textulariida (10 morphospecies), Lagenida (2 morphospecies), and Allogromida (2 morphospecies). For the ~ 1,000 bp sequence alignment see appendix 9.7.1

Amplification of the complete SSU rRNA gene in the planktonic foraminifera proved extremely problematic, and though certain taxa amplified very well in PCR (primarily the non-spinose planktonic taxa), others failed to amplify even with extensive optimisation of the method (the majority of spinose planktonic taxa). An approximately 3,000 bp fragment of the SSU rRNA gene**,** representing almost its complete length, was successfully amplified and sequenced for 13 morphospecies of planktonic foraminifera (order Globigerinida) (see appendix 9.1 for details). Of these, 2 were spinose planktonic, 8 were non-spinose macroperforate, 1 was non-spiral planktonic, and 2 were non-spinose microperforate. These were aligned together with existing sequences for 22 morphospecies of benthic foraminifera (all those for which complete SSU rRNA gene sequences currently exist on GenBank), covering the orders

Rotaliida (7 species), Milliolida (10 species), Textulariida (4 species), and Allogromida (1 species) (see appendix 9.1 for details). For the ~ 3,000 bp sequence alignment see appendix 9.7.8

5.4.1.2 Summary of molecular data

A summary of the molecular data for both datasets is shown in Table 5.1. Using the Likelihood ratio test (LRT), the GTR+Γ model was found to be optimal for both datasets (see appendix 9.2.1).

From the ~ 1,000 bp partial fragment of the SSU rRNA gene, 407 nucleotide sites could be unambiguously aligned across all taxa for use in phylogenetic analyses. A total of 178 (44 %) variable sites were found, of which 137 (34 %) were parsimony-informative. The highest base frequency in the 407 bp of the SSU rRNA gene was for G (0.276) followed by T (0.253), then A (0.245) and finally C (0.227).

From the ~ 3,000 bp almost complete SSU rRNA gene, 1002 nucleotide sites could be unambiguously aligned across all taxa for use in phylogenetic analyses. A total of 344 (34 %) variable sites were found, of which 208 (51 %)were parsimony-informative. The highest base frequency in the 1002 bp of the SSU rRNA gene was for A (0.280) followed by G (0.274), then T (0.242) and finally C (0.203).

Table 5.1. Summary of molecular data for the foraminiferal SSU rRNA gene

Data set	Optimal model	# of variable sites		Mean base frequencies				# of parsimony informative sites	
				A	C	G	T		
		#	%					#	%
407 bp	GTR + Γ	178	44	0.245	0.227	0.276	0.253	137	34
1002 bp	GTR + Γ	344	34	0.280	0.203	0.274	0.242	208	51

Figures calculated in PAUP*

5.4.1.3 Corrected pairwise p-distances

Within the 407 bp dataset, pairwise distances (corrected) across all taxa ranged from 0 to 0.479, and within the 1002 bp dataset, ranged from 0 to 0.353. For both datasets, the greatest range in distances occurred within the spinose planktonic foraminifera (407 bp dataset: 0 – 0.479; 1002 bp dataset: 0 to 0.353). The smallest ranges in distance occurred within the non-spinose microperforate planktonic foraminifera (407 bp dataset: 0 - 0.010; 1002 bp dataset: 0.012) and within the non-spiral planktonic foraminifera (407 bp dataset: 0.003) (see also appendix 9.4).

Mean corrected pairwise distances, both within and between the major foraminiferal groups, are shown in tables 5.2 (407 bp dataset) and 5.4 (1002 bp dataset). Table 5.3 shows the mean corrected pairwise distances (407 bp dataset) with certain unusually rapidly evolving foraminiferal lineages (discussed in section 5.4.3) excluded, to eliminate them as a potential source of error when evaluating between group distances. Mean corrected pairwise distances within and between the major foraminiferal groups (tables 5.2 & 5.3: 407 bp dataset, table 5.4: 1002 bp dataset), indicate substantial distances between the spinose planktonic foraminifera and both the non-spinose (macroperforate, microperforate, & non-spiral) planktonic and benthic

foraminifera. Mean distances between the non-spinose (macroperforate, microperforate, & non-spiral) planktonic foraminifera and the benthic foraminifera are smaller. The highest mean distances were actually evident within the spinose and non-spinose macroperforate planktonic groups, reflecting the extreme ranges of rates of evolution found within planktonic foraminiferal groups (see section 5.4.3).

Table 5.2. Mean corrected pairwise distances within and between the major foraminiferal groups, based on 407 bp of the SSU rRNA gene

407 bp dataset	Spinose	Macroperforate	Microperforate	Non-spiral	Benthic
Spinose	0.2127				
Macroperforate	0.1998	0.0586			
Microperforate	0.1619	0.0491	0.0033		
Non-spiral	0.1689	0.0508	0.0030	0.0027	
Benthic	0.1859	0.0700	0.0296	0.0303	0.0491

Corrected pairwise distances calculated in PAUP* using maximum likelihood with a GTR + Γ model.

Table 5.3. Mean corrected pairwise distances within and between the major foraminiferal groups, based on 407 bp of the SSU rRNA gene, with rapidly-evolving taxa excluded

407 bp dataset	Spinose	Macroperforate	Microperforate	Non-spiral	Benthic
Spinose	0.1147				
Macroperforate	0.1218	0.0182			
Microperforate	0.1086	0.0223	0.0033		
Non-spiral	0.1125	0.0227	0.0030	0.0027	
Benthic	0.1259	0.0305	0.0117	0.0114	0.0196

Corrected pairwise distances calculated in PAUP* using maximum likelihood with a GTR + Γ model. Figures shown represent the relative rates within and between foraminiferal groups after the exclusion of certain unusually rapidly evolving taxa (spinose planktonic; *Globigerina bulloides*, *Globigerinoides sacculifer, Turborotalita quinqueloba*, non-spinose macroperforate planktonic; *Globorotalia menardii, Globorotalia ungulata, Globorotalia truncatulinoides,* and Benthic order Milliolida) (see section 5.4.3), in order to eliminate them as a potential source of error.

Table 5.4. Mean corrected pairwise distances within and between the major foraminiferal groups, based on 1002 bp of the SSU rRNA gene

1002 bp dataset	Spinose	Macroperforate	Microperforate	Non-spiral	Benthic
Spinose	0.2841				
Macroperforate	0.2364	0.0716			
Microperforate	0.1985	0.0589	0.0124		
Non-spiral	0.1971	0.0592	0.0157	N/A	
Benthic	0.2333	0.1059	0.0712	0.0681	0.0728

Corrected pairwise distances calculated in PAUP* using maximum likelihood with a GTR + Γ model.

5.4.1.4 Evaluating the sequence data for saturation

Prior to phylogenetic analysis, the sequence data was examined for evidence of substitution saturation, which if severe may remain uncorrected by the chosen model of evolution, leading to inaccuracies in the phylogeny produced. For both the 407 bp and 1002 bp datasets, transition and transversion distances were plotted against uncorrected distances (fig. 5.2a, 5.2b). For both datasets, transition and transversion substitutions can be seen to increase linearly, indicating that saturation has not been reached. As highly divergent sequences are more prone to substitutions than closely related sequences, saturation (which generally occurs in transitions before transversions) would be observed as a curve and eventual plateau in the line of best fit (Salemi, 2009). The lack of saturation was confirmed by the plots of transition distance against transversion distance, which also showed a linear relationship for both datasets (fig. 5.2c, 5.2d). For both types of plots (figs. 5.2a, 5.2b, & 5.2c, 5.2d), the transition distances were higher than the transversion distances, as would be expected due to the generally more frequent occurrence of transitions relative to transversions (Salemi, 2009).

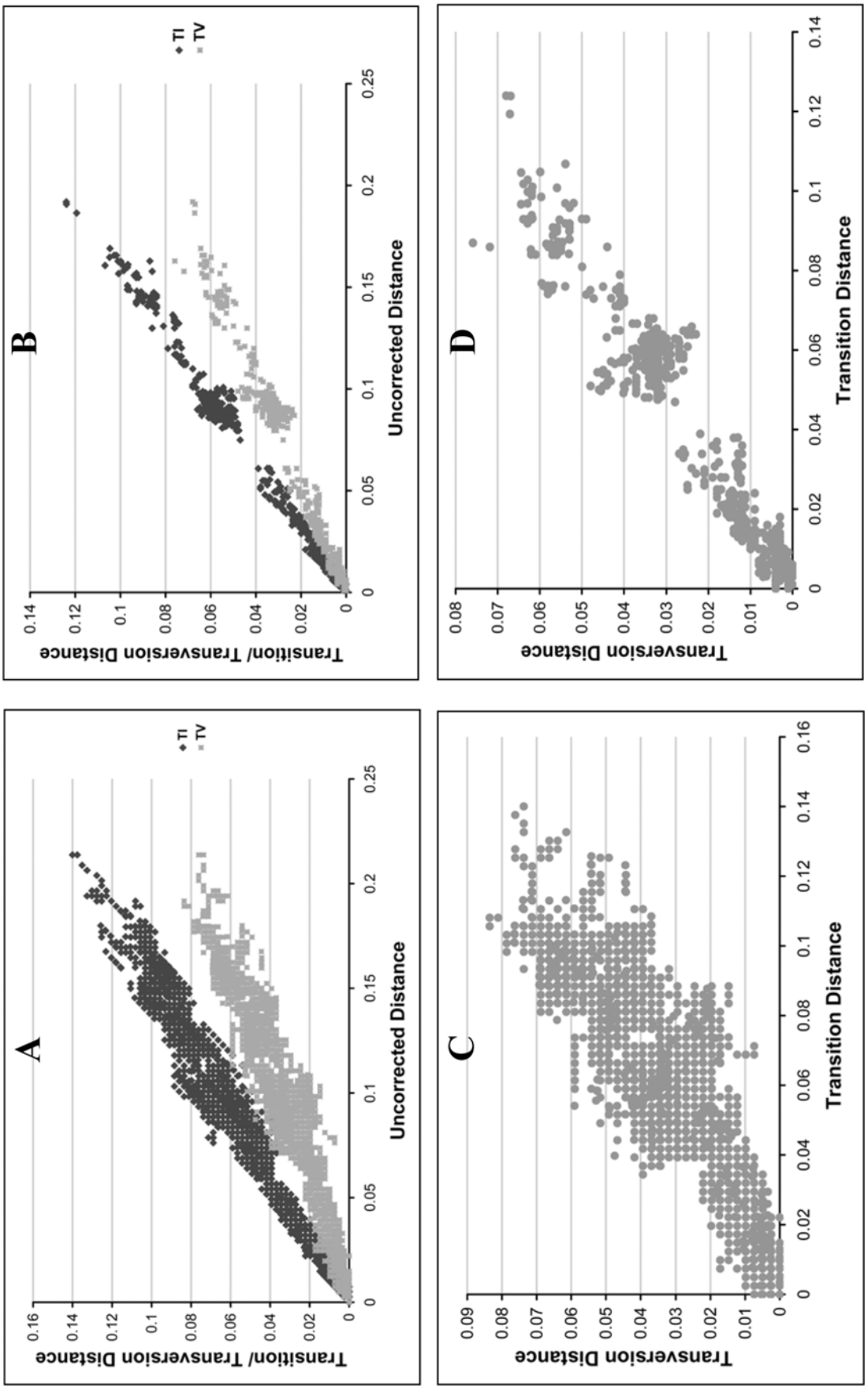


Figure 5.2. Plots of uncorrected pairwise transition (ti) and transversion (tv) distances against pairwise total uncorrected distances for A) 407 bp and B) 1002 bp of the SSU rRNA gene in the Foraminifera and plots of uncorrected pairwise transition (ti) distances against transversion (tv) distances for C) 407 bp and D) 1002 bp sites of the SSU rRNA gene in the Foraminifera.

5.4.2 Phylogenetic relationships of the planktonic foraminifera

5.4.2.1 Phylogenetic analyses using an ~ 1,000 bp partial 3´ terminal fragment of the SSU rRNA gene

A comprehensive phylogeny of the foraminifera, based on the analysis of 407 bp of the SSU rRNA gene is shown in figure 5.3, including examples of all planktonic foraminiferal morphospecies and genotypes sequenced to date plus representatives of the major groups of benthic taxa (1 per family). All methods of phylogenetic reconstruction employed were largely consistent in their inferred trees.

Within the foraminiferal phylogeny, the planktonic taxa do not form a monophyly, but fall in separate groups throughout the tree, appearing in at least 3, but possibly up to 5 locations. The first group is comprised of the spinose planktonic foraminifera (Globigerinidae: *Globigerinella siphonifera*, *Globigerinella calida*, *Orbulina universa*, *Globigerinoides sacculifer*, *Globoturborotalita rubescens* (pink), *Globigerinoides ruber*, *Globigerinoides conglobatus*, *Globigerina bulloides*, *Turborotalita quinqueloba*, *Globigerina falconensis* and Hastigerinidae: *Hastigerina pelagica*), which fall in a monophyletic clade (p= 0.94 BI, 46 % ML, 29 % NJ; Fig. 5.3). There is little resolution at the base of the clade, which appears as a polytomy. Within the clade there are 5 groupings: 1) *O. universa* & *G. sacculifer* (p= 0.60 BI, 27 % ML, - NJ; Fig. 5.3), 2) *G. siphonifera* & *G. calida* (p= 1.00 BI, 96 % ML, 92 % NJ; Fig. 5.3), 3) *G. rubescens* (pink), *G. ruber*, & *G. conglobatus* (p= 1.00 BI, 93 % ML, 92 % NJ; Fig. 5.3), 4) *G. bulloides*, *T. quinqueloba*, & *G. falconensis* (p= 1.00 BI, 78 % ML, 74 % NJ; Fig. 5.3), and 5) *Hastigerina pelagica*.

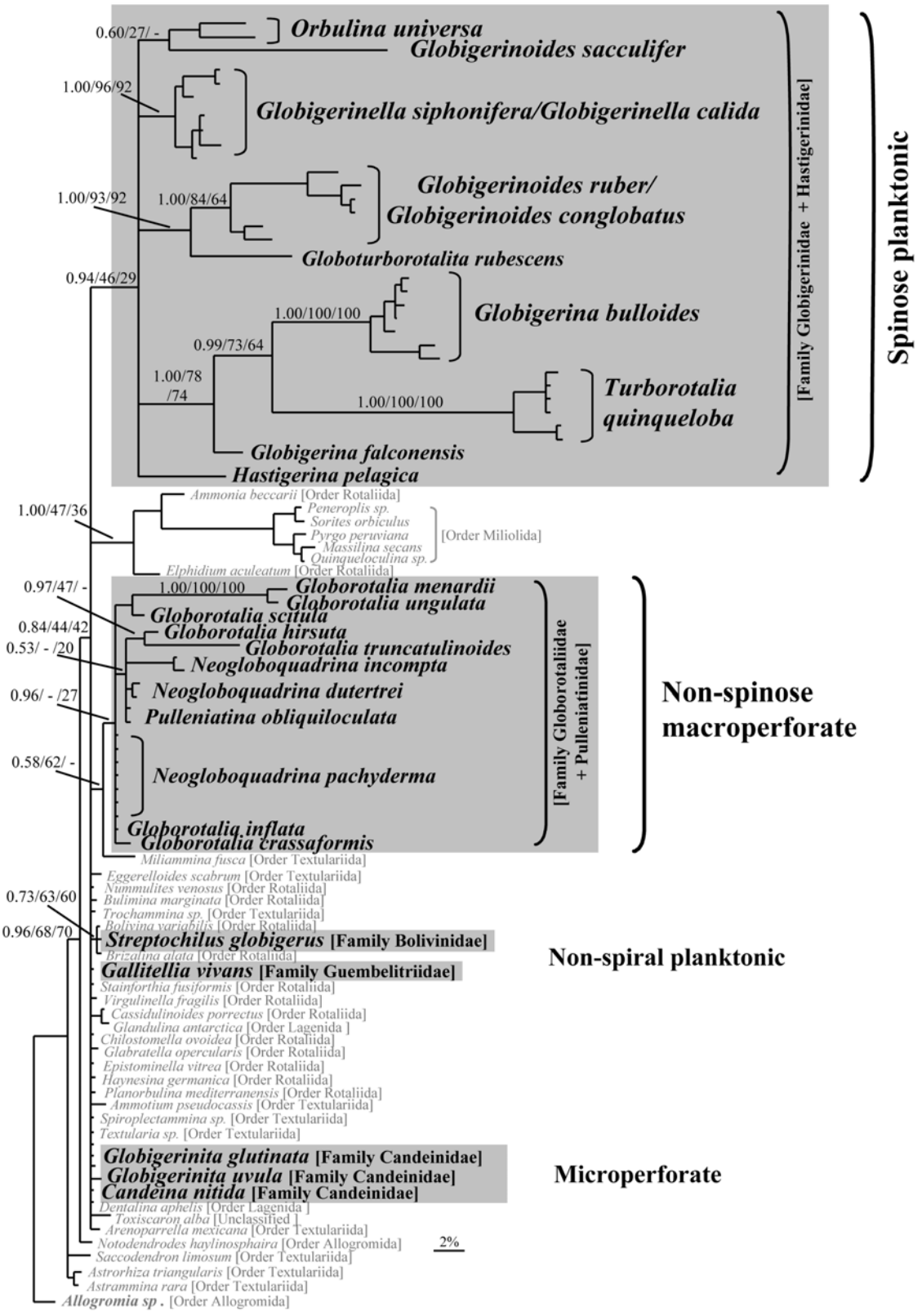


Figure 5.3. Bayesian inference SSU rDNA phylogeny of the benthic and planktonic foraminifera. The phylogeny is based on the partial 3' terminal fragment of the SSU rRNA gene (407 unambiguously aligned nucleotide sites) and is rooted on the benthic foraminifer *Allogromia sp.* Bayesian posterior probabilities (from last 1000 trees, obtained within MrBayes) and bootstrap support, derived from ML and NJ methods (expressed as a percentage, 1000 replicates) are shown on the tree (BI posterior probabilities/ ML bootstraps/ NJ bootstraps). The scale bar corresponds to a genetic distance of 2 %. Benthic foraminiferal taxa are shown in grey text, and planktonic foraminifera are shown in black. The major planktonic foraminiferal taxonomic groupings are highlighted on a grey background.

The second group is comprised of the non-spinose macroperforate planktonic foraminifera (Globorotaliidae: *Globorotalia* (*menardii, ungulata, truncatulinoides, crassaformis, hirsuta, scitula and inflata*), *Neogloboquadrina* (*incompta, dutertrei* and *pachyderma*) and Pulleniatinidae: *Pulleniatina* (*obliquiloculata*)). These fall in a monophyletic clade, which despite low bootstrap support (p= 0.96 BI, - ML, 27 % NJ; Fig. 5.3), is recovered with all methods of phylogenetic reconstruction.

The remaining planktonic foraminifera fall amongst the benthic foraminiferal taxa, in an area of the tree with little resolution. According to shell morphology these can be split into two groups: the microperforate planktonic foraminifera (Candeinidae: *Globigerinita glutinata, Globigerinita uvula & Candeina nitida*) and the non-spiral planktonic foraminifera (Bolivinidae: *Streptochilus globigerus* & Guembelitriidae: *Gallitellia vivans*).

The microperforate planktonic foraminifera (Candeinidae: *G. glutinata, G. uvula & C. nitida*) do not form a coherent group in the phylogeny here, though the evolutionary distances between them are very small (*G. glutinata & G. uvula* = 0.0075; *G. glutinata & C. nitida* 0.0006; *G. uvula & C. nitida* = 0.0075) (appendix. 9.4.2).

The two extant non-spiral planktonic foraminifera, *Streptochilus globigerus* (order Bolivinidae) and *Gallitellia vivans* (order Guembelitriidae), fall separately in the phylogeny (fig. 5.3). Biserial morphospecies *S. globigerus* falls together with benthic species *Bolivina variabilis* and *Brizalina alata* (p= 0.96 BI, 63 % ML, 60 % NJ; Fig. 5.3), and triserial morphospecies *Gallitellia vivans* falls alone. The evolutionary distance between *S. globigerus* and *G. vivans* is 0.0027, compared to a distance of

0.00 between *S. globigerus* and benthic morphospecies, *Bolivina variabilis* and a distance of 0.00 between *G. vivans* and benthic morphospecies, *Stainforthia fusiformis* (appendix 9.4.3).

Despite the clear topological separation of the planktonic foraminiferal groups (spinose, non-spinose macroperforate, non-spinose microperforate, and non-spiral) within the 407 bp SSU rRNA phylogeny, a planktonic foraminiferal monophyly could not be rejected in Kishino–Hasegawa (KH) likelihood tests (table 5.5). A constrained monophyly of the spinose planktonic and non-spinose macroperforate planktonic foraminifera produced the best tree according to the KH test (–ln L 3431.69653, best tree versus –ln L 3465.04514 for the unconstrained MB tree shown in fig. 5.3), though the result was not significant ($P = 0.115$) (table 5.5).

Table 5.5. Kishino–Hasegawa test of alternative phylogenetic hypotheses for the 407 bp dataset

Tree		-ln L	Diff -ln L	P
1	Unconstrained MB tree	3465.04514	33.34861	0.115
2	Planktonic foraminiferal monophyly	3442.35658	10.66005	0.236
3	Planktonic foraminiferal monophyly minus non-spiral taxa	3433.35428	1.65776	0.648
4	Spinose and non-spinose macroperforate planktonic foraminiferal monophyly	3431.69653	(best)	
5	Spinose planktonic and *G. menardii*/ *G. ungulata* monophyly	3441.27470	9.57818	0.397
6	Spinose and non-spinose microperforate planktonic foraminiferal monophyly	3436.35306	4.65654	0.606
7	Spinose planktonic, non-spinose microperforate planktonic, and non-spiral foraminiferal monophyly	3437.63788	5.94136	0.585

KH test using RELL bootstrap, two-tailed test, with 1000 bootstrap replicates, performed in PAUP* for 407 bp of the SSU rRNA gene. * $P < 0.05$

5.4.2.2 Phylogenetic analyses using the ~ 3,000 bp almost complete SSU rRNA gene

Phylogenetic analyses based on the almost complete (~3,000 bp fragment) SSU rRNA gene were carried out, incorporating 13 morphospecies of planktonic foraminifera (those for which the ~ 3000 bp fragment could be successfully obtained), plus the 21 benthic foraminiferal morphospecies for which full-length SSU rRNA sequences are currently available on GenBank (see appendix 9.1 for details). Phylogenetic trees were constructed using Bayesian inference (BI; fig. 5.4), maximum likelihood (ML; fig. 5.5), and neighbour-joining (NJ; fig. 5.6).

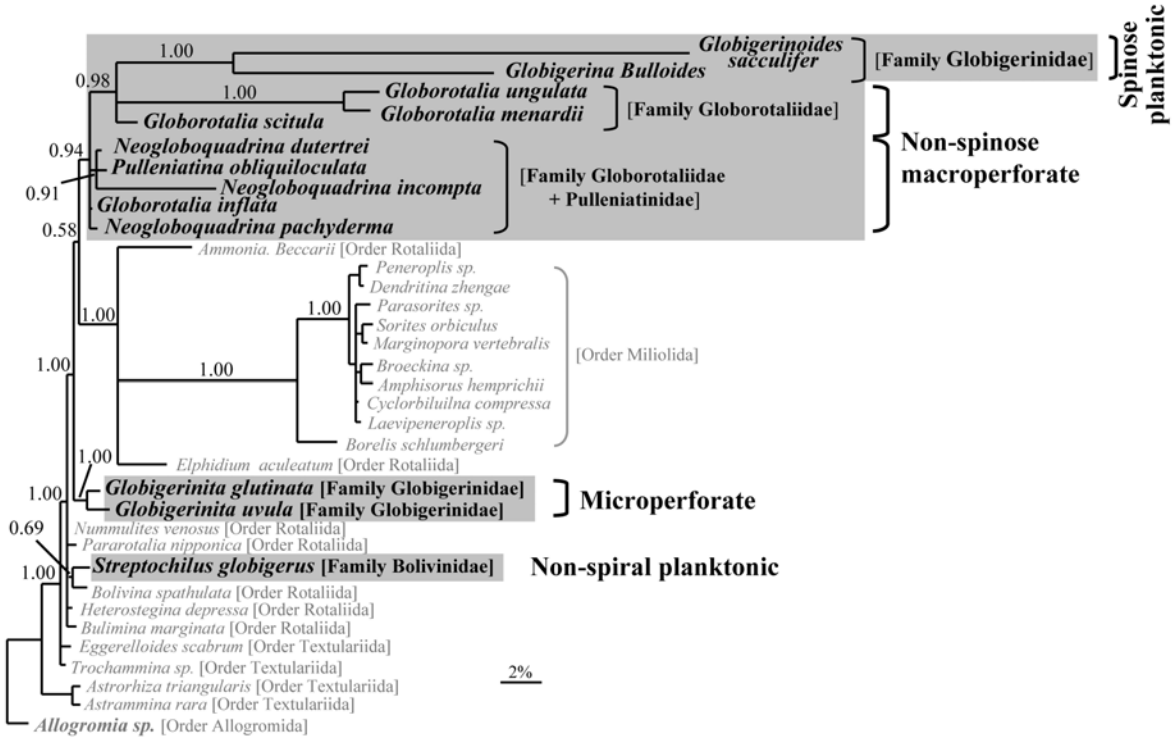


Figure 5.4. Bayesian inference phylogeny of the benthic and planktonic foraminifera, based on the almost complete SSU rRNA gene (1002 unambiguously aligned nucleotide sites). The phylogeny is rooted on the benthic foraminifer *Allogromia sp.* Bayesian posterior probabilities (from last 1000 trees, obtained within MrBayes) are shown on the tree. The scale bar corresponds to a genetic distance of 2 %. Benthic foraminiferal taxa are shown in grey text, and planktonic foraminifera are shown in black. The major planktonic foraminiferal taxonomic groupings are highlighted on a grey background.

There are some inconsistencies in the phylogenies produced using the different methods (figs. 5.4 – 5.6), with the positioning of some poorly supported groupings remaining inconclusive. However, other relationships within the phylogeny are more informative.

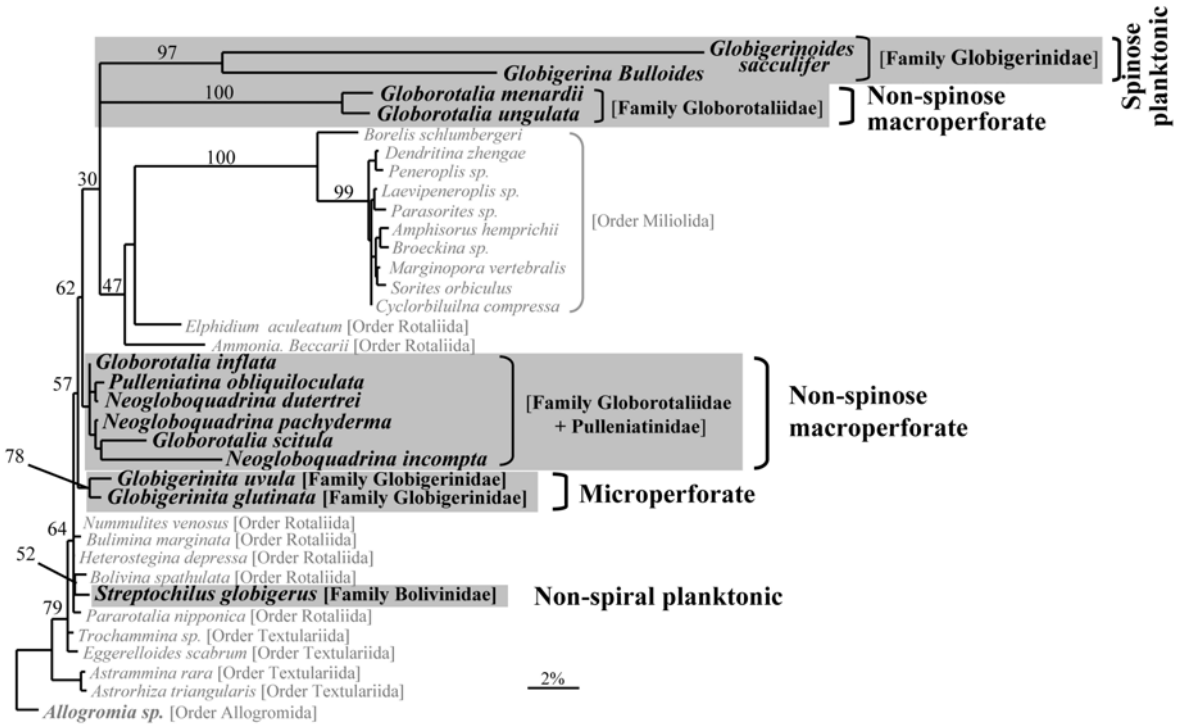


Figure 5.5. Maximum likelihood phylogeny of the benthic and planktonic foraminifera, based on the almost complete SSU rRNA gene (1002 unambiguously aligned nucleotide sites). The phylogeny is rooted on the benthic foraminifer *Allogromia sp.* ML bootstrap support values (expressed as a percentage, 1000 replicates) are shown on the tree. The scale bar corresponds to a genetic distance of 2 %. Benthic foraminiferal taxa are shown in grey text, and planktonic foraminifera are shown in black. The major planktonic foraminiferal taxonomic groupings are highlighted on a grey background.

Only two morphospecies of spinose planktonic foraminifera, *Globigerinoides sacculifer* and *Globigerina bulloides* were successfully amplified and sequenced for the almost complete (~3,000 bp) SSU rRNA gene. The pair fall together in a strong group in all three phylogenies (figs. 5.4 – 5.6. p= 1.00 BI; 97 % ML; 91 % NJ). In the BI phylogeny, the spinose planktonic foraminifera and the non-spinose

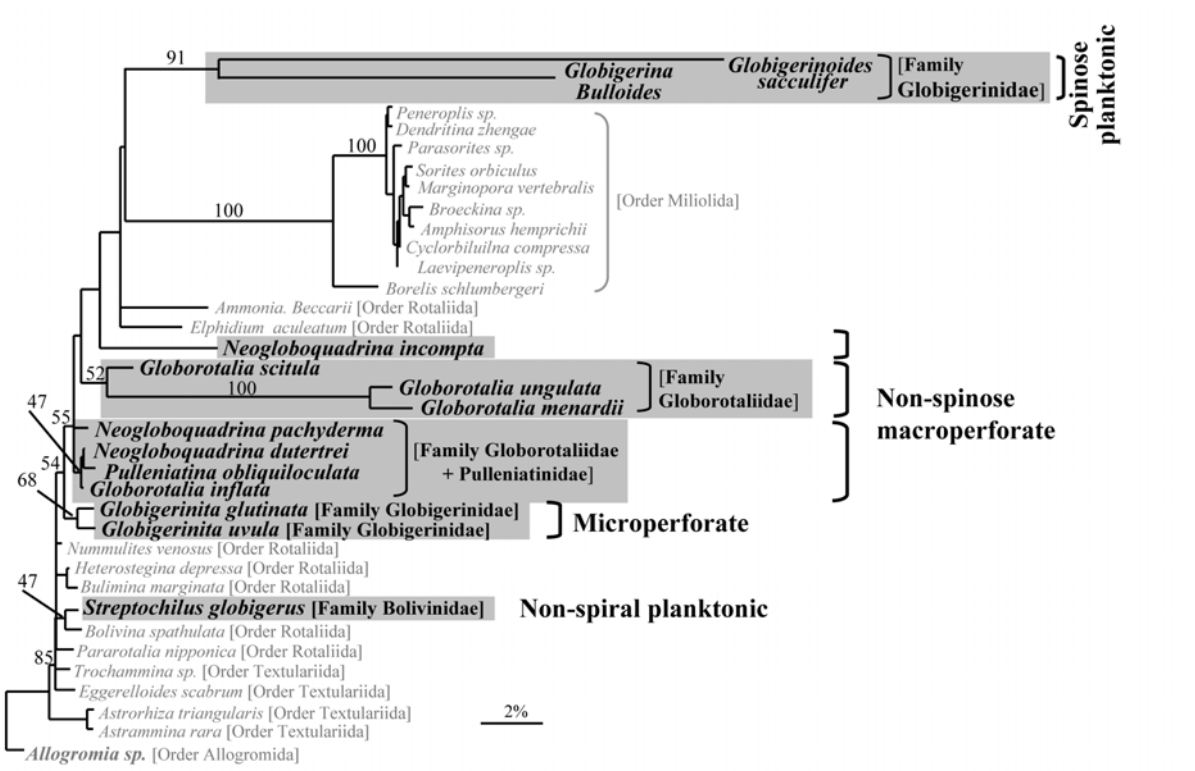


Figure 5.6. Neighbour-joining phylogeny of the benthic and planktonic foraminifera, based on the almost complete SSU rRNA gene (1002 unambiguously aligned nucleotide sites). The phylogeny is rooted on the benthic foraminifer *Allogromia sp.* ML bootstrap support values (expressed as a percentage, 1000 replicates) are shown on the tree. The scale bar corresponds to a genetic distance of 2 %. Benthic foraminiferal taxa are shown in grey text, and planktonic foraminifera are shown in black. The major planktonic foraminiferal taxonomic groupings are highlighted on a grey background.

macroperforate planktonic foraminifera form a monophyletic grouping (fig. 5.4. p=0.94 BI). However, in the ML tree (fig. 5.5), the spinose taxa fall together with only two of the macroperforates, *G. menardii* and *G. ungulata*, along with a group of benthics (the Milliolida plus two rotalliids; *Ammonia beccarii* & *Elphidium aculeatum*). The three lineages join in a polytomy, with little resolution and only poor support (fig. 5.5. 30 % ML). In the NJ phylogeny the spinose taxa again fall alongside the same group of benthics, but with little support (fig. 5.6) and the non-spinose macroperforate species fall away from the spinose taxa, although not in a cohesive group.

The macroperforate planktonic taxa do not form a monophyletic group with any of the tree-building methods used. The topologies are highly inconsistent with regards to the positioning of individual morphospecies, and their interrelationships are poorly resolved. However, a consistent relationship is observed between *Pulleniatina obliquiloculata* and *Neogloboquadrina dutertrei,* which fall together with every method (fig. 5.4. p= 0.91 BI, (also with *N. incompta*); fig. 5.5. 88 % ML; fig. 5.6. 86 % NJ) and also between *Globorotalia menardii* and *Globorotalia ungulata*, which fall together on a long branch with every tree-building method used (figs. 5.4 – 5.6. p= 1.00 BI; 100 % ML; 100 % NJ), though the position of this branch varies.

With every method used, the non-spinose macroperforate planktonic taxa, a group of benthics (the Milliolida plus two rotalliids; *Ammonia beccarii & Elphidium aculeatum*), and the spinose planktonic taxa fall together (figs. 5.4 – 5.6. p= 1.00 BI; 57 % ML; 54 % NJ).

The two microperforate morphospecies, *Globigerinita glutinata* and *Globigerinita uvula* fall together with every tree reconstruction method (figs. 5.4 – 5.6. p= 1.00 BI; 78 % ML; 68 % NJ), and consistently fall prior to the divergence of the non-spinose macroperforate planktonic taxa, certain benthics (the Milliolida plus two rotalliids; *Ammonia beccarii & Elphidium aculeatum*), and the spinose planktonic taxa.

The only non-spiral planktonic morphospecies included, *Streptochilus globigerus* consistently falls together with the benthic foraminifer *Bolivina variabilis* (figs. 5.4 – 5.6. p= 0.69 BI; 52 % ML; 47 % NJ), away from the other planktonic foraminifera.

In Kishino–Hasegawa (KH) likelihood tests of the 1002 bp dataset (appendix 9.5), the Bayesian inference tree was highlighted as the best tree (-1n L 4594.79599), the ML tree had a lower score, but not significantly so (-1n L 4599.52616, $P = 0.662$), and the NJ tree was significantly worse (-1n L 4611.68090, $P < 0.05$). As with the 407 bp dataset, a planktic foraminiferal monophyly could not be rejected in KH tests (table 5.6), though the evolutionary distances between some of the planktonic groups, (e.g. the spinose and non-spinose macroperforate taxa = 0.2364) were substantial (table 5.4). The best tree produced was one in which the non-spiral planktonic foraminifera were separate from the remaining planktonic taxa, though with no significance.

Table 5.6. Kishino–Hasegawa test of alternative phylogenetic hypotheses for the 1002 bp dataset

	Tree	-ln L	Diff. -ln L	P
1	Unconstrained MB tree	4594.79599	6.50404	0.258
2	Planktonic foraminiferal monophyly	4599.45634	11.16439	0.291
3	Planktonic foraminiferal monophyly minus non-spiral taxa	4588.29195	(best)	
4	Spinose and non-spinose macroperforate planktonic foraminiferal monophyly	4589.68957	1.39763	0.429
5	Unconstrained NJ tree	4611.68090	23.38895	0.020*

KH test using RELL bootstrap, two-tailed test, with 1000 bootstrap replicates, performed in PAUP* for 1002 bp of the SSU rRNA gene. * P < 0.05

5.4.3 Rates of rDNA evolution in the foraminifera

Pairwise comparisons of rates of rDNA evolution between major groups in the foraminiferal phylogeny were made using the relative rate test (RRT) (Sarich & Wilson, 1967), performed in the GRate package (K. Müeller, unpublished: appendix 9.3). Rates were firstly examined within the spinose and the non-spinose planktonic foraminifera (tables 5.7 & 5.8), and secondly between the major taxonomic groups of planktonic foraminifera (tables 5.9 & 5.10).

5.4.3.1 Variation in rates of rDNA evolution within the spinose and non-spinose planktonic foraminifera

Relative rate tests, based on the 407 bp dataset, were used to examine the rates of rDNA evolution within the two major groups of planktonic foraminifera, the spinose planktonic foraminifera (Globigerinidae & Hastigerinidae) (table 5.7) and the non-spinose planktonic foraminifera (encompassing macroperforate, microperforate, and non-spiral taxa) (table 5.8).

Rates of evolution within the spinose planktonic group were found to be relatively constant, with the exception of 3 significantly faster evolving lineages; *Globigerina bulloides*, *Turborotalita quinqueloba*, and *Globigerinoides sacculifer* (table 5.7). The greatest rate difference was between *T. quinqueloba* and *Globigerinella siphonifera*/ *Globigerinella calida.* Rates of evolution within the non-spinose planktonic foraminifera (encompassing macroperforate, microperforate, and non-spiral taxa) were found to be more variable (table 5.8). Again, certain lineages were found to be evolving at a significantly faster rate than the rest (*Globorotalia menardii/ Globorotalia ungulata* & *Globorotalia truncatulinoides*), whilst others were evolving significantly slower (microperforates, *Globigerinita glutinata* & *Candeina nitida*).

Table 5.7. Relative rates of substitution between the spinose planktonic foraminiferal taxa

Taxon 1	Taxon 2										
	O. uni	*G. sacc*	*G. sip*	*G. cal*	*G. ruber*	*G. cong*	*G. rubesc*	*G. bull*	*T. quinq*	*G. falc*	*H. pel*
O. uni	-	-0.125**	0.021	0.021	-0.029	-0.028	-0.045	-0.135**	-0.209**	-0.004	0.003
G. sacc	0.046	-	0.145**	0.145**	0.096*	0.097	0.080	-0.010	-0.084	0.121*	0.128**
G. sip	0.024	0.047	-	0.000	-0.050	-0.048	-0.065	-0.155**	-0.230**	-0.025	-0.018
G. cal	0.026	0.046	0.009	-	-0.050	-0.048	-0.065	-0.156**	-0.230**	-0.025	-0.018
G. ruber	0.031	0.049	0.028	0.028	-	0.002	-0.016	-0.106**	-0.180**	0.025	0.032
G. cong	0.036	0.053	0.030	0.029	0.022	-	-0.017	-0.107*	-0.182**	0.023	0.030
G. rubesc	0.036	0.055	0.031	0.031	0.031	0.033	-	-0.090*	-0.164**	0.041	0.048
G. bull	0.042	0.057	0.042	0.043	0.044	0.049	0.046	-	-0.074	0.131**	0.138**
T. quinq	0.052	0.066	0.056	0.056	0.052	0.057	0.059	0.053	-	0.205**	0.212**
G. falc	0.025	0.048	0.023	0.025	0.030	0.035	0.034	0.037	0.053	-	0.0107
H. pel	0.025	0.049	0.025	0.025	0.030	0.033	0.035	0.045	0.055	0.029	-

Degree of substitutional rate divergence within the spinose planktonic foraminifera, based on 407 bp of the SSU rRNA gene (shown above the diagonal). Rate divergence was assessed by the relative rate test (Sarich & Wilson, 1967), performed in the GRate package (Müller, K, unpublished), using Maximum likelihood estimates of substitutions per site (with a GTR + Γ model) (appendix 9.3). The reference taxon was benthic species, *Allogromia sp*. A positive value indicates that taxon 1 is evolving faster than taxon 2, and inversely for a negative value. Standard errors (shown below the diagonal) were estimated via bootstrapping (300 replicates) (Efron, 1982, Felsenstein, 1985). The significance of differences between groups, tested using a two-tailed z-test, is indicated by *p=0.05, **p=0.01.

Table 5.8. Relative rates of substitution between the non-spinose planktonic foraminiferal taxa

Taxon 1	Taxon 2															
	G. men	*G. ung*	*G. Scit*	*G. hir*	*G. trunc*	*N. inc*	*N. dut*	*P. obli*	*N. pac*	*G. infl*	*G. crass*	*S. glob*	*G. viv*	*G. glu*	*G. uvula*	*C. nitida*
G. men	-	0.016	0.101**	0.091**	0.050	0.087**	0.103**	0.108**	0.111**	0.114**	0.104**	0.118**	0.117**	0.120**	0.117**	0.120**
G. ung	0.010	-	0.085**	0.075*	0.034	0.071*	0.087**	0.092**	0.095**	0.098**	0.088**	0.101**	0.100**	0.104**	0.101**	0.104**
G. Scit	0.032	0.030	-	-0.010	-0.051*	-0.014	0.002	0.007	0.010	0.013	0.003	0.017	0.016	0.019*	0.016	0.019*
G. hir	0.032	0.031	0.011	-	-0.041*	-0.004	0.012	0.017	0.020*	0.023*	0.013	0.027*	0.026*	0.029**	0.026*	0.029**
G. trunc	0.037	0.035	0.020	0.020	-	0.037	0.053**	0.058**	0.061**	0.064**	0.054*	0.068**	0.067**	0.070**	0.067**	0.070**
N. inc	0.033	0.031	0.013	0.013	0.020	-	0.017	0.021	0.024*	0.027*	0.018	0.031*	0.030*	0.034**	0.030*	0.034*
N. dut	0.032	0.031	0.010	0.010	0.020	0.012	-	0.005	0.008	0.011	0.001	0.014	0.013	0.017*	0.014	0.017*
P. obli	0.032	0.030	0.010	0.009	0.020	0.011	0.004	-	0.003	0.006	-0.004	0.009	0.008	0.012	0.009	0.012
N. pac	0.032	0.030	0.008	0.010	0.021	0.012	0.008	0.007	-	0.003	-0.007	0.007	0.006	0.009	0.006	0.009
G. infl	0.032	0.030	0.008	0.010	0.021	0.012	0.006	0.004	0.005	-	-0.010	0.003	0.002	0.006	0.003	0.006
G. crass	0.032	0.031	0.010	0.012	0.022	0.013	0.008	0.007	0.008	0.005	-	0.013	0.012	0.016*	0.013	0.016*
S. glob	0.032	0.030	0.010	0.011	0.023	0.013	0.008	0.007	0.007	0.005	0.008	-	-0.001	0.003	-0.000	0.003
G. viv	0.032	0.031	0.010	0.011	0.022	0.013	0.007	0.007	0.007	0.005	0.007	0.003	-	0.004*	0.000	0.004*
G. glu	0.032	0.031	0.009	0.011	0.022	0.013	0.007	0.006	0.007	0.004	0.007	0.003	0.002	-	-0.003	-0.000
G. uvula	0.032	0.030	0.011	0.013	0.022	0.014	0.009	0.009	0.009	0.007	0.009	0.006	0.006	0.006	-	0.003
C. nitida	0.032	0.031	0.009	0.011	0.022	0.013	0.007	0.006	0.007	0.004	0.007	0.003	0.002	0.000	0.006	-

Degree of substitutional rate divergence within the non-spinose planktonic foraminifera, based on 407 bp of the SSU rRNA gene (shown above the diagonal). Rate divergence was assessed by the relative rate test (Sarich & Wilson, 1967), performed in the GRate package (Müller, K, unpublished), using Maximum likelihood estimates of substitutions per site (with a GTR + Γ model) (appendix 9.3). The reference taxon was benthic species, *Allogromia sp*. A positive value indicates that taxon 1 is evolving faster than taxon 2, and inversely for a negative value. Standard errors (shown below the diagonal) were estimated via bootstrapping (300 replicates) (Efron, 1982, Felsenstein, 1985). The significance of differences between groups, tested using a two-tailed z-test, is indicated by *p=0.05, **p=0.01.

The greatest rate difference was between *Globorotalia menardii* and *Globigerinita glutinata/ Candeina nitida*.

5.4.3.2 Variation in the rate of rDNA evolution between the 5 major foraminiferal taxonomic groups

The relative rates of rDNA evolution were also compared between the five major taxonomic groups within the foraminiferal phylogeny, the spinose planktonic (Globigerinidae & Hastigerinidae), non-spinose macroperforate planktonic (Globorotaliidae & Pulleniatinidae), non-spinose microperforate planktonic (Candeinidae), non-spiral planktonic, and benthic foraminifera (table 5.9). An additional RRT was made, excluding any unusually fast-evolving taxa from each group, to ensure that the results were not skewed by their presence (table 5.9). Relative rate tests were performed using both the original 407 bp dataset (table 5.9), and the extended 1002 bp dataset (table 5.10). Differences in substitutional rates between groups were found to be greater using the 1002 bp dataset than when using the 407 bp dataset, though the significant differences in rates between groups were generally consistent regardless of the number of nucleotide sites used.

A great deal of rate variation was evident between the major taxonomic groups of foraminifera. The spinose planktonic foraminiferal taxa (Globigerinidae & Hastigerinidae) displayed significantly higher rates of evolution than any of the other groups of foraminifera, both planktonic and benthic, even with the exclusion of the faster evolving spinose morphospecies (*G. bulloides*, *T. quinqueloba*, & *G. sacculifer)* (tables 5.9 & 5.10).

Table 5.9. Relative rates of substitution between 5 major foraminiferal groups, based on 407 bp of the SSU rRNA gene

Taxon group 1	Taxon group 2				
	Spinose planktonic	Non-spinose macroperforate planktonic	Non-spinose microperforate planktonic	Non-spiral planktonic	Benthic
Spinose planktonic	-	0.134** (0.027*)	0.174** (0.041**)	0.171** (0.037*)	0.154** (0.035*)
Non-spinose macroperforate planktonic	0.023 (0.014)	-	0.039** (0.015*)	0.037** (0.012*)	0.020* (0.009)
Non-spinose microperforate planktonic	0.025 (0.015)	0.009 (0.006)	-	-0.003 (-0.003)	-0.020** (-0.006**)
Non-spiral planktonic	0.025 (0.015)	0.009 (0.006)	0.002 (0.002)	-	-0.017** (-0.003)
Benthic	0.024 (0.014)	0.009 (0.006)	0.005 (0.002)	0.005 (0.002)	-

Degree of substitutional rate divergence between groups of foraminiferal taxa based on 407 bp of the SSU rRNA gene (shown above the diagonal). Rate divergence was assessed by the relative rate test (Sarich & Wilson, 1967), performed in the GRate package (Müller, K, unpublished), using maximum likelihood estimates of substitutions per site (with a GTR + Γ model) (appendix 9.3). The reference taxon was benthic species, *Allogromia sp*. A positive value indicates that taxon group 1 is evolving faster than taxon group 2, and inversely for a negative value. Figures in brackets represent the relative rates between groups after the exclusion of unusual rapidly evolving taxa (spinose planktonic; *Globigerina bulloides*, *Globigerinoides sacculifer, Turborotalita quinqueloba*, non-spinose macroperforate planktonic; *Globorotalia menardii, Globorotalia ungulata, Globorotalia truncatulinoides,* and Benthic order Milliolida). Standard errors (shown below the diagonal) were estimated via bootstrapping (300 replicates) (Efron, 1982, Felsenstein, 1985). The significance of differences between groups, tested using a two-tailed z-test, is indicated by *p=0.05, **p=0.01.

Rates in the non-spinose macroperforate taxa (Globorotaliidae & Pulleniatinidae) were significantly slower than in the spinose group, but faster than in the microperforate and non-spiral planktonic taxa (tables 5.9 & 5.10), even with the exclusion of the faster evolving *G. menardii, G. ungulata* & *G. truncatulinoides* from the macroperforate group. With the 407 bp dataset, macroperforate rates of evolution were significantly higher than in the benthic taxa (table 5.9), however, with all faster taxa removed from the 407 bp dataset, and in the 1002 bp dataset (table 5.10), rates of evolution in the macroperforates were equivalent to those in the benthic foraminifera.

Table 5.10. Relative rates of substitution between 5 major foraminiferal groups, based on 1002 bp of the SSU rRNA gene

Taxon group 1	**Taxon group 2**				
	Spinose planktonic	Non-spinose macroperforate planktonic	Non-spinose microperforate planktonic	Non-spiral planktonic	Benthic
Spinose planktonic	-	0.146**	0.183**	0.188**	0.148**
Non-spinose macroperforate planktonic	0.019	-	0.037**	0.042**	0.003
Non-spinose microperforate planktonic	0.020	0.007	-	0.005	-0.034**
Non-spiral planktonic	0.020	0.007	0.004	-	-0.039**
Benthic	0.020	0.009	0.008	0.008	-

Degree of substitutional rate divergence between groups of foraminiferal taxa based on 1002 bp of the SSU rRNA gene (shown above the diagonal). Rate divergence was assessed by the relative rate test (Sarich & Wilson, 1967), performed in the GRate package (Müller, K, unpublished), using maximum likelihood estimates of substitutions per site (with a GTR + Γ model) (appendix 9.3). The reference taxon was benthic species, *Allogromia sp*. A positive value indicates that taxon group 1 is evolving faster than taxon group 2, and inversely for a negative value. Standard errors (shown below the diagonal) were estimated via bootstrapping (300 replicates) (Efron, 1982; Felsenstein, 1985). The significance of differences between groups, tested using a two-tailed z-test, is indicated by *p=0.05, **p=0.01.

Both with and without the rapidly evolving taxa excluded, the non-spinose microperforate taxa appeared to be evolving at a significantly slower rate than all groups, with the exception of the non-spiral planktonics, which had a roughly equivalent rate of evolution (tables 5.9 & 5.10).

The non-spiral planktonic taxa showed significantly slower rates of substitution than in the spinose planktonic and non-spinose macroperforate planktonic taxa, and equivalent rates to those seen in the microperforate planktonic and benthic foraminifera, even with unusually fast-evolving lineages removed (tables 5.9 & 5.10).

5.5 Discussion

5.5.1 Amplification of the ~3,000 bp, almost complete SSU rRNA in the foraminifera

Phylogenetic analyses of the foraminifera, based on the traditionally used ~1,000 bp partial 3′ terminal region of the SSU rRNA gene, are commonly afflicted with problems of poor resolution and low bootstrap support in deep-lineage relationships, and inconsistencies in the phylogenetic positioning of certain taxa. Extreme rate heterogeneity in foraminiferal rDNA evolution may in part be responsible, however, limited data may also be a factor, given that only ~500 bp can usually be retained for phylogenetic analyses, 407 bp under the stringent conditions used in this study. In order to alleviate these problems, the primary aim of this work was to sequence the complete foraminiferal SSU rRNA gene (~ 3,000 bp in length), substantially increasing the number of sites available for phylogenetic analyses.

It proved extremely difficult, however, to obtain full-length SSU rDNA sequences for the planktonic foraminifera, with high failure rates in PCR. It is likely that poor DNA extraction and preservation techniques, and the resultant presence of inhibitory substances in the PCR reactions and degradation of the sample DNA are in part responsible for such failures, however, there was also a marked difference in success rates between non-spinose and spinose planktonic samples.

The design of anti-sense PCR primers in the 3′ terminal region of the SSU rRNA gene is made easy by the availability of extensive sequence information for both benthic and planktonic taxa. At the 5′ end of the gene, in contrast, sequence data is available for only a handful of benthic foraminifera, plus other eukaryote taxa, on which the

universal primers of White *et al*. (1990) were based. High sequence homology between the benthic and non-spinose planktonic taxa, allows 5′ region sense primers designed on the former, to work well on the latter (although minor sequence variations were observed even in some non-spinose taxa in this region), however, it appears that they are a poor match for the significantly more divergent spinose taxa, for which amplification in most species proved impossible. With the exception of two species (*Globigerinoides sacculifer* & *Globigerina bulloides*) all attempts to amplify planktonic taxa failed, despite a high throughput of samples of every species, from various geographic localities, extensive re-designing of primers, and much optimisation of the method.

Even for those specimens that did amplify, the process proved extremely labour intensive, with 3 rounds of PCR (in some cases with multiple 3° reactions needed to pool enough PCR product), cloning necessary for most samples (x3 replicates each), and sequencing reactions undertaken with many primers. The eventual outcome was that an ~3,000 bp fragment, or almost the complete length of the SSU rRNA gene (minus ~200 bp and ~20 bp at the 5′ and 3′ ends respectively) was successfully amplified for 13 morphospecies of planktonic foraminifera, including 8 non-spinose macroperforate species (*Globorotalia menardii, Globorotalia ungulata, Globorotalia scitula, Neogloboquadrina dutertrei, Pulleniatina obliquiloculata, Neogloboquadrina pachyderma, Neogloboquadrina incompta, & Globorotalia inflata,* 2 non-spinose microperforate species (*Globigerinita glutinata & Globigerinita uvula*), 1 non-spiral species (*Streptochilus globigerus*), and 2 spinose species (*Globigerinoides sacculifer & Globigerina bulloides*). These were added to the 22 pre-existing complete SSU rDNA sequences published for benthic taxa for phylogenetic analysis. The unusually

large genetic distances seen between foraminiferal taxa inevitably meant that only a relatively small percentage of the entire gene (1002 bp) could be reliably aligned across all taxa, though this more than doubled the number of sites available for phylogenetic analysis, compared to traditional analyses.

Phylogenetic analyses based on the almost complete (~3,000 bp) SSU rRNA gene (1002 bp dataset) (fig. 5.4 – 5.6), are likely to have been affected by the poor taxon sampling resulting from the difficulties amplifying the complete gene in the planktonic foraminiferal taxa. Bootstrap support is low in certain areas of the trees, which in turn has led to inconsistent topologies being produced between the various methods of tree reconstruction employed (fig. 5.4; Bayesian Inference (BI), fig. 5.5; Maximum Likelihood (ML), fig. 5.6; Neighbour-joining (NJ)). In addition to poor taxon sampling, the extreme homogeneity of rates in rDNA evolution observed in the foraminifera may be partly responsible for such effects.

5.5.2 Evolutionary origins of the planktonic foraminifera

Despite the assumption in traditional classifications that the planktonic foraminifera represent a monophyletic lineage, first appearing in the Mid-Jurassic (Caron & Homewood, 1983; Görög, 1994; Loeblich & Tappan, 1974), the phylogeny based on the partial ~ 1,000 bp fragment of the SSU rRNA gene (407 bp dataset) (fig. 5.3), provides strong indications that they are in fact polyphyletic in origin, arising from up to 5 independent lineages, each representing an evolutionary move from the benthic to planktonic environment. The lineages correspond to three major morphological groups; the spinose planktonic (Globigerinidae & Hastigerinidae), non-spinose macroperforate planktonic (Globorotaliidae & Pulleniatinidae), and non-spinose

microperforate planktonic (Candeinidae) foraminifera, the independent origins of which have been indicated in past phylogenetic studies (Aurahs *et al*., 2009; Darling *et al*., 1997, 1999, 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001). In addition to these are two further groups, represented by the biserial, non-spiral morphospecies *Streptochilus globigerus* (seen here in a comprehensive foraminiferal phylogeny for the first time), and the triserial non-spiral morphospecies *Gallitellia vivans*, the independent origin of which has been proposed by Ujiié *et al*., (2008).

5.5.2.1 The spinose and non-spinose macroperforate planktonic groups

Two of the largest morphologically distinct groups within the planktonic foraminifera are the spinose planktonic and non-spinose macroperforate planktonic taxa. Though both possess the macroperforate test wall structure that is characteristic of the majority of planktonic foraminifera, they differ significantly in other respects. The test of the spinose taxa is primarily globular in form, bearing elaborate radiating spines, a likely adaptation to the planktonic mode of life, while the non-spinose macroperforate taxa lack spines and possess a more flattened, or sometimes carinate smooth test (Hemleben *et al*., 1989; Kennett & Srinivasan, 1983).

In traditional paleontological terms it was thought that the main familial representatives of these two groups, the globigerinids (spinose) and the globorotaliids (non-spinose) shared a common origin (Hart, 1980; Caron, 1983; Bolli, 1986), arising from a single 'globigerinid-like' ancestor in the Mid-Jurassic (Loeblich & Tappan, 1974; Caron & Homewood, 1983). Fossil records place the first spinose globigerinids, 65 Ma ago, after the K/T crisis (Hemleben, 1991), much earlier than the first globorotaliids, which appeared only ~22 Ma, during the Miocene, following the

Eocene-Oligocene crisis (Kennett & Srinivasan, 1983). The Globorotaliidae were thought to have diverged from a Globigerinidae ancestor in the Neogene (Cifelli, 1982; Pearson, 1993), with evolution therefore progressing from globular, globigerinid-like ancestors to carinate, Globorotaliidae-like endmembers (Hart, 1980; Caron, 1983; Bolli, 1986). However de Vargas *et al* (1997) point out that the fossil evidence for a passage from the spinose honeycomb test wall structure to a non-spinose smooth wall is extremely weak. Furthermore, it seems unlikely that such highly adaptive features such as the radial spines, which favour life in a planktonic environment, would be lost in the macroperforate taxa, to be replaced by a smooth form, more consistent with the benthic foraminiferal test.

The spinose planktonic and non-spinose macroperforate planktonic lineages are particularly well represented in the 407 bp phylogeny (fig. 5.3). Each falls as a monophyletic group, recovered consistently with all methods of tree reconstruction, though with relatively low bootstrap support. As in past studies, the precise position of these groups among the benthic taxa cannot be determined, due to poor resolution at the base of the clades, though there may be some association between the non-spinose macroperforate taxa and the benthic morphospecies, *Miliammina fusca* (weakly supported in 0.58 BI posterior probabilities & 62 % ML bootstraps, fig 5.3).

The two groups are not as clearly defined in the phylogenies derived from the almost complete SSU rRNA gene (~ 3,000 bp) (1002 bp dataset), which offer conflicting results, throwing their independent origins into doubt. In the 1002 bp NJ phylogeny, the spinose and non-spinose taxa are separated, supporting their independence (fig. 5.6), however, in the BI tree (fig. 5.4) (found to be optimal in KH tests, appendix 9.5),

the spinose and non-spinose macroperforate taxa fall in a monophyletic group. In the ML tree (fig. 5.5), two microperforates, *G. menardii* and *G. ungulata* are grouped together with the spinose taxa, though this may be due to the long branch attraction (LBA) phenomenon (Bergsten 2005; Olsen, 1987) (these two globorotaliids are also misplaced in the 407 bp NJ tree, falling basal to the spinose planktonic group, together with a number of faster-evolving benthic taxa). It is impossible to identify, from these results, the true relationship between these taxa with any certainty. Only by overcoming the difficulties in amplifying the complete SSU rRNA gene in the planktonic foraminifera (particularly in the spinose taxa), could the problem of poor taxon sampling be overcome, to produce more informative results.

With both the 407 bp & 1002 bp datasets, a monophyletic relationship between the spinose planktonic and non-spinose macroperforate taxa or indeed a complete planktonic foraminiferal monophyly could not be rejected in KH tests (tables 5.5 & 5.6). Though this does throw some doubt on the multiple origins of the planktonic foraminifera, it is also likely that the data itself is insufficient to provide conclusive results, making all hypotheses put forward in the KH test equally likely. For the 407 bp dataset, too few sites are available for analysis, and for the 1002 bp dataset the issue of poor taxon sampling again applies. The presence of excessively long branches within the phylogenies, from those taxa with unusually high rates of evolution may also be problematic.

Despite ambiguities in the phylogenetic analyses, additional molecular data gives compelling evidence against a common origin of the globigerinids and globorotaliids. The foraminifera display unusually variable rates of rDNA evolution, being 50 to 100

times faster in the spinose planktonic Globigerinida, than in some benthic lineages (Pawlowski *et al*., 1997). In this study, relative rates tests (tables 5.9 & 5.10) reveal that rates of substitution in the spinose planktonic taxa are indeed significantly faster than in the benthic taxa, but also significantly faster than in all other groups of planktonic foraminifera, including the non-spinose macroperforate group. Rates of evolution in the macroperforate taxa, excluding certain unusually fast evolving lineages (*G. menardii, G. ungulata, G. truncatulinoides*), are more consistent with those of the benthic foraminifera (tables 5.9 & 5.10), setting them apart from the spinose planktonic taxa. Rates of evolution appear to be more stable within the spinose planktonic group, than in the non-spinose planktonic foraminifera (macroperforate, microperforate, and non-spiral) (tables 5.7 & 5.8). de Vargas *et al*., (1998) also calculated that rates of evolution in the spinose globigerinids are relatively constant, with a mean of 4.3 sub/site/10^9 years, whereas the globorotaliids display more variable rates, ranging from a mean of 1 sub/site/10^9 yrs in the slower evolving taxa (*G. inflata, N. dutertrei, G. hirsuta*), to 7 sub/site/10^9 yrs in the faster evolving lineages (*G. menardii, G. truncatulinoides*). The faster rates of evolution observed in the spinose taxa contrasts the taxonomic rates, calculated from the fossil record, which indicated that the globorotaliids had undergone a significantly more rapid evolutionary turnover than the spinose globigerinids (Stanley *et al*., 1988). The two types of rates are dependent, however, on very different factors. (de Vargas *et al*.,1998).

There are a number of possible explanations for the evolutionary rate homogeneity observed in the foraminifera, a number of which may be symptomatic of life in the pelagic environment. Generation time can influence the rate of evolution (Catzeflis *et al*., 1987; Gaut *et al*., 1992; Laird *et al*., 1969; Li *et al*., 1987; Martin & Palumbi,

1993), with a greater reproductive turnover leading to faster rates. Reproduction in the benthic foraminifera (by alternation of asexual & sexual generations) is slow, with generation times of up to 1 year. The planktonic foraminifera, by contrast, reproduce sexually, usually in a lunar or semi-lunar cycle (Hemleben *et al*., 1989). Pawlowski *et al*., (1997), however, state that this alone is unlikely to explain the extreme rate acceleration observed in the spinose planktonic taxa. Environmental stresses and relative exposure to mutagens may also affect the rate of evolution (Adelman *et al*., 1988), possibly by altering DNA replication or repair mechanisms (Britten, 1986). The thin, delicate shells of the planktonic foraminifera would provide little protection against UV radiation, for example, and in other organisms such as diatoms and echinoids, pelagic life or proximity to the water surface has certainly been correlated with elevated rates of evolution (Kooistra & Medlin, 1996; Smith *et al*., 1992). As the ancestors of the spinose planktonic foraminifera entered the plankton far earlier than those of the non-spinose macroperforate taxa (Kennett & Srinivasan, 1983), their longer exposure to the planktonic domain may have resulted in their extreme rates of evolution.

A high genetic similarity is also observed between the non-spinose macroperforate planktonic taxa and the benthic foraminifera. The macroperforate planktonic taxa show an evolutionary distance of only 0.0700 from the benthic foraminifera, compared to a far greater distance of 0.1859 between the spinose planktonic taxa and the benthic foraminifera. In addition, certain substitutional changes, and an insertion/deletion event in the SSU rRNA gene, have been found to be specific to the spinose taxa (Darling *et al*., 1997). The benthic and non-spinose planktonic (globorotaliid) species possess 2 extra bases in the C1 conserved region of the SSU

rRNA gene, which are lacking in the spinose planktonic taxa. The close genetic homogeneity of the macroperforate and benthic taxa (also observed by de Vargas *et al*., 1997) thus supports the more recent origin of the macroperforate taxa from an independent benthic ancestor.

5.5.2.2 The non-spinose microperforate and non-spiral planktonic foraminifera

The remaining planktonic species fall separately from the spinose and macroperforate planktonic taxa amongst the benthic foraminifera, in an area of the tree where rates of substitution are generally low, and relationships are generally poorly resolved as a result (fig. 5.3). They can be separated from one-another on the basis of test morphology into two groups, the non-spinose microperforate and non-spiral planktonic foraminifera (Kennett & Srinivasan, 1983; Hemleben *et al*., 1989). Morphology alone suggests they are not related to the other planktic lineages, as they lack the large perforations of the test, common to the other planktonic taxa (Hemleben *et al*., 1989).

The non-spinose microperforate species, *Globigerinita glutinata, Globigerinita uvula, Candeina nitida* belong to the family Candeinidae (Decrouez, 1989; Loeblich & Tappan, 1992), and are characterised by their small size and smooth microperforate test (Hemleben *et al*., 1989). The monophyly of this group is rarely proven in phylogenetic analyses (Aurahs *et al*., 2009; Darling *et al*., 2006 Stewart *et al*., 2001), and despite the extremely small evolutionary distance between them (407 bp dataset: *G. glutinata* & *G. uvula* = 0.0075; *G. glutinata* & *Candeina nitida* 0.0006; *G. uvula* & *Candeina nitida* = 0.0075, appendix 9.4.2), the morphospecies fell separately among the benthic taxa in the current analyses based on 407 bp (fig. 5.3). In the 1002 bp

phylogenies (figs. 5.4, 5.5, & 5.6), however, *G. glutinata* and *G. uvula* consistently fell together, as in Ujiié *et al*. (2008). As in past studies utilising the ~1,000 bp partial 3´ terminal fragment of the SSU rRNA gene, poor resolution in the 407 bp phylogeny (fig. 5.3), makes the benthic ancestry of the microperforate Candeinidae impossible to ascertain. The group position in the 1002 bp phylogenies was consistent though, placing it basal to the large group of spinose planktonic, non-spinose macroperforate planktonic, and certain benthic taxa (figs, 5.4, 5.5, & 5.6).

Additional molecular data support the independence of the non-spinose microperforate taxa from the other planktonic foraminiferal groups. Rates of evolution in the microperforate taxa are significantly slower than in the spinose and non-spinose macroperforate groups, and are even significantly slower than that of the benthic taxa (tables 5.9 & 5.10). They are also separated from the other planktonic groups, with the exception of the non-spiral taxa, by large evolutionary distances, sharing a greater genetic homogeneity with the benthic taxa (tables 5.2 & 5.3), supporting the null dissimilarity found between *G. glutinata* and most species of the Rotaliida or Textulariida by de Vargas *et al*. (1997). Both of these factors indicate a recent divergence from benthic taxa, which is consistent with the fossil record estimate for the first appearance of the Candeinidae ~34 Ma ago, during the Eocene-Oligocene crisis (de Vargas *et al*., 1997). It should be noted that this may not have been the first time that microperforate morphospecies had made the transition from benthos to plankton, as other minute microperforate planktonic forms were evident in the fossil record following the K/T extinctions (early Paleogene) (Brinkhuis & Zachsriasse, 1988; Li & Radford, 1991; Lui & Olsson, 1992).

Separate to the microperforate Candeinidae, but also falling amongst the benthic taxa phylogenetically, are the non-spiral planktonic foraminifera (fig. 5.3), represented by the only extant morphospecies of their kind, *Streptochilus globigerus* (family Chiloguembelinidae) and *Gallitellia vivans* (family Guembelitriidae). These have been traditionally placed in the superfamily, Heterohelicacea and are characterised by a microperforate wall structure and biserial and triserial test, respectively (Loeblich & Tappan, 1987).

Rates of evolution in the non-spiral planktonic taxa are comparable to those in the majority of benthic foraminifera and also the microperforate Candeinidae (tables 5.9 & 5.10). Their evolutionary distance from the microperforate Candeinidae is relatively small (0.0030) compared to their distance from the non-spinose macroperforate planktonic taxa (0.0508) and the spinose planktonic group (0.1689) (see table 5.2).

However, despite sharing the morphological characteristic of a non-spiral test, molecular evidence suggests that these morphospecies may actually represent two independent transitions from the benthic to planktonic mode of life, rather than belonging to a single microperforate lineage. They do not group together in the 407 bp phylogeny (fig. 5.3), and though this alone does not prove their independence, the fact that both species share a greater genetic homology to other benthic taxa than to each other does. The distance between *Streptochilus globigerus* and *Gallitellia vivans*, for example is 0.0027 (appendix 9.4.3), compared to a distance of 0.00, or complete sequence homology, between *Streptochilus globigerus* and benthic species *Bolivina variabilis*. *Streptochilus globigerus* also consistently clusters with *B.*

variabilis in phylogenetic analyses (figs. 5.3, 5.4, 5.5, & 5.6) (Darling *et al*., 2009). Comparison of the 1,000 bp partial SSU rDNA sequences of 5 clones from the S. *globigerus* specimens to sequences of *B. variabilis* from various geographical localities revealed almost identical variable elements to those in *B. variabilis* from the Kenyan coastal region (see alignment in appendix 9.7.9, data also presented in Darling *et al*., 2009). It now seems likely that *S. globigerus* and the benthic species *B. variabilis* actually represent the same biological species (within the family Bolivinidae), one that has adopted a tychopelagic lifestyle, utilising both benthic and planktonic habitats. Such an adaptation reveals insights to the possible mechanisms by which the passage from benthic to planktonic life may occur in the foraminifera.

Gallitellia vivans too has been shown to cluster closely with two triserial benthic species, *Stainforthia* and *Virgulinella* in an SSU rDNA phylogeny (Ujiié *et al*., 2008). Though not resolved in the phylogenetic tree here (fig. 5.3), the relationship is reflected in the 0.00 distance from *Gallitellia vivans* to sister species *Stainforthia* (appendix 9.4.3). Ujiié *et al*., (2008) estimate that the divergence of *G. vivans* from the benthic *Stainforthia* lineage took place ~18 Ma ago, in the early Miocene.

5.5.3 The future of foraminiferal phylogenetics

The lack of sufficient information provided by the traditionally used 1,000 bp partial 3′ terminal fragment of the SSU rRNA gene, has resulted in poor resolution and low bootstrap support of the major clades in foraminiferal phylogenetic analyses (fig. 5.3) (Darling *et al*., 1999, 2000, de Vargas *et al*., 1997; Pawlowski *et al*., 1997; Stewart *et al*., 2001). It was hoped that these problems would be alleviated by the use of the complete gene, for reconstructing phylogenies, however, poor taxon sampling due to difficulties in the PCR amplification of the complete gene in certain taxa has limited the success of this strategy. There are certain questions still unanswered regarding the origins of the planktonic foraminifera from the benthic taxa, leaving a clear need to obtain supporting data from other independent genes. Data from other genetic markers could corroborate the topologies and evolutionary distances produced during analyses of the SSU rRNA gene. Ideally, a concatenated data set should be formed, based on several genes. A multi-gene approach to phylogenetic analysis would provide a far more accurate picture of the evolutionary origins of the planktonic foraminifera and the relationships of the closely related genotypes within morphospecies. It is important to have data from a variety of genetic markers that differ in their rate of change and thus their usefulness in resolving evolutionary relationships at different taxonomic levels.

However, our ability to amplify and sequence new genes is severely limited by the extreme lack of genetic data available for the foraminifera. We currently know almost nothing about the foraminiferal genome and only a handful of foraminiferal genes have been sequenced so far, mostly for benthic taxa. Protein coding genes such as tubulin, ubiquitin and RNA polymerase II largest subunit (RPB1) could be utilised, so

far sequenced for a small number of benthic species (Pawlowski *et al.*, 1999; Archibald *et al.*, 2003; Longet *et al.*, 2003; Habura *et al.*, 2005) and 2 planktonic species (*Globigerinella siphonifera* and *Globigerinita glutinata*) in the case of RPB1 (Longet & Pawlowski, 2007). The actin gene, for which a well-represented phylogeny already exists for the benthic foraminifera (Flakowski *et al.* 2005), has been sequenced for a single planktonic foraminifer, *Globigerinella siphonifera* (Flakowski *et al.* 2005) and represents another option. Whilst amplification of these nuclear markers has proven successful in the benthic foraminifera, it could be more problematic in the planktonic foraminifera, which unlike their multinucleate benthic relatives carry only a single copy of their genome. The problem could possibly be circumvented by the use of gametogenic specimens (as in Darling *et al.*, 1996a,b, 1997).

The identification of new genetic markers could be achieved by creating an expressed sequence tag (EST) library, a quick and inexpensive way to identify unknown genes and to map their positions within a genome (Theodorides *et al.*, 2002, Whitton *et al.*, 2004; Davison & Blaxter 2005; Papanicolaou *et al.*, 2005). Alternatively, the new genome sequencing technique developed by 454 Life Sciences Corporation could be utilised, which boasts a 100-fold throughput over previous sequencing technologies (Margulies *et al.*, 2005). In order to achieve such goals, it will first be essential to establish a reliable supply of foraminiferal DNA, which could be accomplished through laboratory culturing (see chapter 6). In addition to providing the template for genomic work, a sustained supply of genetic material would be invaluable for PCR optimisation & primer development, the lack of which, due to unavailability of samples, severely limits our current work.

Extensive difficulties have also been encountered in the PCR amplification of the foraminiferal SSU rRNA gene, with high failure rates resulting in poor taxon sampling, particularly for the almost complete gene (~3,000 bp fragment). In addition to primers being a poor match to certain taxa (discussed earlier), the DNA extraction method currently employed, using the buffer of Holzmann *et al*. (1996), may result in the transfer of inhibitory substances into the PCR reaction, and long-term storage of samples in the extraction buffer, sometimes without freezing, may also lead to degradation of the template DNA. The procurement of fresh samples, and the development of an effective DNA extraction and storage method will be necessary to overcome such problems (see chapter 7).

5.6 Conclusions

According to the phylogenetic analyses presented here, the planktonic foraminifera are represented by up to 5 extant lineages, each of which may represent an independent move from benthic ancestors to the planktonic mode of life. It is likely that the move from benthos to the plankton was not a single unusual event, but one that has occurred numerous times throughout geologic history, the necessary adaptations developing in parallel in several groups of benthic taxa. A body of evidence consisting of molecular, morphological, and biological data support the independence of these major planktonic foraminiferal groups.

Phylogenies constructed from the traditionally used ~1,000bp partial terminal 3′ fragment of the SSU rRNA gene, from which 407 bp could be reliably aligned, were unfortunately subject to the same shortcomings as in previous studies, with poor resolution and low bootstrap support for the major clades. Furthermore, such

problems could not be resolved, as hoped, by the use of the complete SSU rRNA gene in phylogenetic analysis, as high PCR failure rates resulted in poor taxon sampling. Further work is needed to resolve such issues, and to confirm the validity of the current findings regarding the independent origins of the 5 planktonic foraminiferal lineages. The use of new genetic markers will be invaluable in accomplishing this task and will hopefully allow the avoidance of the problems associated with the use of foraminiferal ribosomal genes in phylogenetic analysis, largely the result of their extreme and variable rates of evolution. Efforts will also continue to obtain the complete SSU rDNA sequences for the remaining spinose planktonic taxa, using fresh samples and an efficient new method of DNA extraction (see chapter 7).

5.7 References

Adelman, R., Saul, R. L., & Ames, 1988. Oxidative damage to DNA: Relation to species metabolic rate and life span. *Proc. Natl. Acad. Sci.* USA, **85**, 2706-2708

Anderson, O. R., & Bé, A. W. H. 1976. The ultrastructure of a planktonic foraminifer *Globigerinoides sacculifer* (Brady), and its symbiotic dinoflagellates. *Journal of Foraminiferal Research*, **6**, 1-21

Archibald, J. M., Longet, D., Pawlowski, J., & Keeling, P. J. 2003. A novel Polyubiquitin structure in Cercozoa and Foraminifera: evidence for a new eukaryotic supergroup. *Molecular Biology and Evolution*, **20** (1), 62-66

Aurahs, R, Grimm, G. W., Hemleben, V., Hemleben, C., & Kucera, M. 2009 Geographical distribution of cryptic genetic types in the planktonic foraminifer *Globigerinoides ruber. Molecular Ecology*, **18**, 1692 – 1706

Banner, F. T., & Lowry, F. M. D. 1985. The stratigraphical record of planktonic foraminifera and its evolutionary implications. *Spec. Pap. Paleontol*., **33**, 117-130

Bergsten, J. 2005. A review of long-branch attraction. *Cladistics* **21**(2), 163-193

Bolli, H. M. 1986. Evolutionary trends in Planktic Foraminifera from early Cretaceous to Recent, with special emphasis on selected tertiary lineages. *Soc. Nat. Elf. Aquitaine* BCREDP, **10**, 555-577

Bolli, H. M., Loeblich Jr., A. R., & Tappan, H. 1957. Planktonic foraminiferal families Hantkeninidae, Orbulinidae, Globorotaliidae, and Globotruncanidae. *Bull. U.S. Nat. Mus*, **215**, 1-50

Brinkhuis, H., & Zachariasse, W. J. 1988. Dinoflagellate cysts, sea level changes in planktonic foraminifers across the Cretaceous-Tertiary boundary at El Haria, Northwest Tunisia. *Marine Micropaleontology*, **13**, 153-191

Britten, R. J. 1986. Rates of DNA sequence evolution differ between taxonomic groups. *Science*, **231**, 1393-1398

Caron, M. 1983. La spéciation chez les foraminifères planctiques: une résponse adaptée aux contraintes de l'environment. *Zitteliana*, 10, 671-676

Caron, M., & Homewood, P. 1983. Evolution of early planktic foraminifers. *Marine Micropaleontology*, **7**, 453-462

Catzeflis, F. M., Sheldon, F. H., Ahlquist, J. E., & Sibley, C. G. 1987. DNA-DNA hybridization evidence of the rapid rate of rodent DNA evolution. *Mol. Biol. Evol*., **4**, 242-253

Cifelli, R. 1982. Early occurrences and some phylogenetic implications of spiny, honeycomb textured planktonic foraminifera. Journal of Foraminiferal Research, 12, 105-115

Culver, S. J. 1991. Early Cambrian Foraminifera from West Africa. *Science*, **254**, 689-691

Culver, S. J. 1993. Foraminifera. In: Fossil prokaryotes and protists (ed. Lipps, J. H.). Blackwell. Sci. Publ., Oxford, London, Edinburgh, Melbourne, Paris, Berlin, Vienna

Cushman, J. A. 1948. *Foraminifera: Their classification and Economic Use*, 4th Ed., Harvard University Press, Cambridge

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996a. The isolation and amplification of the 18s ribosomal RNA gene from planktonic foraminifers using gametogenic specimens. *In: Microfossils and oceanic environments* (eds.

Moguilevsky, A & Whatley, R.): University of Wales, Aberystwyth Press, 3.1, 249-259

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996b. Molecular phylogeny of the planktonic foraminifera. *Journal of Foraminiferal Research*, **26** (4), 324-330

Darling, K. F., Thomas, E., Kasemann, S. A., Seears, H. A., Smart, C. W., & Wade, C. M. 2009. Surviving mass extinction by bridging the benthic/planktic divide. *Proc. Natl. Acad. Sci.* USA, **106** (31), 12629-33

Darling, K. F., Kucera, M., Kroon, D., & Wade, C. M. 2006. A resolution for the coiling direction paradox in *Neogloboquadrina pachyderma*. *Paleoceanography,* **21**, PA2011, doi:10.1029/2005PA001189

Darling, K. F., Wade, C. M., Steward, I. A., Kroon, D., Dingle, R., & Leigh Brown, A. J. 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature***, 405**, 43-47

Darling, K. F., Wade, C. M., Kroon, D. & Leigh Brown, A. J. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. *Marine Micropaleontoogy,* **30**, 251-266

Darling, K. F., Wade, C. M., Kroon, D., Leigh Brown, A. J. & Bijma, J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulation. *Paleoceanography,* **14**, 3-12

Davison, A. & Blaxter, M. L. 2005. An expressed sequence tag survey of gene expression in the pond snail *Lymnaea stagnalis*, an intermediate vector of *Fasciola hepatica*. *Parasitology,* **130,** 539-552

de Vargas, C., & Pawlowski, J. 1998. Molecular versus taxonomic rates of evolution in planktonic foraminifera. *Molecular Phylogenetics and Evolution*, **9** (3), 463-469

de Vargas,C., Zaninetti, L., Hilbrecht, H. & Pawlowski, J. 1997. Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record. *Journal of Molecular Evolutio,n* **45** (3), 285-294

De Weaver, P., Dumitrica, P., Caulet, J. P., Nigrini, C., & Caridroit, M. 2001. Skeletal structures and shapes. In: Radiolarians in the sedimentary record (eds. De Weaver, P., Dumitrica, P., Caulet, J. P., Nigrini, C., & Caridroit, M.). Gordon and Breach Science Publishers, 13 pp

Decrouez, D. 1989. Generic ranges of Foraminiferida. *Revue de Paléobiologie*, **8**, 263-321

d'Orbigny, A. 1826. Tableau Méthodique de la Classe des Céphalopodes. *Annales des Sciences Naturelles, Paris (Série 1)*, **7**, 245-314

Douglas, R., & Savin, S. M. 1972. Depth stratification in Tertiary and Cretaceous planktonic Foraminifera. *Geol. Soc. Am., Abstr. with Programs, 1972 Ann. Meet.*, **4** (7), 491

Efron, B. 1982. The Jackknife, Bootstrap, and other resampling plans. *CBMS-NSF Regional conference series in applied mathematics*, Society for industrial and applied mathematics, Philadelphia

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution,* **17**, 368-376

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39: 783-79

Flakowski, J., Bolivar, I., Fahrni, J., & Pawlowski, J. 2005. Actin phylogeny of foraminifera. *Journal of Foraminiferal Research*, **35** (2), 93-102

Fogg, C. E. 1965. *Algal Cultures and Phytoplankton Ecology*. University of Wisconsin Press, Madison and Milwaukee, Wisconsin.126 pp

Gaut, B. S., Muse, S. V., Clark, W. D., & Clegg, M. T. 1992. Relative rates of nucleotide substitution at the *rbc*L locus of monocotyledonous plants. *Journal of Molecular Evolution*, **35**, 292-303

Görög, A. 1994. Early Jurassic planktonic foraminifera from Hungary. *Micropaleontology*, **40**, 255-260

Habura, A., Wegener, L., Travis, J. L., & Bowser, S. S. 2005. Structural and Functional Implications of an Unusual Foraminiferal *b*-Tubulin. *Mol. Biol. Evo*l. **22** (10), 2000-2009

Hart, M. B. 1980. A water depth model for the evolution of the planktonic Foraminifera. *Nature*, **286** (5770), 252-254

Hemleben, C., Mühlen, D., Olsson, R. K., & Berggren, W. A. 1991. Surface texture and the first occurrence of spines in planktonic foraminifera from the early Tertiary. *Geol Jahrbuch*, **128**, 117-146

Hemleben, Ch., Spindler M., & Anderson, O. R. 1989. Modern Planktonic Foraminifera. Springer Verlag, New York

Holzmann, M., & Pawlowski, J. 1996. Preservation of foraminifera for DNA extraction and PCR amplification. *Journal of Foraminiferal Research*, **26** (3), 264-267

Hutchinson, G. E., 1967. *A treatise on Limnology, V. 2, Introduction to Lake Biology and the Limnoplankton.* Wiley, New York, N.Y., 1115 pp

Kennett, J. P., & Srinivasan, M. S. (eds). 1983. *Neogene planktonic foraminifera, a phylogenetic atlas*. Hutchinson Ross Publishing Company, Stroudsburg, Pennsylvania

Kishino, H., & Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution*, **29**, 170-179

Kooistra, W. H. C. F., & Medlin, L. K. 1996. Evolution of the diatoms (Bacillariophyta). *Mol. Phyl. Evol*., **6**, 391-407

Laird, C. D., McConaughy, B. L., & McCarthy, B. J. 1969. Rate of fixation of nucleotide substitutions in evolution. *Nature*, **224**, 149-154

Lanave, C, Preparata G, Saccone C, & Serio G. 1984. A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution,* **20**, 86-93

Larget, B., & Simon, D. L. 1999. Markov chain monte carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution*, **16,** 750-759

Li, Q., & Radford, S. S. 1991. Evolution and biogeography of Paleogene microperforate planktonic foraminifera. *Paleogeo., Paleoclim., Paleoecol.*, **83**, 87-115

Li, W. H., Tanimura, M., & Sharp, P. M. 1987. An evaluation of the molecular clock hypothesis using mammalian DNA sequences. *Journal of Molecular Evolution*., **25**, 330-342

Lipps, J. H. 1966. Wall structure, systematics, and phylogeny studies of Cenozoic planktonic Foraminifera. *Journal of Paleontology*, **40**, 1257-1274

Liu, C., & Olsson, R. K. 1992. Evolutionary radiation of microperforate planktonic foraminifera following the K/T mass extinction event. *J Foram Res,* **22**, 328–346

Loeblich, A. R., & Tappan, H. 1987. *Foraminiferal Genera and Their Classification*, v. 1-2, Van Nostrand Reinhold, New York

Loeblich, A. R., & Tappan, H. 1992. Present Status of foraminiferal classification. In: *Studies in Benthic Foraminifera* (eds. Takayangi, Y & Saito, T), Proceedings of the Fourth International Symposium on Benthic Foraminifera, Sendai, 1990 (Benthos '90), Tokai University Press, Tokyo, Japan, pp. 93-102

Loeblich, Jr., A. R., & Tappan, H. 1974. Recent advances in the classification of the Foraminiferida. In: *Foraminifera*, 1 (eds. Hedley, R. H., & Adams, C. G.). Academic Press, London, p1-53

Longet, D., & Pawlowski, J. 2007. Higher-level phylogeny of Foraminifera inferred from the RNA polymerase II (RPB1) gene. *European Journal of Protistology*, **43**, 171-177

Longet, D., Archibald, J. M., Keeling, P. J., & Pawlowski, J. 2003. Foraminifera and Cercozoa share a common origin according to RNA polymerase II phylogenies. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 1735-1739

Lui, C., & Olsson, R. K. 1992. Evolutionary radiation of microperforate planktonic foraminifera following the K/T mass extinction event. *J. Foraminiferal Res*., **22**, 328-346

Margulies, M. *et al*. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376- 380

Martin, A. P., & Palumbi, S. R. 1993. Body size, metabolic rate, generation time and the molecular clock. *Proc. Natl. Acad. Sci. USA*, **90**, 4087-4091

Mayr, E. 1960. The emergence of evolutionary novelties. In: *Evolution after Darwin* (ed. Tax, S), University of Chicago Press, Chicago, III, 1, 349-380

Norris, R. D. 1991. Biased extinction and evolutionary trends. *Paleobiology*, **17** (4), 388-399

Olsen**,** G. 1987. Earliest phylogenetic branching: comparing rRNA-based evolutionary trees inferred with various techniques. *Cold Spring Harb. Symp. Quant. Biol.* **LII**, 825–837

Olsson, R. K., Hemleben, C., Berggren, W. A., & Huber, B. T. 1999. Atlas of Paleocene Planktonic Foraminifera. *Smithsonian Contributions to Paleobiology*. Smithsonian Institution Press, Washington, DC, No.85.

Olsson, R. K., Hemleben, C., Berggren, W. A., & Liu, C. 1992. Wall texture classification of planktonic foraminifera genera in the lower Danian. *Journal of Foraminiferal Research*, **22**, 195-213

Papanicolau, A., Joron, M., McMillan, W. O., Blaxter, M. L. & Jiggins, C. D. 2005 Genomic tools and cDNA derived markers for butterflies. *Molecular Ecology* **14,** 2883-2897

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C., & Bowser, S. S. 1999. Molecular evidence that *Reticulomyxa filosa* is a freshwater naked foraminifer. *Journal of Eukaryotic Microbiology*, **46** (6), 612-617

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C., Gouy, M. & Zaninetti, L. 1997. Extreme differences in rates of molecular evolution of foraminifera revealed by comparison of ribosomal DNA sequences and the fossil record. *Mol. Biol. Evol.* **14** (5), 498-505

Pearson, P. N. 1993. A lineage phylogeny for the Paleogene planktonic foraminifera. *Micropaleontology*, **39**, 193-232

Petrushevskaya, M. G., Cachon, J. & Cachon, M. 1976. Comparative morphological study of Radiolaria: Foundations of a new taxonomy. *Journal of Zoology,* **55**, 485-496

Ronquist, F., & Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572-1574

Ruttner, F. 1963. *Fundamentals of Limnology*. (Translated by Frey, D. G., & Fry, F. E. J.) University of Toronto Press, Toronto, Ont., 3rd ed., 295 pp

Saito, T., Thompson, P. R., & Breger, D. 1981. *Systematic index of recent and Pleistocene planktonic foraminifera*. University of Tokyo Press

Saitou, N., & Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., **4**, 406-425

Salemi, M. 2009. Genetic distances and nucleotide sunstitution models: practice. In: *The phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing* (eds. Lemey, P., Salemi, M., & Vandamme, A-M). Cambridge University Press, Cambridge UK

Sarich, V. M. & Wilson, A. C. 1967. Immunological time scale for hominid evolution. *Science,* **158**, 1200-1203

Schönborn, W. 1962. Über Planktismus und Zyklomorphose bei *Difflugia limnetica* (Levander) Penard. *Limnologica*, **1**, 21-34

Smith, A. B., Lafay, B., & Christen, R. 1992. Comparative variation of morphological and molecular evolution through geologic time: 28S ribosomal RNA versus morphology in echinoids. *Philos. Trans. R. Soc. Lond*. B, **338**, 365-382

Smith, S. W., Overbeek, R., Woese, R., Gilbert, W. & Gillevet, P. M., 1994. The genetic data environment and expandable GUI for multiple sequence analysis. *Comput. Appl. Biosci*., **10**, 671-675

Staden, R. Beal, K. F. & Bonfield, J. K. 2000. The Staden package, 1998. Methods in Molecular Biology **132**: 115-130

Stanley, S. M., Wetmore, K. L., & Kennett, J. P. 1988. Macroevolutionary differences between two major clades of Neogene planktonic foraminifera. *Paleobiology*, **14**, 235-249

Stewart, I. A., Darling, K. F., Kroon, D., Wade, C. M., & Troelstra, S. R. 2001. Genotypic variability on subarctic Atlantic planktic foraminifera. *Marine Micropaleontology*, **43**, 143-153

Tappan, H. & Loeblich Jr, A. R. 1973. Evolution of the Oceanic Plankton. *Earth-Science Reviews*, **9**, 207-240

Tappan, H. & Loeblich, A. R. Jr. 1988. Foraminiferal evolution, diversification, and extinction. *Journal of Paleontology,* **62** (5), 695-714

Theodorides, K., De Riva, A., Gómez-Zurita, J., Foster, P. G., & Vogler, A. P. 2002. Comparison of EST libraries from seven beetle species: towards a framework for phylogenomics of the Coleoptera. *Insect Mol. Biol.* **11,** 467-475

Ujiié, Y., Kimoto, K., & Pawlowski, J. 2008. Molecular evidence for an independent origin of modern triserial planktonic foraminifera from benthic ancestors. *Marine Micropaleontology*, **69**, 334-340

Vickerman, K. 1992. The diversity and ecological significance of Protozoa. *Biodiversity and Conservation*, **1**, 334-341

Wernli, R. 1988. Les protoglobigérines (foraminifères) du Toarcien et de l'Aalénien du Domuz Dag (Taurus Occidental, Turquie). *Ecologae geol Helv*, **81**, 661-668

White, T. J, Burns, T, Lee, S, & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (eds Innis MA, Gelfland DH, Sninsky JJ, White TJ), Harcourt Brace Jovanovich, San Diego, pp 315 – 322

Whitton, C., Daub, J., Thompson, M. & Blaxter, M. 2004. Expressed sequence tags: medium-throughput protocols. *Method. Mol. Biol.* **270,** 75–92

Yang, Z. 1993. Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates vary over sites. *Molecular Biology and Evolution,* **10**, 1396-1401

6 Culturing foraminifera: the first step towards developing a model organism

6.1 Introduction

6.1.1 The need for laboratory cultures of foraminifera

Our ability to amplify and sequence new genes in the foraminifera is severely limited by the extreme lack of genetic data available for the group. There is a pressing need to identify new markers for this important group, both to enhance phylogenetic analyses of the foraminifera and for use in population genetic studies. Being unicellular organisms, each foraminiferan provides only a limited source of DNA to work with, leaving a need to pool individuals. Laboratory culturing could provide an invaluable source of the raw genetic material needed for a range of molecular applications.

To date, phylogenetic studies of the foraminifera have focused primarily on a single gene, the small subunit ribosomal RNA (SSU rRNA) gene (Darling *et al.*, 1997, 1999, 2000, 2004; Wade *et al.,* 1996b; de Vargas *et al.,* 1997, 1999, 2001, 2002; Pawlowski *et al.,* 1997). Some sequencing has been carried out of the tubulin, ubiquitin and RNA polymerase II largest subunit (RPB1) genes, for only a small number of benthic species (Pawlowski *et al.*, 1999; Archibald *et al.*, 2003; Longet *et al.*, 2003; Habura *et al.*, 2005) and Longet *et al.* (2007) gained some sequences of RPB1 in 2 species of planktonic foraminifera, *Globigerinella siphonifera* and *Globigerinita glutinata.* The actin gene has been more thoroughly sequenced, in benthic taxa, providing a well-represented phylogeny (Flakowski *et al.* 2005), though it has only been sequenced for

a single species of planktonic foraminifera, *Globigerinella siphonifera* (Flakowski *et al.* 2005).

While the SSU rRNA gene has proven to be an enormously useful marker for investigating foraminiferal evolutionary relationships, only a short region of ~1000bp is generally used in phylogenetic analyses and many relationships within the foraminifera remain unresolved due to insufficient information. In addition, inconsistencies are often seen among the phylogenies produced using different tree-building methods. There is therefore a clear advantage to be gained from the introduction of new genes for use in phylogenetic reconstructions, to lend support to existing phylogenies and to provide greater resolution to the trees produced.

Population genetic studies of the foraminifera have focused solely the SSU rRNA gene, thanks to the unusually high rate of change observed in the rRNA genes of planktonic foraminifera (Darling *et al.*, 1999, 2000, 2003, 2004, 2006; de Vargas *et al.*, 1999, 2001, 2002; Stewart *et al.*, 2001). However, the resolution is often weak, highlighting an obvious need to identify more variable markers, for example mitochondrial genes (Avise *et al.*, 1987), that are better suited for examining evolutionary relationships between closely related foraminiferal species and among within-morphospecies genetic types.

As discussed in chapter 5, new markers for investigating foraminiferal evolution could be identified by the construction of an expressed sequence tag (EST) library (Theodorides *et al.*, 2002, Whitton *et al.*, 2004; Davison & Blaxter 2005; Papanicolaou *et al.*, 2005), or by full genome sequencing in microfabricated high-

density picolitre reactors (as developed by the 454 Life Sciences Corporation) (Margulies *et al.*, 2005). However, in order to carry out either of the molecular methods above, you first need a large and continual source of genetic material. Foraminifera are single-cell organisms, and thus to gain enough genetic material, many individuals need to be pooled. Pooling many individuals collected from the field is possible, however, minor genetic variation could lead to sequence ambiguities. In order to gain many identical copies of the genome it would therefore be more desirable to set up a clonal culture system, in a controlled laboratory environment, starting from a single, asexually reproducing individual. If the whole foraminiferal genome was eventually obtained from such a clonal system, it could pave the way to their use as a model organism, particularly for the study of evolutionary processes.

In practical terms, the maintenance of a continual culture of foraminifera in the laboratory, would eliminate the need for repeated collection trips to the field, which can be inconvenient if you are based a great distance from the sea, and would provide a constant source of material for several molecular uses.

A large supply of readily available DNA would allow the extensive testing of PCR and sequencing primers, prior to use on important samples, something that has been impossible until now. This would be of great benefit when developing primers to amplify and sequence new genes as molecular markers and would also allow improvements to be made to primers currently used on genes such as the SSU rRNA and Actin genes, especially in taxa that have proven difficult to amplify.

In addition to developing primers for use on individual genetic markers, a large source of genetic material could also allow for genomic studies of the foraminifera, something that has not yet been attempted in either the planktonic or benthic taxa. Here the whole genome would be mapped, something that is increasingly being undertaken for other organisms, for example early work on bacteriophage λ (Sanger *et al*., 1982), and more recently work on many species including members of the Bacteria, e.g. *Escherichia coli* (Blattner *et al*., 1997), Archaea, e.g. *Archaeoglobus fulgidus* (Klenk *et al*., 1997), and Eukaryota, e.g. *Mus musculus* (Waterston *et al*., 2002).

6.1.2 Culturing foraminifera

A number of species of benthic foraminifera have been successfully maintained or cultured under laboratory conditions (reviewed in Anderson *et al*., 1991). Some are simply maintained in the laboratory, meaning that they are kept alive for sustained periods, from weeks to years, but do not reproduce. This can be achieved relatively easily. Culturing, on the other hand, refers to the continuous growth and reproduction of several generations in the lab, which can prove more difficult. The methods used for culturing foraminifers vary greatly, some involving sophisticated equipment that provide a highly tailored environment, and others that are simple in design, easy to set up and less time-consuming.

Sophisticated systems such as circulating and re-circulating marine aquaria and other flow-through systems provide a highly successful means of maintaining foraminifera. A gentle flow of water is provided and the systems are illuminated, aerated, temperature regulated and the pH and salinity usually adjusted daily. Glass covers are

used to reduce evaporation and slow salinity shifts. Lutze & Wefer (1980) used a simple circulating aquarium to culture and observe asexual reproduction in the larger foraminifera, *Cyclorbiculina compressa.* Re-circulating systems provide the additional advantage of reducing the recruitment of new organisms into the aquarium that could interfere with the foraminiferal population. Species such as *Ammonia beccarii*, *Rosalina leei* and *Bolivina vaughni* have been kept alive and reproducing for years (reviewed in Anderson *et al.*, 1991) using such systems. However, it has been found that the gravel needed to maintain proper filtration in circulating and re-circulating marine aquaria does make it difficult to harvest and examine the foraminifera (Arnold, 1974). Commercially available chemostats may be used as an alternative. These provide a continuous flow of fresh sterile media into the foraminiferal culture, and may be superior to other flow-through systems. Lee *et al.* (1991b) successfully used a chemostat system to conduct nutritional experiments on the larger foraminifera, *Amphistegina lobifera*, *Amphisorus hemprichii* and *Marginopora kudakajimensis*. The disadvantage of all of these sophisticated systems is that specialist equipment is required to set them up, which may be costly or difficult to make.

Many people instead choose a more simplistic approach to maintaining or culturing foraminifera. Petri dishes or beakers make suitable culture vessels for small-scale studies. Light, temperature, salinity and pH can be manipulated manually, using basic lab equipment. Stouff *et al.* (1999a,b) successfully cultured *Ammonia tepida* and *Ammonia beccarii* in petri dishes and beakers, with a little sediment, and natural seawater changed monthly. *Ammonia tepida* has also been cultured by Morvan *et al.* (2004), using simple petri dishes and filtered seawater. Takata *et al.* (2009) used a

basic method to investigate the substrate preferences of *A. beccarii* and Lee *et al.* (1991b) maintained cultures of 3 species of symbiont-bearing, large foraminifera (*A. lobifera*, *A. hemprichii* and *M. kudakajimensis)* using plastic tissue-culture flasks and salinity-adjusted natural seawater.

In this study 3 different species of benthic foraminifer were cultured in the laboratory, using a number of basic culturing methods. The methods and conditions used were experimented with and optimised during the course of the study, to best encourage growth and reproduction in these foraminifera.

Ultimately, it would also be desirable to culture a species of planktonic foraminifera but this is likely to prove considerably more problematic. A number of species of planktonic foraminifera have been maintained in laboratory studies, for example *Orbulina universa* (Mashiotta *et al*., 1997; Russell *et al*., 2004, Uhle & Macko, 1999), *Globigerina bulloides* (Mashiotta *et al*., 1997; Russell *et al*., 2004), *Globigerinella siphonifera* (Bijma *et al*., 1998), and *Globigerinoides sacculifer* (Bé, 1980; Spero & Lea, 1993), however these were not truly cultured. Whilst benthic foraminifera reproduce mainly by asexual multiple fission, planktonic foraminifera reproduce entirely by sexual reproduction (Murray, 2006). To date, they have not been kept in continuous culture from generation to generation, but have only been maintained during development from young stages to maturity and gamete release. As Hemleben *et al*. (1989) stated, these can only be considered as maintenance cultures. In addition, the sexual nature of planktonic foraminiferal reproduction would make it impossible to establish a genetically identical ‘mono-culture’, something that could be achieved

with benthic taxa, as they can reproduce asexually for many generations, allowing a culture to be established rapidly.

6.1.3 Choosing a target species

6.1.3.1 Life cycle of the foraminifera

Benthic foraminifera typically reproduce by a classical dimorphic life cycle, consisting of a regular alternation between sexual and asexual generations (Goldstein, 1999; Lee *et al*., 1991a) (see chapter 1, fig. 1.3). The haploid, megalospheric gamont releases gametes (~ 1-4 μm), which are fertilized to produce a zygote, eventually giving rise to a diploid microspheric agamont (sexual reproduction). The agamont then produces haploid megalospheric young, by multiple fission (asexual reproduction), and the cycle begins again. A biological dimorphism usually exists between the gamont (sexual), which has a single nucleus and a megalospheric test, characterised by a large proloculus (1st chamber) but a relatively small overall diameter, and the agamont (asexual), which is multinucleate and has a microspheric test, characterised by a smaller proloculus, but relatively larger overall test diameter (Goldstein, 1999). Exceptions to this rule do exist in some species, where this size relationship may be inverted. This dimorphic life cycle was first recognised by Lister (1895) in a study of the benthic foraminifer, *Elphidium crispum*, and was later confirmed by Schaudinn (1895). The alternation of generations may be obligatory (fixed) in some foraminifera, for example *Elphidium crispum* (Lister, 1985; Jepps 1942) and *Glabratella sulcata* (Grell, 1958), or facultative (flexible) in others, e.g. *Ammonia tepida* (Bradshaw, 1957; Goldstein & Moodley, 1993) and *Saccammina alba* (Goldstein, 1988). The facultative system is often referred to as a biologically trimorphic life cycle, and involves successive asexual reproduction that inserts a third,

biologically distinct form between the agamont and gamont generations, the megalospheric schizont (chapter 1, fig. 1.3; Stouff *et al*., 1999a). Schizonts reproduce by multiple fission, to produce either another schizont generation, or megalospheric gamonts, at which point the cycle is closed. Schizogony may become cyclic, with several generations of schizonts following one another (Dettmering *et al.*, 1998). Trimorphism was first suggested as a reproductive strategy in foraminifera by Rhumbler (1909) and was subsequently proposed to be the dominant life cycle in larger foraminifera (Leutenegger 1977; Lee *et al.*, 1991a). Confirmation of this reproductive strategy came when primary successive asexual reproduction was recorded in laboratory cultures of *Heterostegina depressa* (Röttger *et al.*, 1986, 1990), and has since been documented in other species such as the larger foraminifer *Amphistegina gibbosa* (Dettmering *et al.*, 1998; Harney *et al.*, 1998), and in *Ammonia tepida* (Stouff *et al.*, 1999a). In contrast to the variety of reproductive strategies seen in the benthic foraminifera, only sexual reproduction has ever been recorded in the planktonic taxa (Goldstein, 1999; Murray, 2006). Hemleben *et al*. (1989) suggest that the agamont and multiple fission have been lost, leaving a 'gamic' life cycle.

The dimorphic or sometimes trimorphic life cycles of the benthic foraminifera makes them particularly well suited to culture in the laboratory, with large numbers of offspring being produced asexually. This may be particularly true of those species with a trimorphic life cycle, where the alternation of generations is facultative, and successive asexual generations may be produced by schizogony. It has been postulated that, in species with a trimorphic life cycle, alternation of asexual and sexual generations is more common in favourable conditions, and that low densities or environmental stress may trigger successive asexual generations (cyclic schizogony)

(Harney *et al.*, 1998; Röttger, 1990). The advantage of this strategy is that through asexual reproduction, by multiple fission, large numbers of generally larger, megalospheric offspring are produced, acquiring their symbionts directly from the parent. The same advantage is not afforded to microspheric agamont offspring, as the tiny gametes that form them carry no symbionts, and must be acquired from the environment. Successive asexual generations can quickly increase the population size and propagate genetically identical offspring of the genotype that survived the stress. In culture systems, where stress is often apparent, this mechanism could be harnessed to rapidly produce large populations of genetically identical individuals.

6.1.3.2 Foraminifera and symbionts

A significant problem faced in molecular studies of the foraminifera is the occurrence of potentially contaminating endosymbionts, commensals and prey items in the samples. The presence of algal endosymbionts is prevalent in certain benthic species of foraminifera from the families Peneroplidae, Soritidae, Alveolinidae (order Milliolida), Amphisteginidae, Calcarinidae and Nummulitidae (order Rotaliida) (Hallock, 1988; Lee & Anderson, 1991). Some families of smaller rotaliid foraminifers (e.g. Asterigerinidae) may also contain a few members that host endosymbionts, however, these have yet to be studied in detail (Hallock, 1999). In addition to these true symbiont-bearing species, some benthic foraminifera of the families Nonionidae, Elphidiidae, and Rotaliellidae are known to sequester chloroplasts from algal food sources, though they are eventually digested, and must be constantly replenished (Goldstein, 1999).

Within the planktonic foraminifera many species bear true symbionts, including, *Orbulina universa*, *Globigerinella siphonifera, Turborotalita humilis,* and all species from the genus *Globigerinoides*. A number also harbour facultative (non-permanent) symbionts, including, *Globigerinita glutinata*, *Neogloboquadrina dutertrei*, *Pulleniatina obliquiloculata*, *Globorotalia inflata* and *Globorotalia menardii* (Hemleben *et al*., 1989).

When establishing a culture to gain genetic material for molecular work there would be an obvious advantage to selecting a species of foraminifera that bears no symbionts. The benthic species *Ammonia tepida* was chosen as the best candidate. This is a species found commonly on the British coast that has been shown to reproduce under laboratory conditions in previous studies (de Nooijer *et al.*, 2009; Morvan *et al.*, 2004; Stouff *et al.,* 1999a). *Ammonia tepida* belongs to the order Rotaliina, which contains 3 families with symbiont-bearing members, however *A. tepida* is reportedly symbiont-barren (J. Murray, Pers. Comm.). Another two species, *Bolivina variabilis* (order Rotaliina) and *Cornuloculina balkwilli* (order Milliolida) were also investigated. Neither is thought to bear symbionts (J. Murray, Pers. Comm.), though *Cornuloculina balkwilli* belongs to the suborder Miliolina, of which many members are symbiont-bearing.

The eventual aim would also be to culture a member of the planktonic foraminifera. Here *Globigerina bulloides* would be the ideal choice for molecular study, as it is unusual in lacking symbionts and has also been maintained in the laboratory to the point of gametogenesis in the past (Darling *et al*., 1996a,b; Mashiotta *et al*., 1997; Russell *et al*., 2004; Wade *et al*., 1996). Other planktonic species lacking symbionts could also be considered, including *Hastigerina pelagica*, *Neogloboquadrina*

pachyderma, Globorotalia truncatulinoides and *Globorotalia hirsuta* (Hemleben *et al*., 1989). Nevertheless, difficulties associated with culturing planktonic foraminifera may well preclude the development of a culture system.

If endosymbionts were present in the species selected, the genetic material could still be used, however, it would be important to screen any sequences obtained to ensure that they were foraminiferal and not contaminant in origin.

6.1.4 The target species

6.1.4.1 Ammonia tepida

Ammonia tepida (Cushman), of the order Rotaliina (fig. 6.1a,b,c) was chosen as the most suitable species for use in culturing experiments. It is a cosmopolitan species of benthic foraminifera found abundantly in estuaries throughout Britain and has a relatively large size (~ 400 μm) (Murray, 1979), making it easy to pick from sediments. The cytoplasm of this species has a bright orange colouration, which gives an excellent indication of the health of each individual. *Ammonia tepida* is characterised by a biconvex test, rounded in outline. The spiral side has a low conical form with flush sutures. Chambers are trochospirally coiled, with 6 to 9 in the last whorl. On the umbilical side, the sutures are depressed, and the umbilicus open, unlike its close relative *Ammonia beccarii*, in which the umbilicus is occupied by a calcite boss. 6 to 8 chambers may be visible on the umbilical side. The aperture appears as a slit at the base of the last chamber (Murray 1979). In British estuaries, *A. tepida* tolerates a diurnal salinity variation of 0-35 ‰, with an optimal temperature range of 15-20 °C (Murray 1979).

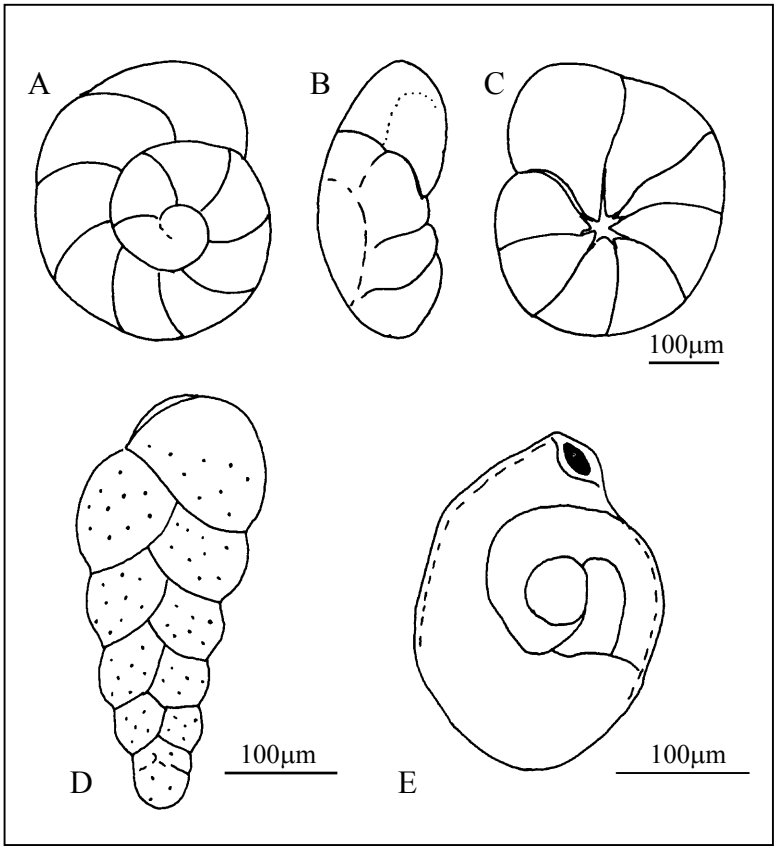


Figure 6.1. Diagrams of benthic foraminifera. A, B, C, *Ammonia tepida* (Cushman), Suborder Rotaliina. A=spiral view, B= apertural view, C=umbilical view; D, *Bolivina variabilis* (Williamson), Suborder Rotaliina, E, *Cornuloculina balkwilli* (Macfadyen), Suborder Miliolina. Adapted from Murray (1979).

Ammonia tepida has been the subject of many laboratory studies, focusing on its adaptation to different ecological conditions (Bradshaw, 1957, 1961; Schnitker, 1974), morphological variation (Schnitker, 1974; Walton and Sloan, 1990) and life cycle (Schnitker, 1974; Goldstein and Moodley, 1993; Goldstein.1997; Stouff *et al*. (1999a). It has long been considered a species that easily reproduces in laboratory cultures, by asexual reproduction, a process that was documented well by Stouff *et al.* (1999a) and utilised in studies by de Nooijer *et al.* (2009) and Morvan *et al.* (2004).

Ammonia tepida has a trimorphic life cycle (Stouff *et al.*, 1999a) (see chapter 1, fig. 1.3), making it particularly ideal for culturing. Unlike the obligatory (fixed) dimorphic life cycles of many benthic species, the facultative (flexible) nature of this life cycle means that the sexual/asexual pattern can be broken, and successive asexual generations of offspring can be produced to quickly increase the population size. *Ammonia tepida* forms an easily identifiable reproductive cyst just prior to asexual reproduction, which could be particularly useful as an indicator of the mode of reproduction taking place. The shape of the asexual reproductive cyst (flattened cyst, below the umbilical surface of the test) is easily distinguishable from that of other cysts produced during the life cycle such as the growth cyst (covering the entire test) and the sexual reproductive cyst (rounded, compact cyst, completely encasing test) (Goldstein & Moodley, 1993; Stouff *et al.*, 1999a). It is hoped that a culture of genetically identical individuals can be easily established by asexual reproduction, to provide a clean source of genetic material for downstream applications.

During the course of the experiment two further species of benthic foraminifera, *Bolivina variabilis* and *Cornuloculina balkwilli* were also identified as potential subjects for culturing. Both originated from the seawater collected in the field alongside the sediment, but soon established well in the stock populations of mixed species being maintained alongside the experimental cultures of *A. tepida*.

6.1.4.2 Bolivina variabilis

Bolivina variabilis (Williamson), of the order Rotaliina (fig. 6.1d), also known as *Brizalina variabilis*, is a marine inner shelf benthic foraminiferal species that is sometimes transported into the muddy parts of estuaries. It has a dark orange

cytoplasm, which again is a good indicator of health. It has a compressed biserial test, comprised of calcitc, radially arranged crystallites, perforate in nature and often appearing translucent. The chamber walls bear coarse pores, each set in a conical depression. The sutures are depressed, straight, and oblique to the margin. The aperture is terminal, with a toothplate. The average length of an adult specimen is 500 μm (Murray, 1979). The life cycle of *B. variabilis* has not specifically been documented, though as a benthic species it is most likely to be dimorphic, with both sexual and asexual generations. Whether or not it can undergo multiple asexual generations (a facultative / trimorphic life cycle) is unclear.

6.1.4.3 Cornuloculina balkwilli

Cornuloculina balkwilli (MacFadyen) (fig. 6.1e), of the suborder Miliolina, is thought to be an inner shelf marine species, which is transported into the mouths of muddy estuaries (Murray, 1979). It has a porcellaneous, white, calcitic wall. The test is oval in outline, compressed, with each chamber forming roughly two thirds of a whorl. The aperture is simple and terminal. The average maximum size is 150 μm (Murray, 1979). C*ornuloculina balkwilli* is considered to be epifaunal, attached to the substrate, but also able to move freely (Sturrock & Murray, 1981). As in *B. variabilis,* the specific life cycle of *C. balkwilli* is not known, though it is most likely to be dimorphic, or possibly even trimorphic.

6.2 Aims and Objectives

The primary objective of this work was to produce a continually reproducing, genetically identical culture of the benthic foraminifer, *Ammonia tepida* to provide a reliable source of genetic material for use in genomic work and other molecular applications. *Ammonia tepida* is well suited to this work as it is easy to collect in the UK, has a relatively large size for a benthic foraminifer, and has been cultured in laboratory studies previously, reproducing for successive generations (de Nooijer *et al.* 2009; Morvan *et al.* 2004; Stouff *et al.* 1999a). In addition it is a symbiont-barren species, which reduces the chance of contaminant DNA interfering with subsequent genomic work. Culture experiments were also carried out on two additional species of benthic foraminifera, *Bolivina variabilis* and *Cornuloculina balkwilli*, neither of which have been cultured before. A series of experiments were performed to ascertain the optimal conditions under which to culture these foraminifera in the lab.

6.3 Methods

6.3.1 Collection of benthic foraminifera

Live, benthic foraminifera were collected from Brancaster, Norfolk in May 2007 and May 2009. Sediment was collected at low tide from tidal mudflats, from the green, algal-rich surface layer, where foraminifera are abundant. The sediment was sieved (212 μm) and washed through with seawater. Sea temperature and salinity were recorded on location at both low and high tide.

6.3.2 Picking foraminifera in the lab

Thin layers (~50 mm) of the sieved sediment, containing the live foraminifera, were placed in plastic tubs, covered with a 5 cm deep layer of fresh seawater (collected at high tide on the day of the foraminifera collection), with a loose lid to prevent evaporation. During the first 2 hours, the sediment was washed twice with fresh seawater, allowing it to settle in between. Obvious large organisms, such as nematode worms, were removed as they can die easily, causing contamination of the water. After 24 hours, healthy, live forams could be seen, by eye, to have migrated to the surface of the sediment, clinging to small pieces of plant matter and to the sides of the tubs. These were removed by pipette to a 19 cm diameter petri dish containing fresh seawater (salinity ~ 27 – 30 ‰). This mixed culture, containing a small amount of the original surficial sediment, was maintained for the duration of the experimental period as a stock population. Individuals of the target species, *Ammonia tepida*, were then selected for use in culturing experiments. Only those with healthy bright yellow cytoplasm were used.

Two other species, *Bolivina variabilis* and *Cornuloculina balkwilli*, were also used in culturing experiments. These originated in seawater from the collection site (see section 6.4.1 of the results) but were found thriving in the petri dishes containing the stock populations of mixed species. Again, only healthy individuals were selected. These were easily identified in *B. variabilis*, which has a dark orange cytoplasm, but slightly more difficult to see in *C. balkwilli* which has an opaque shell giving it a paler appearance.

6.3.3 Culturing experiments

A number of different experiments were carried out to assess both the optimal conditions under which to culture benthic foraminifera in the lab, and to identify the species best suited for this use. Experiments were developed over time, using the results of each to determine the next experiment undertaken. For all culturing experiments samples were kept at 23 °C (ambient laboratory temperature), with lighting of 2 x 20w for twelve hours per day. Light and dark cycles were regulated by an automated timer. Water salinity was maintained between 27 – 30 ‰, within the natural range of the estuary collection site.

6.3.3.1 Stock populations

Stock populations containing a mixture of the foraminiferal species found at the collection site and a little estuarine sediment sieved to 212 μm, were kept throughout the course of the experimental period to provide a possible source of additional specimens. Their condition was observed over time, as a point of interest. They were maintained using un-filtered natural seawater, taken from the collection site on an incoming tide.

6.3.3.2 *Culture experiments with Ammonia tepida*

The largest specimens of *A. tepida*, with the healthiest orange colouration to their cytoplasm, were selected from those collected for use in the following culture experiments.

6.3.3.2.1 Experiment 1: *Ammonia tepida* in artificial seawater enriched with f/2 nutrients

Tests were carried out to determine whether promoting the growth of an algal food alongside the *A. tepida* specimens could be beneficial. Single adult specimens of *A. tepida* were transferred to 19 cm diameter petri dishes. All were kept in artificial seawater, some enriched with f/2 nutrients (Guillard & Ryther, 1962; Guillard, 1975) (20 replicates), and some without (40 replicates). Two thirds of the water was replaced weekly. 500 μl of food, in the form of the algae, *Dunaliella tertiolecta* was added weekly.

6.3.3.2.2 Experiment 2: *Ammonia tepida* in natural seawater with a leaf substrate

On collection of fresh foraminiferal specimens from the field, it was observed that *A. tepida* had a tendency to migrate up and cling to portions of leaf substrate in the sieved mud. It therefore seemed logical to replicate these conditions in the lab in an attempt to promote natural behaviour, and in particular reproduction. Moreover, experiment 1 revealed problems of excessive salt crystallisation at the bottom of the petri dish with artificial seawater so all specimens were kept in natural seawater, filtered to 11 μm (Whatman filter paper 1 quantitative 32 cm). Single adult specimens of *A. tepida* were transferred to 19 cm diameter petri dishes (10 replicates) Two thirds of the water was replaced weekly. 500 μl of food, in the form of the algae, *Dunaliella tertiolecta* was added weekly. As a substrate, all were provided with plant matter taken from the surface mud of the collection site. Leaf matter was carefully cleaned to remove organisms and checked for foraminifera. For half, the leaf matter was microwaved (30 seconds, 600 w) as an additional precaution to remove microorganisms or juvenile forams. Cultures were maintained for 6 weeks.

6.3.3.2.3 Experiment 3: *Ammonia tepida* in natural seawater with a mixed food algal lawn

Tests were carried out to see whether *A. tepida* survival in culture could be improved by providing mixed food. The diatom *Phaeodactylum tricornutum* (Blades Biological Ltd.) was introduced as a new food source alongside the existing food, *D. tertiolecta*. The *P. tricornutum* provided had been inoculated onto a gel media. Two treatments were set up. In treatment A, a diatom lawn of *D. tertiolecta* was pre-grown in 19 cm petri dishes containing a thin layer of f/2 nutrient (with silicates) enriched agar (Guillard & Ryther, 1962; Guillard, 1975) for 2 weeks prior to the culture experiment. Artificial seawater containing f/2 media (with silicates) was used to promote diatom

growth but was removed before the foraminiferal specimens were added. In treatment B, empty petri dishes were used at the start of the culturing period. For each treatment, single adult specimens of *A. tepida* were added to the petri dishes (50 replicates), and multiple-specimen cultures containing 50 *A. tepida* were also set up (3 replicates). The foraminifera were kept in natural seawater, filtered to 11 μm (Whatman filter paper 1 quantitative 32 cm), with two thirds of the volume being replaced weekly. All specimens were fed weekly with *D. tertiolecta* algae (500 μl). Specimens in treatment B were also fed *P. tricornutum* (a small piece of inoculated gel, ground up).

6.3.3.2.4 Experiment 4: *Ammonia tepida* in natural seawater with mixed liquid food

Following the results of the feeding test in experiment 3 (see results section), a mixed food diet was again used for this next test, but the *Phaeodactylum tricornutum* diatoms were now provided in liquid form. Single adult specimens of *A. tepida* were added to empty 19 cm petri dishes (50 replicates). Multiple-specimen cultures containing 50 *A. tepida* each were also set up (3 replicates). The foraminifera were kept in natural seawater, filtered to 11 μm (Whatman filter paper 1 quantitative 32 cm), with two thirds of the volume replaced weekly. Specimens were fed weekly on *D. tertiolecta* (500 μl) and 5 drops of a liquid culture of *P. tricornutum* (Sciento, Manchester).

6.3.3.2.5 Experiment 5: *Ammonia tepida* in 7 cm beakers of natural seawater with mixed liquid food

In the previous experiments (1-4) evaporation from the petri-dish culture vessels had been a particular problem. These were now replaced with deeper plastic beakers. Single adult specimens of *A. tepida* were added to empty 7 cm plastic beakers (30 replicates). Multiple-specimen cultures containing 25 *A. tepida* each (5 replicates) were also set up. The foraminifera were kept in 50 ml natural seawater, filtered to 11 μm (Whatman filter paper 1 quantitative 32 cm), with two thirds of the volume replaced every 2 weeks. Specimens were fed as in experiment 4.

6.3.3.3 Culture experiments with Bolivina variabilis and Cornuloculina balkwilli

Experiments 1-5 had focused on the primary species of interest, *A. tepida*, however two other species, *Bolivina variabilis* and *Cornuloculina balkwilli* had established themselves in the stock cultures and were also identified as possible targets of culturing experiments. Specimens with the healthiest orange colouration to their cytoplasm were selected from those collected for use in the following culture experiments. Where it is indicated that adult specimens were used, the largest specimens collected were selected.

6.3.3.3.1 Experiment 6: *Bolivina variabilis* bulk cultures in 7 cm beakers of natural seawater with mixed liquid food

25 adult specimens of *Bolivina variabilis* were added to empty 7 cm plastic beakers (10 replicates). The foraminifera were kept in 50 ml natural seawater, filtered to 0.2 μm (Whatman) (determined to be the best filter size following experiments 1-5), with two thirds of the volume replaced every 2 weeks. Specimens were fed weekly with *D. tertiolecta* (500 μl) and 5 drops of a liquid culture of *P. tricornutum*.

6.3.3.3.2 Experiment 7: *Cornuloculina balkwilli* bulk cultures in 7 cm beakers of natural seawater with mixed liquid food

25 adult specimens of *Cornuloculina balkwilli* were added to empty 7 cm plastic beakers (10 replicates). The foraminifera were maintained and fed as in experiment 6.

6.3.3.3.3 Experiment 8: *Bolivina variabilis* individual samples

To assess whether a culture could be established from a single individual by asexual reproduction, 10 individual specimens of *B. variabilis* were placed in separate 7 cm plastic beakers. 5 of the specimens were mature adults, and 5 were juveniles (~ 8 chambers). Multiple-specimen cultures containing 20 *B. variabilis* each were also set up (2 replicates) as a control. The foraminifera were maintained and fed as in experiment 6.

6.3.3.3.4 Experiment 9: *Cornuloculina balkwilli* individual samples

10 individual specimens of *C. balkwilli* were placed in separate 7 cm plastic beakers. 5 of the specimens were mature adults, and 5 were juveniles (~ 160 μm). Multiple-specimen cultures containing 20 *C. balkwilli* each were also set up (2 replicates) as a control. The foraminifera were maintained and fed as in experiment 6.

6.4 Results

6.4.1 Observations of stock populations

Stock populations of mixed species from the collected sediment were kept for the duration of the experimental period, to provide a possible source of fresh specimens. Observations were made of the condition of these individuals during the course of the experimental period. By far the most dominant species of benthic foraminifera found in the 212 μm sieved estuarine sediment was *Ammonia tepida,* of the order Rotaliina.

Over time, however, it became apparent that two species of foraminifera, that had not originally been numerous in the sediment were taking over in numbers in the stock populations, particularly as the *A. tepida* started to die out. These were identified as the biserial benthic, *Bolivina variabilis* of the order Rotaliina and *Cornuloculina balkwilli* of the suborder Miliolina. As only one or two specimens of these species had been identified in the collected sediment it seemed likely that they might have been seeded from the unfiltered seawater used to maintain the stock populations. This was confirmed by filtering seawater through a 0.2 μm Whatman filter; examination of the filter revealed the presence of juveniles of both foraminiferal species.

A process of succession seemed to take place within the stock populations, with *B. variabilis* first taking a hold as the *A. tepida* died out and later, especially where numbers of *B. variabilis* were low, *C. balkwilli* flourished. Both species seemed to thrive in the lab and as a result, were selected in addition to *A. tepida* for culturing.

6.4.2 Culture experiments with *Ammonia tepida*

The specimens of *Ammonia tepida* collected in the field varied in size, but the largest individuals, with an average test diameter of 400 μm were selected for culturing. On the spiral side of the test, 20 – 25 chambers were visible (fig. 6.2a), and on the umbilical side 8 - 9 chambers could usually be observed (fig. 6.2b). Cytoplasm, with a healthy yellow colouration, could usually be seen in all but the last of the chambers.

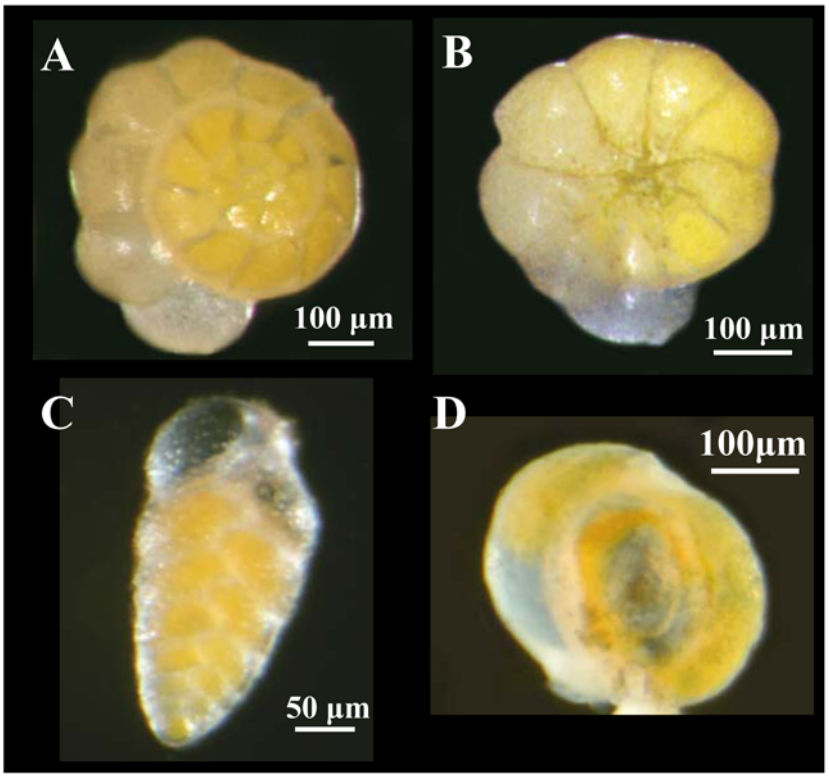


Figure 6.2. Photographs of benthic foraminifera taken under a light microscope. A, B, *Ammonia tepida* (Cushman) A = spiral view, B = umbilical view. Suborder Rotaliina; C, *Bolivina variabilis* (Williamson), Suborder Rotaliina; D, *Cornuloculina balkwilli* (Macfadyen), Suborder Miliolina.

6.4.2.1 Experiment 1: Ammonia tepida in artificial seawater enriched with f/2 nutrients

After 1 week, the single adult specimens of *A. tepida* kept in artificial seawater with no f/2 nutrients and fed *Dunaliella tertiolecta,* appeared pale. Specimens kept in

artificial seawater with f/2 nutrients generally had dark green matter attached to their underside and green colouration could be seen within the last 4 – 6 chambers of their shells. Two individuals in the f/2-enriched water were dead, leaving empty shells.

After 3 weeks, of the 40 individuals kept in artificial seawater with no f/2 nutrients, only 12 were healthy, 20 were pale in colouration, and 8 were dead. Of the 20 kept in nutrient enriched artificial seawater, 9 were healthy, 2 were pale, and 9 were dead. A large amount of green algal matter could be seen covering the bottom of these petri dishes, and in some cases, the forams themselves.

After 6 weeks, all of the specimens of *A. tepida* in both treatments were dead, none having reproduced. As a result of using artificial seawater, excessive salt crystallisation was noted in all dishes throughout the duration of the experiment. Natural seawater, filtered to remove organisms and debris, was therefore used in subsequent experiments.

6.4.2.2 Experiment 2: Ammonia tepida in natural seawater with a leaf substrate

After 2 days, the foraminifera had a healthy colouration and almost all were attached to the plant matter provided, by their umbilical side. After 2 weeks all of the foraminifera were still healthy and the 5 provided with an un-treated natural leaf substrate were all still attached to the leaves. All of the foraminifera provided with a microwaved leaf substrate, however, had dropped off and were on the bottom of the dish. After 4 weeks, the foraminifera from both treatments were pale in colouration and after 6 weeks all had died.

6.4.2.3 Experiment 3: Ammonia tepida in natural seawater with a mixed food algal lawn

After 1 week, the *A. tepida* kept on a pre-grown diatom lawn (*P. tricornutum*) and fed with additional *D. tertiolecta* algae were found to be attached to the bottom of the petri dishes and feeding tracks could be seen through the lawn. For most, a green colouration could be seen in the last 4 – 6 chambers of their tests. By week 3, however, the algae and diatoms had grown excessively and were beginning to overwhelm the foraminifera. Most forams were covered by sheets of green algae, most likely the *D. tertiolecta*, and had become pale in colour. After 3 months, all specimens were dead.

The foraminifera kept without an algal lawn were initially healthy and many secreted a cyst (formed during growth and reproduction) about or below their test within the first week of culture. Most survived for the 3-month duration of the experiment, however, none reproduced.

6.4.2.4 Experiment 4: Ammonia tepida in natural seawater with mixed liquid food

After 2 days, all foraminifera had a healthy yellow colouration, and most had attached to the bottom or sides of the petri dish with pseudopodia extended. In the multi-specimen cultures, many of the individuals had attached to one-another. Natural cyst formation was evident in some specimens, in a few cases completely encasing the test, but for most forming only below the umbilical surface (fig. 6.3b), causing the foram to be slightly raised from the bottom of the dish.

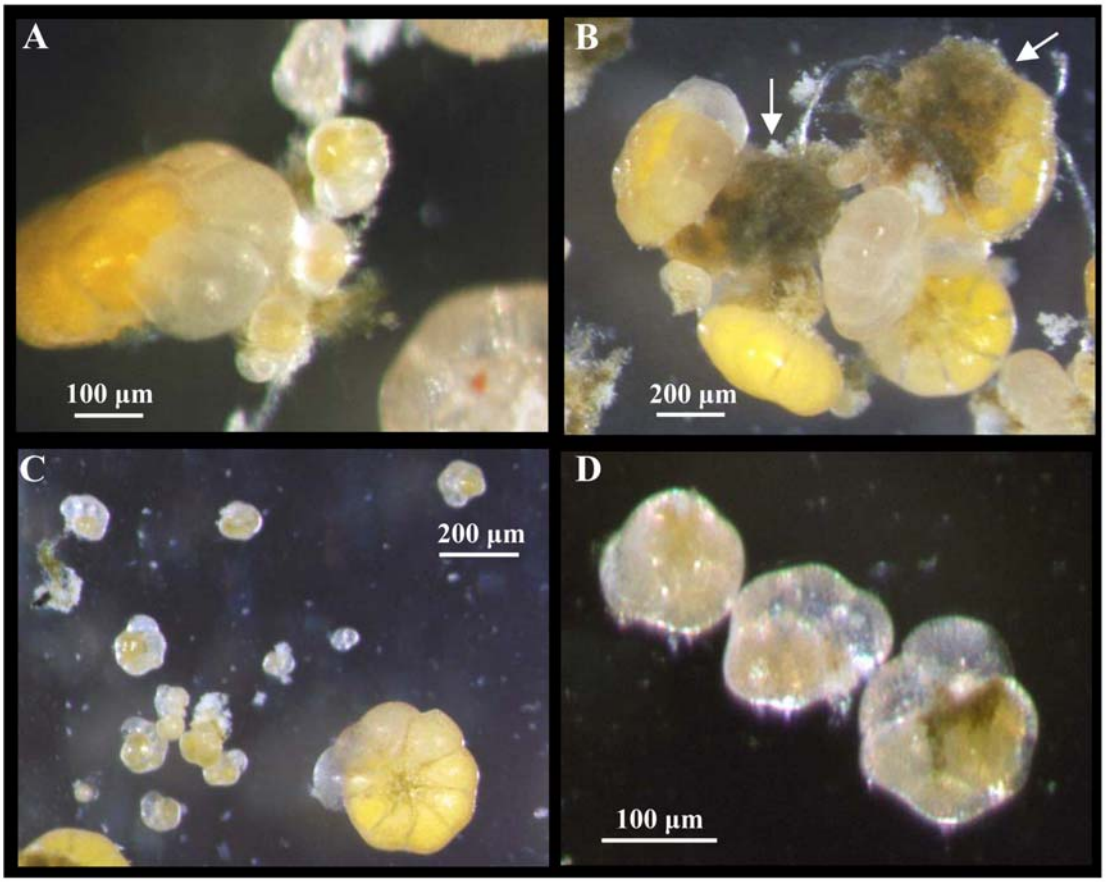


Figure 6.3. Light microscope photographs of the benthic foraminifer *Ammonia tepida* in culture. A, Adult specimen with juveniles from another parent attached; B, Adult specimens showing asexual reproductive cyst formation (indicated by arrows). Juveniles from another parent are also present; C, Adult specimen and juveniles from mixed broods, showing different stages of development; D, Juvenile specimens showing, 4, 5, and 6 chambers (left to right).

After 1 week, reproduction had taken place in two of the multiple-specimen cultures, with approximately 20 - 25 juveniles per brood. Juveniles comprised of a large proloculus, or first chamber (~ 50 µm), and 4 subsequent chambers, with a mean size of 145µm. Most had attached to either the bottom of the petri dish or to the mature foraminifera (fig 6.3a, 6.3b).

At 2 weeks, one of the individually kept specimens had reproduced. The adult had died, leaving an empty shell surrounded by juveniles. The brood size was consistent with those seen in the multi-specimen cultures and each juvenile had approximately 4

chambers, and an average size of 120 μm. The juvenile brood was separated into 2 petri dishes to be maintained.

After 2 months, further reproductive events had occurred in the multiple-specimen cultures, consistent with only a couple of individuals reproducing. Figures 4c and 4d show the different sizes of juvenile present, from 2 chambers (large proloculi and 1 additional chamber; size 66 μm) up to 7 chambers (size 155 μm). No further reproduction had been seen in any of the individually kept specimens, though the juveniles produced in the second week had now grown to a size of approximately 12 chambers.

At 3 months, the cultures were no longer healthy, with a great deal of algal material present in each (derived from the *D. tertiolecta* and *P. tricornutum* added as food). The water was cloudy and evaporation had led to higher salinities. Most of the specimens were dead and the cultures had not reached large population sizes. In the multi-specimen cultures only 1 – 3 specimens in 50 had reproduced, and the same was reflected in the individually kept specimens, where only 1 in 50 had reproduced. The juveniles produced died before reaching a mature, reproductive size.

6.4.2.5 Experiment 5: Ammonia tepida in 7 cm beakers of natural seawater with mixed liquid food

After 1 month, reproduction had occurred in 3 out of 5 of the multi-specimen beakers, again with small brood sizes of approximately 20 - 25 juveniles each. In beakers containing single individuals, all were alive with a healthy colouration, but none had reproduced.

By 3 months, the *A. tepida* were still healthy and multiple reproductive events had clearly taken place in the multi-specimen cultures, as was evident in the different sized groups of juveniles present, some only 4 chambers in size, others now 8 or 9 chambers and some resembling small adults. No reproduction had taken place in any of the single-specimen beakers, though the adults were still alive and healthy. Conditions in the beakers were found to be much better than in the petri dishes previously used. Less evaporation was seen and the water stayed clearer.

At approximately 4 months, a problem occurred with the air-conditioning of the lab, causing a sudden rise in temperature. Most of the specimens died off suddenly at this point. By 6 months only one or two specimens were alive in the mixed-specimen cultures and all of the single specimens had died. No reproduction had been observed in any of the individually kept specimens.

6.4.3 Culture experiments with *Bolivina variabilis* and *Cornuloculina balkwilli*

The largest specimens of *B. variabilis* collected in the field, and selected for culturing were approximately 290 – 320 μm in size, with around 14 chambers. The cytoplasm was a bright orange/yellow in colour and occupied all but the last 1 – 2 chambers of the test (fig. 6.2c). The test wall was almost transparent, with pores clearly visible.

The largest specimens of *C. balkwilli* were approximately 300 – 320 μm in size. They had a more opaque test, giving a slightly paler appearance than the other two species (fig. 6.2d).

6.4.3.1 Experiment 6: Bolivina variabilis bulk cultures in 7 cm beakers of natural seawater with mixed liquid food

After 4 months, the specimens of *B. variabilis* were extremely healthy, with a bright orange colouration (fig. 6.4a) and reproduction had occurred in 7 of the 10 cultures. In two of these, huge populations of *B. variabilis* now existed, with hundreds of juveniles of varying sizes, indicating multiple reproductive events (fig. 6.4b). Average sizes were as follows: 6 chambers ~ 65 μm, 8 chambers ~90 μm, 12 chambers ~ 180 μm. Nearly all of the forams were attached to portions of an algal substrate that had naturally established over the duration of the experiment, presumably from the algal and diatom food that was added (fig. 6.4c).

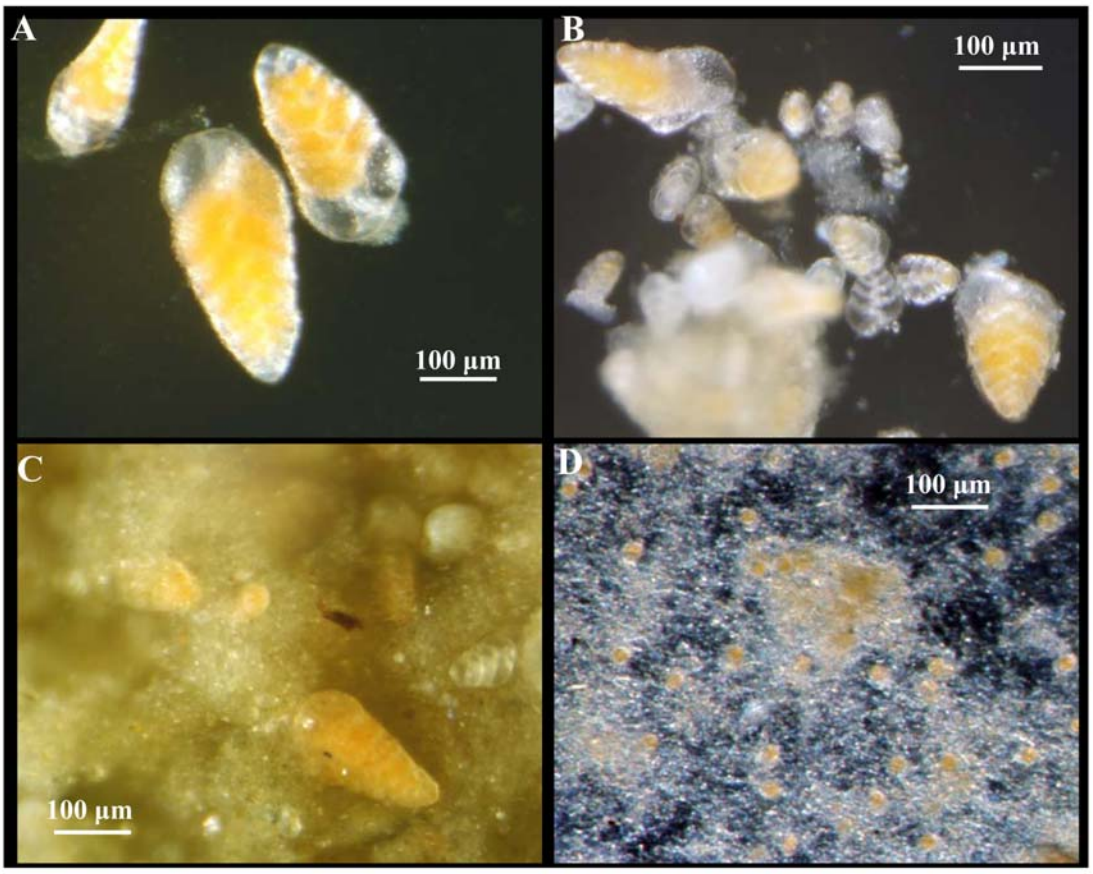


Figure 6.4. Light microscope photographs of the benthic foraminifer *Bolivina variabilis* in culture. A, Healthy adult specimens; B, A random sample of specimens from the culture population, showing different stages of development; C, Specimens attached to an algal substrate; D, newly released juveniles, attached to an algal substrate.

Most were attached by the aperture end of their shell, with the tip raised. Some empty shells could be seen, especially of larger specimens. Some juveniles, at ~ 20μm in size, were only just visible under the greatest microscope magnification, indicating that reproduction had only just taken place (fig. 6.4d). These juveniles were scattered over a small area, and were again attached firmly to the algal substrate. A single reproductive event had likely taken place in each of the other 4 successful cultures, leading to small, localised populations of 50 – 100 individuals on the algal mats.

After 6 months, one of the most successful cultures still contained a large, thriving population. Individuals of many different sizes were present, indicating that multiple reproductive events had occurred over time. Some of the previous batch of juveniles had now grown to almost adult size. A large mass of algal substrate that had formed during the experimental period was covered in thousands of individuals. Huge numbers were alive, with a healthy orange colouration though there were empty shells of all sizes mixed in with them. Interestingly, in the other culture that had been particularly successful at 4 months, almost the whole colony had died off, leaving only a few large individuals. Of the remaining cultures, 2 were reasonably healthy but 6 contained almost no living specimens. Most showed signs that 1 – 2 reproductive events had taken place at some point, before the samples died off.

6.4.3.2 Experiment 7: Cornuloculina balkwilli bulk cultures in 7 cm beakers of natural seawater with mixed liquid food

After 4 months, reproduction had occurred in all 10 of the bulk sample cultures of *C. balkwilli*, and healthy and thriving populations had formed (fig. 6.5a, 6.5b). Most beakers supported a population of many hundreds of individuals, far larger than in the *B. variabilis* cultures. Again, specimens were attached, in huge numbers, to portions

of algal substrate that had formed during the experimental period, (fig. 6.5d) and only a few were found loose in the beakers. A variety of different sizes were present, indicating multiple reproductive events (figs. 6.5a, 6.5c). The smallest juveniles were approximately 60 – 70 μm in size. After 1 week they had grown to ~ 100 μm. Some had grown to ~ 220 μm, about half the size of the specimens originally put in.

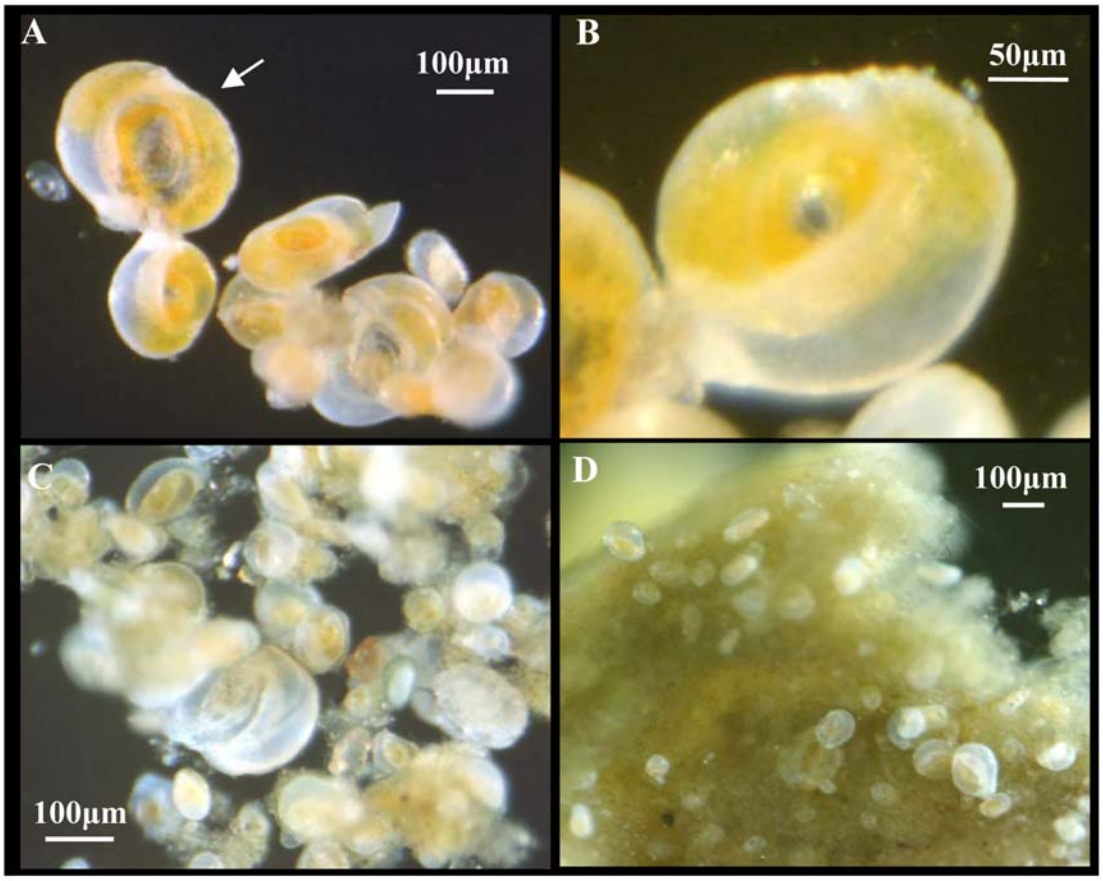


Figure 6.5. Light microscope photographs of the benthic foraminifer *Cornuloculina balkwilli* in culture. A, Adult specimen (indicated by an arrow) together with immature specimens of different sizes; B, healthy, immature specimen; C; Many different sized specimens from the culture population, indicating multiple reproductive events; D, Juvenile specimens attached to an algal substrate

After 6 months, 4 of the cultures were still healthy and thriving, each containing many different sizes of *B. variabilis.* Very small juveniles present in each indicated that reproduction was still taking place. The remaining 6 cultures appeared less healthy, with many pale individuals and a greater proportion of empty shells. It was clear that

though successful populations had been present at some point, they had now suffered a crash. However, evidence of recent reproduction could still be seen in 4 of them.

In all of the cultures small juveniles greatly outnumbered the larger specimens, and many larger specimens had died, leaving empty shells.

6.4.3.3 Experiment 8: Bolivina variabilis individual samples

After 3 months, in the individual specimen cultures, 1 of the 5 immature specimens had died, but those remaining were healthy and had grown significantly. All 5 mature specimens were still live and healthy. No reproduction was evident in any of these single specimen beakers.

5 or 6 specimens from each of the multi-specimen control cultures (20 each) had died but the rest were live and healthy. In one of the 2 cultures a small number of juveniles were found (~ 10) most likely as the result of a single reproductive event, and in the other, no reproduction had occurred.

After 6 months, however, only one of the individual specimens, which had started as a juvenile, remained alive, and this had never reproduced.

Only 6 of the 20 original specimens in the first of the multi-specimen control cultures was still alive, and despite reproduction having occurred in the first 3 months, only a single juvenile was now alive. In the other multi-specimen culture, no reproduction had occurred and only 5 adults were still living. A thin film of algae was present in both beakers, though no large lumps had formed.

6.4.3.4 Experiment 9: Cornuloculina balkwilli individual samples

After 3 months, in the single specimen cultures, 3 of 5 of those that started as adults had died and the others were healthy, though none had reproduced. Surprisingly, in the cultures started from single immature specimens, thriving populations had established in 4 out of 5. The remaining specimen had died. Hundreds of individuals of many different sizes were now present in the 4 successful cultures.

Conditions in the two multi-specimen cultures of *C. balkwilli* were very different. In the first, the samples were not thriving and roughly half had died with no reproduction having taken place. In the second, a huge population had established, with over a thousand individuals of all sizes present, indicating many reproductive events. Unlike in previous cultures of *C. balkwilli* no algal substrate had formed during the course of the experiment (algae formed a substrate naturally in some culture vessels, from the food added, and was not created intentionally) for specimens to attach to and yet the population was still as successful.

After 6 months, of the single specimen cultures, all those that had started as mature adults were now dead, none having reproduced. The 4/5 successful cultures from individual immature specimens (as recorded at 3 months) were all still thriving and reproducing. All contained thousands of individuals. One of these cultures was extremely healthy, however, the other showed some signs of algal overgrowth, in the form of a thin film covering the specimens.

All specimens from the first of the multi-specimen control cultures had perished. In the other, a large population with individuals of many different sizes was still present,

though it did not appear as healthy as at the 3-month point. A thin film of algae was present, but no large masses.

6.5 Discussion

6.5.1 Optimising conditions for culturing benthic foraminifera in the lab

Methods used for maintaining and culturing benthic foraminifera in the lab vary a great deal, and no one set of conditions can be said to be ideal. It was therefore important to experiment with the conditions used, and to tailor them to the requirements of the species being used. A simple approach was taken, using basic culture vessels, and a variety of media and foods. The aim was to attain the best conditions possible with the equipment available.

6.5.1.1 Water

Experiment 1 quickly showed that the benthic foraminifer, *A. tepida*, did not survive well in artificial seawater, a response that has also been noted in the benthic foraminifera *Amphistegina hemprichii* and *Amphistegina lobifera* (Lee *et al.*1991b). Specimens became pale in colour after only 1 week, an indication of poor health in benthic foraminifera (Lee *et al.*1991b), and no specimens survived past 6 weeks. Salt crystals were evident in most of the culture vessels after the first week of experimentation. The artificial seawater was made up from a powder, using a specified volume of distilled water. It is likely, however, that excessive evaporation from the petri dishes caused the salt concentration to become elevated, leading to precipitation. This could have exacerbated the problems caused by using artificial seawater, which crystallises to some extent even in a sealed container.

Using an f/2 nutrient enriched media to promote microalgal growth alongside the foraminifera, as suggested by Lee *et al*. (1970, 1975), was also unsuccessful

(experiment 1). The inclusion of f/2 nutrients led to petri dishes becoming overgrown with algae, which was detrimental to the foraminifera, quickly overwhelming them.

It was quickly decided that natural seawater, taken from the collection site on an incoming tide, would be a better alternative. Initially, the natural seawater was filtered through an 11 μm filter paper, to remove any foraminifera or other biota, whilst allowing large volumes of water to be processed quickly. However, small juveniles of *B. variabilis* and *C. balkwilli* began appearing in the stock cultures of mixed foraminifera, where they had been almost absent to begin with, as well as in some of the early cultures of *A. tepida.* It was subsequently determined that these had originated from the tanks of collected seawater and in all subsequent culture experiments the seawater was therefore filtered to 0.2 μm, as in de Nooijer *et al.* (2009) and Morvan *et al.* (2004). Using a 0.2 μm filter prevents the tiny gametes of foraminifera from transferring through to the culture vessels, though it does also make it extremely difficult to process large volumes of water.

The use of natural rather than artificial seawater proved immediately successful, with the *A. tepida* specimens retaining their healthy orange colouration for a much longer period of time (experiments 2 & 3). These specimens did not reproduce, however, and their health could not be maintained long-term indicating that other factors in the culturing process were still sub-optimal for culturing this species.

Frequent changing of the water was also found to be important to the survival of the cultures. When culturing foraminifera in petri dishes or beakers, half of the volume of water is usually replaced at regular intervals, varying from every 2 days (Lutze &

Wefer, 1980) up to weekly (Lee *et al.*, 1991b) or even monthly (Stouff *et al.*, 1999a, b). Cultures have been shown to be more successful when the medium is changed frequently (Lee *et al.*, 1991b), though the process can be time consuming. Both in this study, with the water changed every 1 – 2 weeks, and in the studies of Stouff *et al.* (1999a, b), with the water changed monthly, brood sizes in *A. tepida* were small, at approximately 20. de Nooijer *et al.* (2009), conversely, reported brood sizes of 50 – 200 in *A. tepida*, when the media was changed every 2 days. It is likely that more frequent changing of the media would be beneficial, especially when evaporation from the vessels is a problem, as was observed during this study.

6.5.1.2 Substrate

In their natural habitat, many benthic foraminifera will often attach to pieces of substrate, in the form of plant material, algae, wood or rock (Anderson *et al.*, 1991; Lutze & Wefer, 1980). In the lab, it was observed that foraminifera in the freshly collected sieved sediment, migrated overnight to take up positions at the tips of any pieces of detritus and leaf matter present. It therefore seemed natural to test whether a substrate could be beneficial in promoting natural behaviour such as feeding and reproduction.

Substrates are sometimes used in foraminiferal culturing experiments, and may be natural or artificial (Lutze & Wefer, 1980; Stouff *et al.*, 1999b). To create a substrate, leaves found in the collected sediment were treated in two different ways. The leaves were cleaned meticulously under a dissection microscope to remove any foraminifera present, and half were then sterilised by microwave as an extra precaution to remove any biota. In petri dishes, the specimens of *A. tepida* quickly attached to the leaves

(experiment 2), however, those on the sterile substrate detached again after only a short time. This perhaps indicates that attachment is dependent on the presence of microorganisms, a food source for the foraminifera and that without them the substrate becomes unattractive. Indeed, it has been shown in the benthic species *Ammonia beccarii*, that a free-living lifestyle is adopted in specimens kept on an artificial substrate and that stress may be introduced under such conditions (Takata *et al.*, 2009). Despite seeing some success, with specimens attaching to the untreated leaves, none of the individuals reproduced and all became unhealthy and died after 6 weeks, most likely as a result of other conditions being poor.

Even when not added intentionally, substrates comprising of algal, diatom and bacterial material tended to form in the culture vessels. Both *B. variabilis* and *C. balkwilli* formed huge thriving populations on these masses (experiments 6 & 7) (figs 5c, 5d, 6d), though *C. balkwilli* was found to prosper equally well in an empty petri dish (experiment 9). It seems then that while these species of benthic foraminifera certainly have a tendency towards inhabiting substrates, they may not be necessary for their survival and growth in culture, provided sufficient food is present.

6.5.1.3 Feeding

One possible explanation for the lack of longevity and reproduction seen in the *A. tepida* cultures is incorrect feeding, though other factors were undoubtedly also involved. Within the Foraminifera, diet varies a great deal and is specific to each individual species. The food provided for a species in culture can vary, as was shown in a review by Anderson *et al.* (1991), and there is no definitive guide to the diet of the three species of foraminifera used here.

A number of different methods can be chosen for feeding foraminifera. Heat-killed algae will be readily consumed (Grell, 1954; Bradshaw, 1957), though they begin to decay, promoting the growth of bacteria after only a few hours. Foraminifera can be inoculated onto previously grown lawns of algae, whose growth have been promoted with a nutrient enriched seawater medium for several days (Anderson *et al.*, 1991). Live food can be simply dropped in to foraminifera kept in plain seawater, or food organisms can be encouraged to grow along with the foraminifera (Anderson *et al.*, 1991). Mixtures of food species have been found to promote more vigorous growth and reproduction of foraminifera than single species (Lee *et al.*, 1966; Muller & Lee, 1969), although availability and cost can limit what food is ultimately used.

Many foraminifera have been successfully fed in culture on species from the algal genus *Dunaliella* (Faber & Lee, 1991; Grell, 1954; Hedley, 1964; Muller, 1975), and *A. tepida* itself has been shown to consume *Dunaliella salina* (de Nooijer *et al.*, 2009). A species that has not specifically been used before, *Dunaliella tertiolecta,* was available in the lab and so became the first food to be tested.

In *A. tepida* fed purely on *D. tertiolecta* (experiments 1 & 2), green material was soon evident within the last 4-6 chambers of the test in many individuals, a promising indication of successful feeding, however, the specimens failed to thrive. Attempts made to grow the food source in the same media as the foraminifera (experiment 1) quickly failed as the *A. tepida* could not survive in the enriched artificial seawater and were quickly overwhelmed by algal overgrowth.

A new food-source was then tested, in the form of the diatom *Phaeodactylum tricornutum*, impregnated onto an agar gel. *Phaeodactylum tricornutum* has been shown to form part of the diet of a number of foraminifera (Arnold, 1954; Lee *et al.*, 1969; Murray, 1963) and was successfully used to feed *A. tepida* by Morvan *et al.* (2004). The *P. tricornutum* was initially grown as a lawn on the bottom of the petri dishes for 2 weeks prior to the forams being added, using an f/2 nutrient enriched media (experiment 3). Liquid *D. tertiolecta* algae were also added as an additional food source during the experimental period. Feeding tracks were seen in the lawn soon after the forams were added, a positive sign that the strategy was working. After 3 weeks, however, the diatoms and algae had grown excessively and soon overwhelmed the forams. Many of the foraminifera were covered over by the algae and no-longer mobile. The specimens became pale and died within 3 months. Algal overgrowth has been shown to inhibit foraminiferal growth and reproduction (Arnold, 1954, 1974), with metabolic by-products of the algae becoming poisonous to the foraminifera (Lee *et al.* 1991b). Specimens kept without the diatom lawn and fed with a combination of *D. tertiolecta* and fragments of the *P. tricornutum* agar (experiment 3) survived well for the 3 month duration of the experiment, and were still healthy after this time, however, they failed to reproduce. Observations indicated that because the *P. tricornutum* was restricted to the pieces of agar, it was not accessible enough for the foraminifera to feed on.

Healthy growth and reproductive success was finally gained in cultures of *A. tepida* when a mixture of *D. tertiolecta* and *P. tricornutum,* in liquid form, were used for feeding (experiments 4 & 5). This feeding strategy was adopted for subsequent

culturing experiments on *B. variabilis* and *C. balkwilli*, proving successful for both (experiments 6 – 9).

It should be noted that the DNA of the food organisms added to the culture system could become a source of contamination during subsequent molecular procedures. The genomic DNA of the food organisms would therefore need to be sequenced alongside that of the foraminifera (or the sequence gained from GenBank) for comparison and elimination from further analysis (the same would apply if any symbionts or commensals were present). In addition, to minimise contamination from prey particles, all foraminiferans would be kept without food for a period of time prior to the extraction of their RNA.

6.5.1.4 Culture vessel

Success in the *A. tepida* cultures was in part dependent on the type of culture vessel used. Despite petri dishes being commonly used for culturing foraminifera (Morvan *et al.*, 2004; Stouff *et al.*, 1999a,b; Takata *et al.*, 2009), it was found here that evaporation from them, particularly during the summer months, was a major factor in the failure of culture experiments. Seawater levels dropped quickly, becoming cloudy and clogged with algae and bacteria (experiments 1 – 4). In experiment 4, the *A. tepida* did reproduce, but deteriorating conditions in the petri dishes led to most of the foraminifera and offspring dying within 3 months.

Most foraminifera are adapted to life within a species-specific optimal salinity range. Significant divergences from these levels can lead to reduced growth (Arnold, 1954), deformities in the growing test (Stouff *et al.*, 1999b), and can delay or even prevent

reproduction (Bradshaw, 1961, 1957). *Ammonia tepida* can tolerate a diurnal salinity variation of 0 – 35 ‰ (Murray, 1979), as would be found in its natural estuary environment. The collected water used in the lab had a salinity of 30 ‰, however excessive evaporation from the culture vessels could easily have raised this beyond tolerable levels.

Crystallisation jars or plastic beakers are an alternative sometimes used when culturing foraminifera (Stouff *et al.*, 1999a; Lee *et al.*, 1991b). When 7 cm beakers covered with a loose lid were used here in place of the petri dishes, an immediate improvement in culturing success was seen (experiments 5 – 9). Greater volumes of water could be used (50 ml instead of 20 ml), and evaporation was greatly reduced. Salinity was easily maintained within a range of 27 – 30 ‰ and the culture conditions were a lot more stable. Although some algal growth still occurred, it was far less overwhelming. In experiments 7 – 9, where 7 cm beakers were in use, multiple reproductive events were finally observed in all three species of benthic foraminifera being cultured and in the case of *B. variabilis* and *C. balkwilli*, populations were still thriving at the end of the experimental period.

Nevertheless, despite this eventual success, overall observations indicated that permanently keeping a foraminiferal culture in any one vessel eventually leads to excessive algal growth and contamination by waste products. In future work a system of transferring the foraminifera to fresh culture vessels at regular intervals (~ every 3 months) or re-establishing cultures from a subset of the original culture will therefore be introduced.

6.5.1.5 Light & Temperature

Light is an important requirement for the survival of benthic foraminifera, particularly in those carrying endosymbionts. Bleaching of the cytoplasm occurs rapidly if specimens are kept in the dark (Lee *et al.*, 1991b) and in symbiont bearing species, growth rate decreases (Kuile & Erez, 1984), and long-term survival is impossible (Lee *et al.*, 1991b). None of the species utilised in the culture experiments undertaken here are believed to harbour symbionts, though they were still observed to bleach if kept in the dark. Using artificial lighting, controlled by a timer, it was easy to maintain a suitable level of light for an appropriate number of hours each day. A cycle of 12 hours light and dark was used, as in other studies (Krüger *et al.*, 1997; Maréchal-Abram *et al.*, 2004; Stouff *et al.*, 1999a,b). Light is therefore unlikely to have been a factor in the failure of any of the culture experiments.

A constant temperature was more difficult to maintain, as samples were kept on a lab bench, which was subject to temperature variations. The thermal tolerance limit of benthic foraminifera is usually far narrower than that of salinity tolerance (Arnold, 1954) and should therefore be controlled carefully. Both the temperature response of benthic foraminifera and the lethal temperature limit is species specific (Bradshaw, 1961). The optimal temperature range for *A. tepida* is approximately 15 – 20 °C (Murray, 1979), and culturing is usually carried out at 20 – 21 °C (Morvan *et al.*, 2004; Stouff *et al.*, 1999a, b; Takata *et al.*, 2009), though temperatures as high as 25 °C have been used (de Nooijer *et al.*, 2009). For this study, the lab temperature should have been maintained at 23 °C, already higher than the optimum, however, problems with the air conditioning meant that sudden rises in temperature occurred more than once during the summer months. Such increases would clearly be

detrimental, also causing raised salinities as a result of excessive evaporation. The effects were evident in experiment 5 where successfully reproducing multi-specimen cultures of *A. tepida* were maintained in 7 cm beakers for 4 months, until the lab temperature suddenly rose and the cultures were decimated as a result.

Ideally, cultures should be kept in an incubator, where a constant temperature and programmed light cycle could be maintained, however, such equipment was not available at the time of these experiments. More sophisticated culturing set-ups such as circulating and re-circulating marine aquaria (Anderson *et al.*, 1991; Lutze & Wefer, 1980), or chemostats (Lee *et al.*, 1991b) would doubtless improve the success and reliability of culturing experiments, however, it is at least proven here that a continuously reproducing culture can be obtained using a very simplistic approach.

6.5.2 Performance of three species of benthic foraminifera in culture

The original aim of this study was to gain a continuously reproducing culture of the benthic foraminiferan, *Ammonia tepida*. This species was chosen as it has been successfully cultured in the laboratory in the past, and is known to reproduce asexually (de Nooijer *et al.*, 2009; Morvan *et al.*, 2004; Stouff *et al.*, 1999a). It would therefore be possible to grow a large culture population, starting from a single individual, which could then be used as a DNA source for molecular applications.

6.5.2.1 Ammonia tepida in culture

In culture, the specimens of *A. tepida* initially displayed healthy, normal behaviour. Most individuals quickly became orientated with their umbilical side to the surface of the culture vessel with pseudopodia extended. Many became encysted (a process that

occurs during growth and reproduction), some so completely that their test was no longer visible. In the presence of the algae *Dunaliella tertiolecta*, most specimens soon showed a green colouration within the last 4 – 6 chambers of their test, a promising sign of feeding and when a lawn of the diatom *Phaeodactylum tricornutum* and *D. tertiolecta* was provided, feeding tracks were seen within the first day.

Nevertheless, it proved difficult to attain longevity in the cultures. *Ammonia tepida* responded poorly to artificial seawater and did not survive when fed on a single food item (*D. tertiolecta).* Improvements were seen with the use of natural seawater, the introduction of mixed liquid food (*P. tricornutum* and *D. tertiolecta),* and with the provision of more stable conditions, using 7 cm beakers in place of petri dishes. Species of the genus Ammonia are reported to be particularly stress tolerant (Bouchet *et al.*, 2007; Bradshaw, 1961; Walton & Sloan, 1990), yet here this species seemed particularly sensitive to changes in temperature, and possibly salinity, dying quickly if conditions in the culture vessel deteriorated.

6.5.2.2 Ammonia tepida reproduction in culture

Reproduction was first observed after 1 week, in the multi-specimen cultures of *A. tepida,* kept in natural seawater with a mixed liquid food (experiment 4). Single specimens from two of the populations had produced young, with approximately 20 - 25 juveniles per brood, as is consistent with other culture studies (Morvan *et al.*, 2004; Stouff *et al.*, 1999a, b). The average proloculus size was 50 - 100 μm, consistent with proloculus size found in schizonts by Stouff *et al.* (1999a), indicating that asexual reproduction had occurred. Stouff *et al.* (1999a) reports that proloculus size in

gamonts is smaller, at 25 – 50 μm, though this size difference may not apply in other species.

A further indication that asexual reproduction was taking place in the *A. tepida* cultures came when asexual cyst formation was noted in a number of specimens (experiment 4). Some specimens were fully encysted, completely enclosing the test, indicating either growth or sexual reproduction, however, many specimens formed a cyst only under the umbilical surface of the test (fig 4b), indicative of asexual reproduction (Stouff *et al.*, 1999a). The shape of the developing proloculi are sometimes recognisable as bulges in these cysts, however, under the magnifications used they could not be distinguished.

Juveniles were usually first observed at the 4-chamber stage. Though they are released from the adult with only 2 chambers, they develop 3 chambers within 24 hours, and 4 chambers very quickly after that (Stouff *et al.*, 1999a). It is therefore unusual to observe young at the 3-chamber stage, and 2-chamber young are often hidden within the reproductive cyst, which is cast off from the adult after schizogony. 5 chambers are formed 1 week after release, and in 2 weeks 8 – 9 chambers can be seen. It may take up to 3 months for an individual to reach an adult size, with around 14 – 20 chambers (Stouff *et al.*, 1999a).

Asexual reproduction was confirmed in week 2 of experiment 4, when juveniles were produced by one of the individually kept specimens. Reproduction in culture from a single individual of *A. tepida* has been reported previously (Stouff *et al.*, 1999a), hence it was seen as the ideal species to use here. The adult specimen had died,

leaving behind an empty shell surrounded by a small brood of juveniles (4-5 chambers). It is quite common for the adult to die after schizogony, though roughly 10 % do survive. Those that survive schizogony are not usually observed to reproduce subsequently (Stouff *et al.*, 1999b).

The offspring from the individually kept specimen that reproduced by schizogony were separated into 2 vessels to be maintained. From these, it was hoped that by successive asexual reproduction, thriving cultures would be established. This is a mechanism observed in nature to rapidly increase population size under conditions of stress (Harney *et al.*, 1998; Röttger, 1990) (see introduction). Unfortunately, the populations were decimated as a result of a sudden temperature rise in the lab and no other individually kept specimens were seen to reproduce.

Whilst reproduction was successfully observed in *A. tepida* in the lab, reproduction by schizogony from a single individual only occurred once. In addition, brood size was always small (~ 20 individuals) and only 1-3 in every 50 individuals reproduced. Brood sizes are usually smaller in laboratory culture than in the natural environment (Lee *et al.*, 1991b) and may be the result of stress due to sub-optimal conditions. In the field, severe stress was found to cause a reduction in brood size when *Amphistegina gibbosa* reproduced by multiple fission, as well as variation in the size and shape of juveniles (Harney *et al.*, 1998). Indeed, larger brood sizes have been reported in *A. tepida* in culture, with the media being changed every 2 days rather than weekly (de Nooijer *et al.*, 2009).

With reproduction being so infrequent and brood sizes being small, at least under the conditions in the culture experiments undertaken here, it would take an unfeasibly long time for the population to reach a desirable size, but ultimately, it was the poor resistance to variable conditions in the culture vessels that rendered *A. tepida* unsuccessful as a culture subject. Further optimisation of the culturing method and better control of conditions could possibly rectify this situation. This could be achieved by more regular changing of the water (once a week or more), experimenting with different food sources, transfer to fresh culture vessels regularly to avoid algal overgrowth, and the use of an incubator to regulate light and temperature. In addition, it has been noted that mature specimens of *A. tepida* can survive schizogony, but are not usually seen to reproduce subsequently (Stouff *et al.*, 1999b). As only the largest of the collected specimens were selected for culturing, it may be that they had already reproduced. Indeed, it was observed in *C. balkwilli* (experiment 9), that only those specimens placed in culture at an immature stage of development went on to reproduce, and it may be that the same would be found should smaller, immature specimens of *A. tepida* be selected for culturing. This test was not performed here with *A. tepida*, as all of the specimens had died by the time this finding became apparent in *C. balkwilli.* It would, however, be interesting to compare culture success between mature and immature specimens of *A. tepida* in the future.

6.5.2.3 Bolivina variabilis in culture

Bolivina variabilis survived very well in culture initially (experiment 6). Specimens were healthy, with a bright orange colouration and large populations established rapidly. Rather than affixing to the bottom of the culture vessel, specimens had a tendency to gather together on clumps of substrate, where they thrived. The multi-

specimen cultures proliferated for 4 months, however, at the end of the 6 month period many of the populations had been decimated. The crash likely coincided with a sudden increase in temperature in the lab as in *A. tepida*. Unlike the *A. tepida* cultures, however, where nearly all specimens died, 3 populations of *B. variabilis* survived the poor conditions, one of which remained very strong.

When the culturing experiment was repeated with individually kept specimens and two multi-specimen controls, it was uninterrupted by rises in temperature (experiment 8). Most specimens survived well for the first 3 months. All of the living individuals had a good healthy colouration and those that had started as juveniles had grown significantly. After 6 months, however, far fewer individuals were left alive, although those that remained were still healthy. It may be that most specimens had simply reached the end of their natural life span, however, a thin film of algae was present in most beakers, possibly indicating that over time *B. variabilis* becomes affected by the waste products of algal overgrowth. Bouchet *et al.* (2007) reported that *A. tepida* was highly tolerant of unfavourable conditions and that *B. variabilis* was not. Here it was found that although the *B. variabilis* cultures did decline eventually, they survived better than *A. tepida*.

6.5.2.4 Bolivina variabilis reproduction in culture

4 months into the culture period reproduction had occurred in 7 out of 10 of the multi-specimen trial cultures (experiment 6). Two large populations had established, with hundreds of juveniles of different sizes, indicating multiple reproductive events. In comparison to *A. tepida*, brood size must be far larger in *B. variabilis*, for such populations to have formed. Indeed other species of benthic foraminifera have been

recorded as producing large numbers of young in culture, for example 500 – 600 schizonts in *Amphistegina lobifera* (Lee *et al*., 1991b) and 500 – 1000 in *Cyclorbiculina compressa* (Lutze & Wefer, 1980). By 6 months, thousands of individuals were present in the cultures, suggesting that individuals grow quickly to reproductive maturity. This together with the large brood size could make *B. variabilis* a good candidate for culturing large populations quickly.

However, subsequent culture experiments with *B. variabilis* were less successful. Reproduction occurred in only 1 of the 2 multi-specimen cultures in experiment 8, and this was only a small brood (~10). Moreover, only one juvenile survived to the 6-month point. Furthermore, none of the individually kept specimens reproduced, though all were healthy. Despite the early success, and healthy growth of *B. variabilis* in culture, this lack of reproduction from a single individual may make *B. variabilis* an unsuitable candidate for culturing a large homogeneous population.

6.5.2.5 Cornuloculina balkwilli in culture

It was difficult to assess the health of *C. balkwilli* specimens in culture as their slightly opaque test gives them a naturally pale appearance in comparison to *A. tepida* and *B. variabilis*. Cytoplasm could be seen, however, within the living specimens (fig. 6.5a, 6.5b) and increasing population size indicated good health. Again, in this species, individuals gathered on any substrate present (experiment 7), though they were also found to thrive in the absence of algal matter (experiment 9). *Cornuloculina balkwilli* seemed more resilient to altering conditions in the culture vessels and though some reduction in population size was evident at elevated temperatures (during experiment 7), on the whole, the cultures survived well. They also re-established well once

conditions stabilised, possibly aided by their larger population sizes. There was some evidence of algal overgrowth affecting the cultures in experiment 9. After 6 months, 1 of the 2 multi-specimen cultures was completely decimated and there was evidence of a fine algal film over the other cultures. Most, however, were unaffected by these conditions, establishing populations of thousands of individuals.

Cornuloculina balkwilli may be an opportunistic species, as was demonstrated in the stock populations. A process of succession was evident, with *Ammonia tepida* being the most numerous initially, but with worsening conditions, dying out to be replaced by *Bolivina variabilis*. These too eventually succumbed, at which point *C. balkwilli* began to thrive. If the *B. variabilis* population remained strong in a particular vessel, *C. balkwilli* did not establish. A superior ability of *C. balkwilli* to survive variable conditions in the lab is indicated, making it a better candidate for culturing.

6.5.2.6 Cornuloculina balkwilli reproduction in culture

Of all 3 species, *C. balkwilli* was clearly the most successful at reproducing in culture. After 4 months, reproduction had occurred in all 10 of the multi-specimen trial cultures (experiment 7), and the populations were large and healthy. Hundreds of individuals were present, of many different sizes, indicating multiple reproductive events. Many more of the original specimens must have reproduced than in the *A. tepida* cultures. Specimens were heavily crowded making it impossible to determine how many young were produced per brood, however, from the population size, it seems reasonable to assume that it is significantly more than in either *A. tepida* or *B. variabilis*. The number of large empty shells present suggested that, as in the other 2 species, the adult specimens frequently died following schizogony.

The populations of *C. balkwilli* not only established well, but also had longevity. Many were still healthy at 6 months and even in those with signs of poor health, reproduction was still taking place. This is unlike *A. tepida* and *B. variabilis*, where reproduction ceased immediately in poor conditions.

Most importantly, *C. balkwilli* successfully reproduced in culture from a single individual (experiment 9). Interestingly, it was found that only those individuals that began as immature specimens reproduced (4/5). Of those that were mature at the start of the culture, 3 died, and 2 remained healthy but failed to reproduce. It has been observed in *Ammonia tepida* that mature adults surviving prior reproduction don't usually reproduce again (Stouff *et al.*, 1999b), and if this were the case in *C. balkwilli* it could explain the findings here. Large populations established from each of the individually kept *C. balkwilli* and were still healthy and thriving after 6 months. They will continue to be maintained and will eventually form the basis of future genomic work.

6.6 Conclusions

Optimal conditions for culturing were established when 7 cm beakers were used containing 50 ml of natural seawater filtered to 0.2 μm. Two thirds of the volume of seawater should be replaced at least once a week, though more frequent changing would be advantageous. Observations indicate that algal overgrowth will almost always occur in the culture vessels given enough time, even with regular changing of the water. For future studies it would therefore be better to transfer the samples to a fresh vessel, or to seed new cultures using a few individuals from the original culture, every few weeks. Specimens survived well when fed a mixed liquid food (*Dunaliella tertiolecta & Phaeodactylum tricornutum*) weekly. A light/dark cycle of 12 hours was maintained, however, the temperature in the lab was too high at 23 °C and above. A fixed temperature of 20 °C would be preferable. The use of a constant light and temperature incubator would greatly enhance the success of culturing experiments.

Cornuloculina balkwilli was found to be the most suitable species for obtaining a continuous culture from a single individual. Though *Ammonia tepida* reproduced from a single individual, reproduction was infrequent and the brood sizes too small. An inability to survive fluctuating conditions in the lab made it particularly unsuitable for culturing. *Bolivina variabilis* formed large populations in culture, but failed to reproduce from individually kept specimens.

A continuously reproducing culture of *C. balkwilli* was obtained and will be maintained and ultimately used as a source of RNA & DNA for EST library construction/ genomic sequencing and other molecular work in the future.

6.7 References

Anderson, O. R., Lee, J. J., & Faber Jr, W. W. 1991. Collection, maintenance and culture methods for the study of living foraminifera. In *Biology of Foraminifera* (ed. J.J Lee & O. R. Anderson), Academic press (San Diego) pp 335-357

Archibald, J. M., Longet, D., Pawlowski, J. & Keeling, P. J. 2003. A novel polyubiquitin structure in Cercozoa and Foraminifera: Evidence for a new eukaryotic supergroup. *Mol. Biol. Evol.* **20**, 62-66

Arnold, Z. M. 1954. Culture methods in the study of living Foraminifera. *Paleontology,* **28**, 404-416

Arnold, Z. M. 1974. Field and laboratory techniques for the study of living foraminifera. In: *Foraminifera* (ed. R. H. Hedley & C. G. Adams), vol 1, pp. 153-206. Academic Press, London

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A., & Saunders, N. C. 1987. Intraspecific Phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecological Systematics,* **18**, 489-522

Bé, A. W. H. 1980. Gametogenic calcification in a spinose planktonic foraminifer, *Globigerinoides sacculifer* (Brady). *Marine Micropaleontology,* **5**, 283-310

Bijma, J., Hemleben, C., Huber, B. T., Erlenkeuser, H., & Kroon, D. 1998. Experimental determination of the ontogenetic stable isotope variability in two morphotypes of *Globigerinella siphonifera* (d'Orbigny). *Marine Micropaleontology,* **35**, 141-160

Blattner, F. R., Plunkett, G. 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F. *et al*. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science,* **277**, 1453–1474

Bouchet, V. M. P., Debenay, J. P., Sauriau, P. G., Radford-Knoery, J., & Soletchnik, P. 2007. Effects of short-term environmental disturbances on living benthic foraminifera during the Pacific oyster summer mortality in the Marennes-Oléron Bay (France). *Marine Environmental Research,* **64** (3), 358-383

Bradshaw, J. S. 1957. Laboratory studies of the rate of growth of the Foraminifera "*Streblus beccarii* (Linné) var. *tepida* (Cushman)". *Journal of Paleontology,* **31** (6), 1138-1147

Bradshaw, J. S. 1961. Laboratory experiments on the ecology of foraminifera. *Contr. Cushman. Found. Res.*, **12** (3), 87-106

Burki, F., Nikolaev, S.I., Bolivar, I., Guiard, J., & Pawlowski, J. 2006. Analysis of expressed sequence tags from a naked foraminiferan Reticulomyxa filosa. *Genome,* **49** (8), 882-887

Burki, F., Shalchian-Tabrizi, K., Minge, M., Skjaeveland, A., Nikolaev, S. I., Jakobsen,K.S., & Pawlowski,J. 2007. Phylogenomics reshuffles the eukaryotic supergroups. *PLoS ONE,* **2** (8), e790

Darling, K. F., Kroon, D., Wade, C. M. & Leigh Brown, A. J. 1996b. Molecular evolution of planktic foraminifera. *Journal of Foraminiferal Research,* **26**, 324-330

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996a. The isolation and amplification of the 18s ribosomal RNA gene from planktonic foraminifers using gametogenic specimens. In**:** *Microfossils and oceanic environments* (eds.

Darling, K. F., Kucera, M., Wade, C. M., von Langen, P., & Pak, D. 2003. Seasonal distribution of genetic types of planktonic foraminifer morphospecies in the

Santa Barbara Channel and its paleoceanographic implications. *Paleoceanography,* **18** (2) 1032

Darling, K. F., Wade, C. M., Kroon, D. & Leigh Brown, A. J. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. Mar. *Micropaleontol*., **30**, 251-266

Darling, K. F., Wade, C. M., Kroon, D., Leigh Brown, A. J. & Bijma, J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulation. *Paleoceanography,* **14**, 3-12

Darling, K.F., Kucera, M., Kroon, D., & Wade, C.M., 2006. A resolution for the coiling direction paradox in *Neogloboquadrina pachyderma*. *Paleoceanography* **21**, PA2011, doi:10.1029/2005PA001189.

Darling, K.F., Kucera, M., Pudsey, C.J., & Wade, C.M., 2004. Molecular evidence links cryptic diversification in polar plankton to Quaternary climate dynamics. *Proc. Natl. Acad. Sci. USA*. **101**, 7657-7662.

Darling, K.F., Wade, C.M., Steward, I.A., Kroon, D., Dingle, R., & Leigh Brown, A.J., 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature,* **405**, 43-47.

Davison, A. & Blaxter, M. L. 2005. An expressed sequence tag survey of gene expression in the pond snail *Lymnaea stagnalis*, an intermediate vector of *Fasciola hepatica*. *Parasitology,* **130**, 539-552

de Nooijer, L. J., Langer, G., Nehrke, G., & Bijma, J. 2009. Physiological controls on seawater uptake and calcification in the benthic foraminifer *Ammonia tepida*. *Biogeosciences Discussions,* **6**, 7083-7102

de Vargas, C., Bonzon, M., Rees, N.W., Pawlowski, J., & Zaninetti, L. 2002. A molecular approach to diversity and biogeography in the planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Marine Micropaleontology,* **870**, 1-16

de Vargas, C., Norris, R., Zaninetti, L., Gibb, S. W., & Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proc. Natl. Acad. Sci. USA*., **96**, 2864-2868

de Vargas, C., Renaud, S., Hilbrecht, H., & Pawlowski, J. 2001. Pleistocene adaptive radiation in *Globorotalia truncatulinoides*: genetic, morphologic, and environmental evidence. *Paleobiology,* **27**(1), 104-125.

de Vargas, C., Zaninetti, L., Hilbrecht, H. & Pawlowski, J. 1997. Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record. *J. Mol. Evol.,* **45** (3), 285-294

Dettmering, C., Rottger, R., Hohenegger, J., & Schmalijohann, R. 1998. The trimorphic life cycle in foraminifera: Observations from cultures allow new evaluation. *European Journal of Protistology,* **34** (4), 363-368

Faber, W. W. Jr., & Lee, J. J. 1991. Feeding and growth of *Peneroplis planatus* (Fichel and Moll) Montfort. *Symbiosis,* **10**, 63-82

Flakowski, J., Bolivar, I, Fahrni, J. & Palowski, J. 2005. Actin phylogeny of foraminifera. *Journal of Foraminiferal Research,* **35**, 93-102

Goldstein, S. T. 1988. On the life cycle of *Saccammina alba* Hedley, 1962. *Journal of Foraminiferal Research*, **18**, 311-325

Goldstein, S. T. 1997. Gametogenesis and the antiquity of reproductive pattern in the Foraminiferida. *Journal of Foraminiferal Research,* **27** (4), 319-328

Goldstein, S. T. 1999. Foraminifera: A biological Overview. In *Modern Foraminifera* (Ed. Gupta, B. K. S), Kluwer Academic Publishers, p37-55

Goldstein, S. T. & Moodley, L. 1993. Gametogenesis and life cycle of the foraminifer Ammonia beccarii (Linné) forma tepida (Cushman). *Journal of Foraminiferal Research,* **23** (4), 213-220

Grell, K. G. 1954. Der Generationswechsel der Polythalmen Foraminifera Rotaliella heterocaryotica. *Archiv der Protistk,* **100**, 211-235

Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates. pp 26-60. In *Culture of Marine Invertebrate Animals* (ed. W.L. Smith and M.H. Chanley), Plenum Press, New York, USA.

Guillard, R.R.L. & Ryther, J.H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. *Can. J. Microbiol*., **8**, 229-239.

Habura, A, Wegener, L., Travis, J. L. & Bowser, S. S. 2005. Structural and functional implications of an unusual foraminiferal β-tubulin. *Mol. Biol. Evol.,* **22**, 2000-2009

Hallock, P. 1988. Interoceanic differences in Foraminifera with symbiotic algae: A result of nutrient supplies? *Proceedings, 6th International Coral Reef Symposium*, Townsville, Australia, **3**, 251-254

Hallock, P. 1999. Symbiont-Bearing Foraminifera. In *Modern Foraminifera* (Ed. Gupta, B. K. S), Kluwer Academic Publishers, p123-139

Harney, J. N., Hallock, P., & Talge, H. K. 1998. Observations on a trimorphic life cycle in *Amphistegina gibbosa* populations from the Florida Keys. *Journal of Foraminiferal Research,* **28** (2), 141-147

Hedley, R. H. 1964. The Biology of Foraminifera. In *Internat. Rev. Gen. Exper. Zool. I.* (ed. W. J. Felts & R. J. Harrison) pp. 1-45. Academic Press, London

Hemleben, Ch., Spindler M., Anderson, O. R. 1989 Modern Planktonic Foraminifera. Springer Verlag, New York, 363pp

Jepps, M. W. 1942. Studies on *Polystomella* Lamarck (Foraminifera). *Journal of the Marine Biological Association of the United Kingdom*, **25**, 607-666

Klenk, H. P., Clayton, R. A., Tomb, J-F., White, O., Nelson, K. E., Ketchum, K. A., Dodson, R. J., Gwinn, M., Hickey, E. K., Peterson, J. D. *et al*. 1997. The complete genome sequence of the hyperthermophilic, sulphate- reducing archaeon *Archaeoglobus fulgidus*. *Nature,* **390**, 364–370

Krüger, R., Röttger, R., Lietz, R., & Hohenegger, J. 1997. Biology and reproductive processes of the larger foraminiferan *Cycloclypeus carpenteri* (Protozoa, Nummulitidae). *Arch. Protistenkd.,* **147**, 3-7-321

Kuile, B, ter, & Erez, J. 1984. *In situ* growth rate experiments on the foraminifera Amphistegina lobifera and Amphisorus hemprichii. *Journal of Foraminiferal Research,* **14**, 262-276

Leutenegger, S. 1977. Reproduction cycles of larger foraminifera and depth distribution of generations: *Utrecht Micropaleontological Bulletin,* **15**, 26-34

Lee, J. J., & Anderson, O. R. 1991. Symbiosis in Foraminifera. In *Biology of Foraminifera* (ed. J. J. Lee, & O. R. Anderson), Academic Press, New York, pp. 157-220

Lee, J. J., Faber, W. W. Jr., Anderson, O. R., & Pawlowski, J. 1991a. Life cycles of foraminifera. In: *Biology of Foraminifera* (ed. J. J. Lee, & O. R. Anderson), Academic Press, New York, pp. 285-334

Lee, J. J., McEnery, M., Pierce, S., Freudenthal, H. D. & Muller, W. A. 1966. Tracer experiments in feeding littoral foraminifera. *J. Protozool.,* **13**, 659-670

Lee, J. J., McEnery, M.E., Kennedy, E. M. & Rubin, H. 1975. A nutritional analysis of a sublittoral spiphytic diatom assemblage from Long Island salt marsh. *J. Phycol,* **11**, 14-49

Lee, J. J., Muller, W. A., Stone, R. J., McEnery, M. E., & Zucker, W. 1969. Standing crop of Foraminifera in sublittoral epiphytic communities of a Long Island salt marsh. *Marine Biology,* **4**, 44-61

Lee, J. J., Sang, K., ter Kuile, B., Strauss, E., Lee, P. J., & Faber, Jr., W. W. 1991b. Nutritional and related experiments on laboratory maintenance of 3 species of symbiont-bearing, large foraminifera. *Marine Biology,* **109** (3), 417-425

Lee, J. J., Tietjen, J. H., Stone, R. J., Muller, W. A., Rullman, J. & McEnery, M. 1970. The cultivation and physiological ecology of members of salt marsh epiphytic communities. *Helgolander. Wiss. Meeres,* **20**, 136-156

Lister, J. J. 1895. Contributions to the life history of the Foraminifera. *Philosophical Transactions of the Royal Society of London, Series B*, **186**, 401-453

Longet, D., & Pawlowski, J. 2007. Higher-level phylogeny of Foraminifera inferred from RNA polymerase II (RPB1) gene. *European Journal of Protistology,* **43**, 171-177

Longet, D., Archibald, J. M., Keeling, P. J. & Pawlowski, J. 2003. Foraminifera and Cercozoa share a common origin according to RNA polymerase II phylogemies. *Int. J. Syst. Evol. Micr.,* **53**, 1735-1739

Lutze, G. F., & Wefer, G. 1980. Habitat and asexual reproduction of *Cyclorbiculina compressa* (Orbigny), Soritidae. *Journal of Foraminiferal Research,* **10** (4) 251-260

Marechal-Abram, N., Debenay, J. P., Kitazato, H., & Wada, H. 2004. Cadmium partition coefficients of cultured benthic foraminifera *Ammonia beccarii*. *Geochemical journal,* **38** (3), 271-283

Margulies, M. *et al*. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature,* **437**, 376- 380

Mashiotta, T. A., Lea, D. W., & Spero, H. J. 1997. Experimental determination of cadmium uptake in shells of the planktonic foraminifera *Orbulina universa* and *Globigerina bulloides*: Implications for surface water paleoreconstructions. *Geochimica et Cosmochimica Acta,* **61** (19), 4053-4065

Morvan, J., Le Cadre, V., Jorissen, F., & Debenay, J. P. 2004. Foraminifera as potential bio-indicators of the "*Erika*" oil spill in the Bay of Bourgneuf: Field and experimental studies. *Aquatic Living Resources,* **17**, 317-322

Muller, W. A. 1975. Competition for food and other niche-related studies of three species of salt marsh foraminifera. *Marine Biology,* **31**, 339-351

Muller, W. A. & Lee, J. J. 1969. Apparent indispensability of bacteria in foraminiferan nutrition. *J. Protozool,* **16**, 471-478

Murray, J. W. 1963. Ecological experiments on Foraminiferida. *J. Mar. Biol. Assoc. U.K.,* **43**, 621-642

Murray, J. W. 1979. British Nearshore Foraminiferids. In: *Synopses of the British Fauna* (eds. D. M. Kermack & R. S. K, Barnes), No. 16

Palumbi, S. R. 1996. Nucleic Acids II: The polymerase Chain Reaction. In *Molecular Systematics, second edition* (eds. D. M. Hillis, C.,Moritz, & B. K Mable), pp 205-247

Papanicolau, A., Joron, M., McMillan, W. O., Blaxter, M. L. & Jiggins, C. D. 2005 Genomic tools and cDNA derived markers for butterflies. *Molecular Ecology,* **14**, 2883-2897

Pawlowski, J., Bolivar, I., Fahrni, J. F., Cavalier-Smith, T., & Gouy, M. 1996. Early origin of Foraminifera suggested by SSU rRNA gene sequences. *Mol. Biol. Evol.,* **13** (3), 445-450

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C., Gouy, M. & Zaninetti, L. 1997. Extreme differences in rates of molecular evolution of foraminifera revealed by comparison of ribosomal DNA sequences and the fossil record. *Mol. Biol. Evol.,* **14** (5), 498-505

Pawlowski, J., Bolivar, I., Fahrni, J., de Vargas, C. & Bowser, S. S. 1999. Naked foraminiferans revealed. *Nature,* **399**, 27

Rhumbler, L. 1909. Die Foraminiferen (Thalamorphoren) der plankton-Expedition, Teil. In *Ergebnisse der Plankton-Expedition der Humboldt-Stiftung, v. 3,* (ed. V. Hensen), Lipsius und Tischer, Kiel, p131

Röttger, R., Fladung, M., Schmaljohann, R., Spindler, M., & Zacharias, H. 1986. A new hypothesis: The so-called megalospheric schizont of the larger foraminifer, *Heterostegina depressa* D'Orbigny, 1862, is a separate species. *Journal of Foraminiferal Research,* **16**, 141-149

Röttger, R., Kruger, R., & De Ruk, S. 1990. Trimorphism in foraminifera (Protazoa): Verification of an old hypothesis. *European Journal of Protistology,* **25**, 226-228

Russell, A. D., Hönisch, B., Spero, H. J., & Lea, D. W. 2004. Effects of seawater carbonate ion concentration and temperature on shell U, Mg, and Sr in cultured planktonic foraminifera. *Geochimica et Cosmochimica Acta,* **68** (21), 4347-4361

Schaudinn, P. 1895. Uber den Dimorphisms bei Foraminiferen. *Sitzungsberichte der Gesellschaft naturforschender Freunde zu Berlin*, 5, 87-97

Schnitker, D. 1974. Ecotypic variation in *Ammonia beccarii* (Liné). *Journal of Foraminiferal Research*, **4** (4), 217-223

Spero, H. J., & Lea, D. W. Intraspecific stable isotope variability in the planktic foraminifera G*lobigerinoides sacculifer*: Results from laboratory experiments. 1993. *Marine Micropaleontology,* **22**, 221-234

Stewart, I.A., Darling, K.F., Kroon, D., Wade, C.M., & Troelstra, S.R., 2001. Genotypic variability on subarctic Atlantic planktic foraminifera. *Marine Micropaleontology,* **43**, 143-153

Stouff, V., Geslin, E., Debenay, JP., & Lesourd, M. 1999b. Origin of morphological abnormalities in *Ammonia* (Foraminifera): Studies in laboratory and natural environments. *Journal of Foraminiferal Research,* **29** (2), 152-170

Stouff, V., Lesourd, M., & Debenay, J. P. 1999a. Laboratory observations on asexual reproduction (schizogony) and ontogeny of *Ammonia tepida* with comments on the life cycle. *Journal of Foraminiferal Research,* **29** (1), 75-84

Sturrock, S., & Murray, J. W. 1981. Comparison of low energy and high energy marine middle shelf foraminiferal faunas: Celtic Sea and western English Channel. In: *Microfossils from Recent and fossil shelf seas* (Neale, J. W., & Brasier, M. D, eds). Ellis Horwood, Chichester, 251-260

Takata, H., Dettman, D. L., Seto, K., Kurata, K., Hiratsuka, J., & Khim, B. 2009. Novel habitat preference of *Ammonia "Beccarii"* forma 1 in a macrobenthos community on hard substrates in the Ohashi River, Southwest Japan. *Journal of Foraminiferal Research,* **39** (2), 87-96

Theodorides, K., De Riva, A., Gómez-Zurita, J., Foster, P. G., & Vogler, A. P. 2002. Comparison of EST libraries from seven beetle species: towards a framework for phylogenomics of the Coleoptera. *Insect Mol. Biol.,* **11**, 467-475

Uhle, M. E., & Macko, S. A. 1999. The fate of nitrogen in the *Orbulina universa* foraminifera-symbiont system determined by nitrogen isotope analyses of shell-bound organic matter. *Limnology and Oceanography,* **44** (8), 1968-1977

Wade, C. M., Darling, K. F., Kroon, D. & Leigh Brown, A. J. 1996. Early evolutionary origin of the planktic foraminifera inferred from SSU rDNA sequence comparisons. *Journal of Molecular Evolution.,* **43**, 672-677

Walton, W. R., & Sloan, B. J. 1990. The genus *Ammonia* Brünnich, 1772: its geographic distribution and morphological variability. *Journal of Foraminiferal Research,* **20**, 128-156

Waterston, R. H., Lindblad-Toh, K., Birney, E., *et al.* 2002. "Initial sequencing and comparative analysis of the mouse genome". *Nature,* **420** (6915), 520–62

White, T.J., Burns, T., Lee, S., & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (eds. Innis M. A., Gelfland D. H., Sninsky J. J., White T. J.). Harcourt Brace Jovanovich, San Diego, pp 315 – 322

Whitton, C., Daub, J., Thompson, M. & Blaxter, M. 2004. Expressed sequence tags: medium-throughput protocols. *Method. Mol. Biol.,* **270**, 75–92

Wray, C. G., Langer, M. R., DeSalle, R., Lee, J. J., & Lipps, J. H. 1995. Origin of the foraminifera. *Proc. Natl. Acad. Sci. USA,* **92**, 141-145

7 Development of a method for the extraction of DNA from within intact foraminiferal shells using a lysis buffer

7.1 Introduction

7.1.1 The need for a new method of extracting foraminiferal DNA

The identification of planktonic foraminiferal morphospecies from the characteristics of their calcium carbonate shells is central to their use as palaeoproxies of past climate change. Planktonic foraminifera are unicellular protists, and since it is difficult to observe the features of the cell itself, the characteristics of the shell or 'test' are used for their identification. A wealth of morphological variation can be seen between the shells of different planktonic foraminiferal morphospecies, and in some cases a level of morphological plasticity has even been reported within individual morphospecies (Bijma & Hemleben, 1994; de Vargas *et al*., 1999, 2001; Huber *et al*., 1997).

For genetic studies of living planktonic foraminifera, samples collected via siphoning or plankton net from a ship are initially identified from their shell morphology using a binocular microscope, with photographs taken for reference. The process can be difficult, with vibrations and movements of the ship often reducing the quality of the photographs. To extract and preserve DNA from the sample, currently the whole specimen is crushed in a lysis buffer containing 50 mM Tris buffer (pH 8.6), 2 mM EDTA, 0.1 % Triton X-100, and 0.5 % Na deoxycholate and incubated for 1 hour at 60 °C (Holzmann *et al*. 1996). The solution is then used directly in PCR without a subsequent phenol-chloroform/ chloroform extraction or ethanol precipitation, and the

shell is destroyed during the process making it impossible to refer back to morphology at a later stage.

The work in this study will focus on the development of a method of extracting DNA from foraminifera whilst leaving the delicate $CaCO_3$ shell intact. The ability to preserve the shell would be of great benefit, as it would allow direct examination of the morphological features of individual specimens being used in genotype studies. This could provide more accurate insights into the links between low-level genetic variation and the minor morphological plasticity observed within planktonic foraminiferal morphospecies. Such information could greatly enhance the accuracy of past climate models, which rely on matching morphologically distinct species to the particular environmental conditions to which they are adapted.

The design of a new cell lysis/ DNA extraction method could also greatly improve the yield and quality of the DNA obtained from foraminifera. Currently, the method widely employed for the extraction of foraminiferal DNA, based on the work of Holzmann *et al*. (1996), produces highly variable results. PCR amplifications following these extractions can be problematic, producing weak bands or even failing completely, though at other times, the results are good (chapters 3 & 4). Much of this difficulty is likely to be the result of the way in which the buffer is used. A lysis buffer is usually employed to rupture the cell wall, releasing DNA, which is then purified using phenol-chloroform or chloroform extraction along with alcohol precipitation, using ethanol or isopropanol. The buffer of Holzmann *et al*. (1996), however, is typically used to store samples in long-term, with the solution then being used directly in PCR. Over time degradation of the stored DNA takes place, rendering

PCR amplification unsuccessful. In addition, any inhibitory substances present in the lysis buffer itself that would normally be removed in a phenol-chloroform/ chloroform and alcohol precipitation stage, remain present and are transferred through to the PCR. In this study, it is hoped that by testing new buffers, using a range of available reagents, and adding a chloroform extraction and alcohol precipitation stage, the factors responsible for the higher than usual failure of foraminiferal PCR amplifications may be overcome.

7.1.2 Lysis buffers and DNA extraction methods

Lysis buffers are widely used for the extraction of crude DNA from a variety of sources, from soil microbes (Bruce et el., 1992; Steffan et el., 1988; Tsai and Olson 1991; Zhou et el., 1996) to clinical samples (Fredricks et el., 2005; Noordhoek et el., 2009) and forensic specimens (Bienvenue et el., 2006; Gill et el., 1985). There is no standard method for such DNA extraction procedures, and the chemical content of the lysis buffers varies greatly. It is generally accepted that extraction methods using a lysis buffer in conjunction with mechanical grinding of the specimen produce the greatest yields of crude DNA (Fredricks et el., 2005; Miller et el., 1999; Moré et el, 1994). Freeze-thaw treatments can also be also used alongside chemical lysis (Hurt et el., 2001; Tsai & Olson 1991; Erb & Wagner-Döbler 1993), though yields have been found to be slightly lower than those for lysis buffer with mechanical grinding (Kuske et el, 1988; Leff et el., 1995; Zhou et el., 1996). The need to retain the intact shell of the foraminifera, after DNA extraction, however, precludes the use of such methods. Instead, a method using a lysis buffer plus a simple high temperature incubation will be employed (Bruce et el., 1992; Smith & Tiedje 1992). Whilst it has been suggested that DNA yields from such methods may be lower (Zhou et el., 1996), the more gentle

approach avoids the often severe problem of DNA shearing that can result from mechanical grinding (Leff et el., 1995; Ogram et el., 1987; Zhou et el., 1996). The length and heat of the incubation period will be manipulated along with the chemical content of the lysis buffer to obtain the highest yield of crude DNA possible, whilst avoiding damage to the shell.

7.1.3 Lysis buffer components

Lysis buffers may contain a variety of chemical reagents, each of which performs a specific role. They include a buffer, for example Tris pH 8.5 (Hurt et el., 2001; Tsai and Olson 1991), to maintain the solution at a constant pH, preventing the denaturation of the DNA that may otherwise occur under extremes of pH (pH <3 or >10). NaCl may also be included (Tebbe & Vahjen 1993; Tsai and Olson 1991) to induce cells with an osmotic shock, thus breaking down the cell membrane to release the genetic material. Both Tris and NaCl are included in the new buffers.

A detergent is added to promote cell lysis (by disruption of the hydrophobic attraction between membrane phospholipids), solubilisation of membrane proteins and lipids, and denaturation (denaturing detergents only). The buffers tested will contain one of two denaturing detergents, Sodium dodecyl sulfate (SDS) (Kuske et el., 1998; Tebbe & Vahjen 1993), or Sodium *N*-lauroyl sarcosine (Sarkosyl) (Chakravorty & Tyagi., 2001; Hurt et el., 2001). The lysis buffer traditionally used for extraction of DNA from planktonic foraminifera (Holzmann *et al*., 1996) contains a less potent, a non-denaturing detergent, Triton X-100.

Some of the new buffers developed in this study contain Guanidinium isothiocyanate (GITC), which is frequently used in DNA extraction procedures (Maciel et el., 2009; Noordhoek et el., 2009). The Guanidine and thiocyanate ions denature proteins and lyse the cell. Isopropanol may be added (Erb & Wagner-Döbler 1993, Hurt et el., 2001) to precipitate DNA and help dissolve the Guanidinium isothiocyanate. Urea, a non-charged chaotrope that disrupts noncolvalent bonds thereby denaturing proteins of the cell wall, may also be included (Walker, 2005).

The lysis buffers developed in this study include a range of reducing agents, which reduce disulphide bonds, irreversibly denaturing proteins, to lyse the cell. They include β-mercaptoethanol (β-ME) (Chakravorty & Tyagi., 2001), Dithiothreitol (DTT) (Bienvenue et el., 2006), and 2-Aminethanethiol Hydrochloride. Reducing agents may have strong odours, must be handled in a fume cabinet and must be added fresh to the buffer as they degrade quickly in solution.

There are a number of other reagents that may be included in a lysis buffer, which are not used in this study. These include enzymes such as lysozyme (Maciel et el., 2009; Tsai and Olson 1991), which digests the polysaccharide component of bacterial cell walls, and Proteinase K (Maciel et el., 2009; Tebbe et el., 1993), which digests contaminating proteins and degrades nucleases that might otherwise damage the target DNA. Neither was used here as their required freezing for storage makes them impractical for use on board a ship. Chelating agents, for example, ethylenediaminetetraacetic acid (EDTA) (Chakravorty & Tyagi., 2001; Frostegård et el., 1999; Holzmann *et al*., 1996; Tsai and Olson 1991), may also be included to

inhibit protease and deoxyribonuclease (DNase) activity by chelating magnesium ions', though these may be damaging to the $CaCO_3$ shell (discussed later).

In many DNA extraction procedures, a post cell-lysis stage using phenol-chloroform/ chloroform is added to remove residual proteins and isolate DNA from the buffer solution (Luna et el., 2006; Maciel et el., 2009; Sambrook *et al*., 1989; Tsai & Olson, 1991; Erb & Wagner-Döbler 1993). After mixing to form an emulsion, centrifugation is used to separate the liquid into an organic phase (containing cell debris and protein matter) and an aqueous phase, which contains the target DNA. The DNA is then precipitated using ice-cold isopropanol or ethanol. A DNA pellet is obtained by centrifugation, which can then be re-suspended in an elution buffer or distilled water. This final stage purifies the DNA, removing substances that could interfere with downstream molecular applications such as PCR.

7.1.4 Designing a DNA extraction protocol: maximising performance and overcoming potential problems

In order to develop a method of extracting DNA efficiently from individual foraminiferal specimens whilst leaving the $CaCO_3$ shell intact, it was necessary to find reagents that would maximise performance and provide high yields of crude DNA for use in PCR. The DNA should be undamaged by the process, leading to high success in PCR, without interference from inhibitory substances. The new lysis buffer would have to be effective enough to penetrate the shell of the foraminifer, without damaging the shell itself, even if the sample were stored in the buffer for an extended period, something that may be necessary in the field or on a ship.

A significant problem faced when designing DNA extraction procedures is that a number of the components commonly used are known to have an inhibitory effect on

Table 7.1. Compounds commonly included in DNA extraction procedures and the concentrations at which they cause inhibition to PCR

Compound		Final concentration in PCR that gives		
		No inhibition	Inhibition	Reference
Phenol		0.1 %	0.5 %	1
Chloroform		5 %	n.d	1
Isopropanol		0.5 %	1 %	1
Ethanol		2.5 %	5 %	1
Lysozyme		n.d	0.5 mg/ml	1
Proteinase K		0.5 mg	n.d	1
NaCl		0.3 %	0.5 %	1
EDTA		0.1 mM	1 mM	1
DTT		10 mM	n.d	1
Guanidinium isothiocyanate		20 mM	100 mM	1
Urea		n.d	0.5M	2
Detergents				
SDS	I	0.005 %	0.01 %	1
CTAB	I	0.001 %	0.01 %	1
Na-sarkosyl	I	0.01 %	0.05 %	1
Na-deoxycholate	I	n.d	< 0.06 %	3
Nonidet P40	N	n.d	> 5 %	3
Triton X-100	N	1 %	2 %	1
Tween-20	N	2 %	10 %	1
N-Octylglucoside	N	n.d	< 0.4 %	3

Figures in the 'No inhibition' column indicate levels at which tested compounds caused absolutely no inhibition to the PCR. Figures in the 'Inhibition' column indicate the level at which inhibition was definitely detected. n.d. = not determined, I = ionic, N = non-ionic. Adapted from Rossen *et al*., 1992. References: (1) Rossen *et al*., 1992; (2) Gelfland *et al*., 1990, (3) Weyant et el., 1990.

PCR, when carried over into the reaction mix (Table 7.1, Rossen *et al*., 1992; Saunders *et al*., 1999; Tijssen, 1993; Weyant et el., 1990). The extraction method of Holzmann *et al*. (1996) includes no phenol-chloroform/ chloroform extraction and ethanol precipitation stage to purify the DNA, which makes the process quick and easy but may leave inhibitory substances in the solution that could interfere with the PCR.

One of the key components of the lysis buffer of Holzmann *et al.* (1996) is the chelating agent EDTA (ethylenediaminetetraacetic acid). EDTA is a commonly used compound in molecular biology that will strongly complex with most metal ions. In a lysis buffer, it prevents divalent cation-dependent proteases and DNase from degrading the DNA, however, it is also a known inhibitor of PCR (table 7.1, Al-Soud & Rådström 2001; Rossen *et al*., 1992). The inhibitory effect results from its ability to chelate Mg^{2+}, which is necessary for the activity of DNA polymerase (McPherson et el., 1991) and is also required by the nucleotides (dNTPs) and primers in a PCR reaction (Dieffenbach & Gabriela, 1995). EDTA is tolerated in PCR reactions at concentrations equal to and below 0.1 mM. Above this level problems may start to occur, and inhibition is certainly caused at concentrations of 1 mM and above (table 7.1; Rossen *et al*., 1992, Khosravinia & Remesha, 2007, Kreader, 1996). Since the DNA being extracted from a foraminifer originates from a single cell, low DNA concentrations in the solution often mean that up to 5 µl of template (lysis buffer used directly) is added into the PCR reaction (see chapter 3 & 4). If only1 µl of extraction buffer were carried over to a 50 µl PCR reaction as the template, it would lead to a concentration of 0.04 mM EDTA, which is below the inhibitory threshold of 0.1 mM (table 7.1), however, if 5 µl of extraction solution were used as a template, an EDTA concentration of 2 mM would result in the PCR reaction, which is twice the level known to cause PCR inhibition (table 7.1). The problem is not necessarily impossible to solve. If an excess of EDTA is carried over into the PCR, the problem can usually be overcome by increasing Mg^{2+} concentrations in the reaction (Khosravinia & Remesha, 2007).

Perhaps a more significant disadvantage of including EDTA in the new lysis buffers would be that in addition to chelating Mg^{2+}, EDTA also strongly chelates calcium ions and could therefore cause severe damage to the $CaCO_3$ foraminiferal shell that we are trying to preserve. In fact, EDTA based buffers have been used specifically for extracting DNA from within stromatolites, by inducing dissolution of their $CaCO_3$ structure (Wade & Garcia-Pichel, 2003). $CaCO_3$ damage was not an issue in previous genetic studies of the foraminifera, where the shell was crushed as part of the extraction procedure, however, for these new trial buffers, EDTA was excluded to avoid damage to the foraminiferal shells.

During DNA extraction, detergents are often used to promote cell lysis and denaturation (Tijssen, 1993). Some methods utilise mild non-ionic detergents such as Triton X-100, as was used in the Holzmann *et al*. (1996) lysis buffer. However, it has been indicated that non-ionic detergents (Triton X-100, Tween 20, Nonidet P40, *N*-Octylglucoside) are far less effective in DNA extraction than the stronger ionic detergents (SDS, Sarkosyl, Na-deoxycholate), sometimes providing very poor results (Simmon *et al*., 2004). In order to penetrate the shell of the foraminifera, stronger ionic detergents were used in the new trial buffers. A potential downside of using ionic detergents (Sodium dodecyl sulfate (SDS), Na-sarkosyl, Na-deoxycholate) is that they are far more inhibitory to PCR (causing denaturation of the polymerase) than non-ionic ones (Triton X-100, Tween 20, Nonidet P40, *N*-Octylglucoside). Non-ionic detergents generally cause no inhibition at concentrations of up to 5 %, whilst ionic detergents can only be used at low concentrations (e.g. SDS < 0.01 %) (table 7.1, Weyant *et al*., 1990).

Some of the other components of the 9 new lysis buffers (Isopropanol, Guanidinium isothiocyanate, Urea) have also been reported to have some inhibitory effect on PCR, however, they are effective reagents that are commonly used in extraction procedures and should be suitable if used at the right concentrations. Moreover, the addition of a chloroform extraction and ethanol precipitation stage should ensure that any remaining inhibitory substances are removed from the final DNA product before PCR is carried out, bringing them below the concentrations at which inhibition of PCR may be caused (see table 7.1) (Rossen *et al*., 1992; Weyant *et al*., 1990). It is the lack of this step that has potentially led to some of the problems experienced when using the Holzmann *et al*. (1996) method, where PCR results are often poor (chapters 3 & 4). By cleaning up the extracted DNA before use, it also allows more effective chemicals to be used in the new buffers, without the risk of them inhibiting the downstream PCR.

As much as possible the new lysis buffers have been re-designed to find better performing reagents, whilst avoiding high concentrations of inhibitory substances. 12 trial lysis buffers were developed incorporating a range of reagents in different combinations, including Guanidium Isothiocyanate, Isopropanol, Urea, Tris buffer, NaCl, the detergents Sarkosyl and SDS, and the reducing agents β-mercaptoethanol (β-ME), Dithiothreitol (DTT) and 2-Aminethanethiol.

7.1.5 Testing the new lysis buffers

Initial testing of the new buffers and DNA extraction method was carried out on a benthic foraminiferan, *Ammonia tepida*. *Ammonia tepida* is a common British intertidal species, found in the surface sediments of muddy estuaries (Murray, 1979).

It was chosen because of its relatively large size compared to other species of benthic foraminifera (~ 400 μm), and its ease of collection. Samples were incubated in 12 new lysis buffers, created from a variety of reagents, and the incubation temperature and duration manipulated to optimise the effectiveness of these buffers.

7.2 Aims and objectives

The aim of this work was to design a lysis buffer that would allow the DNA of single-celled foraminifera to be extracted effectively whilst leaving the $CaCO_3$ shell intact. The shell should be undamaged, even after prolonged storage in the buffer, allowing its morphology to be studied in greater detail at a later date. The conditions of the incubation period (temperature and duration) were manipulated to optimise the effectiveness of the buffers. Testing different reagents in the buffers allowed for the selection of the most effective compounds and revealed any that led to shell degradation or PCR inhibition. A chloroform extraction and ethanol precipitation was added to limit the transfer of inhibitory substances into the PCR reaction. It was hoped that in designing a new method for cell lysis/ DNA extraction, improvements would be seen in the success of PCR amplification in the foraminifera.

7.3 Methods

7.3.1 Collection of benthic foraminifera

Fresh specimens of foraminifera were required to test the effectiveness of the newly designed lysis buffers at removing viable DNA from within the shell. Live, benthic foraminifera were collected from Brancaster, Norfolk in May 2007. Sediment was collected from tidal mudflats, from the green, algal-rich surface layer, where foraminifera are abundant. The sediment was sieved to 212 μm and washed through with seawater. Sea temperature and salinity were recorded on location at both low and high tide.

7.3.2 Picking foraminifera in the lab

Thin layers (~50 mm) of the sieved sediment, containing the live foraminifera, were placed in several plastic tubs, covered with a 5 cm deep layer of fresh seawater (collected at high tide on the day of the foraminifera collection), and a loose lid to prevent evaporation. During the first 2 hours, the sediment was washed twice with fresh seawater, allowing it to settle in between. Obvious large organisms, such as nematode worms, were removed as such organisms had previously been observed to die easily, their decay contaminating the water. After 24 hours, healthy, live forams could be seen, by eye, to have migrated to the surface of the sediment, clinging to small pieces of plant matter and to the sides of the tubs. These were removed by pipette to a petri dish containing fresh seawater (salinity ~ 27 – 30 ‰). Large, healthy individuals of the chosen species, *Ammonia tepida,* were selected and placed in 1.5 ml tubes. Care was taken to collect individuals of a similar size.

7.3.3 Buffer solutions

Three main buffer solutions were tested (table 7.2a), from each of which, 4 final solutions were made, each containing a different reducing agent (Table 7.2b). In total 12 trial lysis buffers were applied to the *Ammonia tepida* foraminiferal test samples.

Table 7.2. Reagents included in the 12 trial lysis buffer solutions and their concentrations

A)

BUFFER	
1	
Tris buffer pH8.5	100 mM
NaCl	100 mM
Guanidinium Isothiocyanate	4 M
Isopropanol	17 %
Sarkosyl	1 %
2	
Tris buffer pH8.5	100 mM
NaCl	100 mM
Sarkosyl	1 %
Urea	8 M
3	
Tris buffer pH8.5	100 mM
NaCl	100 mM
SDS	2 %
Urea	7 M

B)

Reducing agent		
A	No reducing agent	-
B	β-mercaptoethanol	0.1 M
C	DTT	50 mM
D	2-Aminethanethiol Hydrochloride	0.1 M

A) Reagents included in the 3 main buffer solutions (1M TRIS/NaCl stock solution made to pH 8.5). B) The reducing agents added to these main buffer solutions to create the final 12 trial lysis buffers.

The lysis buffer of Holzmann *et al*. (1996) was also used as a control (table 7.3). This buffer has been used routinely to extract and store foraminiferal DNA from single individuals in almost all molecular studies on the foraminifera to date. Samples were crushed in the buffer and incubated at 60 °C for 1 hour, to extract DNA from the cell.

Table 7.3. Reagents included in the control lysis buffer

Control lysis buffer	
Tris buffer pH 8.6	50 mM
EDTA	2 mM
Triton X-100	0.1 %
Na deoxycholate	0.5 %

Taken from Holzmann *et al*. (1996)

Each new lysis buffer was then tested on the freshly collected samples of the benthic foraminifer, *Ammonia tepida*. Specimens were placed into 1.5 ml tubes into which the buffers were added, and placed on a heat block for incubation. Optimisation tests were performed using a range of temperatures and incubation durations to find the conditions that would promote the most effective action of the new buffers.

7.3.4 Cell lysis: optimising conditions for incubation in the lysis buffers

7.3.4.1 Incubation temperature

Tests were carried out to determine the optimal temperature at which to incubate foraminiferal samples in the lysis buffer solutions, both in the presence and the absence of a reducing agent. Prior to testing, photographs of the *Ammonia tepida* specimens were taken under a Nikon SMZ1500 microscope using a Nikon

DXM1200F camera, to establish a record of their appearance before cell lysis. A healthy orange cytoplasm colouration was noted in all specimens.

6 solutions were used, 3 with no reducing agent (1A, 2A, 3A, table 7.1), plus, the same solutions with the reducing agent β-mercaptoethanol added (1B, 2B, 3B, table 7.1). 200 µl of each buffer was added to four 1.5 ml tubes, each containing 3 *Ammonia tepida.* For each buffer, the first tube remained at room temperature (23 °C), whilst the others were heated to 60 °C, 95 °C or 100 °C for 1 hour. At the end of each incubation period the samples were removed from the lysis buffers for examination, and photographed again as described above. The effectiveness of the lysis buffers was then determined by observation of the amount of cytoplasm (orange colouration) lost from within the shells of the foraminifera. The ultimate aim was to remove all cellular material from the shell, as would be indicated by a complete loss of orange colouration.

7.3.4.2 Incubation length

Whilst testing the subset of buffers above to determine the optimal incubation temperature, an incubation length of 1 hour was found to be insufficient to promote the complete removal of cellular material from the shells of the foraminifera. A new test was therefore carried out to find the optimal length of incubation.

200 µl of each of the 12 trial buffers was added to 1.5 ml tubes, containing 3 *Ammonia tepida.* For each buffer one tube of forams was then incubated for 2, 4, 8, 16, or 24 hours at 75 °C. This 75 °C temperature was selected based on the temperature test (above), as it fell directly between 60 °C, which seemed insufficient to remove all

cellular material from the shell (at least in the 1 hour incubation used), and 90 °C, which caused unwanted precipitation in the buffers. Again, the samples were observed and photographed under the microscope (as described above) after incubation and compared to un-treated foraminifera photographed at the same time. The effectiveness of the buffers was determined by the amount of cytoplasm (orange colouration) removed from the shells.

7.3.5 Testing the effectiveness of the 12 trial buffers under optimal incubation conditions

The tests described above established the optimal temperature (75 °C) and incubation length (24 hrs) under which to use the new lysis buffers. The optimums were chosen based on observation of cytoplasm removal from the shells, and represent the conditions that suit the overall use of all of the buffers.

The next step was to test the integrity of the shells and the quality of the DNA produced following incubation in the new lysis buffers under these optimal conditions. Specimens of *Ammonia tepida* were incubated in all 12 buffers, to achieve cell lysis. The DNA suspended in the buffer solution was then purified using chloroform extraction and ethanol precipitation. For half of the samples this process was carried out only after they had been stored in the buffers for 6 weeks following incubation. This was to simulate the extended storage in buffers that could be necessary in the field or on a ship, before a lab could be reached to complete the chloroform extraction and ethanol precipitation stage. The effectiveness of the lysis buffers was assessed by measurement of crude DNA yield produced, and by carrying out PCR amplification (described below). The shells were also inspected for damage.

7.3.5.1 Cell lysis incubation

For each of the 12 final buffer solutions 200 µl was added to a 1.5 ml microcentrifuge tube containing a single *Ammonia tepida* specimen (20 replicates). These were heated to 75 °C for 24 hrs (determined to be optimal in the temperature and incubation length experiments described above). Following incubation, the liquid solution from half of the samples in each buffer type (10 replicates) was transferred to a fresh tube, leaving the empty shell behind for later use. The DNA was purified immediately using chloroform extraction and alcohol precipitation (described below). The other half (10 replicates) were stored at room temp for 6 weeks, at which point the liquid was removed, the DNA purified, and the shell retained for later use.

Following incubation and removal of the buffer solution, the empty shells were stored dry for 4 months before being removed and checked for structural integrity under a binocular microscope. Photographs were taken as described above (section 7. 2 .4 .1).

An additional 20 specimens were crushed into 50 µl of the control lysis buffer (Holzmann *et al*., 1996). Half were stored at room temp for 6 weeks, and half were prepared for immediate use.

7.3.5.2 Post-cell lysis chloroform extraction and alcohol precipitation

After incubation in the buffer solution (+/- 6 weeks storage), 1 µl of tRNA 1 µg/µl was added to the sample followed by 200 µl Chloroform-Isoamyl Alcohol (24:1). The tube was inverted for 10 minutes and then centrifuged for 10 minutes at 13000 rpm. The aqueous layer was retained (~200 µl), to which was added 2.5 volumes (~500 µl) ice-cold ethanol 95 % and 0.1 volumes (~20 µl) 3M Sodium acetate (NaOAc).

Samples were stored overnight at –80 °C and then centrifuged for 20 minutes at 13000 rpm. The supernatant was removed and the pellet washed in 500µl ethanol 70 % before being centrifuged for 5 minutes at 13000 rpm. The remaining ethanol was removed and the sample dried for 15 minutes at 45 °C. The DNA was then re-suspended in 50 µl Tris buffer. The total amount of DNA present after extraction (crude DNA yield) was measured using a Thermo Scientific NanoDrop 1000.

7.3.5.3 Statistical Analysis of DNA yield

A one-way between groups analysis of variance (ANOVA) (Harris, 1994) was conducted within the SPSS v. 17.0 package (SPSS, 2009) to assess the effect of buffer type on the mean yield of crude DNA obtained. Post-hoc comparisons using Tukey's HSD test (Hsu, 1996) (performed within the SPSS v. 17.0 package: SPSS, 2009) were used to indicate differences in mean scores between individual buffers.

7.3.5.4 DNA amplification

To test the suitability of the DNA for use in downstream applications PCR amplification of an approximately 500 bp region of the terminal 3′ end of the foraminiferal SSU rRNA gene was carried out using a nested PCR approach. 3 µl of extracted DNA was used as the template for the first round of PCR, using primers C5 (5′-GTAGTATGCACGCAAGTGTGA-3′) and 138 (5′-TGATCCTGCAGGTTCACCTAC-3′) (Medlin *et al*., 1988). 1 µl of product from the first round was used as the template in the second round using primers 2082F (5′-TGAAACTTGAAGGAATTGACGGAAG-3′) and 2514R 5′ (5′-GGCATCACAGACCTGTTATTGCC-3′) (modified from primers NS5 and NS6, White *et al*. 1990) (for primer sequences and positions see chapter 2, section

2.2.2). PCR amplification was performed using 0.5 units Taq polymerase (Qiagen), with 0.2 μM each primer, 200 μM dNTPs, and 3mM magnesium chloride in a 50 μl final volume (see chapter 2, section 2.2.2 for details). Thermal cycling (with a Perkin Elmer cycler) was performed with cycling parameters as described in chapter 2, tables 2.3 and 2.4. Positive controls using 3 μl of DNA extracted using the traditional lysis buffer method of Holzmann *et al*. (1996) plus negative controls using distilled water were included during each round. Gel electrophoresis was carried out using a 1.5 % agarose gel, with gels visualised and photographed using a Bio-Rad gel doc system (see chapter 2, section 2.3 for details).

7.4 Results

7.4.1 Cell lysis: optimising conditions for incubation in the lysis buffers

7.4.1.1 Temperature

The optimal temperature at which to incubate samples in the buffers, to promote cell lysis and the removal of cellular material from the shell, was determined by heating benthic foraminifera in a small subset of the new buffers for 1 hour under a range of temperatures. Colour loss could be seen in some of the specimens after incubation, as can be seen in the microscope photographs (fig. 7.1), however, none of the shells were completely emptied of cellular material, regardless of the temperature used. Greater colour loss could be seen at higher temperatures and in addition, buffers containing a reducing agent (1B, 2B, and 3B) were more effective than those without (1A, 2A, and 3A). Precipitation was noted in most tubes at 95 °C and 100 °C. Some damage of the shells was noted at 100 °C, particularly in buffer 3B. No precipitation or shell damage was observed at 60 °C or below.

A temperature of 75 °C was chosen for subsequent incubations. This fell below the temperature at which unwanted precipitation occurred in the buffers (90 °C), but was higher than the next lowest temperature tested (60 °C), which was ineffective at removing cellular material. As none of the shells were completely emptied of cellular material, even at 90 or 100 °C after 1 hour, it was clear that the length of incubation would need to be raised, and this was tested next.

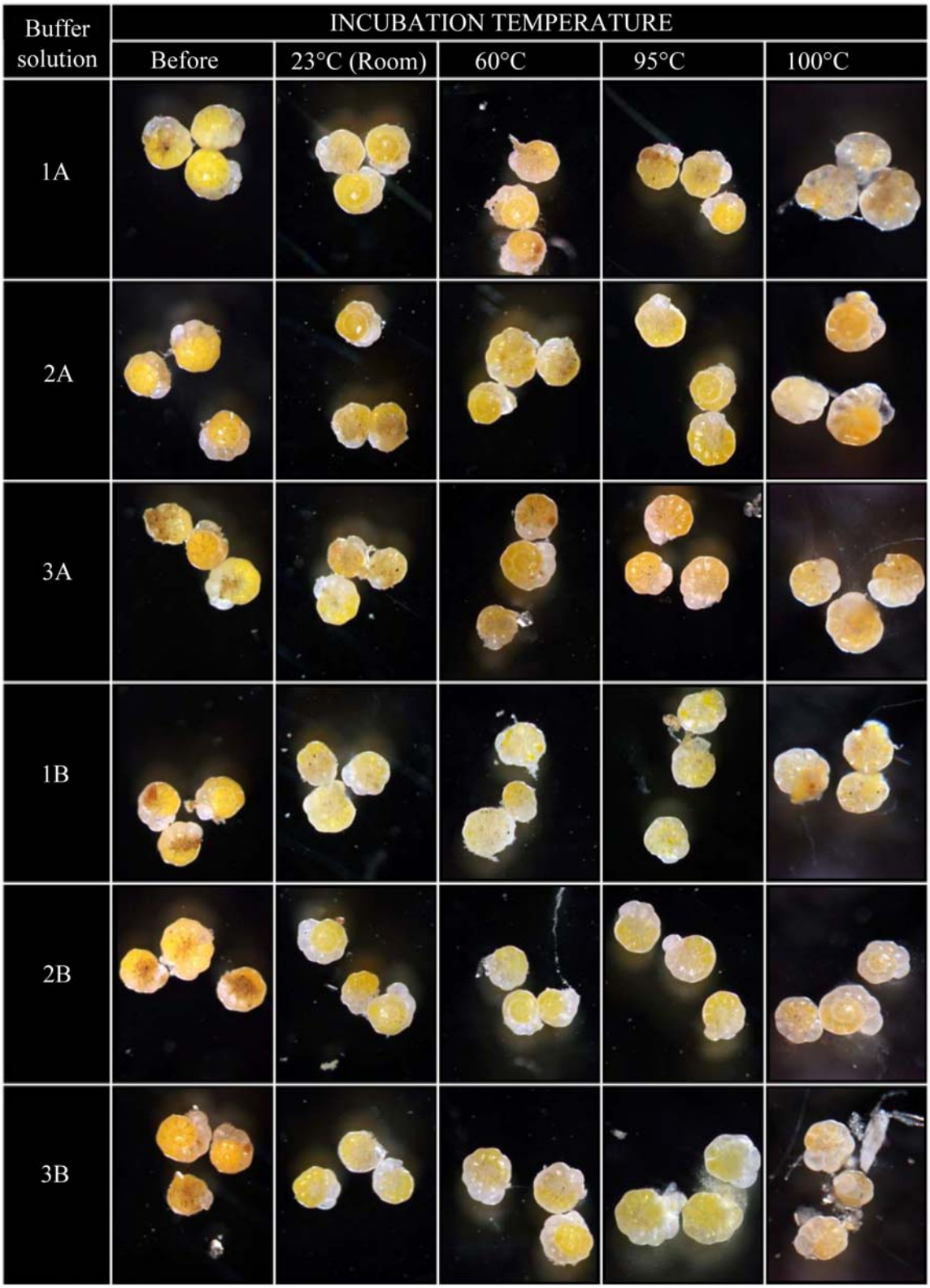
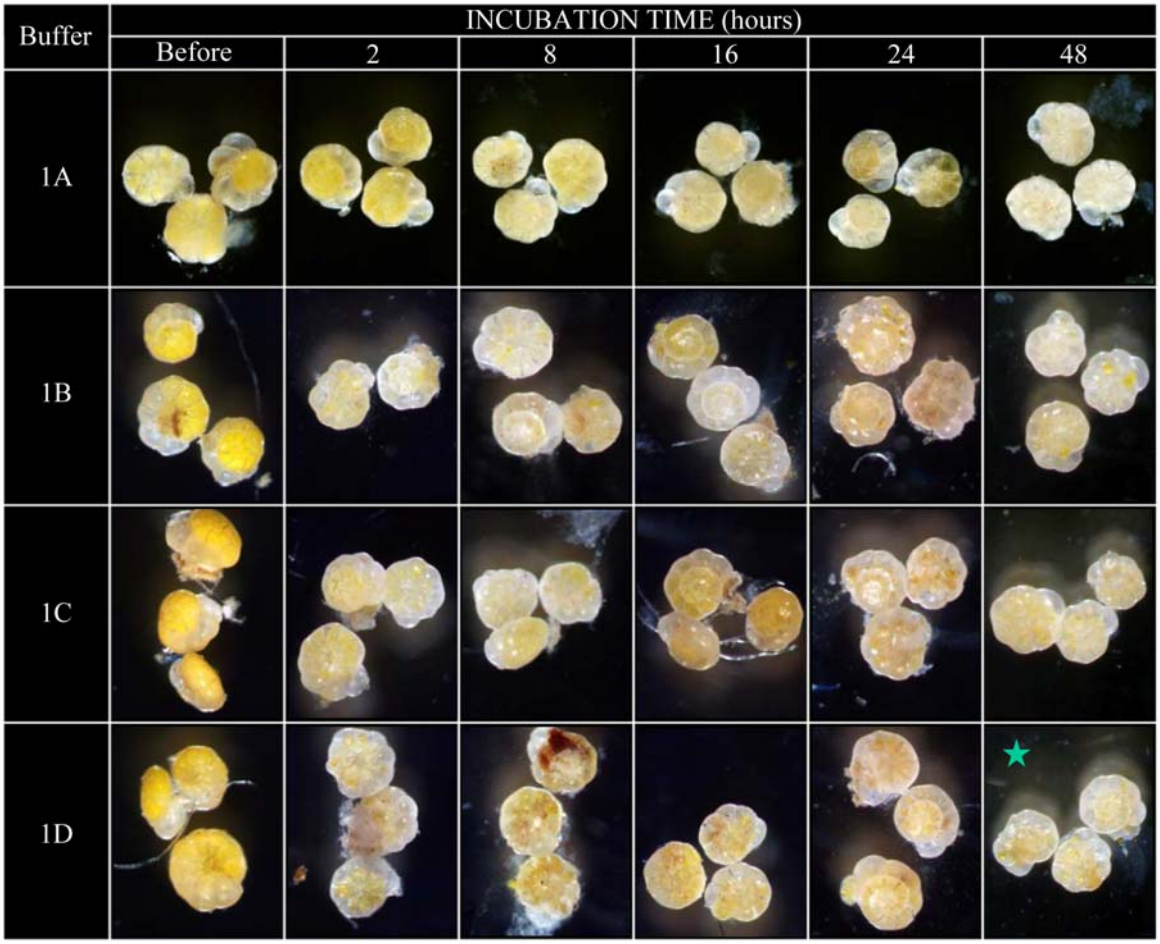
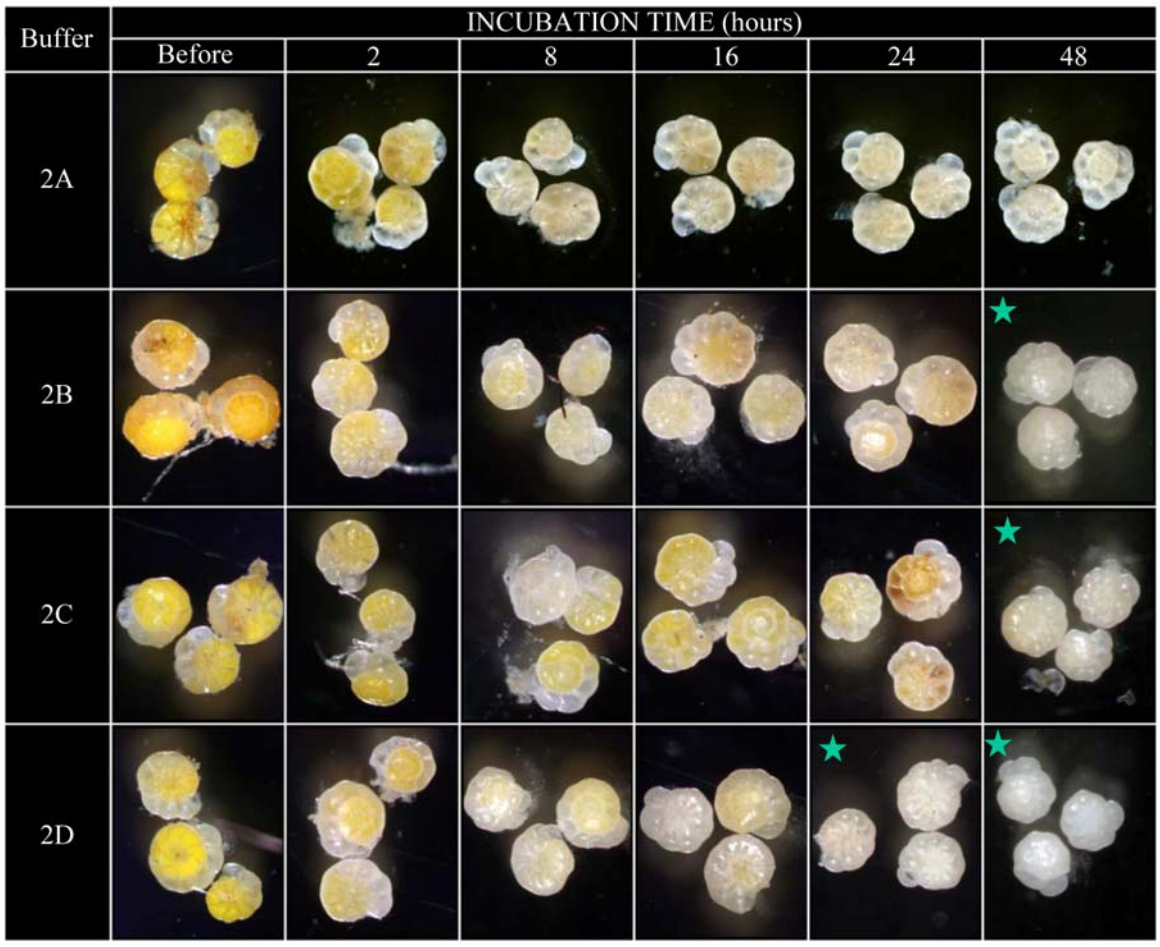


Figure 7.1. Benthic foraminifera photographed after incubation in 6 lysis buffers, 3 with no reducing agent (1A, 2A, 3A) and 3 with ßME (β-mercaptoethanol) (1B, 2B, 3B) for 1 hour at varying temperatures. Removal of cellular material from the shell is indicated by a loss of the orange colouration.

7.4.1.2 Incubation length

To determine the optimal length of incubation, foraminifera were heated in the 12 new buffers to 75 °C, for a range of times, from 2 to 48 hours. Loss of colouration from the shells was taken as an indicator that cell lysis was occurring and that cellular material was being removed into solution (fig. 7.2). Evacuation of cellular material was evident sooner in specimens incubated in buffers containing a reducing agent than those in buffers lacking a reducing agent (2 hours compared to 8 hours). The buffers varied greatly in the number of hours taken for all material to be removed from the shells. Shells in buffers 1A, 1B, 1C, 1D, 3A and 3B still contained some remnants of cytoplasm after 48 hours of incubation. Shells in buffers 2A, 2B, 2C and 3C still contained some material after 24 hours incubation but were clear by 48 hours. Shells in buffer 2D took 24 hours to be clear of cytoplasm and those in buffer 3D were empty after only 16 hours. No damage to the shells was caused by buffers 1A, 1B, 1C, 2A, 3A and 3B. Minor damage was caused to shells in buffers 1D, 2B, 2C, and 3C after 48 hours and to shells in buffer 2D after 24 hours. For shells in buffer 3D, minor damage was seen after 16 and 24 hours, with major damage seen after 48 hours incubation. An incubation length of 24 hours was chosen for subsequent tests, with the aim of reaching a compromise between maximum removal of cellular material and minimal damage to the shell.


Buffer
INCUBATION TIME (hours)
Before
2
8
16
24
48
1A
1B
1C
1D


Buffer
INCUBATION TIME (hours)
Before
2
8
16
24
48
2A
2B
2C
2D

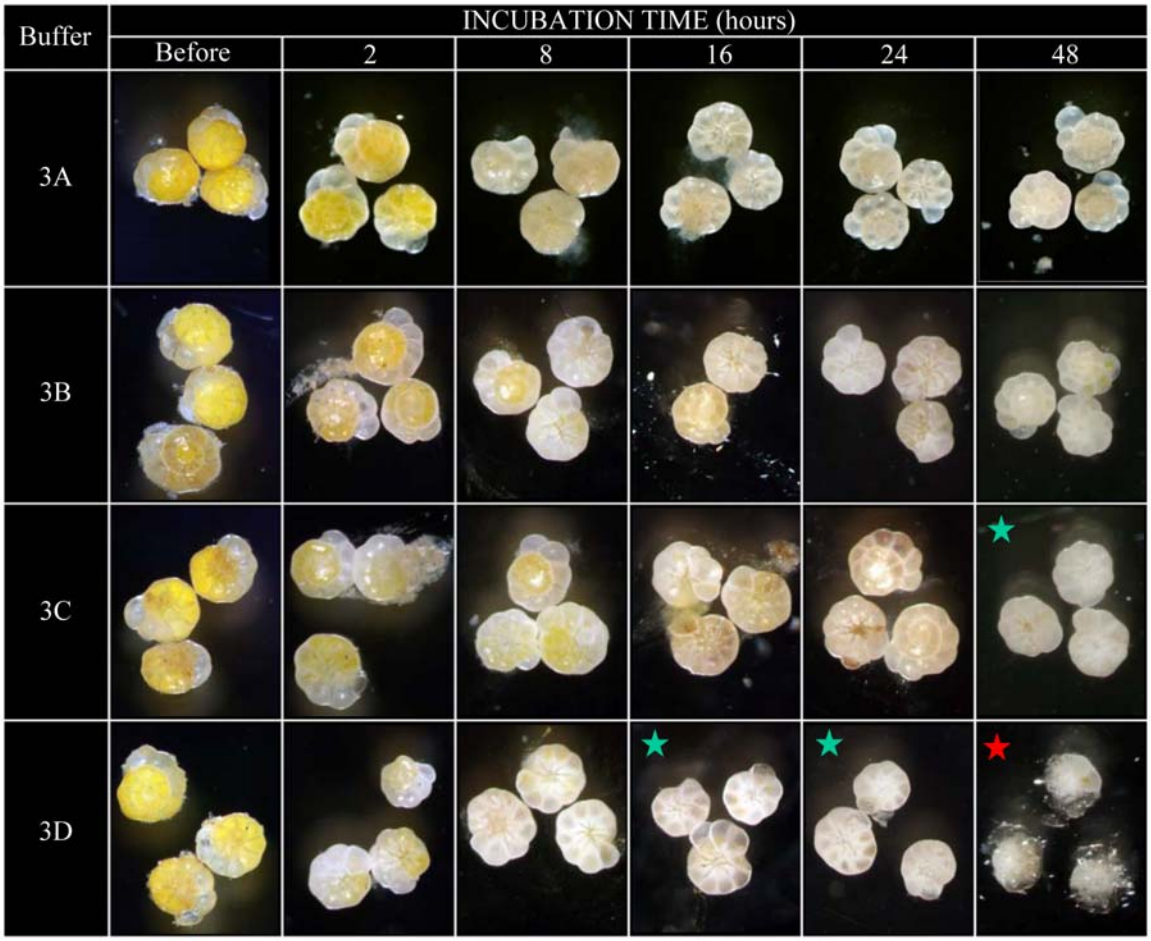


Figure 7.2. Photographs of foraminifera taken after incubation in the 12 trial lysis buffers (1A, 1B, 1C, 1D, 2A, 2B, 2C, 2D, 3A, 3B, 3C, 3D) at 75 °C for varying lengths of time. Removal of cellular material from the shell is indicated by a loss of the orange colouration. Damage to the shells is shown by a star, the colour indicating the severity of damage (green = minor, orange = moderate, red = severe).

7.4.2 Effectiveness of the 12 trial buffers under optimal incubation conditions

Having assessed the effectiveness of the new buffers at removing cellular material from within the foraminiferal shell, by observing the loss of their orange colouration, it was now important to assess their effectiveness in terms of the yield of crude DNA gained and success in PCR. For the next tests individual foraminifera were incubated in each of the 12 trial buffers (10 replicates each) under the optimal conditions of 75 °C for 24 hours. For half of the samples, the buffer solution was transferred to a fresh 1.5 ml tube following incubation, and a post cell lysis chloroform extraction and ethanol precipitation stage performed immediately. The empty shells were left to dry at room temperature (21 °C) in the original tubes. For the other half of the samples, the buffer solution was only removed after 6 weeks, to simulate the period of storage that could be necessary in the field or on board a ship. A chloroform extraction and ethanol precipitation was performed at this point and the shells left to dry as above.

7.4.2.1 Crude DNA yield

Mean yields of crude DNA extracted (ng/µl) are presented in fig. 7.3. A one-way between groups analysis of variance (ANOVA) (Harris, 1994) showed a statistically significant difference in crude DNA yield between the different buffers at the $p<0.05$ level (appendix 9.8.2). The effect size, calculated using eta squared was 0.6 (appendix 9.8.3). Post-hoc comparisons using Tukey's HSD test (Hsu, 1996) indicate which individual buffers differed significantly in mean crude DNA yield (significant at the $p = 0.05$ level) (appendix 9.8.4).

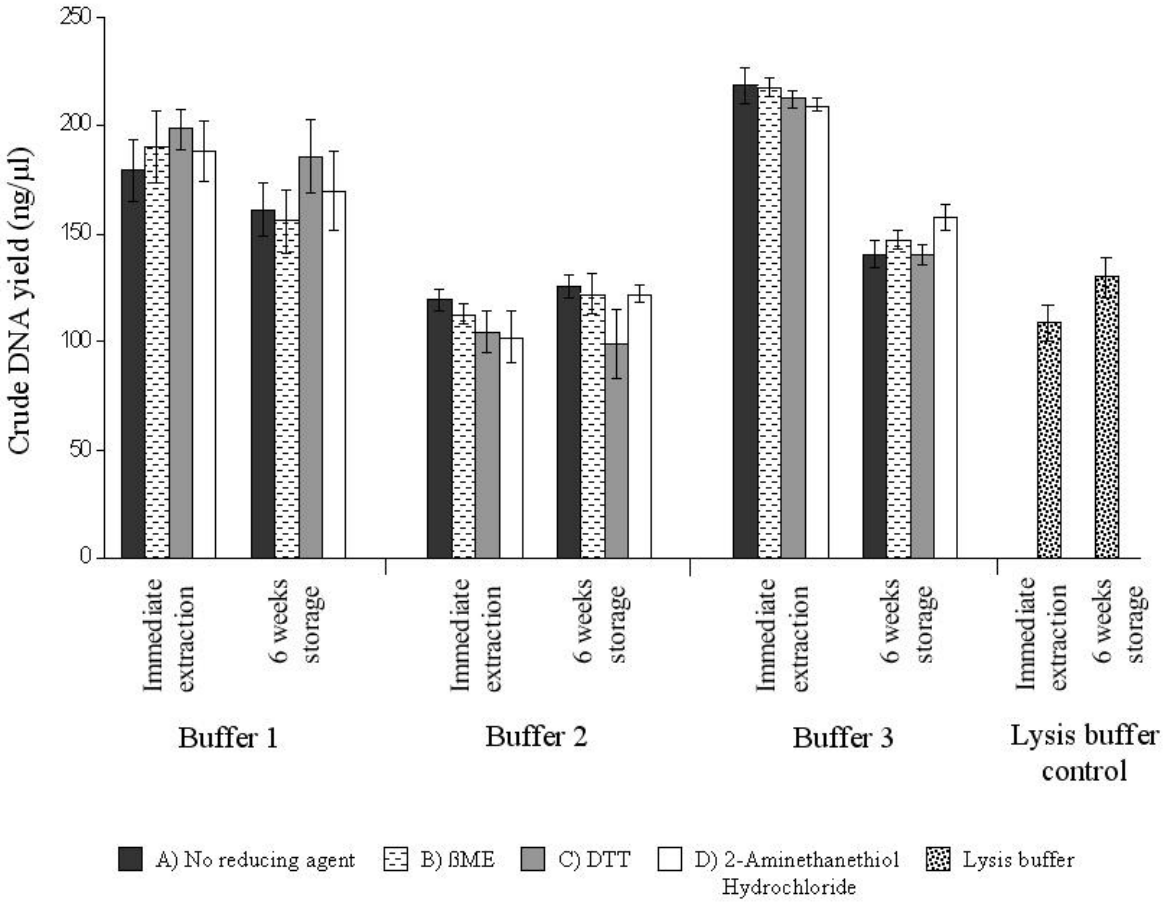


Figure 7.3. Graph showing the mean yield of crude DNA obtained following incubation in the 12 lysis buffers and subsequent chloroform extraction/ ethanol precipitation. Values are shown for chloroform extraction/ ethanol precipitation carried out immediately after incubation and following 6 weeks storage in the buffers. Crude DNA yield obtained using the control buffer of Holzmann *et al.* (1996) is also shown

Comparing the basic buffer solutions, the group 3 buffers produced the highest crude DNA concentrations on immediate chloroform extraction/ ethanol precipitation after incubation (significantly higher than the DOC lysis buffer of Holzmann *et al*. (1996), used as standard in genetic studies of the foraminifera and here as a control), however a significant loss of yield took place after storage at room temperature for 6 weeks (buffer 3 versus lysis buffer control: fig 7.3, Tukey HSD, appendix 9.8.4). The group 1 buffers produced the next highest yields on immediate chloroform extraction/

ethanol precipitation, again significantly higher than the DOC lysis buffer of Holzmann *et al*. (1996) (buffer 1 versus lysis buffer control: fig. 7.3, Tukey HSD, appendix 9.8.4). Following 6 weeks storage, these yields had only dropped by an insignificant amount (fig. 7.3, Tukey HSD, appendix 9.8.4), remaining higher than for the control buffer (though not all significantly so; Tukey HSD, appendix 9.8.4). Buffer 2 produced the lowest mean yield of crude DNA on immediate chloroform extraction/ ethanol precipitation but showed little change after 6 weeks storage. Group 2 buffer DNA yields were comparable to those gained using the Holzmann *et al*., 1996 buffer (buffer 2 versus lysis buffer control: fig. 7.3, Tukey HSD, appendix 9.8.4). The type of reducing agent included in the buffer (A, B, C, D) had no significant effect on mean yield of crude DNA (fig. 7.3, Tukey HSD, appendix 9.8.4). The highest overall mean crude DNA yield was produced by incubation in buffer 3A followed by immediate chloroform extraction/ ethanol precipitation (218 ng/µl), whilst the lowest was from buffer 2C after 6 weeks storage (99 ng/µl).

7.4.2.2 Success in PCR

There was a great deal of variation in the level of success of PCR after DNA extraction from the buffers (fig. 7.4). All group 1 buffers performed extremely well in PCR, both after immediate chloroform extraction/ ethanol precipitation and for most after 6 weeks storage, with every sample (3 replicates for each buffer) producing strong bands (except buffer 1D after 6 weeks storage, which had 1 strong band, 1 weak band and one failure).

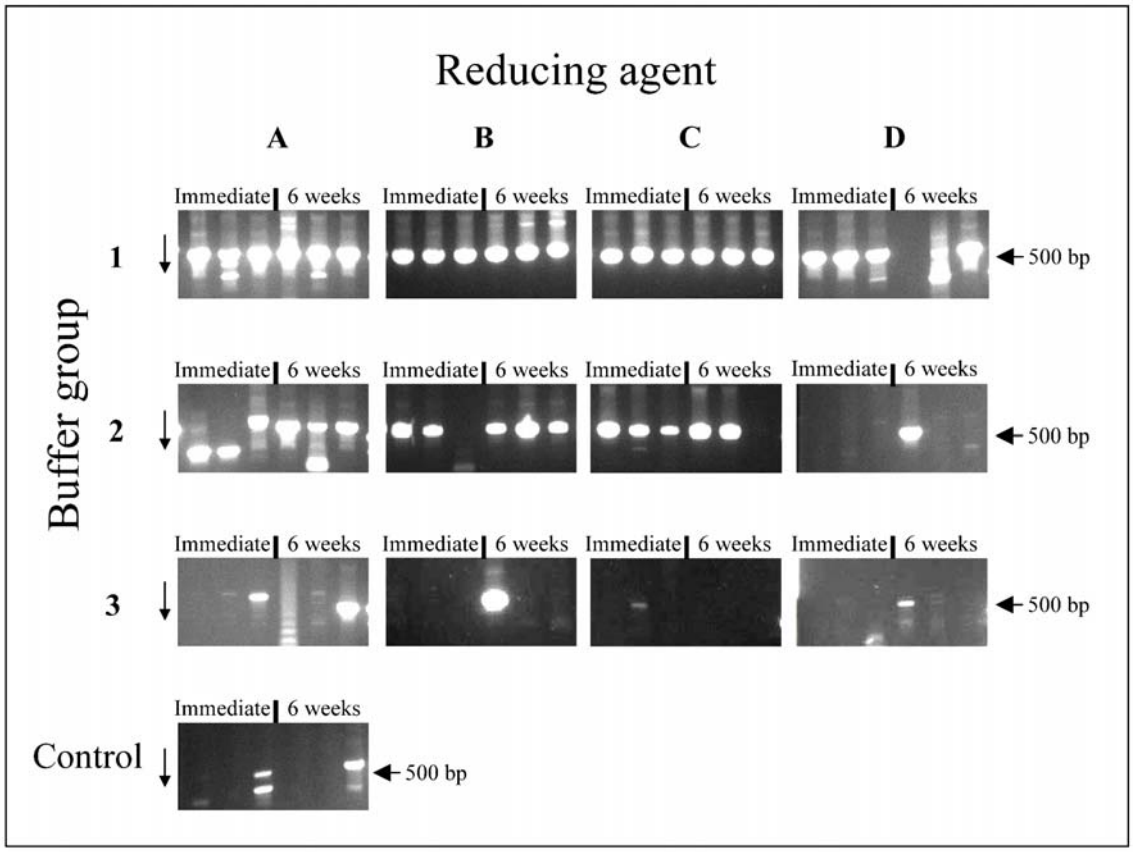


Figure 7.4. Agarose gels showing the intensity of bands produced from the PCR amplification of an ~ 500 bp fragment of the SSU rRNA gene using DNA extracted with each of the 12 buffers (1A, 1B, 1C, 1D, 2A, 2B, 2C, 2D, 3A, 3B, 3C, 3D). Transfer to the chloroform extraction/ ethanol precipitation stage was carried out either immediately after cell lysis or following 6 weeks storage of samples in the lysis buffers, as indicated. All bands above the 500 bp marker (determined using a 100 bp DNA ladder: New England Biolabs) are foraminiferal in origin, whilst those below are likely to be from the amplification of algal symbionts. For the control, samples were crushed in 50 µl of the Holzmann *et al*. (1996) lysis buffer and incubated at 60 °C for 1 hour. Material was then taken directly from the buffer for use in PCR both immediately and after 6 weeks storage in the solution. Negative controls were included for each round of PCR, and found to be blank when run on the agarose gel.

The group 2 buffers did not perform quite as well in PCR as the group 1 buffers. On immediate chloroform extraction/ ethanol precipitation after incubation, successful foraminiferal bands were produced in only 1 & 2 replicates out of 3 for buffers 2A and 2B respectively, although for samples stored for 6 weeks following incubation and prior to chloroform extraction/ ethanol precipitation, both buffers produced 3 strong bands. Buffer 2C produced 3 reasonable bands on immediate chloroform extraction/ ethanol precipitation after incubation but only 2 bands after 6 weeks storage. None of

the samples incubated in buffer 2D worked in PCR on immediate chloroform extraction/ ethanol precipitation, though 1 sample worked for 2D after 6 weeks storage, producing a strong band.

The group 3 buffers performed very poorly in PCR. After immediate chloroform extraction/ ethanol precipitation nearly all samples failed in PCR. Buffer 3A produced 1 reasonable band, 3C produced 1 very weak band, and buffers 3B and 3D failed to amplify. After 6 weeks storage, buffers 3A and 3B produced 1 band each, 3D produced 1 weak band, and buffer 3C failed completely.

Success of the PCR amplification was generally correlated with the buffer group (1, 2, or 3) used. With regards to the reducing agent included, no reducing (A), β-ME (B), or DTT (C) produced better results than the reducing agent 2-Aminethanethiol Hydrochloride within the successful buffer solutions (1 & 2).

In comparison to the control samples, prepared using the lysis buffer of Holzmann *et al*. (1996), the performance in PCR of the group 1 and 2 buffers was far superior. Only 2 of the control samples were successful in PCR, one used immediately after incubation, and the other after 6 weeks storage.

7.4.2.3 Damage to shells

Following cell lysis, the buffer solution was removed from the tubes (either immediately or following 6 weeks storage in the buffer) to be taken on to the DNA purification stage by chloroform extraction and ethanol precipitation. The empty shells were left in the tubes to dry at room temperature (21 °C), and then stored dry for 6 months. After this period they were examined for damage (fig. 7.5).

All of the buffer 1 solutions performed very well (fig. 7.5). Buffer 1A (containing no reducing agent) caused no damage to the shells and buffers 1B, 1C, and 1D (containing the reducing agents β-ME, DTT & 2-Aminethanethiol Hydrochloride respectively) caused only minor damage to 1 or 2 shells. Storage for 6 weeks in these buffers did not cause the level of damage to increase.

The buffer 2 solutions showed some variation in the amount of shell damage they caused (fig. 7.5). Buffer 2A (no reducing agent) caused no damage at all, even after 6 weeks storage. Buffer 2B (β-ME) caused no damage to shells that were removed immediately after incubation, and only minor damage to 1 shell after 6 weeks storage. Buffer 2C (DTT) caused some minor damage to 1 shell both on immediate removal after incubation and after storage in the buffer for 6 weeks. Buffer 2D (2-Aminethanethiol Hydrochloride) did not perform well. Though it caused only minor to moderate damage to shells that were removed immediately after incubation, those shells stored in the buffer for 6 weeks were completely dissolved.

Figure 7.5. Photographs of empty foraminiferal shells, stored dry for 6 months following treatment with the 12 new lysis buffers. The two columns represent specimens that were removed from the buffers immediately after incubation and those that were stored in the buffers for 6 weeks before being removed. Damage to the shells is highlighted by a star, with the colour indicating the level of damage (green = minor, orange = moderate, red = severe)

All of the buffer 3 solutions performed very poorly with regards to shell damage. All four (3A, 3B, 3C, 3D, containing no reducing agent, β-ME, DTT & 2-Aminethanethiol Hydrochloride respectively) caused moderate to severe damage to the shells when removed immediately from the buffers after incubation. 6 weeks storage in buffers 3A, 3B, and 3C did not cause the severity of the damage to increase, however, shells stored in buffer 3D for 6 weeks were dissolved completely.

Within each buffer group (1, 2 and 3) buffers containing no reducing agent caused the least damage to the shells (1A, 2A, 3A). Buffers, 1B and 2B, containing βME, caused only minor damage to some of the shells, though buffer 3B, which also contained βME caused moderate to severe damage to some shells. Again, buffers 1C and 2C, which contained DTT, caused only very minor damage, whereas buffer 3C, which also contained DTT caused moderate to severe damage. Buffers 2D, and 3D, which contained the reducing agent 2-Aminethanethiol Hydrochloride caused the most shell damage, though buffer 1D, which also contained 2-Aminethanethiol Hydrochloride caused little damage.

7.5 Discussion

7.5.1 Determining the optimal incubation conditions for cell lysis

Having designed the new buffers, it was important to determine the optimal conditions for their use. Testing the buffers at a range of temperatures, from room temp to 100 °C for 1 hour, indicated that higher temperatures increased the effectiveness of the buffers, promoting cell lysis and removal of cellular material from the shell (fig. 7.1). However, at temperatures above 95 °C there was a significant amount of evaporation from the solutions leading to precipitation and crystallisation. Such an effect is undesirable making it both difficult to remove the liquid containing the DNA and causing possible damage to the shell. Damage was evident in shells incubated in buffer 3B at 100 °C. A temperature of 75 °C was chosen for subsequent incubations, with the aim of keeping the temperature as high as possible, whilst avoiding precipitation.

During the temperature test, an incubation of 1 hour had been insufficient to cause complete lysis of the cell and dispersal of the cytoplasm into solution (fig. 7.1). Incubation of forams in the 12 trial buffers for a range of times at 75 °C (fig. 7.2) showed that increasing the incubation time led to more effective removal of cellular material from the shell. Performance differed between buffers, the best causing complete evacuation of the shell by 16 hours (buffer 3D), and the worst still containing material at 48 hours (buffers 1A, 1B, 1C, 1D, 3A and 3B). For some buffers, shell damage was caused at longer incubation times. Buffers 1A, 1B, 1C, 2A, 3A, and 3B caused no shell damage at all, buffers 1D, 2B, 2C, and 3C caused minor damage after 48 hours. Buffer 2D caused minor damage after only 16 hours, and major damage after 48 hrs.

Taking into account the overall effectiveness of the buffers after different incubation periods as well as any damage caused to the shells, 24 hours at 75 °C was selected for use in subsequent incubations. This incubation temperature is slightly higher than the 60 °C at which crushed foraminifera are traditionally incubated in lysis buffer (Holzmann *et al*., 1996), and the length of incubation is considerably longer at 24 hours compared to 1 hour (Holzmann *et al*., 1996).

The temperature and length of incubation used in DNA extraction procedures can vary a great deal. In most bacterial studies a lower temperature of around 37 °C is commonly used (Tsai & Olson, 1991; Smith & Tiedje, 1992), and although temperatures as high as 70 °C can be used, the length of incubation is only usually between 30 minutes to 1 hour (Bruce *et al*., 1992; Kuske *et al*., 1998). A similarly low temperature has been used to extract DNA from lymphocytes (37 °C), but with an overnight incubation (Godschalk *et al*., 1998). DNA extractions from blood cells or dried blood samples vary from 10 to 90 minutes at 56 °C (Persat *et al*., 2009; de Vange Panteleeff *et al*., 1999), or may be carried out overnight at 60 °C, (Tani *et al*., 2008). DNA extractions from tissues use temperatures of around 50-55 °C, with incubations from a few hours to overnight (Sambrook *et al*., 1989; Stoffberg *et al*., 2010). Higher temperatures can be used, for example, when extracting DNA from difficult substrates such as feathers. Here incubation temperatures of 70-75 °C are standard, but samples are usually incubated for less than an hour (Gebhardt *et al*., 2009; Malagó *et al*, 2002).

For this study, the fact that the cellular material had to be extracted from within an intact foraminiferal shell necessitated the use of a high temperature and long incubation time.

7.5.2 Performance of the new lysis buffers

When assessing the success of each buffer it is important to consider a range of factors, such as crude DNA yield after DNA extraction, performance in PCR, and damage caused to the $CaCO_3$ shells. It is also useful to observe how these factors change if foraminifera are stored in the buffers for a long period of time (e.g. 6 weeks) as they might be after a collection trip.

There was a considerable amount of variation in overall performance between the different buffers, which will be discussed firstly in terms of the main buffer groups 1, 2, and 3, and secondly according to the individual reducing agents added (A, B, C, D).

7.5.2.1 Group 1 buffers

All of the group 1 buffers (1A, 1B, 1C, 1D) performed well overall, despite early indications that they were not effective enough. During tests to determine the optimal length of incubation (fig 7.2), some residual cellular material had been evident in the shells after the longest incubation time tested (48 hours at 75 °C), though as a positive they also caused little damage to the shells. When incubated under the selected conditions of 75 °C for 24 hours, however, the effectiveness of the group 1 buffers, as assessed by crude DNA yield and success in PCR amplification, was extremely good, possibly indicating that total removal of cytoplasm from the shell is not essential. The amount of crude DNA produced on immediate chloroform extraction/ ethanol

precipitation after incubation was high for all of the group 1 buffers and remained high for those samples stored in the buffers for 6 weeks (fig. 7.3). In PCR, all of the group 1 buffers were highly successful; the samples chloroform extracted/ ethanol precipitated immediately after incubation all producing strong bands (x3 replicates for each buffer) (fig. 7.4), and nearly all producing strong bands after 6 weeks storage (with the exception of buffer 1D, containing the reducing agent 2-Aminethanethiol hydrochloride). In comparison to the traditional foraminiferal DNA extraction method (Holzmann *et al*., 1996) (used here as a control), the group 1 buffers produced higher yields of crude DNA and were far superior in terms of PCR success (most of the control samples failed in PCR).

No precipitation was noted in any of the group 1 buffers. Out of all of the groups of buffers, those based on buffer 1 caused the least overall damage to the foraminiferal shells, both in those that were removed from the buffers immediately after incubation and in those that were stored in the buffers for six weeks before being dried out (fig. 7.5). Buffers 1A and 2A caused no shell damage at all.

It is clear that these group 1 buffers are highly suitable for extracting DNA from foraminifera for use in PCR, whilst keeping the shell intact, and that their performance may be unhindered when storing samples in them for longer periods (6 weeks at least), making them an ideal choice for use in the field or on a ship.

7.5.2.2 Group 2 buffers

The group 2 buffers (2A, 2B, 2C, 2D) varied in their performance. During the test for optimising incubation length (fig. 7.2), shells in buffers 2B and 2C were empty of cytoplasm within 48 hours, although minor shell damage was evident, and those in buffer 2D were empty in only 24 hours, though again with minor damage to the shells. Precipitation occurred in buffer 2B from 8 hours onwards and in buffer 2C after 24 hours. Precipitation is undesirable, making it difficult to separate the empty shell from the buffer at the end of the incubation, and possibly leading to damage of the shell. In addition, the buffer becomes thickened and difficult to pipette.

The group 2 buffers produced the lowest yields of crude DNA overall, though the yield was not significantly diminished when samples were stored in the buffers for six weeks (fig. 7.3, Tukey HSD, appendix 9.8.4). The performance in PCR was varied. On immediate chloroform extraction/ ethanol precipitation after cell lysis, buffers 2A, 2B, 2C and 2D, produced 1, 2, 3, and 0 bands respectively (fig. 7.4). Strangely, for buffers 2A, 2B, and 2D the PCRs were slightly more successful for samples that had been stored in the buffers for 6 weeks after incubation, with, 3, 3, and 1 bands being produced respectively (fig. 7.4). This perhaps suggests that the 24 hr incubation time used is insufficient for these buffers. DNA yields were comparable to those obtained using the control buffer (Holzmann *et al*., 1996), though PCR success was better (with the exception of buffer 2D).

Buffer 2A (containing no reducing agent) caused no damage to the foram shells, even after 6 weeks storage (fig. 7.5). Buffers 2B and 2C caused only minor shell damage, however, buffer 2D, which also had the poorest PCR result, caused severe damage to

the shells, completely dissolving them after they were stored in the buffer for six weeks. Buffer 2D is clearly not suitable for future use and for the remaining group 2 buffers performance was relatively poor, though there was little apparent deterioration following long-term storage.

7.5.2.3 Group 3 buffers

The performance of the group 3 buffers varied somewhat, but was probably least favourable overall. During tests to find the optimal length of incubation (fig. 7.2), buffer 3B failed to cause the complete evacuation of cytoplasm from the foraminiferal shells, however, caused no shell damage. Shells in buffer 3C were emptied of cytoplasm after 48 hours but also sustained minor damage. Buffer 3D was the fastest acting of all the 12 buffers, the shells being empty after only 16 hours, however, minor shell damage was sustained in only 16 hours, and severe shell damage was evident after 48 hours in the buffer. Some precipitation was noted in buffer 3D at 16, 24, and 48 hours

The group 3 buffers produced the highest mean yields of crude DNA of all the buffers, on immediate chloroform extraction/ ethanol precipitation after incubation. However, there was a significant loss of yield when shells were stored in the buffers for 6 weeks before chloroform extraction/ ethanol precipitation (fig. 7.3, Tukey HSD, appendix 9.8.4). It should be noted though that despite this loss, the mean yields were still higher than those of the group 2 buffers, even when these were extracted immediately. The group 3 buffers produced significantly higher yields of crude DNA than the control buffer (Holzmann *et al*., 1996), on immediate chloroform extraction/ ethanol

precipitation, but only equivalent yields to the control after 6 weeks storage (fig. 7.3, Tukey HSD, appendix 9.8.4).

In terms of PCR success the group 3 buffers performed extremely poorly, as did the control buffer (fig. 7.4). On immediate chloroform extraction/ ethanol precipitation, buffers 3A and 3C produced 1 weak band each, while 3B and 3D failed completely. As with the group 2 buffers, PCR seemed slightly more successful for samples stored for 6 weeks in the buffers before being chloroform extracted/ ethanol precipitated, again suggesting that an incubation time longer than the 24 hrs used may be beneficial when using these particular buffers. Buffers 3A and 3B then produced strong bands for 1 in 3 samples each, buffer 3D produced a weak band for 1 in 3 samples, and samples in buffer 3C failed completely.

The group 3 buffers clearly produced the worst results in terms of damage to the $CaCO_3$ shells (fig. 7.5), all causing moderate to severe damage both on immediate removal from the buffers after incubation and after storage for 6 weeks. The worst of all 12 buffers for shell damage was buffer 3D, which caused severe damage when shells were removed immediately, and completely dissolved the shells stored in it for 6 weeks.

Based upon the testing undertaken at the present time, the group 3 buffers performed the worst for both PCR and shell damage, indicating that they should not be considered for further use.

7.5.2.4 The DOC lysis buffer control

Using the traditional method of foraminiferal DNA extraction (Holzmann *et al*. 1996), the yield of crude DNA produce was respectable, however, almost all of the PCR reactions failed, indicating that PCR inhibition had taken place. This is a problem that has surfaced regularly during lab work for the biogeographical and phylogenetical studies of this thesis (chapters 3, 4, & 5). It is possible, however, that such effects could be avoided with the addition of a post cell lysis chloroform extraction and alcohol precipitation stage, though the method does not afford the advantage of preserving the foraminiferal shell.

Overall, the results indicate that the success of DNA extraction and shell preservation is strongly influenced by the buffer group used (1, 2, & 3), and therefore the component reagents used to create them.

7.5.2.5 What makes certain buffers more effective than others?

A marked difference in PCR amplification success could be seen between samples incubated in the different lysis buffers groups. Samples incubated in all buffer 2 and 3 solutions produced poorer PCR results than those incubated in the buffer 1 solutions. Inhibition by the detergent Sarkosyl can be ruled out as a cause of poor PCR performance, as it is present in both the successful group 1 buffers and the poor group 2 buffers. The buffer 2 solutions could simply be inadequate at extracting the DNA from the foraminifera, leading to low yields of crude DNA (fig. 7.3), and therefore poor PCR results (fig. 7.4). However, the group 3 buffers produced high yields of crude DNA, yet produced the poorest results in PCR of all. Urea (present in buffer groups 2 & 3) is likely to have caused some inhibition to PCR (Gelfland *et al*., 1990),

and in addition, does not mix well in solution, explaining why these buffers sometimes precipitated when those from group 1 did not. The detergent SDS was present in the poorly performing group 3 buffers alone, and may have caused the most severe PCR inhibition. Inhibition was evident despite the use of the chloroform/ ethanol precipitation stage, which should have brought the SDS concentration in the final DNA product below its reported inhibition concentration of 0.01 % (Rossen *et al*., 1992, also section 7.1.4: table 7.1). Though the alternative detergent, Sarkosyl (used in buffers 1 & 2) also reputedly has some inhibitory effect on PCR, it may be used at higher concentrations before these become apparent (Rossen *et al*., 1992, section 7.1.4: table 7.1). In addition to their inhibitory effect on PCR, SDS and Urea in combination (in buffer group 3), caused high levels of damage to the foraminiferal shells.

Of course it may simply be that the reagents unique to the group 1 buffers, Guanidinium Isothiocyanate (GITC) and Isopropanol, greatly enhance their performance. Interestingly, Guanidinium Isothiocyanate is a known PCR inhibitor (table 7.1, Rossen *et al*., 1992), but seems to have had no effect here. It is likely that the process of chloroform extraction/ethanol precipitation and subsequent dilution in the PCR mix was sufficient to bring the concentration down below its 100 mM inhibition threshold. GITC DNA extraction has recently been used successfully on specimens of the planktonic foraminifera *Orbulina universa*, leaving the shell intact (Morard *et al*., 2009).

The varying amount of damage caused to the shells of the foraminifera by the different buffers may also be traced to particular reagents. The fact that the group 3

buffers cause significantly greater levels of damage is likely to be due to the presence of the detergent SDS. This is the only chemical unique to this buffer group. Sarkosyl, the detergent present in buffer groups 1 and 2 seems to be a better alternative.

7.5.3 The effects of including a reducing agent

Reducing agents may be added to lysis buffers (Bienvenue et el., 2006; Chakravorty & Tyagi., 2001) to promote cell lysis, by reducing disulphide bonds and thus denaturing the proteins of the cell membrane. As mechanical lysis of the foraminiferal cell was impossible due to the need for shell preservation, reducing agents were added to some of the new buffers with the aim of increasing their effectiveness (buffers 1B, 2B, 3B (β-mercaptoethanol); buffers 1C, 2C, 3C (DTT); buffers 1D, 2D, 3D (2-Aminethanethiol hydrochloride)). Some of the trial buffers were left with no reducing agent (buffers 1A, 2A, 3A) to allow for comparison. In addition to the influence of the overall buffer choice (1, 2, or 3), the reducing agent added to each of these was also found to affect performance in terms of the removal of cellular material from the foraminiferal shells, PCR success, and damage to the shells.

7.5.3.1 Removal of cellular material from the foraminiferal shell

When manipulating the duration of the incubation at 75 °C (fig. 7.2), specimens incubated in buffers containing a reducing agent (buffers 1B, 2B, 3B, 1C, 2C, 3C, 1D, 2D, 3D) showed signs of cytoplasm evacuation from their shells far earlier than specimens incubated in buffers lacking a reducing agent (buffers 1A, 2A, 3A) (2 hours compared to 8 hours). This perhaps indicates a potential a role of reducing agents in minimising the length of incubation needed. In terms of the time taken for the complete removal of cellular material from the foraminiferal shells (fig. 7.2), there

was little difference in performance within the group 1 buffers, regardless of the reducing agent used. Of the group 2 & 3 buffers, those containing β-ME (2B & 3B), worked only as well as the buffers lacking a reducing agent (buffers 2A & 3A). DTT appears to have enhanced the action of the group 3 buffer (3C) slightly, with all cellular material being evacuated in 48 hours, compared to buffer 3A, containing no reducing agent, or buffer 3B, containing β-ME, which left some residual material after this time. The addition of the reducing agent 2-Aminethanethiol hydrochloride to the group 2 and 3 buffers (2D, 3D), accelerated the complete removal of material from the shells particularly well, with total evacuation occurring in only 24 (buffer 2D) and 16 hours (buffer 3D). However, the inclusion of this particular reducing agent was detrimental in other respects, as is discussed below.

In addition, it should be noted that the complete removal of cellular material from the shell might not be necessary to obtain high yields of good quality DNA for PCR. The group 1 buffers, for example, left some residual material after 48 hours at 75 °C, but performed extremely well in terms of DNA yield and PCR success.

The inclusion of a reducing agent could be used to lower the required incubation time for cell lysis, an advantage when processing large numbers of samples. However, other aspects of performance must also be considered.

7.5.3.2 DNA yield and success in PCR

Interestingly, there was found to be no apparent correlation between the reducing agent used and the yield of crude DNA produced after DNA extraction (fig. 7.3, Tukey HSD, appendix 9.8.4), seeming instead to be more dependent on the buffer

group used (1, 2, or 3). Though it was assumed that a reducing agent would be needed to promote cell lysis, leading to high yields of DNA, the lysis buffers lacking a reducing agent (1A, 2A, 3A) actually produced DNA yields equal to the other buffers (fig. 7.3; incubation at 75 ^{o}C for 24 hours), perhaps indicating that for a 24 hour incubation at 75 °C, a reducing agent may be unnecessary.

Success in PCR was predominately dependent on the buffer group used (1, 2, or 3) (fig. 7.5), however, particularly poor results were gained from buffers containing the reducing agent 2-Aminethanethiol hydrochloride. When combined with buffer 1, these detrimental effects were only evident after long-term storage of samples in the buffer (fig. 7.5: 1D, 6 weeks: only 1 strong band), however, when combined with buffer 2 (buffer 2D), PCR failure was high, even on immediate chloroform extraction/ ethanol precipitation (fig. 7.5: 2D, Immediate: no bands, 6 weeks: only 1 strong band). The success of buffers 1B and 2C indicate that the reducing agents β-ME and DTT have no detrimental effect on the PCR.

7.5.3.3 Shell damage

The point at which performance of the different reducing agents begins to differ significantly is when damage to the $CaCO_3$ shell of the foraminiferan is concerned. Again, it is clear that the level of shell damage is also dependent on buffer group (1, 2, & 3) to a certain extent, with the greatest amount of damage evident in samples incubated in the group 3 buffers (fig. 7.5), however, within each buffer group the least damage was caused by buffers containing no reducing agent (fig. 7.5; buffers 1A, 2A, 3A), with buffers 1A and 2A causing no damage at all, even after specimens had been stored in them for 6 weeks. Overall, the level of damage caused by the buffers

containing the reducing agents β-ME and DTT was similar, being only minor when they were included in the groups 1 (fig. 7.5; buffers 1B, 1C) & 2 (fig. 7.5; buffers 2B, 2C) buffers, but moderate within buffer group 3 (fig. 7.5; buffers 3B, 3C). By far the most severe damage was caused by the reducing agent 2-Aminethanethiol hydrochloride, when included in the group 2 & 3 buffers (fig. 7.5; buffers 2D, 3D). Damage was particularly severe when specimens were stored in these buffers for 6 weeks, which resulted in the shells being entirely dissolved. Despite the more rapid action of the reducing agent-containing buffers in evacuating cellular material from the shells, the results here perhaps indicate some detrimental effects of their inclusion, particularly in the case of 2-Aminethanethiol Hydrochloride.

7.5.4 Selecting the best lysis buffer

Of the three buffer groups tested (1, 2, & 3), group 1 produced the most favourable results overall. Though groups 2 & 3 were possibly more efficient at removing cellular material from the foraminiferal shells during incubation (fig. 7.2), they did not perform as well in other respects (buffer 2: poorer DNA yield: fig. 7.3, slightly poorer PCR performance: fig. 7.4, greater shell damage: fig. 7.5, Buffer 3: poorer PCR performance: fig. 7.3, greater shell damage: fig. 7.5). The group 1 buffers (containing GITC and Isopropanol) produced high yields of crude DNA, and performed the best in the PCR, outperforming the other new buffers (2 & 3) and the control buffer of Holzmann *et al*. (1996). They also caused little damage to the delicate $CaCO_3$ shells of the foraminifera, leaving them intact for future use. Most importantly, the performance of the group 1 buffers was unhindered when samples were stored in them for an extended period of time (6 weeks), as could be required in the field or onboard a ship.

The inclusion of a reducing agent in the lysis buffer may accelerate the removal of cellular material from the shells (fig. 7.2), however, in all other respects it may not be necessary (figs 7.3 & 7.4: DNA yield & PCR success), and might even be detrimental (fig. 7.5; shell damage).

Buffer 1A, containing no reducing agent, was found to be the best candidate for further use, producing a good yield of crude DNA, excellent results in PCR and causing no damage to the shells. Buffers 1B, and 1C (containing the reducing agents βME and DTT respectively), could also be good candidates, as these started to remove cytoplasm from the shells faster than buffer 1A, produced good yields of crude DNA and were extremely successful in PCR. They did, however, cause minor damage to some shells, probably due to their component reducing agents (fig. 7.5). None of the buffers containing 2-Aminethanethiol hydrochloride are likely to be suitable for future use. Though the inclusion of 2-Aminethanethiol hydrochloride appears to speed up the evacuation of cytoplasm from the shell during incubation, the detrimental effects such as poor PCR success and severe damage to the foraminiferal shells heavily outweigh this benefit.

7.6 Conclusions

For the purpose of extracting DNA from foraminifera, whilst preserving the $CaCO_3$ shell for morphological study, the most successful new lysis buffer was buffer 1A (incubated for 24 hours at 75 °C), made from 100mM TRIS, 10mM NaCl, 4M Guanidinium Isothiocyanate, 17 % Isopropanol and 1 % Sarkosyl, and containing no reducing agent. Buffer 1A yielded high levels of crude DNA, and produced extremely strong results in PCR, both on immediate chloroform extraction/ ethanol precipitation after incubation, and after 6 weeks storage. The yield of DNA obtained was higher than that from the traditional foraminiferal DNA extraction method (Holzmann *et al*., 1996), and in terms of PCR success was far superior. The foraminiferal shells remained intact after incubation in buffer 1A, even when stored in the buffer for 6 weeks, and were in good condition following dry storage for 6 months. The excellent results gained using this buffer, even when samples were stored in it for a long period before removal of the shell and the final chloroform extraction and alcohol precipitation stage, gives it great utility in the field, where extended storage could be necessary.

Buffers 1B and 1C, also outperformed the traditional method of foraminiferal DNA extraction (Holzmann *et al*. 1996), in terms of DNA yield and PCR success, and could be suitable for future use. These have the same basic composition as buffer 1A, but with the addition of the reducing agents β-ME and DTT (50 mM) respectively. Results suggest that the addition of a reducing agent accelerates the rate at which cellular material is removed from the foraminiferal shell during incubation (first signs seen in 2 hours compared to 8 hours), suggesting a possible mechanism by which the incubation time could be decreased, however minor shell damage was also evident

with these buffers. Furthermore, reducing agents must be added fresh to the buffer before use, and have to be handled in a fume cupboard. As the addition of a reducing agent offers no overall improvement in performance it is therefore preferable to exclude them from the buffer.

Certain reagents were found to be detrimental to PCR success (Urea & SDS), and to foraminiferal shell integrity (Urea, SDS, & 2-Aminethanethiol hydrochloride), and are therefore unsuitable for use in the extraction of DNA from intact foraminiferal shells.

For future work, it may be beneficial to test some of the trial buffers again, using lower temperatures and shorter incubation times, to see if some of their detrimental affects could be avoided. A faster acting lysis buffer could certainly be of benefit when processing large numbers of samples. There are also many other combinations of reagents in use for lysis buffers that could be tested. Finally, it will be essential to apply the new DNA extraction method to specimens of planktonic foraminifera, which are harder to collect and maintain in the lab.

7.7 References

Al-Soud, W. A., & Rådström, P. 2001. Purification and characterization of PCR-inhibitory components in blood cells. *Journal of Clinical Microbiology,* **39** (2), 485 – 493

Bienvenue, J. M., Duncalf, N., Marchiarullo, D., Ferrance, J. P., & Landers, J. P. 2006. *Journal of Forensic Sciences* **51** (2) 266 – 273

Bijma, J, & Hemleben, C. 1994. Population dynamics of the planktic foraminifer *Globigerinoides sacculifer* (Brady) from the central Red Sea. *Deep-Sea Research*, **41** (3) 485 – 510

Bruce, K. D., Hiorns, W. D., Hobman, J. L., Osborn, A. M., Strike, P., & Ritchie, D. A. 1992. Amplification of DNA from native populations of soil bacteria by using the Polymerase Chain Reaction. *Applied and Environmental Microbiology* **58** (10) 3413 - 3416

Chakravorty, S., & Tyagi, J. S. 2001. Novel use of guanidinium isothiocyanate in the isolation of *Mycobacterium tuberculosis* DNA from clinical material. *FEMS Microbiology Letters* **205**, 113 – 117

de Vargas, C., Norris, R., Zaninetti, L., Gibb, S. W., & Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proc. Natl. Acad. Sci. USA* **96**, 2864-2868

de Vargas, C., Renaud, S., Hilbrecht, H., & Pawlowski, J. 2001. Pleistocene adaptive radiation in *Globorotalia truncatulinoides*: genetic, morphologic, and environmental evidence. *Paleobiology* **27**(1), 104-125

deVange Panteleeff, D., John, G., Nduati, R., Nbori-Ngacha, D., Richardson, B., Kreiss, J., & Overbaugh, J. 1999. Rapis method for screening dried blood samples on filter paper for Human Immunodeficiency Virus Type 1 DNA. *Journal of Clinical Microbiology*, **37** (2), 350-353

Dieffenbach, C. W. & Gabriela, S. 1995. PCR Primer: A laboratory Manual, Cold Spring Harbour Laboratory Press, New York

Erb, R. W., & Wagner-Döbler, I. 1993. Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and Polymerase Chain Reaction. *Applied and Environmental Microbiology* **59** (12), 4062 - 4073

Fredricks, D. N., Smith, C., & Meier, A. 2005. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. *Journal of Clinical Microbiology* **43** (10), 5122 – 5128

Frostegård, Å., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X., & Simonet, P. 1999. Quantification of bias related to the extraction of DNA directly from soils. *Applied and Environmental Microbiology* **65** (12), 5409 – 5420

Gebhardt, K. J., Brightsmith, D., Powell, G., & Waits, L. P. 2009. Molted feathers from clay licks in Peru provide DNA for three large macaws (*Ara ararauna, A. chloropterus*, and *A. macao*. *Journal of Field Ornithology* **80** (2), 183-192

Gelfland, D. H., & White, T. J. 1990. Thermostable DNA polymerases. In: *PCR Protocols: a guide to methods and applications* (eds. Innis M. A., Gelfland, D. H., Sninsky, J., & White, T. J.). . San Diego: Academic Press. pp. 129 - 141

Gill, P., Jeffreys, A. J., & Werrett, D. J. 1985. Forensic application of DNA 'fingerprints'. *Nature* **318** (6046), 577 – 579

Godschalk, R. W. L., Maas, L. M., Kleinjans, J. C. S., & Van Schooten. 1998. Influences of DNA Isolation and RNA Contamination on Carcinogen-DNA Adduct Analysis by P-Postlabeling. *Environmental and Molecular Mutagenesis* **32**, 344-350

Harris, R. J. 1994. *ANOVA: An analysis of variance primer*. Itasca, I11: Peacock

Holzmann, M., & Pawlowski, J. 1996. Preservation of foraminifera for DNA extraction and PCR amplification. *Journal of Foraminiferal Research*, **26** (3), 264-267

Hsu, J. C. 1996. *Multiple Comparisons: Theory and Methods*. Chapman & Hall/CRC, Boca Raton, London, New York, Washington, D. C. pp. 119-121

Huber, B. T., Bijma, J., & Darling, K. F. 1997. Cryptic speciation in the living planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Paleobiology* **23**, 33-62

Hurt, R. A., Qui, X., Wu, L., Roh, Y., Palumbo, A. V., Tiedje, J. M., & Zhou, J. 2001. Simultaneous recovery of RNA and DNA from soils and sediments. *Applied and Environmental Microbiology* **67** (10), 4495 - 4503

Khosravinia, H., & Ramesha, K.P. 2007. Influence of EDTA and magnesium on DNA extraction from blood samples and specificity of polymerase chain reaction. *African Journal of Biotechnology* **6** (3), 184 – 187

Kreader, C. A. 1996. Relief of Amplification Inhibition in PCR with Bovine Serum Albumin or T4 Gene 32 Protein. *Applied and Environmental Microbiology* **62** (3), 1102 – 1106

Kuske, C. R., Banton, K. L., Adorada, D. L., Stark, P. C., Hill, K K., & Jackson, P. J. 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Applied and Environmental Microbiology* **64** (7), 2463 – 2472

Leff, L. G., Dana, J. R., McArthur, J. V., & Shimkets, L. J. 1995. Comparison of methods of DNA extraction from stream sediments. *Applied and Environmental Microbiology* **61** (3), 1141 - 1143

Luna, G. M., Dell'Anno, A., & Danovaro, R. 2006. DNA extraction procedure: a critical issue for bacterial diversity assessment in marine sediments. *Environmental microbiology* **8** (2) 308 – 320

Maciel, B. M., Santos, A. C. F., Dias, J. C. T., Vidal, R. O., Dias, R. J. C., Gross, E., Cascardo, J. C. M., & Rezende, R. P. 2009. Simple DNA extraction protocol for a 16S rDNA study of bacterial diversity in tropical landfarm soil use for bioremediation of oil waste. *Genetics and Molecular Research* **8** (1), 375 – 388

Malagó Jr., W., Franco, H. M., Matheucci Jr, E., Medaglia, A., & Henrique-Silva, F. 2002. Large scale sex typing of ostriches using DNA extraction from feathers. *BMC Biotechnology* **2**, 19

McPherson, M. J., Quirke, P., & Taylor, G. R. 1991. PCR: A Practical Approach, vol. 1, Oxford University Press, Oxford

Medlin, L., Elwood, H. J., Stickeland, S., & Sogin, M. L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, **71** (2), 491-499

Miller, D. N., Bryant, J. E., Madsen, E. L., & Ghiorse, W. C. 1999. Evaluation of optimisation of DNA extraction and purification procedures for soil and sediment samples. *Applied and Environmental Microbiology* **65** (11), 4715 - 4724

Morard, R., Quillévéré, F., Escarguel, G., Ujiie, Y., de Garidel-Thoron, T., Norris, R. D., & de Vargas, C. 2009. Morphological recognition of cryptic species in the

planktonic foraminifer O*rbulina universa*. *Marine Micropaleontology* **71**, 148-165

Moré, M. I., Herrick, J. B., Silva, M. C., Ghiorse, W. C., & Madsen, E. L. 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Applied and Environmental Microbiology* **60** (5), 1572 – 1580

Murray, J. W. 1979. British Nearshore Foraminiferids. In *Synopses of the British Fauna* (ed. D. M. Kermack & R. S. K, Barnes), No. 16

Noordhoek, G. T., Kaan, J. A., Mulder, S., Wilke, H., & Kolk, A. H. 1995. Routine application of the polymerase chain reaction for detection of Mycobacterium tuberculosis in clinical samples. *Journal of Clinical Pathology* **48**, 810 – 814

Ogram, A., Sayler, G. S., & Barkay, T. 1987. The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods* **7**, 56 - 66

Persat, A., Marshall, L. A., & Santiago, J. G. 2009. Purification of Nucleic Acids from Whole Blood Using Isotachophoresis. *Analytical Chemistry* **81**, 9507-9511

Rossen, L., Norskov, P., Holmstrom, K., & Rasmussen, O. F. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA extraction solutions. *Int. J. Food. Nicrobiol.*, **17**, 37-45

Sambrook, J., Fritsch, E. F., & Maniatis, T. 1989. *Molecular cloning: A laboratory Manual, second edition*. Cold Spring Harbour Laboratory Press, New York

Saunders, G. C., Parkes, H. C., & Primrose, S. B. 1999. Analytical Molecular Biology: Quality and Validation. Laboratory of the Government Chemist (Great Britain)

Simmon, K. E., Steadman, D. D., Durkin, S., Baldwin, A., Jeffrey, W. H., Sheridan, P., Horton, R., & Shields, M. S. 2004. Autoclave method for rapid preparation of bacterial PCR-template DNA. *Journal of Microbiological Methods*, **56**, 143-149

Smith, G. B., & Tiedje, J. M. 1992. Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. *Applied and Environmental Microbiology* **58** (1), 376 – 384

SPSS for Windows, v. 17.0. 2009. Chicago: SPSS Inc

Steffan, R. J., Goksøyr, J., Bej, A. K., & Atlas, R. M. 1988. Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology* **54** (12), 2908 – 2915

Stoffberg, S., Jacobs, D. S., Mackie, I. J., & Matthee, C. A. 2010. Molecular phylogenetics and historical biogeography of *Rhinolophus* bats. *Molecular Phylogenetics and Evolution* **54**, 1-9

Tani, H., Tada, Y., Sasai, K., & Baba, E. 2008. Improvement of DNA extraction method for dried blood spots and comparison of four PCR methods for detection of *Babesia gibsoni* (Asian genotype) infection in canine blood samples. *Journal of Veterinary Medical Science* **70** (5), 461-467

Tebbe, C. C., & Vahjen, W. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Applied and Environmental Microbiology* **59** (8), 2657 – 2665

Tijssen, P. 1993. Hybridization with nucleic acid probes. In: *Laboratory Techniques in Biochemistry and Molecular Biology* (ed. van der Vliet, G. M.), vol. 24, pp. 191-195. Elsevier

Tsai, Y., & Olson, B. H. 1991. Rapid method for direct extraction of DNA from soils and sediments. *Applied and Environmental Microbiology* **57** (4), 1070 - 1074

Wade, B. D., & Garcia-Pichel, F. 2003. Evaluation of DNA extraction methods for molecular analyses of microbial communities in modern calcareous microbialites. *Geomicrobiology Journal* **20**, 549-561

Walker, J. M (Editor). 2005. The Proteomics Protocols Handbook. Humana Press

Weyant, R. S., Edmunds, P., & Swaminathan, B. 1990. Effects of ionic and non-ionic detergents on the *Taq* polymerase. *BioTechniques* **9**, 308 - 309

Zhou, J., Bruns, M. A., & Tiedje, J. M. 1996. DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* **62** (2), 316 – 322

8 Summary and Conclusions

Since the discovery of 'cryptic' genetic diversity within the planktonic foraminiferal morphospecies (Darling *et al*., 1997, 1999, 2004, 2006, 2007; de Vargas *et al*., 1997, 1999, 2001, 2002; Huber *et al*., 1997), biogeographical surveys of genotype distribution have become essential to our understanding of foraminiferal diversification and speciation and give insight into the possible effects of hidden diversity on their role as paleoproxies for past climate change.

Here, biogeographical surveys were carried out in two markedly different areas of the global ocean, the tropical Arabian Sea, and the transitional/sub-polar North Atlantic Ocean. For each region, the biogeographical distribution of the planktonic foraminiferal morphospecies and genetic types was explored, and their positions within a comprehensive phylogeny of the foraminifera (containing a broad range of both planktonic and benthic taxa) determined.

The Arabian Sea (tropical province; 24 °C – 30 °C), situated at the top of the Indian Ocean, represents a unique marine environment, with a circulation that is completely reversed biannually due to seasonally reversing monsoon winds (Schott *et al*., 1983; Swallow, 1984). Whilst long being an area of interest for the study of the planktonic foraminifera, this was the first time that an investigation of their genetic diversity in the region had been carried out. Three hundred and sixty three specimens of planktonic foraminifera were collected from 8 stations along a cruise transect in the Arabian Sea during the summer (SW) monsoon of 2003. Partial ~ 500 bp small subunit rRNA gene sequences were successfully amplified for 213 individual

specimens. Twenty different genotypes were recognised from 13 different mixed layer morphospecies (spinose: *Globoturborotalita rubescens* (pink), *Globigerinoides ruber, Globigerinoides sacculifer*, *Globigerinella siphonifera, Globigerina bulloides*, *Orbulina universa*, *Turborotalita quinqueloba*; non-spinose: *Pulleniatina obliquiloculata*, *Neogloboquadrina dutertrei*, *Globorotalia menardii*, *Globorotalia ungulata*; microperforate: *Globigerinita glutinata*; and bi-serial: *Streptochilus globigerus*). Of these, three morphospecies, *G. rubescens* (pink), *G. ungulata* and *S. globigerus,* were sequenced for the first time and four new genotypes of *G. ruber*, *G. siphonifera*, *T. quinqueloba* and *G. glutinata* were identified. Partial (~1,000 bp) SSU rDNA sequences were obtained for all new types and added to a comprehensive foraminiferal phylogeny.

During the SW monsoon, pronounced environmental conditions lead to a strong disparity between the northern and southern mixed layer water masses of the Arabian Sea, in terms of both primary productivity (phytoplankton growth) and biogeochemistry (Wiggert *et al*., 2002; Banse, 1987: Banse & English, 2000). A distinct difference in the distribution and ecology of the planktonic foraminifera of the Arabian Sea mixed layer was evident at this time, with morphospecies and genotypes being segregated between the high salinity, more eutrophic north and the lower salinity, oligotrophic south.

In complete contrast to the tropical Arabian Sea, sampling across the North Atlantic Ocean, allowed for the study of a typical high latitude morphospecies assemblage. Though home to a lower diversity of planktonic foraminiferal morphospecies (the result of less vertical stratification and niche partitioning), the morphospecies found

here are highly adapted to, and thrive in the colder setting. Most of the morphospecies display a clear bipolar, anti-tropical distribution globally (Bé & Tolderlund, 1971; Darling & Wade, 2008), however, localised variation in the biogeographical distribution of genetic types within the morphospecies was still evident.

Seven hundred and ninety nine specimens of planktonic foraminifera were collected from 27 stations along a cruise transect in the North Atlantic Ocean (July 2004), spanning from Scotland to Newfoundland, and traversing both the subpolar (5-10 °C) and transitional (10-18 °C) provinces. Partial (~500 bp) small subunit rRNA gene sequences were successfully amplified for 164 individual specimens. Eight different genotypes were recognised from 6 mixed layer morphospecies (spinose: *Globigerina bulloides*, *Orbulina universa*, *Turborotalita quinqueloba*; non-spinose: *Neogloboquadrina pachyderma, Neogloboquadrina inflata*; microperforate: *Globigerinita uvula)*, though no novel genotypes were discovered. The phylogenetic placement of the North Atlantic taxa within the foraminifera was consistent with previous studies. The geographical distribution of planktonic foraminiferal SSU rDNA genetic types supported previous evidence of ecological partitioning (Darling *et al*., 2003 2006, 2008; de Vargas *et al*., 1999; Stewart *et al*., 2001).

Ecological partitioning of the planktonic foraminiferal genetic types was evident in both the tropical Arabian Sea and transitional/ sub-polar North Atlantic Ocean. The ability of individual genetic types to become specialized and adapted to life in regionally distinct ecosystems is a likely driver of divergence and speciation of foraminifera in the open ocean, running counter to the apparent lack of barriers to

gene flow, though competitive exclusion may also play some role in their distribution (Aurahs *et al*., 2009).

Genotyping additionally reveals geographical connectivity to other regions of the oceans, providing clues to present and past ocean circulation, evolutionary drivers and the evolutionary history of foraminiferal species (Darling *et al*, 2000, 2004, 2006, 2007; de Vargas *et al*., 1999, 2001, 2002). Despite limited sampling of the tropics and sub-tropics, some ecological perspective could be gained in comparing central Arabian Sea mixed layer genotypes with those in other regions of the tropical and subtropical global ocean. Segregation between the warm water genotypes of the Atlantic and Indo-Eastern Pacific Oceans, e.g. the highly divergent *G. bulloides* Types Ib (Atlantic Ocean) and Type Ia (Indo-Eastern Pacific), and *G. siphonifera* Type $Ia_{(2)}$ which appears isolated in the Indo-Eastern Pacific, indicated the geographical isolation of genetic types by vicariance. The African landmass represents a likely barrier to the dispersal of tropical/sub-tropical specialists, with the cool and inhospitable waters around the South African Cape impeding their transit between the major oceans (Darling & Wade, 2008). Some cosmopolitan morphospecies/ genetic types (e.g. *G. sacculifer*, *G. siphonifera* Type $Ia_{(1)}$) have clearly overcome this barrier, allowing gene flow to occur on a global scale.

Biogeographical analysis of the high latitude morphospecies reveals the existence of genetically homogeneous populations of some genotypes at the northern and southern hemisphere poles (*G. bulloides* Types IIa, IIb, *T. quinqueloba* Types IIa, IIc, IId, and *N. incompta* Type I) (Darling *et al*., 2000, 2006, 2008), and points to the continual transit of individuals across the inhospitable tropics. The mechanism by which gene

flow is occurring, however, remains unknown. Nevertheless, despite the high dispersal potential of the planktonic foraminifera, the global biogeography of some genetic types, coupled with phylogenetic evidence indicates that allopatric diversification has also taken place. Certain high latitude genetic types of planktonic foraminifera are found only in the Southern Hemisphere (Antarctic & Southern Oceans) (*Globigerina bulloides* Type IIc (subpolar), and *N. pachyderma* Types II, III (subpolar), IV (polar), V & VI (Benguela upwelling)) (Darling *et al*., 2003, 2004, 2007, 2008), whilst others are found only in the Northern Hemisphere (*N. pachyderma* Type I (polar), *T. quinqueloba* Type IIb (subpolar/polar) (Arctic & North Atlantic Oceans), and *N. pachyderma* Type VII, *N. incompta* type II, *T. quinqueloba* Type IId, and *G. bulloides* Types IIe & IId (North Pacific Ocean) (Darling *et al*., 2003, 2007, 2008; Stewart *et al*., 2001). The most likely explanation is that geographical isolation of these cold-water types occurred during interglacial periods, following periods of a broader distribution during colder times.

The second major topic covered in this thesis addressed the phylogenetic relationships of the planktonic foraminifera and their origins in the benthic foraminifera. In contrast to the assumed monophyly of the foraminifera, as derived from micropaleontological records and traditional classifications (Caron & Homewood, 1983; Decrouez, 1989; Görög, 1994; Kennett & Srinivasan, 1983; Loeblich & Tappan, 1974, 1987, 1992) molecular phylogenetic analyses of the SSU rRNA gene suggest multiple origins of the planktonic foraminifera from different benthic ancestors (Aurahs *et al*., 2009; Darling *et al*., 1997, 1999, 2000, 2006; de Vargas *et al*., 1997; Stewart *et al*., 2001). However, such phylogenetic analyses have traditionally utilised only a partial ~1,000 bp 3′ terminal fragment of the SSU rRNA

gene, resulting in poor resolution of the deep relationships within the phylogenies, and an unclear picture of the precise origins of the major planktonic groups within the benthic foraminifera.

The aim of the work here was to overcome these previous difficulties, in order to determine how many independent extant lineages exist within the planktonic foraminifera and to elucidate their origins within the benthic foraminifera. Firstly the phylogenetic relationships of the planktonic foraminifera were re-explored using the traditional partial ~1,000 bp terminal 3′ fragment of the SSU rRNA gene. Sequences were aligned across 61 foraminiferal morphospecies, 27 of which were planktonic (order Globigerinida: 11 spinose planktonic (32 sequences), 11 non-spinose macroperforate (20 sequences), 2 non-spiral planktonic (2 sequences), and 3 non-spinose microperforate (3 sequences)), and 34 of which were benthic (one from every family in GenBank) (orders; Rotaliida (14 morphospecies), Milliolida (5 morphospecies), Textulariida (10 morphospecies), Lagenida (2 morphospecies), and Allogromida (2 morphospecies).

Phylogenies constructed from the traditionally used ~1,000bp partial terminal 3′ fragment of the SSU rRNA gene, from which 407 bp could be reliably aligned, supported the polyphyletic origins of the planktonic foraminifera, and indicated that they may be represented by up to 5 independent lineages, derived from separate benthic ancestors. The phylogenies were, however, subject to the same shortcomings as in previous studies, with poor resolution and low bootstrap support for the major clades.

Secondly, an approximately 3,000 bp fragment of the SSU rRNA gene**,** representing almost its complete length, was amplified in the planktonic foraminifera for the first time, with the aim of addressing the issues of the poor resolution and support evident in previous phylogenies. The ~3,000 bp SSU rDNA sequence was successfully amplified for 13 morphospecies of planktonic foraminifera (order Globigerinida: 2 spinose planktonic, 8 non-spinose macroperforate, 1 non-spiral planktonic, and 2 non-spinose microperforate), which were aligned together with existing sequences for 22 morphospecies of benthic foraminifera (all those for which complete SSU rRNA gene sequences currently exist on GenBank) (orders Rotaliida (7 species), Milliolida (10 species), Textulariida (4 species), and Allogromida (1 species)).

Phylogenies were constructed from the ~3,000 bp, almost complete SSU rRNA gene, of which 1002 nucleotide sites could be reliably aligned across the foraminifera. However, extreme difficulties encountered in amplifying the complete SSU rRNA gene in the planktonic foraminifera, particularly for the majority of spinose taxa, resulted in poor taxon sampling, which invariably led to some uncertainties in the phylogenies produced using the different methods of tree reconstruction employed (BI, ML, NJ). Consistent groupings were observed between the two spinose planktonic taxa sequenced, *Globigerinoides sacculifer* and *Globigerina bulloides*, between two pairs of non-spinose macroperforate taxa; *Pulleniatina obliquiloculata* / *Neogloboquadrina dutertrei and G. menardii* / *G. ungulata*, and between the non-spinose microperforate taxa *Globigerinita glutinata* and *Globigerinita uvula.* Contrary to the 407 bp dataset, the macroperforate taxa did not form a consistent monophyletic group across all methods of phylogenetic tree reconstruction employed in the analysis of the in the 1002 bp dataset (BI, ML, NJ), most likely as a result of

poor taxon sampling, though with every method used, the non-spinose macroperforate planktonic taxa, a group of benthics (the Milliolida plus two Rotalliids; *Ammonia beccarii* & *Elphidium aculeatum*), and the spinose planktonic taxa fell together.

For both the partial gene and almost complete SSU rRNA gene sequence datasets, Kishino–Hasegawa (KH) RELL tests (Kishino & Hasegawa, 1989) could not reject the possibility of a planktonic foraminiferal monophyly, although a body of additional evidence, consisting of molecular, morphological, and biological data, does support the independence of the major planktonic foraminiferal groups. Data produced during this study highlighted the genetic distances between the major planktonic groups, and the extreme difference in rates of evolution between them. As in previous studies, the non-spinose planktonic foraminifera were found to share a closer affinity to the benthic foraminifera than to the spinose planktonic taxa.

Further work is needed to resolve the issues of poor resolution and bootstrap support in foraminiferal SSU rDNA phylogenies, and to confirm the validity of the current findings regarding the independent origins of the planktonic foraminiferal lineages. There is a clear need to identify new markers for this important group, both to enhance phylogenetic analyses of the foraminifera and for use in population genetic studies. New markers could be identified by the construction of an expressed sequence tag (EST) library (Theodorides *et al.*, 2002, Whitton *et al.*, 2004; Papanicolaou *et al.*, 2005) or by full genome sequencing in microfabricated high-density picolitre reactors (Margulies *et al.*, 2005). Both methods, nevertheless, require a reliable source of DNA from fresh foraminiferal samples.

The objective of the next phase of work, therefore, was to produce a continually reproducing, genetically identical culture of a benthic foraminifer to provide a reliable source of genetic material for use in genomic work and other molecular applications. Culture experiments were carried out using 3 target morphospecies of British intertidal benthic foraminifera: *Ammonia tepida* (order Rotaliina), *Cornuloculina balkwilli* (order Milliolida), and *Bolivina variabilis* (order Rotaliina). All three are easily collected in the U.K. and bear no contaminating algal symbionts. Culturing conditions were optimised with the final method using 7 cm beakers containing 50 ml of natural seawater filtered to 0.2 μm, with two thirds of the volume of seawater replaced at least once a week, and specimens fed on mixed food (*Dunaliella tertiolecta & Phaeodactylum tricornutum*) weekly. It was also observed that the culture vessel should be changed every few weeks, to avoid the problem of algal overgrowth. A light/ dark cycle of 12 hours was found to be suitable, though the lab temperature of 23 °C used in this study was found to be a little high.

Cornuloculina balkwilli proved to be an excellent candidate for laboratory culturing. Specimens reproduced from a single individual, soon establishing large, healthy populations. *Ammonia tepida* reproduced from a single individual, however, reproduction was infrequent and the brood sizes too small, and this species seemed greatly affected by fluctuating environmental conditions in the lab. *Bolivina variabilis* formed large populations in culture, but failed to reproduce from individually kept specimens. A continuously reproducing culture of *C. balkwilli* was successfully obtained and will be maintained and ultimately used as a source of RNA & DNA for EST library construction/ genomic sequencing and other molecular work in the future.

Throughout the molecular work conducted for both the biogeographical (chapters 3 & 4) and phylogenetic studies (chapter 5), it became increasingly apparent that flaws existed in the methods used to extract the foraminiferal DNA from samples. The result was high failure rates of PCR, both when amplifying the traditionally used partial ~1,000 bp terminal 3′ fragment of the SSU rRNA gene, and the ~3,000 bp, almost complete gene. The method widely employed for the extraction of foraminiferal DNA (Holzmann *et al*., 1996), produces highly variable results, often with weak bands or high failure rates. The buffer is typically used to store samples in long-term, with the solution then being used directly in PCR, with no phenol-chloroform or chloroform extraction and alcohol precipitation to purify the DNA.
Such treatment of samples could be leading to degradation of the DNA, and the transfer of inhibitory substances to the PCR.

The final piece of work in this study therefore focused on the design of a new cell lysis/ DNA extraction method to greatly improve the yield and quality of the DNA obtained from the foraminifera. The aim was to develop a method of extracting DNA from foraminiferal specimens whilst leaving their delicate $CaCO_3$ shell intact. In preserving the foraminiferal shell, rather than crushing it, shell morphology could be referred to at a later date, and compared to the genotype information gained for a particular specimen. Such information could greatly enhance the accuracy of past climate models, which rely on matching morphologically distinct species to the particular environmental conditions to which they are adapted.

A range of chemical reagents were used to create three main buffer solutions, each of which was combined with 4 reducing agents, to make 12 final trial buffers in total.

Specimens of the British benthic intertidal foraminiferal morphospecies, *Ammonia tepida*, were incubated in the buffers under varying conditions to establish an optimal method, and to identify the most effective buffer. A Post-cell lysis chloroform extraction and alcohol precipitation stage was added to purify the DNA, removing any traces of substances that could be inhibitory to PCR. Buffer performance was assessed by tests of time taken to evacuate cellular material from the foraminiferal shells, crude DNA yield obtained, success in PCR, and damage to the delicate $CaCO_3$ shell. Performance was compared to that of the control lysis buffer & method of Holzmann *et al*. (1996).

A highly successful method of extracting DNA from within intact foraminiferal shells was achieved by the incubation of samples at 75 °C for 24 hours in a buffer containing 100mM TRIS, 10mM NaCl, 4M Guanidinium Isothiocyanate, 17 % Isopropanol and 1 % Sarkosyl. Performance was excellent, even when samples were stored in the buffer for 6 weeks after incubation, as they might be in the field or onboard ship. Buffers with the same overall composition as the buffer above save for the addition of the reducing agents βME and DTT (50 mM), also performed well, evacuating shells more rapidly than the buffer without a reducing agent, though with some minor damage to the $CaCO_3$ shells. All three buffers outperformed the control buffer (Holzmann *et al*., 1996), particularly in terms of PCR success. The next important step will be to test the successful buffers on specimens of planktonic foraminifera.

8.1 References

Aurahs, R., Göker, M., Grimm, G. W., Hemleben, V., Hemleben, C., Schiebel, R., & Kucera, M. 2009. Using the multiple analysis approach to reconstruct phylogenetic relationships among planktonic foraminifera from highly divergent and length-polymorphic SSU rDNA sequences. *Bioinformatics and Biology Insights*, **2009:3**, 155-177

Banse K. 1987. Seasonality of phytoplankton chlorophyll in the central and southern Arabian Sea. *Deep Sea Research*, **34**, 713-723

Banse K., English D. 2000. Geographical differences in seasonality of CZCS-derived phytoplankton pigment in the Arabian Sea for 1978–1986. *Deep-Sea Research II* **47**, 1623-1677

Bé A. W. H., Tolderlund D. S. 1971. Distribution and ecology of planktonic foraminifera. In: *The Micropaleontology of Oceans* (eds Funnell BM, Riedel WR), Cambridge University Press, London, pp.105-150

Caron, M., & Homewood, P. 1983. Evolution of early planktic foraminifers. *Marine Micropaleontology*, **7**, 453-462

Darling K. F., Kucera M., Kroon D., Wade C. M. 2006. A resolution for the coiling direction paradox in *Neogloboquadrina pachyderma*. *Paleoceanography,* **21**, PA2011, doi:10.1029/2005PA001189

Darling K. F., Kucera M., Pudsey C.J., Wade C. M. 2004. Molecular evidence links cryptic diversification in polar plankton to Quaternary climate dynamics. *Proc. Natl. Acad. Sci. USA*, **101**, 7657-7662

Darling K. F., Kucera M., Wade C. M., von Langen P., Pak D. 2003. Seasonal distribution of genetic types of planktonic foraminifer morphospecies in the Santa Barbara Channel and its paleoceanographic implications. *Paleoceanography,* **18,** (2) 1032-1042

Darling K. F., Kucera M., Wade C.M. 2007. Global molecular phylogeography reveals persistent Arctic circumpolar isolation in a marine planktonic protist. *Proc. Natl. Acad. Sci. USA,* **104**, 5002-5007

Darling K. F., Wade C. M. 2008. The genetic diversity of planktic foraminifera and the global distribution of ribosomal RNA genotypes. *Marine Micropaleontology,* **67**, 216 – 238

Darling K. F., Wade C. M., Kroon D., Leigh Brown A. J. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. *Marine Micropaleontol*ogy, **30**, 251-266

Darling K. F., Wade C. M., Kroon D., Leigh Brown A. J., Bijma J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulation. *Paleoceanography,* **14**, 3-12

Darling K. F., Wade C. M., Steward I. A., Kroon D., Dingle R., Leigh Brown A. J. 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature,* **405**, 43-47

de Vargas C., Bonzon M., Rees N. W., Pawlowski J., Zaninetti L. 2002. A molecular approach to diversity and biogeography in the planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Marine Micropaleontology,* **870**, 1-16

de Vargas C., Norris R., Zaninetti L., Gibb S. W., Pawlowski J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proc. Natl. Acad. Sci. USA,* **96**, 2864-2868

de Vargas C., Renaud S., Hilbrecht H., Pawlowski J. 2001. Pleistocene adaptive radiation in *Globorotalia truncatulinoides*: genetic, morphologic, and environmental evidence. *Paleobiology,* **27,** (1), 104-125

de Vargas C., Zaninetti L., Hilbrecht H., Pawlowski J. 1997. Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record. *Journal of Molecular Evolution,* **45,** (3), 285-294

Decrouez, D. 1989. Generic ranges of Foraminiferida. *Revue de Paléobiologie*, **8**, 263-321

Görög, A. 1994. Early Jurassic planktonic foraminifera from Hungary. *Micropaleontology*, **40**, 255-260

Holzmann, M., and Pawlowski, J. 1996. Preservation of foraminifera for DNA extraction and PCR amplification. *Journal of Foraminiferal Research*, **26** (3), 264-267

Huber B. T., Bijma J., Darling K. F. 1997. Cryptic speciation in the living planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Paleobiology*, **23**, 33-62.

Kennett, J. P., & Srinivasan, M. S. (eds). 1983. *Neogene planktonic foraminifera, a phylogenetic atlas*. Hutchinson Ross Publishing Company, Stroudsburg, Pennsylvania

Kishino, H., & Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution*, **29**, 170-179

Loeblich, A. R., & Tappan, H. 1987. *Foraminiferal Genera and Their Classification*, v. 1-2, Van Nostrand Reinhold, New York

Loeblich, A. R., & Tappan, H. 1992. Present Status of foraminiferal classification. In: *Studies in Benthic Foraminifera* (eds. Takayangi, Y & Saito, T), Proceedings of the Fourth International Symposium on Benthic Foraminifera, Sendai, 1990 (Benthos '90), Tokai University Press, Tokyo, Japan, pp. 93-102

Loeblich, Jr., A. R., & Tappan, H. 1974. Recent advances in the classification of the Foraminiferida. In: *Foraminifera*, 1 (eds. Hedley, R. H., & Adams, C. G.). Academic Press, London, p1-53

Margulies, M. *et al*. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature,* **437**, 376- 380

Papanicolau, A., Joron, M., McMillan, W. O., Blaxter, M. L. & Jiggins, C. D. 2005 Genomic tools and cDNA derived markers for butterflies. *Molecular Ecology,* **14**, 2883-2897

Schott F. 1983. Monsoon response of the Somali Current and associated upwelling. *Progress in Oceanography,* **12**, 357-381

Stewart I. A., Darling K. F., Kroon D, Wade C. M., Troelstra S. R. 2001. Genotypic variability on subarctic Atlantic planktic foraminifera. *Marine Micropaleontology*, **43**, 143-153

Swallow J. C. 1984. Some Aspects of the Physical Oceanography of the Indian Ocean. *Deep-Sea Research*, **31**, 639-650

Theodorides, K., De Riva, A., Gómez-Zurita, J., Foster, P. G., & Vogler, A. P. 2002. Comparison of EST libraries from seven beetle species: towards a framework for phylogenomics of the Coleoptera. *Insect Mol. Biol.,* **11**, 467-475

Whitton, C., Daub, J., Thompson, M. & Blaxter, M. 2004. Expressed sequence tags: medium-throughput protocols. *Method. Mol. Biol.,* **270**, 75–92

Wiggert J. D., Murtugudde R.G., McClain C. R. 2002. Processes controlling interannual variations in wintertime (Northeast Monsoon) primary productivity in the central Arabian Sea. *Deep-Sea Research II*, **49**, 2319-2343

9 Appendix

Appendix 9.1 Foraminiferal taxa included in phylogenetic analyses

Appendix 9.1.1 Benthic foraminiferal taxa included in phylogenetic analyses

Order	Family	Species	GenBank Accession
Allogromida	Lagynidae	*Notodendrodes haylinosphaira*	AJ311214
	Allogromiidae	***Allogromia sp.***	X86093
Lagenida	Glandulinidae	*Glandulina antarctica*	AY179177
	Nodosariidae	*Dentalina aphelis*	AJ972511
Milliolida	Peneropliidae	***Peneroplis sp.***	AJ132368
		Dendritina zhengae†	AJ404297
	Soritidae	***Sorites orbiculus***	AJ404310
		Parasorites sp.	AJ404305
		Marginopora vertebralis†	AJ404312
		Broeckina sp.†	AJ404304
		Amphisorus hemprichii†	AJ404315
		Cyclorbiluilna compressa†	AJ404303
		Laevipeneroplis sp.†	AJ404300
		Borelis schlumbergeri†	AJ404295
	Hauerinidae	*Pyrgo peruviana*	AY179176
	Massilina	*Massilina secans*	Z69606
	Miliolinidae	*Quinqueloculina sp.*	Z69605
Rotaliida	Elphidiidae	*Elphidium aculeatum*	Z69618
		Elphidium williamsoni†	EF534073
	Rotaliacea	***Ammonia beccarii***	X86094
	Rotaliacea	***Pararotalia nipponica***†	AJ508454
	Nummulitidae	***Nummulites venosus***	AJ318226
		Heterostegina depressa†	AJ508453
	Buliminacea	***Bulimina marginata***	DQ408646
	Bolivinacea	*Bolivina variabilis*	AY359140
		Bolivina spathulata†	AJ318227
		Brizalina alata	AF533837
	Turrilinacea	*Stainforthia fusiformis*	AY934745
	Virgulinellacea	*Virgulinella fragilis*	AY359192
	Cassidulinacea	*Cassidulinoides porrectus*	AY934737
	Chilostomellidae	*Chilostomella ovoidea*	AY465842
	Discorbacea	*Glabratella opercularis*	Z69614
	Discorbinellacea	*Epistominella vitrea*	AM491316
	Nonionacea	*Haynesina germanica*	AF190721
	Planorbulinacea	*Planorbulina mediterranensis*	DQ452709
Textulariida	Trochamminacea	***Trochammina sp.***	X86095
	Lituolacea	*Ammotium pseudocassis*	AJ312434
	Spiroplectamminacea	*Spiroplectammina sp.*	AJ504689
	Textulariacea	*Textularia sp.*	Z69612
	Saccaminidae	*Saccodendron limosum*	AJ319988
	Astrorhizidae	***Astrorhiza triangularis***	AJ318224
	Saccaminidae	***Astrammina rara***	AJ318223
	Rzehakinidae	*Miliammina fusca*	AY822040
	Eggerellidae*	***Eggerelloides scabrum***	AJ318228
	Trochamminidae*	*Arenoparrella mexicana*	AJ307741
Unclassified	Unclassified	*Toxiscaron alba*	AJ307749

Classification as shown in GenBank (*some information added from zipcodezoo.com). Species used in the 1002 bp phylogeny for chapter 5 are shown in **bold**. † Used only in the 1002 bp phylogeny.

Appendix 9.1.2 Planktonic foraminiferal taxa included in phylogenetic analyses

Family (Within Order Rotaliida)	Species	Genetic Type	GenBank Accession/ Sample number
Globigerinidae	*Globigerinella siphonifera*	Ia(1)	U65631
	Globigerinella siphonifera	Ia(2)	Sequence: BR14[4]
	Globigerinella siphonifera	IIa(1)	U80788
	Globigerinella siphonifera	IIa(2)	AF102227
	Globigerinella siphonifera	IIa(3)	Sample:AS273[1]
	Globigerinella siphonifera	IIb	AF102228
	Globigerinella calida		Sequence: AC2-P11b[4]
	Orbulina universa	I	U80791
	Orbulina universa	III	AF102229
	Globigerinoides sacculifer		**Sample:AS344[1]**
	Globigerinoides ruber	Pink	U65634
	Globigerinoides ruber	Ia	U80789
	Globigerinoides ruber	Ib(1)	Z69599
	Globigerinoides ruber	Ib(2)	Sample:AS128[1]
	Globigerinoides ruber	IIa	AF102230
	Globigerinoides conglobatus		U80790
	Globoturborotalita rubescens (pink)		Sample:AS07[1]
	Globigerina bulloides	**Ia**	**Sample:AS165[1]**
	Globigerina bulloides	Ib	Z83957
	Globigerina bulloides	IIa	AF250107
	Globigerina bulloides	IIb	AF250109
	Globigerina bulloides	IIc	AF250111
	Globigerina bulloides	IId	AF250106
	Globigerina bulloides	IIe	Sequence:NP19[4]
	Globigerina falconensis		AF387172
	Turborotalita quinqueloba	Ia	AF25250116
	Turborotalita quinqueloba	Ib	Sample:AS66[1]
	Turborotalita quinqueloba	IIa	AF250112
	Turborotalita quinqueloba	IIb	AF250114
	Turborotalita quinqueloba	IIc	AF250115
	Turborotalita quinqueloba	IId	AY241710
Hastigerinidae	*Hastigerina pelagica*		Z83958
Globorotaliidae	***Globorotalia menardii***		**Sample:AS131[1]**
	Globorotalia ungulata		**Sample:AS224[1]**
	Globorotalia scitula		**Sample:Dis87[3]**
	Globorotalia truncatulinoides		Z83968
	Neogloboquadrina pachyderma	**I**	**Sample:NA632[2]**
	Neogloboquadrina pachyderma	II	AY305330
	Neogloboquadrina pachyderma	III	AF250119
	Neogloboquadrina pachyderma	IV	AF250120
	Neogloboquadrina pachyderma	V	AY305332
	Neogloboquadrina pachyderma	VI	EF447102
	Neogloboquadrina pachyderma	VII	EF447103
	Neogloboquadrina dutertrei		U65635
	Neogloboquadrina dutertrei		AY241707
	Neogloboquadrina dutertrei		**Sample:AS96[1†]**
	Globorotalia inflata		Z83971
	Globorotalia inflata		**Sample:Dis63[3†]**
	Globorotalia crassaformis		AY453134
	Neogloboquadrina incompta	**I**	**Sample:NA3[2]**
	Neogloboquadrina incompta	II	AY241711
Pulleniatinidae	*Pulleniatina obliquiloculata*		AY241709
	Pulleniatina obliquiloculata		**Sample:AS108[1]**
Globigerinidae	*Globigerinita glutinata* Type	Ia(1)	AF250105
	***Globigerinita glutinata* Type**	**Ia(2)**	**Sample:AS71[1]**
	Globigerinita glutinata Type	Ia(3)	Sample:AS265[1]
	Globigerinita uvula		**AF387173**

Cont…

Candeinidae	*Candeina nitida*		AB468837
Bolivinidae	***Streptochilus globigerus***		**Sample:AS249[1]**
Guembelitriidae	*Gallitellia vivans*		AB364520

Species used in the 1002 bp phylogeny for chapter 5 are shown in **bold**. † Used only in the 1002 bp phylogeny. [1] RRS Charles Darwin, Cruise CD148, Arabian Sea, July 2003. [2] RV Professor Logachev, Denmark Straight, North Atlantic Ocean, Aug/Sept 1997. [3] RRV Discovery, Cruise 262, North Atlantic, April 2002. [4] Sequences provided by K. Darling. See appendix 9.6 for cruise locations & dates.

Appendix 9.2 The Likelihood Ratio Test (LRT)

Appendix 9.2.1 Log Likelihood Scores

Model	Log Likelihood Score	
	(A) 407 bp	(B) 1002 bp
HKY85	3771.59318	4813.7670
HKY85 + Γ correction	3447.11592	4619.43778
GTR	3779.61526	4786.51536
GTR+ Γ correction	**3437.68980**	**4611.68089**

Log likelihood scores of the 4 models of evolution tested for use in phylogenetic analyses of the foraminifera using (A) 407 unambiguously aligned bp and (B) 1002 unambiguously aligned bp from the SSU rRNA gene, calculated in PAUP* (version 4.0d65; Swofford, 1998). Scores shown in **bold** indicate the optimal model, as determined by the Likelihood Ratio Test (appendix 9.2.2).

Appendix 9.2.2 Likelihood ratio test score

	HKY85	HKY85 + Γ	GTR	GTR+ Γ
HKY85	-	684.95 *	16.04*	667.81*
HKY85 + Γ	388.66*	-	665.00*	18.85*
GTR	54.50*	334.16*	-	683.85*
GTR+ Γ	404.17*	15.51*	349.67*	-

The likelihood ratio test statistic is $\delta = 2(1n\ L_1 - 1n\ L_0)$ where L_1 is the log likelihood under the more complex (parameter-rich) model and L_0 is the log likelihood under the simpler model (Goldman, 1993; Schmidt, 2009; Swofford *et al*., 1996). Above the diagonal corresponds to results for the 407 bp dataset, and below the diagonal results for the 1002 bp dataset. Significance was calculated by applying the difference in log likelihood and the degrees of freedom between nested models to a Chi squared table. * $P < 0.01$

Appendix 9.3 The Relative Rate Test (RRT) using GRate

The following information is taken directly from the supporting material provided with the GRate package, designed by K. Müeller (unpublished):

Relative rate tests (Fig. 1) based on the concept of Sarich & Wilson (Sarich & Wilson, 1967) are used to quantify the degree of rate divergence between groups of taxa. Taxa are partitioned into groups according to taxonomy, ecology, etc.

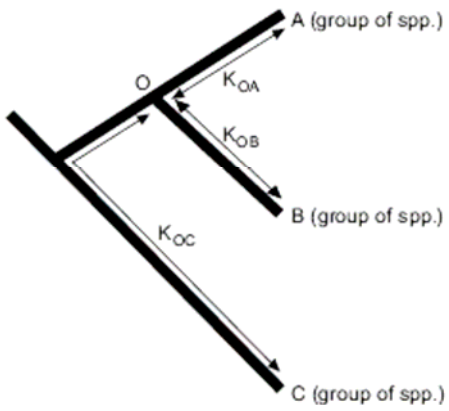


Fig. 1 The relative rate test according to (Sarich & Wilson, 1967). A and B are the sequences to be compared, C is the pre-specified reference taxon. O marks the split between the lineages leading to A and B. K values are the corrected numbers of substitutions per site. Estimates of the difference between K_{OA} and K_{OB} and its standard error allow to accept or refuse rate equality between lineages A and B.

The best fitting model of sequence evolution can be found with Modeltest (Posada & Crandall, 1998). According to the user's choice of the groups to be compared (e.g., pair wise comparisons or comparisons to one reference group), GRate generates a PAUP (Swofford, 1998) command file and later extracts the values needed in the following calculations from a log file.

The ratio between the substitutional rates of a sequence belonging to group A (μ_A) and the rate of a sequence of group B (μ_B) is calculated as

$r = \mu_A/\mu_B = \mu_A t_A/\mu_B t_B = K_{OA}/K_{OB}$

with $t_A = t_B$ = time since divergence, and

$K_{OA}=K_{AC}-((K_{BC}+K_{AC}-K_{AB})/2)$

$K_{OB}=K_{BC}-((K_{BC}+K_{AC}-K_{AB})/2)$.

Differences are calculated as

$d = \mu_A t_A - \mu_B t_B = K_{OA} - K_{OB}$.

To compare the two groups A and B using a third group as outgroup, a combined estimate of r is obtained as

$$\hat{r} = \left(\sum_i \sum_j \sum_k K_{OA}^{(i,j,k)} \right) \Big/ \left(\sum_i \sum_j \sum_k K_{OB}^{(i,j,k)} \right)$$

with $K_{OA(i,j,k)}$ and $K_{OB(i,j,k)}$ being the estimates of K_{OA} and K_{OB} using the i_{th} sequence from group A, the j_{th} sequence from group B, and the k_{th} sequence from the outgroup. If group A, group B, and the outgroup contain o, p, and q sequences, respectively, with N = o p q, the overall d between groups is

$$\hat{d} = \frac{1}{N} \left(\sum_i \sum_j \sum_k K_{OA}^{(i,j,k)} - \sum_i \sum_j \sum_k K_{OB}^{(i,j,k)} \right)$$

The standard error is obtained using the bootstrap (Efron, 1982; Felsenstein, 1985), similar to its application in the calculation of confidence intervals for branch lengths in phylogenetic trees (Dopazo, 1994). For R bootstrap replicates l (d* = d of a bootstrapped data set):

$$\sigma_{\hat{d}} = \sqrt{\frac{1}{R-1} \sum_{l=1}^{B} \left(\left(\frac{1}{R} \sum_{l=1}^{B} \hat{d}_l^* \right) - \hat{d}_l^* \right)^2}$$

Similarly, the standard error of d is obtained by replacing d with r in the above equation. Since the probability distribution does not significantly differ from a normal distribution for large R (can be checked by, e.g., a Kolmogorov-Smirnow test), a two-tailed z-test can be used to evaluate the significance of differences between groups. Moreover, 95 % confidence intervals are supplied based on the normal distribution.

Appendix 9.4 Corrected pairwise distances

Appendix 9.4.1 Range of corrected pairwise distances between foraminiferal SSU rRNA sequences

Dataset	Grouping	Range of distances (Corrected)
407 bp	**Complete phylogeny**	**0 - 0.479**
	Within the spinose planktonic foraminifera	0 - 0.479
	Within the non-spinose planktonic foraminifera (all)	0 - 0.190
	Within the non-spinose macroperforate planktonic foraminifera	0 - 0.190
	Within the non-spinose microperforate planktonic foraminifera	0 - 0.010
	Within the non-spiral planktonic foraminifera	0.003
	Within the benthic foraminifera (all)	0 - 0.157
	Within the benthic foraminifera minus the Milliolida	0 - 0.102
1002 bp	**Complete phylogeny**	**0 - 0.353**
	Within the spinose planktonic foraminifera	0.284
	Within the non-spinose planktonic foraminifera (all)	0.001 - 0.161
	Within the non-spinose macroperforate planktonic foraminifera	0.001 - 0.161
	Within the non-spinose microperforate planktonic foraminifera	0.012
	Within the benthic foraminifera (all)	0.001 - 0.134
	Within the benthic foraminifera minus the Milliolida	0.001 - 0.074

Corrected pairwise distances calculated in PAUP* (Swofford, 1998) using maximum likelihood with a Gtr + Γ model (Rodriguez *et al.*, 1990; Yang 1993).

Microperforates

	G. glutinata	*G. uvula*
G. glutinata		
G. uvula	0.0075	
C. nitida	0.0006	0.0075

Corrected pairwise distances based on 407 bp of the SSU rRNA gene, calculated in PAUP* (Swofford, 1998) using maximum likelihood with a Gtr + Γ model (Rodriguez *et al.*, 1990; Yang 1993).

Non-spirals

	S. globigerus	*G. vivans*
S. globigerus		
G. vivans	0.0027	
B. variabilis	0.00	0.0027
S. fusiformis	0.0025	0.00

Corrected pairwise distances based on 407 bp of the SSU rRNA gene, calculated in PAUP* (Swofford, 1998) using maximum likelihood with a Gtr + Γ model (Rodriguez *et al.*, 1990; Yang 1993).

Appendix 9.5 Kishino–Hasegawa (KH) likelihood tests

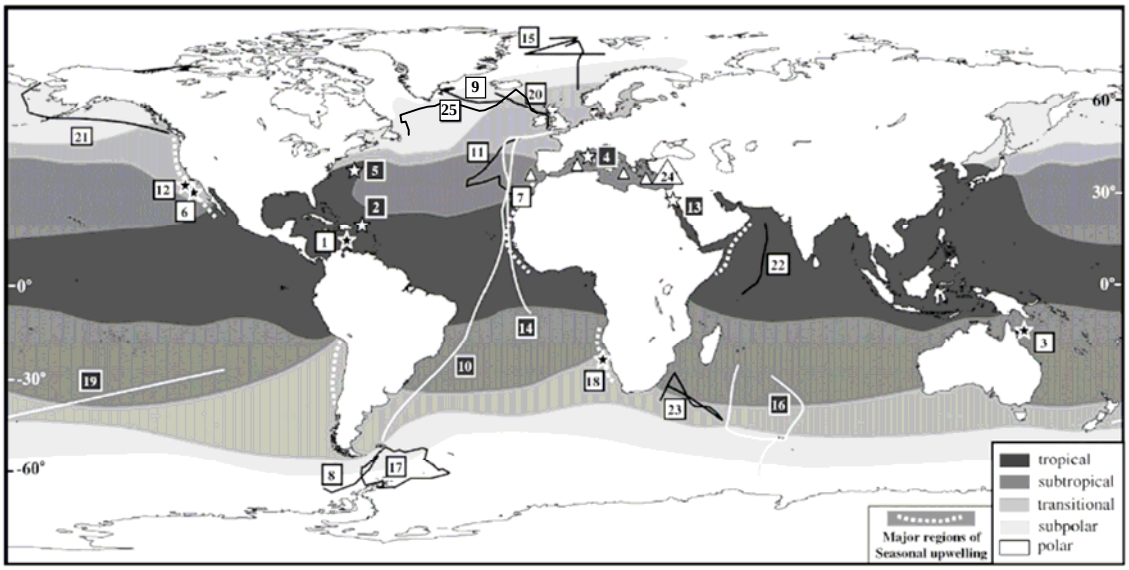
Appendix 9.5.1 KH test between phylogenetic methods for the 1002 bp dataset

Tree		-ln L	Diff. -ln L	P
1	MB	4594.79599	(best)	
2	ML	4599.52616	4.73018	0.662
3	NJ	4611.68090	16.88491	0.048*

KH test using RELL bootstrap (Kishino & Hasegawa, 1989), two-tailed test, with 1000 bootstrap replicates, performed in PAUP* (Swofford, 1998) for 1002 bp of the SSU rRNA gene. * $P < 0.05$

Appendix 9.6 Global sampling sites from which planktonic foraminifera have been collected to date

Appendix 9.6.1 Map showing global sampling sites from which planktonic foraminifera have been collected for molecular study to date



Sites are numbered and marine station and cruise track sampling information is shown in the table below. Point sampling localities with black stars and cruise tracks with black lines and letters were sampled by the Darling *et al.* group. Those shown in white lines, letters and stars were sampled by de Vargas *et al*. The white triangles in the Eastern Atlantic and Mediterranean were sampled by Aurahs *et al*. (abstract published in The Micropalaeontological Society's Foraminifer and Nannofossil Groups Joint Spring Meeting. Integrated Studies of Taxonomy, Ecology and Geochemistry, Angers, France, 2007, pp.10). The five major planktonic foraminiferal faunal provinces (modified from Bé & Tolderlund, 1971), which largely correspond to the main hydrographic regions of the global ocean are shown (see key) (Reproduced from Darling *et al*., 2008).

Appendix 9.6.2 Information on the planktonic foraminiferal sampling localities (field stations and cruises) shown in appendix 9.6.1

No	Field station/cruise	Location	Date	Sampling method	Reference
(1)	Caribbean Marine Biological Institute, Curaçao	Off the west coast of Curaçao, Caribbean Sea	March 1993	Scuba Net (5m)	Darling *et al*. 1996a,b
(2)	Puerto Rico	Caribbean	March 1995	Net (0-100m)	de Vargas *et al*.1997
(3)	Lizard Island Research Station	Off the Great Barrier Reef, Coral Sea	Aug/Sept 1995 & 1997	Net (0-100m)	Darling *et al*. 1997
(4)	Villefranches sur Mer	Mediterranean	Dec 1995	Net (0-100m)	de Vargas *et al*.1997
(5)	Bermuda	Sargasso Sea	April 1996	Net (0-100m)	de Vargas *et al*.1997
(6)	Catalina Marine Science Centre	Off Santa Catalina Island, Southern California Bight	Aug 1996	Scuba	Darling *et al*. 1999
(7)	FS Meteor M37/2 and M38/2	Off the Canary Islands	Dec/Jan 1996/7 April 1997	Pump (6m) Net (0-100m)	Stewart, 2000
(8)	RRV James Clark Ross JR19	Drake Passage/Antarctic Peninsula	Mar/Apr 1997	Pump (6m)	Darling *et al*. 2000
(9)	RV Professor Logachev	Denmark Strait	Aug/Sept 1997	Pump (4.5m) Net (0-200m depth)	Stewart *et al*, 2001
(10)	RRV James Clark Ross AMT-5	UK/Falklands	Sept/Oct 1997	Net (0-175m)	de Vargas *et al*.1999
(11)	FS Poseidon P247	Azores to Canary Islands	Jan 1999	Pump (4.5m)	Stewart *et al*, 2000
(12)	University of California Santa Barbara	The Santa Barbara Channel, California	Jan-Dec 1999	Net (10-50m)	Darling *et al*. 2003
(13)	Eilat	Gulf of Aquaba/Red Sea	May 1999	Net (0-100m)	de Vargas *et al*. 2002
(14)	RRV James Clark Ross AMT-8	UK/Ascension Island	May/June 1999	Net (0-175m)	de Vargas *et al*.2002
(15)	FS Polarstern Arktis XV/1-2	Norwegian Sea and Fram Strait	June-Aug 1999	Pump (6m) Multinets (0-500m)	Darling *et al*. 2004
(16)	Marion Dufresne OISO-4	Southwest Indian Ocean and Southern Ocean	Jan/Feb 2000	Net (0-300m)	de Vargas *et al*. 2004
(17)	RRV James Clark Ross JR 48	Drake Passage Weddell Sea/Scotia Sea	Feb/April 2000	Pump (6m) Nets (0-100m)	Darling *et al*. 2004
(18)	RV Welwitschia	Offshore Namibia	Nov 2001	Net (0-50m)	Darling *et al*. 2004
(19)	R/V Roger Revelle,	Easter Island to New Zealand	Dec/Jan 2001/2.	Net (0-800m)	de Vargas *et al*. 2004
(20)	RRV Discovery D262	North Atlantic from UK to Iceland	April 2002	Pump (5m)	Darling *et al*., 2008
(21)	CCGS Sir Wilfrid Laurier	Canadian Arctic	July 2002	Net (100m)	Darling *et al*. 2007
(22)	RRS Charles Darwin CD148	Arabian Sea	July 2003	Pump (5m)	Darling *et al*., 2008
(23)	RRV Discovery D286	South African Cape to Crozet Islands	Dec 2005	Pump (5m)	Darling *et al*., 2008
(24)	RV Poseidon 321, RV Poseidon 334 RV Meteor 69/1, RV Meteor 71/2 RV Meteor 71/3	Canary Islands-Eastern Mediterranean	May 2005 March 2006 Aug 2006 Dec 2006 Jan 2007	Vertical multinet (0-700m)	Darling *et al*., 2008: Aurahs *et al*. Personal communication (see appendix 9.6.1 legend)
(25)	RRS Charles Darwin CD159	North Atlantic Ocean	July 2004	Pump (6m)	This study

Adapted from Darling *et al*., 2008

Appendix 9.7 SSU rDNA sequence alignments

See supplementary material DVD for the following:

Appendix 9.7.1 **DNA sequence alignment of the partial ~1000 bp terminal 3´ region of the SSU rRNA gene in the foraminifera, showing the 407 unambiguously aligned sites used in phylogenetic alysis**

Appendix 9.7.2 **DNA sequence alignment of the partial ~1000 bp terminal 3´ region of the SSU rRNA gene in *Globigerinella siphonifera* and *Globigerinella calida*, showing the 668 unambiguously aligned sites used in phylogenetic alysis**

Appendix 9.7.3 **DNA sequence alignment of the partial ~1000 bp terminal 3´ region of the SSU rRNA gene in *Globigerinoides ruber* and *Globigerinoides conglobatus*, showing the 589 unambiguously aligned sites used in phylogenetic alysis**

Appendix 9.7.4 **DNA sequence alignment of the partial ~1000 bp terminal 3´ region of the SSU rRNA gene in *Globigerina bulloides*, showing the 669 unambiguously aligned sites used in phylogenetic alysis**

Appendix 9.7.5 **DNA sequence alignment of the partial ~1000 bp terminal 3´ region of the SSU rRNA gene in *Turborotalita quinqueloba*, showing the 669 unambiguously aligned sites used in phylogenetic alysis**

Appendix 9.7.6 **DNA sequence alignment of the partial ~1000 bp terminal 3´ region of the SSU rRNA gene in the Neogloboquadriinids, showing the 666 unambiguously aligned sites used in phylogenetic alysis**

Appendix 9.7.7 **DNA sequence alignment of the partial ~1000 bp terminal 3´ region of the SSU rRNA gene in the *Neogloboquadrina pachyderma* (and other Neogloboquadriinids), showing the 811 unambiguously aligned sites used in phylogenetic alysis**

Appendix 9.7.8 **DNA sequence alignment of the almost complete ~3000 bp SSU rRNA gene in the foraminifera, showing the 1002 unambiguously aligned sites used in phylogenetic alysis**

Appendix 9.7.9 **Streptochilus globigerus and Bolivina variabilis 1000 bp terminal 3´ fragment DNA sequence alignment**

Appendix 9.8 Statistical analysis of crude DNA yield

Appendix 9.8.1 Test of Homogeneity of Variances

Crude DNA Yield			
Levene Statistic	df1	df2	Sig.
3.172	25	234	.000

Performed using the SPSS v17.0 package (SPSS, 2009). Sig. <0.05

Appendix 9.8.2 Oneway Analysis of Variance (ANOVA)

Crude DNA Yield					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	379555.659	25	15182.226	14.197	.000
Within Groups	250242.434	234	1069.412		
Total	629798.093	259			

Oneway ANOVA (Harris, 1994) performed using the SPSS v17.0 package (SPSS, 2009). Sig. <0.05

Appendix 9.8.3 Effect size

Effect Size or the 'strength of Association' indicates the strength of the differences between groups, or the influence of the independent variable (buffer type). The test statistic is given as: Eta^2 = sum of squares between groups/ Total sum of squares. Calculated here:

$Eta^2 = 379555.659 / 629798.093 = 0.6026624$

Using the guidelines of Cohen (1988) the effect size here can be said to be large at 0.6.

Appendix 9.8.4 Tukey's HSD Post Hoc Test: Multiple Comparisons

Tukey's HSD post hoc test (Hsu, 1996) is used here to indicate significant pairwise differences in crude DNA yield.

Crude DNA yield

(I) Buffer	(J) Buffer	Mean Difference (I-J)	Std. Error	Sig.	95 % Confidence Interval	
					Lower Bound	Upper Bound
1A	1B	-10.44000	14.62472	1.000	-64.8898	44.0098
	1C	-19.10800	14.62472	1.000	-73.5578	35.3418
	1D	-9.29700	14.62472	1.000	-63.7468	45.1528
	1A6	18.13300	14.62472	1.000	-36.3168	72.5828
	1B6	23.60900	14.62472	.997	-30.8408	78.0588
	1C6	-6.72000	14.62472	1.000	-61.1698	47.7298
	1D6	9.32300	14.62472	1.000	-45.1268	63.7728
	2A	59.91100*	14.62472	.014	5.4612	114.3608
	2B	66.45400*	14.62472	.002	12.0042	120.9038
	2C	74.80300*	14.62472	.000	20.3532	129.2528
	2D	76.82200*	14.62472	.000	22.3722	131.2718
	2A6	53.54000	14.62472	.061	-.9098	107.9898
	2B6	56.81000*	14.62472	.029	2.3602	111.2598
	2C6	80.11100*	14.62472	.000	25.6612	134.5608
	2D6	57.11900*	14.62472	.027	2.6692	111.5688
	3A	-39.28500	14.62472	.569	-93.7348	15.1648
	3B	-38.39500	14.62472	.617	-92.8448	16.0548
	3C	-33.16900	14.62472	.859	-87.6188	21.2808
	3D	-30.33300	14.62472	.938	-84.7828	24.1168
	3A6	38.58600	14.62472	.607	-15.8638	93.0358
	3B6	31.54200	14.62472	.909	-22.9078	85.9918
	3C6	38.58400	14.62472	.607	-15.8658	93.0338
	3D6	21.38300	14.62472	.999	-33.0668	75.8328
	CB	70.29700*	14.62472	.001	15.8472	124.7468
	CB6	49.16700	14.62472	.144	-5.2828	103.6168
1B	1A	10.44000	14.62472	1.000	-44.0098	64.8898
	1C	-8.66800	14.62472	1.000	-63.1178	45.7818
	1D	1.14300	14.62472	1.000	-53.3068	55.5928
	1A6	28.57300	14.62472	.967	-25.8768	83.0228
	1B6	34.04900	14.62472	.826	-20.4008	88.4988
	1C6	3.72000	14.62472	1.000	-50.7298	58.1698
	1D6	19.76300	14.62472	1.000	-34.6868	74.2128
	2A	70.35100*	14.62472	.001	15.9012	124.8008
	2B	76.89400*	14.62472	.000	22.4442	131.3438
	2C	85.24300*	14.62472	.000	30.7932	139.6928
	2D	87.26200*	14.62472	.000	32.8122	141.7118
	2A6	63.98000*	14.62472	.005	9.5302	118.4298
	2B6	67.25000*	14.62472	.002	12.8002	121.6998
	2C6	90.55100*	14.62472	.000	36.1012	145.0008
	2D6	67.55900*	14.62472	.002	13.1092	122.0088
	3A	-28.84500	14.62472	.964	-83.2948	25.6048
	3B	-27.95500	14.62472	.975	-82.4048	26.4948
	3C	-22.72900	14.62472	.998	-77.1788	31.7208

	3D	-19.89300	14.62472	1.000	-74.3428	34.5568
	3A6	49.02600	14.62472	.147	-5.4238	103.4758
	3B6	41.98200	14.62472	.426	-12.4678	96.4318
	3C6	49.02400	14.62472	.147	-5.4258	103.4738
	3D6	31.82300	14.62472	.902	-22.6268	86.2728
	CB	80.73700*	14.62472	.000	26.2872	135.1868
	CB6	59.60700*	14.62472	.015	5.1572	114.0568
1C	1A	19.10800	14.62472	1.000	-35.3418	73.5578
	1B	8.66800	14.62472	1.000	-45.7818	63.1178
	1D	9.81100	14.62472	1.000	-44.6388	64.2608
	1A6	37.24100	14.62472	.678	-17.2088	91.6908
	1B6	42.71700	14.62472	.389	-11.7328	97.1668
	1C6	12.38800	14.62472	1.000	-42.0618	66.8378
	1D6	28.43100	14.62472	.969	-26.0188	82.8808
	2A	79.01900*	14.62472	.000	24.5692	133.4688
	2B	85.56200*	14.62472	.000	31.1122	140.0118
	2C	93.91100*	14.62472	.000	39.4612	148.3608
	2D	95.93000*	14.62472	.000	41.4802	150.3798
	2A6	72.64800*	14.62472	.000	18.1982	127.0978
	2B6	75.91800*	14.62472	.000	21.4682	130.3678
	2C6	99.21900*	14.62472	.000	44.7692	153.6688
	2D6	76.22700*	14.62472	.000	21.7772	130.6768
	3A	-20.17700	14.62472	1.000	-74.6268	34.2728
	3B	-19.28700	14.62472	1.000	-73.7368	35.1628
	3C	-14.06100	14.62472	1.000	-68.5108	40.3888
	3D	-11.22500	14.62472	1.000	-65.6748	43.2248
	3A6	57.69400*	14.62472	.024	3.2442	112.1438
	3B6	50.65000	14.62472	.109	-3.7998	105.0998
	3C6	57.69200*	14.62472	.024	3.2422	112.1418
	3D6	40.49100	14.62472	.504	-13.9588	94.9408
	CB	89.40500*	14.62472	.000	34.9552	143.8548
	CB6	68.27500*	14.62472	.001	13.8252	122.7248
1D	1A	9.29700	14.62472	1.000	-45.1528	63.7468
	1B	-1.14300	14.62472	1.000	-55.5928	53.3068
	1C	-9.81100	14.62472	1.000	-64.2608	44.6388
	1A6	27.43000	14.62472	.980	-27.0198	81.8798
	1B6	32.90600	14.62472	.868	-21.5438	87.3558
	1C6	2.57700	14.62472	1.000	-51.8728	57.0268
	1D6	18.62000	14.62472	1.000	-35.8298	73.0698
	2A	69.20800*	14.62472	.001	14.7582	123.6578
	2B	75.75100*	14.62472	.000	21.3012	130.2008
	2C	84.10000*	14.62472	.000	29.6502	138.5498
	2D	86.11900*	14.62472	.000	31.6692	140.5688
	2A6	62.83700*	14.62472	.007	8.3872	117.2868
	2B6	66.10700*	14.62472	.003	11.6572	120.5568
	2C6	89.40800*	14.62472	.000	34.9582	143.8578
	2D6	66.41600*	14.62472	.002	11.9662	120.8658
	3A	-29.98800	14.62472	.945	-84.4378	24.4618
	3B	-29.09800	14.62472	.960	-83.5478	25.3518
	3C	-23.87200	14.62472	.997	-78.3218	30.5778
	3D	-21.03600	14.62472	1.000	-75.4858	33.4138
	3A6	47.88300	14.62472	.180	-6.5668	102.3328
	3B6	40.83900	14.62472	.486	-13.6108	95.2888
	3C6	47.88100	14.62472	.180	-6.5688	102.3308
	3D6	30.68000	14.62472	.931	-23.7698	85.1298

	CB	79.59400*	14.62472	.000	25.1442	134.0438
	CB6	58.46400*	14.62472	.020	4.0142	112.9138
1A6	1A	-18.13300	14.62472	1.000	-72.5828	36.3168
	1B	-28.57300	14.62472	.967	-83.0228	25.8768
	1C	-37.24100	14.62472	.678	-91.6908	17.2088
	1D	-27.43000	14.62472	.980	-81.8798	27.0198
	1B6	5.47600	14.62472	1.000	-48.9738	59.9258
	1C6	-24.85300	14.62472	.994	-79.3028	29.5968
	1D6	-8.81000	14.62472	1.000	-63.2598	45.6398
	2A	41.77800	14.62472	.436	-12.6718	96.2278
	2B	48.32100	14.62472	.167	-6.1288	102.7708
	2C	56.67000*	14.62472	.030	2.2202	111.1198
	2D	58.68900*	14.62472	.019	4.2392	113.1388
	2A6	35.40700	14.62472	.768	-19.0428	89.8568
	2B6	38.67700	14.62472	.602	-15.7728	93.1268
	2C6	61.97800*	14.62472	.008	7.5282	116.4278
	2D6	38.98600	14.62472	.586	-15.4638	93.4358
	3A	-57.41800*	14.62472	.025	-111.8678	-2.9682
	3B	-56.52800*	14.62472	.031	-110.9778	-2.0782
	3C	-51.30200	14.62472	.096	-105.7518	3.1478
	3D	-48.46600	14.62472	.163	-102.9158	5.9838
	3A6	20.45300	14.62472	1.000	-33.9968	74.9028
	3B6	13.40900	14.62472	1.000	-41.0408	67.8588
	3C6	20.45100	14.62472	1.000	-33.9988	74.9008
	3D6	3.25000	14.62472	1.000	-51.1998	57.6998
	CB	52.16400	14.62472	.081	-2.2858	106.6138
	CB6	31.03400	14.62472	.922	-23.4158	85.4838
1B6	1A	-23.60900	14.62472	.997	-78.0588	30.8408
	1B	-34.04900	14.62472	.826	-88.4988	20.4008
	1C	-42.71700	14.62472	.389	-97.1668	11.7328
	1D	-32.90600	14.62472	.868	-87.3558	21.5438
	1A6	-5.47600	14.62472	1.000	-59.9258	48.9738
	1C6	-30.32900	14.62472	.938	-84.7788	24.1208
	1D6	-14.28600	14.62472	1.000	-68.7358	40.1638
	2A	36.30200	14.62472	.725	-18.1478	90.7518
	2B	42.84500	14.62472	.383	-11.6048	97.2948
	2C	51.19400	14.62472	.098	-3.2558	105.6438
	2D	53.21300	14.62472	.065	-1.2368	107.6628
	2A6	29.93100	14.62472	.946	-24.5188	84.3808
	2B6	33.20100	14.62472	.858	-21.2488	87.6508
	2C6	56.50200*	14.62472	.032	2.0522	110.9518
	2D6	33.51000	14.62472	.847	-20.9398	87.9598
	3A	-62.89400*	14.62472	.006	-117.3438	-8.4442
	3B	-62.00400*	14.62472	.008	-116.4538	-7.5542
	3C	-56.77800*	14.62472	.030	-111.2278	-2.3282
	3D	-53.94200	14.62472	.056	-108.3918	.5078
	3A6	14.97700	14.62472	1.000	-39.4728	69.4268
	3B6	7.93300	14.62472	1.000	-46.5168	62.3828
	3C6	14.97500	14.62472	1.000	-39.4748	69.4248
	3D6	-2.22600	14.62472	1.000	-56.6758	52.2238
	CB	46.68800	14.62472	.219	-7.7618	101.1378
	CB6	25.55800	14.62472	.992	-28.8918	80.0078
1C6	1A	6.72000	14.62472	1.000	-47.7298	61.1698
	1B	-3.72000	14.62472	1.000	-58.1698	50.7298
	1C	-12.38800	14.62472	1.000	-66.8378	42.0618

	1D	-2.57700	14.62472	1.000	-57.0268	51.8728
	1A6	24.85300	14.62472	.994	-29.5968	79.3028
	1B6	30.32900	14.62472	.938	-24.1208	84.7788
	1D6	16.04300	14.62472	1.000	-38.4068	70.4928
	2A	66.63100*	14.62472	.002	12.1812	121.0808
	2B	73.17400*	14.62472	.000	18.7242	127.6238
	2C	81.52300*	14.62472	.000	27.0732	135.9728
	2D	83.54200*	14.62472	.000	29.0922	137.9918
	2A6	60.26000*	14.62472	.013	5.8102	114.7098
	2B6	63.53000*	14.62472	.005	9.0802	117.9798
	2C6	86.83100*	14.62472	.000	32.3812	141.2808
	2D6	63.83900*	14.62472	.005	9.3892	118.2888
	3A	-32.56500	14.62472	.879	-87.0148	21.8848
	3B	-31.67500	14.62472	.906	-86.1248	22.7748
	3C	-26.44900	14.62472	.987	-80.8988	28.0008
	3D	-23.61300	14.62472	.997	-78.0628	30.8368
	3A6	45.30600	14.62472	.272	-9.1438	99.7558
	3B6	38.26200	14.62472	.625	-16.1878	92.7118
	3C6	45.30400	14.62472	.272	-9.1458	99.7538
	3D6	28.10300	14.62472	.973	-26.3468	82.5528
	CB	77.01700*	14.62472	.000	22.5672	131.4668
	CB6	55.88700*	14.62472	.036	1.4372	110.3368
1D6	1A	-9.32300	14.62472	1.000	-63.7728	45.1268
	1B	-19.76300	14.62472	1.000	-74.2128	34.6868
	1C	-28.43100	14.62472	.969	-82.8808	26.0188
	1D	-18.62000	14.62472	1.000	-73.0698	35.8298
	1A6	8.81000	14.62472	1.000	-45.6398	63.2598
	1B6	14.28600	14.62472	1.000	-40.1638	68.7358
	1C6	-16.04300	14.62472	1.000	-70.4928	38.4068
	2A	50.58800	14.62472	.110	-3.8618	105.0378
	2B	57.13100*	14.62472	.027	2.6812	111.5808
	2C	65.48000*	14.62472	.003	11.0302	119.9298
	2D	67.49900*	14.62472	.002	13.0492	121.9488
	2A6	44.21700	14.62472	.318	-10.2328	98.6668
	2B6	47.48700	14.62472	.192	-6.9628	101.9368
	2C6	70.78800*	14.62472	.001	16.3382	125.2378
	2D6	47.79600	14.62472	.183	-6.6538	102.2458
	3A	-48.60800	14.62472	.159	-103.0578	5.8418
	3B	-47.71800	14.62472	.185	-102.1678	6.7318
	3C	-42.49200	14.62472	.400	-96.9418	11.9578
	3D	-39.65600	14.62472	.549	-94.1058	14.7938
	3A6	29.26300	14.62472	.957	-25.1868	83.7128
	3B6	22.21900	14.62472	.999	-32.2308	76.6688
	3C6	29.26100	14.62472	.957	-25.1888	83.7108
	3D6	12.06000	14.62472	1.000	-42.3898	66.5098
	CB	60.97400*	14.62472	.011	6.5242	115.4238
	CB6	39.84400	14.62472	.539	-14.6058	94.2938
2A	1A	-59.91100*	14.62472	.014	-114.3608	-5.4612
	1B	-70.35100*	14.62472	.001	-124.8008	-15.9012
	1C	-79.01900*	14.62472	.000	-133.4688	-24.5692
	1D	-69.20800*	14.62472	.001	-123.6578	-14.7582
	1A6	-41.77800	14.62472	.436	-96.2278	12.6718
	1B6	-36.30200	14.62472	.725	-90.7518	18.1478
	1C6	-66.63100*	14.62472	.002	-121.0808	-12.1812
	1D6	-50.58800	14.62472	.110	-105.0378	3.8618

	2B	6.54300	14.62472	1.000	-47.9068	60.9928
	2C	14.89200	14.62472	1.000	-39.5578	69.3418
	2D	16.91100	14.62472	1.000	-37.5388	71.3608
	2A6	-6.37100	14.62472	1.000	-60.8208	48.0788
	2B6	-3.10100	14.62472	1.000	-57.5508	51.3488
	2C6	20.20000	14.62472	1.000	-34.2498	74.6498
	2D6	-2.79200	14.62472	1.000	-57.2418	51.6578
	3A	-99.19600*	14.62472	.000	-153.6458	-44.7462
	3B	-98.30600*	14.62472	.000	-152.7558	-43.8562
	3C	-93.08000*	14.62472	.000	-147.5298	-38.6302
	3D	-90.24400*	14.62472	.000	-144.6938	-35.7942
	3A6	-21.32500	14.62472	.999	-75.7748	33.1248
	3B6	-28.36900	14.62472	.970	-82.8188	26.0808
	3C6	-21.32700	14.62472	.999	-75.7768	33.1228
	3D6	-38.52800	14.62472	.610	-92.9778	15.9218
	CB	10.38600	14.62472	1.000	-44.0638	64.8358
	CB6	-10.74400	14.62472	1.000	-65.1938	43.7058
2B	1A	-66.45400*	14.62472	.002	-120.9038	-12.0042
	1B	-76.89400*	14.62472	.000	-131.3438	-22.4442
	1C	-85.56200*	14.62472	.000	-140.0118	-31.1122
	1D	-75.75100*	14.62472	.000	-130.2008	-21.3012
	1A6	-48.32100	14.62472	.167	-102.7708	6.1288
	1B6	-42.84500	14.62472	.383	-97.2948	11.6048
	1C6	-73.17400*	14.62472	.000	-127.6238	-18.7242
	1D6	-57.13100*	14.62472	.027	-111.5808	-2.6812
	2A	-6.54300	14.62472	1.000	-60.9928	47.9068
	2C	8.34900	14.62472	1.000	-46.1008	62.7988
	2D	10.36800	14.62472	1.000	-44.0818	64.8178
	2A6	-12.91400	14.62472	1.000	-67.3638	41.5358
	2B6	-9.64400	14.62472	1.000	-64.0938	44.8058
	2C6	13.65700	14.62472	1.000	-40.7928	68.1068
	2D6	-9.33500	14.62472	1.000	-63.7848	45.1148
	3A	-105.73900*	14.62472	.000	-160.1888	-51.2892
	3B	-104.84900*	14.62472	.000	-159.2988	-50.3992
	3C	-99.62300*	14.62472	.000	-154.0728	-45.1732
	3D	-96.78700*	14.62472	.000	-151.2368	-42.3372
	3A6	-27.86800	14.62472	.975	-82.3178	26.5818
	3B6	-34.91200	14.62472	.790	-89.3618	19.5378
	3C6	-27.87000	14.62472	.975	-82.3198	26.5798
	3D6	-45.07100	14.62472	.282	-99.5208	9.3788
	CB	3.84300	14.62472	1.000	-50.6068	58.2928
	CB6	-17.28700	14.62472	1.000	-71.7368	37.1628
2C	1A	-74.80300*	14.62472	.000	-129.2528	-20.3532
	1B	-85.24300*	14.62472	.000	-139.6928	-30.7932
	1C	-93.91100*	14.62472	.000	-148.3608	-39.4612
	1D	-84.10000*	14.62472	.000	-138.5498	-29.6502
	1A6	-56.67000*	14.62472	.030	-111.1198	-2.2202
	1B6	-51.19400	14.62472	.098	-105.6438	3.2558
	1C6	-81.52300*	14.62472	.000	-135.9728	-27.0732
	1D6	-65.48000*	14.62472	.003	-119.9298	-11.0302
	2A	-14.89200	14.62472	1.000	-69.3418	39.5578
	2B	-8.34900	14.62472	1.000	-62.7988	46.1008
	2D	2.01900	14.62472	1.000	-52.4308	56.4688
	2A6	-21.26300	14.62472	.999	-75.7128	33.1868
	2B6	-17.99300	14.62472	1.000	-72.4428	36.4568

	2C6	5.30800	14.62472	1.000	-49.1418	59.7578
	2D6	-17.68400	14.62472	1.000	-72.1338	36.7658
	3A	-114.08800*	14.62472	.000	-168.5378	-59.6382
	3B	-113.19800*	14.62472	.000	-167.6478	-58.7482
	3C	-107.97200*	14.62472	.000	-162.4218	-53.5222
	3D	-105.13600*	14.62472	.000	-159.5858	-50.6862
	3A6	-36.21700	14.62472	.730	-90.6668	18.2328
	3B6	-43.26100	14.62472	.363	-97.7108	11.1888
	3C6	-36.21900	14.62472	.730	-90.6688	18.2308
	3D6	-53.42000	14.62472	.062	-107.8698	1.0298
	CB	-4.50600	14.62472	1.000	-58.9558	49.9438
	CB6	-25.63600	14.62472	.991	-80.0858	28.8138
2D	1A	-76.82200*	14.62472	.000	-131.2718	-22.3722
	1B	-87.26200*	14.62472	.000	-141.7118	-32.8122
	1C	-95.93000*	14.62472	.000	-150.3798	-41.4802
	1D	-86.11900*	14.62472	.000	-140.5688	-31.6692
	1A6	-58.68900*	14.62472	.019	-113.1388	-4.2392
	1B6	-53.21300	14.62472	.065	-107.6628	1.2368
	1C6	-83.54200*	14.62472	.000	-137.9918	-29.0922
	1D6	-67.49900*	14.62472	.002	-121.9488	-13.0492
	2A	-16.91100	14.62472	1.000	-71.3608	37.5388
	2B	-10.36800	14.62472	1.000	-64.8178	44.0818
	2C	-2.01900	14.62472	1.000	-56.4688	52.4308
	2A6	-23.28200	14.62472	.998	-77.7318	31.1678
	2B6	-20.01200	14.62472	1.000	-74.4618	34.4378
	2C6	3.28900	14.62472	1.000	-51.1608	57.7388
	2D6	-19.70300	14.62472	1.000	-74.1528	34.7468
	3A	-116.10700*	14.62472	.000	-170.5568	-61.6572
	3B	-115.21700*	14.62472	.000	-169.6668	-60.7672
	3C	-109.99100*	14.62472	.000	-164.4408	-55.5412
	3D	-107.15500*	14.62472	.000	-161.6048	-52.7052
	3A6	-38.23600	14.62472	.626	-92.6858	16.2138
	3B6	-45.28000	14.62472	.273	-99.7298	9.1698
	3C6	-38.23800	14.62472	.626	-92.6878	16.2118
	3D6	-55.43900*	14.62472	.040	-109.8888	-.9892
	CB	-6.52500	14.62472	1.000	-60.9748	47.9248
	CB6	-27.65500	14.62472	.978	-82.1048	26.7948
2A6	1A	-53.54000	14.62472	.061	-107.9898	.9098
	1B	-63.98000*	14.62472	.005	-118.4298	-9.5302
	1C	-72.64800*	14.62472	.000	-127.0978	-18.1982
	1D	-62.83700*	14.62472	.007	-117.2868	-8.3872
	1A6	-35.40700	14.62472	.768	-89.8568	19.0428
	1B6	-29.93100	14.62472	.946	-84.3808	24.5188
	1C6	-60.26000*	14.62472	.013	-114.7098	-5.8102
	1D6	-44.21700	14.62472	.318	-98.6668	10.2328
	2A	6.37100	14.62472	1.000	-48.0788	60.8208
	2B	12.91400	14.62472	1.000	-41.5358	67.3638
	2C	21.26300	14.62472	.999	-33.1868	75.7128
	2D	23.28200	14.62472	.998	-31.1678	77.7318
	2B6	3.27000	14.62472	1.000	-51.1798	57.7198
	2C6	26.57100	14.62472	.986	-27.8788	81.0208
	2D6	3.57900	14.62472	1.000	-50.8708	58.0288
	3A	-92.82500*	14.62472	.000	-147.2748	-38.3752
	3B	-91.93500*	14.62472	.000	-146.3848	-37.4852
	3C	-86.70900*	14.62472	.000	-141.1588	-32.2592

	3D	-83.87300*	14.62472	.000	-138.3228	-29.4232
	3A6	-14.95400	14.62472	1.000	-69.4038	39.4958
	3B6	-21.99800	14.62472	.999	-76.4478	32.4518
	3C6	-14.95600	14.62472	1.000	-69.4058	39.4938
	3D6	-32.15700	14.62472	.892	-86.6068	22.2928
	CB	16.75700	14.62472	1.000	-37.6928	71.2068
	CB6	-4.37300	14.62472	1.000	-58.8228	50.0768
2B6	1A	-56.81000*	14.62472	.029	-111.2598	-2.3602
	1B	-67.25000*	14.62472	.002	-121.6998	-12.8002
	1C	-75.91800*	14.62472	.000	-130.3678	-21.4682
	1D	-66.10700*	14.62472	.003	-120.5568	-11.6572
	1A6	-38.67700	14.62472	.602	-93.1268	15.7728
	1B6	-33.20100	14.62472	.858	-87.6508	21.2488
	1C6	-63.53000*	14.62472	.005	-117.9798	-9.0802
	1D6	-47.48700	14.62472	.192	-101.9368	6.9628
	2A	3.10100	14.62472	1.000	-51.3488	57.5508
	2B	9.64400	14.62472	1.000	-44.8058	64.0938
	2C	17.99300	14.62472	1.000	-36.4568	72.4428
	2D	20.01200	14.62472	1.000	-34.4378	74.4618
	2A6	-3.27000	14.62472	1.000	-57.7198	51.1798
	2C6	23.30100	14.62472	.998	-31.1488	77.7508
	2D6	.30900	14.62472	1.000	-54.1408	54.7588
	3A	-96.09500*	14.62472	.000	-150.5448	-41.6452
	3B	-95.20500*	14.62472	.000	-149.6548	-40.7552
	3C	-89.97900*	14.62472	.000	-144.4288	-35.5292
	3D	-87.14300*	14.62472	.000	-141.5928	-32.6932
	3A6	-18.22400	14.62472	1.000	-72.6738	36.2258
	3B6	-25.26800	14.62472	.993	-79.7178	29.1818
	3C6	-18.22600	14.62472	1.000	-72.6758	36.2238
	3D6	-35.42700	14.62472	.767	-89.8768	19.0228
	CB	13.48700	14.62472	1.000	-40.9628	67.9368
	CB6	-7.64300	14.62472	1.000	-62.0928	46.8068
2C6	1A	-80.11100*	14.62472	.000	-134.5608	-25.6612
	1B	-90.55100*	14.62472	.000	-145.0008	-36.1012
	1C	-99.21900*	14.62472	.000	-153.6688	-44.7692
	1D	-89.40800*	14.62472	.000	-143.8578	-34.9582
	1A6	-61.97800*	14.62472	.008	-116.4278	-7.5282
	1B6	-56.50200*	14.62472	.032	-110.9518	-2.0522
	1C6	-86.83100*	14.62472	.000	-141.2808	-32.3812
	1D6	-70.78800*	14.62472	.001	-125.2378	-16.3382
	2A	-20.20000	14.62472	1.000	-74.6498	34.2498
	2B	-13.65700	14.62472	1.000	-68.1068	40.7928
	2C	-5.30800	14.62472	1.000	-59.7578	49.1418
	2D	-3.28900	14.62472	1.000	-57.7388	51.1608
	2A6	-26.57100	14.62472	.986	-81.0208	27.8788
	2B6	-23.30100	14.62472	.998	-77.7508	31.1488
	2D6	-22.99200	14.62472	.998	-77.4418	31.4578
	3A	-119.39600*	14.62472	.000	-173.8458	-64.9462
	3B	-118.50600*	14.62472	.000	-172.9558	-64.0562
	3C	-113.28000*	14.62472	.000	-167.7298	-58.8302
	3D	-110.44400*	14.62472	.000	-164.8938	-55.9942
	3A6	-41.52500	14.62472	.450	-95.9748	12.9248
	3B6	-48.56900	14.62472	.160	-103.0188	5.8808
	3C6	-41.52700	14.62472	.449	-95.9768	12.9228
	3D6	-58.72800*	14.62472	.019	-113.1778	-4.2782

	CB	-9.81400	14.62472	1.000	-64.2638	44.6358
	CB6	-30.94400	14.62472	.924	-85.3938	23.5058
2D6	1A	-57.11900*	14.62472	.027	-111.5688	-2.6692
	1B	-67.55900*	14.62472	.002	-122.0088	-13.1092
	1C	-76.22700*	14.62472	.000	-130.6768	-21.7772
	1D	-66.41600*	14.62472	.002	-120.8658	-11.9662
	1A6	-38.98600	14.62472	.586	-93.4358	15.4638
	1B6	-33.51000	14.62472	.847	-87.9598	20.9398
	1C6	-63.83900*	14.62472	.005	-118.2888	-9.3892
	1D6	-47.79600	14.62472	.183	-102.2458	6.6538
	2A	2.79200	14.62472	1.000	-51.6578	57.2418
	2B	9.33500	14.62472	1.000	-45.1148	63.7848
	2C	17.68400	14.62472	1.000	-36.7658	72.1338
	2D	19.70300	14.62472	1.000	-34.7468	74.1528
	2A6	-3.57900	14.62472	1.000	-58.0288	50.8708
	2B6	-.30900	14.62472	1.000	-54.7588	54.1408
	2C6	22.99200	14.62472	.998	-31.4578	77.4418
	3A	-96.40400*	14.62472	.000	-150.8538	-41.9542
	3B	-95.51400*	14.62472	.000	-149.9638	-41.0642
	3C	-90.28800*	14.62472	.000	-144.7378	-35.8382
	3D	-87.45200*	14.62472	.000	-141.9018	-33.0022
	3A6	-18.53300	14.62472	1.000	-72.9828	35.9168
	3B6	-25.57700	14.62472	.992	-80.0268	28.8728
	3C6	-18.53500	14.62472	1.000	-72.9848	35.9148
	3D6	-35.73600	14.62472	.753	-90.1858	18.7138
	CB	13.17800	14.62472	1.000	-41.2718	67.6278
	CB6	-7.95200	14.62472	1.000	-62.4018	46.4978
3A	1A	39.28500	14.62472	.569	-15.1648	93.7348
	1B	28.84500	14.62472	.964	-25.6048	83.2948
	1C	20.17700	14.62472	1.000	-34.2728	74.6268
	1D	29.98800	14.62472	.945	-24.4618	84.4378
	1A6	57.41800*	14.62472	.025	2.9682	111.8678
	1B6	62.89400*	14.62472	.006	8.4442	117.3438
	1C6	32.56500	14.62472	.879	-21.8848	87.0148
	1D6	48.60800	14.62472	.159	-5.8418	103.0578
	2A	99.19600*	14.62472	.000	44.7462	153.6458
	2B	105.73900*	14.62472	.000	51.2892	160.1888
	2C	114.08800*	14.62472	.000	59.6382	168.5378
	2D	116.10700*	14.62472	.000	61.6572	170.5568
	2A6	92.82500*	14.62472	.000	38.3752	147.2748
	2B6	96.09500*	14.62472	.000	41.6452	150.5448
	2C6	119.39600*	14.62472	.000	64.9462	173.8458
	2D6	96.40400*	14.62472	.000	41.9542	150.8538
	3B	.89000	14.62472	1.000	-53.5598	55.3398
	3C	6.11600	14.62472	1.000	-48.3338	60.5658
	3D	8.95200	14.62472	1.000	-45.4978	63.4018
	3A6	77.87100*	14.62472	.000	23.4212	132.3208
	3B6	70.82700*	14.62472	.001	16.3772	125.2768
	3C6	77.86900*	14.62472	.000	23.4192	132.3188
	3D6	60.66800*	14.62472	.011	6.2182	115.1178
	CB	109.58200*	14.62472	.000	55.1322	164.0318
	CB6	88.45200*	14.62472	.000	34.0022	142.9018
3B	1A	38.39500	14.62472	.617	-16.0548	92.8448
	1B	27.95500	14.62472	.975	-26.4948	82.4048
	1C	19.28700	14.62472	1.000	-35.1628	73.7368

	1D	29.09800	14.62472	.960	-25.3518	83.5478
	1A6	56.52800*	14.62472	.031	2.0782	110.9778
	1B6	62.00400*	14.62472	.008	7.5542	116.4538
	1C6	31.67500	14.62472	.906	-22.7748	86.1248
	1D6	47.71800	14.62472	.185	-6.7318	102.1678
	2A	98.30600*	14.62472	.000	43.8562	152.7558
	2B	104.84900*	14.62472	.000	50.3992	159.2988
	2C	113.19800*	14.62472	.000	58.7482	167.6478
	2D	115.21700*	14.62472	.000	60.7672	169.6668
	2A6	91.93500*	14.62472	.000	37.4852	146.3848
	2B6	95.20500*	14.62472	.000	40.7552	149.6548
	2C6	118.50600*	14.62472	.000	64.0562	172.9558
	2D6	95.51400*	14.62472	.000	41.0642	149.9638
	3A	-.89000	14.62472	1.000	-55.3398	53.5598
	3C	5.22600	14.62472	1.000	-49.2238	59.6758
	3D	8.06200	14.62472	1.000	-46.3878	62.5118
	3A6	76.98100*	14.62472	.000	22.5312	131.4308
	3B6	69.93700*	14.62472	.001	15.4872	124.3868
	3C6	76.97900*	14.62472	.000	22.5292	131.4288
	3D6	59.77800*	14.62472	.014	5.3282	114.2278
	CB	108.69200*	14.62472	.000	54.2422	163.1418
	CB6	87.56200*	14.62472	.000	33.1122	142.0118
3C	1A	33.16900	14.62472	.859	-21.2808	87.6188
	1B	22.72900	14.62472	.998	-31.7208	77.1788
	1C	14.06100	14.62472	1.000	-40.3888	68.5108
	1D	23.87200	14.62472	.997	-30.5778	78.3218
	1A6	51.30200	14.62472	.096	-3.1478	105.7518
	1B6	56.77800*	14.62472	.030	2.3282	111.2278
	1C6	26.44900	14.62472	.987	-28.0008	80.8988
	1D6	42.49200	14.62472	.400	-11.9578	96.9418
	2A	93.08000*	14.62472	.000	38.6302	147.5298
	2B	99.62300*	14.62472	.000	45.1732	154.0728
	2C	107.97200*	14.62472	.000	53.5222	162.4218
	2D	109.99100*	14.62472	.000	55.5412	164.4408
	2A6	86.70900*	14.62472	.000	32.2592	141.1588
	2B6	89.97900*	14.62472	.000	35.5292	144.4288
	2C6	113.28000*	14.62472	.000	58.8302	167.7298
	2D6	90.28800*	14.62472	.000	35.8382	144.7378
	3A	-6.11600	14.62472	1.000	-60.5658	48.3338
	3B	-5.22600	14.62472	1.000	-59.6758	49.2238
	3D	2.83600	14.62472	1.000	-51.6138	57.2858
	3A6	71.75500*	14.62472	.001	17.3052	126.2048
	3B6	64.71100*	14.62472	.004	10.2612	119.1608
	3C6	71.75300*	14.62472	.001	17.3032	126.2028
	3D6	54.55200*	14.62472	.049	.1022	109.0018
	CB	103.46600*	14.62472	.000	49.0162	157.9158
	CB6	82.33600*	14.62472	.000	27.8862	136.7858
3D	1A	30.33300	14.62472	.938	-24.1168	84.7828
	1B	19.89300	14.62472	1.000	-34.5568	74.3428
	1C	11.22500	14.62472	1.000	-43.2248	65.6748
	1D	21.03600	14.62472	1.000	-33.4138	75.4858
	1A6	48.46600	14.62472	.163	-5.9838	102.9158
	1B6	53.94200	14.62472	.056	-.5078	108.3918
	1C6	23.61300	14.62472	.997	-30.8368	78.0628
	1D6	39.65600	14.62472	.549	-14.7938	94.1058

	2A	90.24400*	14.62472	.000	35.7942	144.6938
	2B	96.78700*	14.62472	.000	42.3372	151.2368
	2C	105.13600*	14.62472	.000	50.6862	159.5858
	2D	107.15500*	14.62472	.000	52.7052	161.6048
	2A6	83.87300*	14.62472	.000	29.4232	138.3228
	2B6	87.14300*	14.62472	.000	32.6932	141.5928
	2C6	110.44400*	14.62472	.000	55.9942	164.8938
	2D6	87.45200*	14.62472	.000	33.0022	141.9018
	3A	-8.95200	14.62472	1.000	-63.4018	45.4978
	3B	-8.06200	14.62472	1.000	-62.5118	46.3878
	3C	-2.83600	14.62472	1.000	-57.2858	51.6138
	3A6	68.91900*	14.62472	.001	14.4692	123.3688
	3B6	61.87500*	14.62472	.008	7.4252	116.3248
	3C6	68.91700*	14.62472	.001	14.4672	123.3668
	3D6	51.71600	14.62472	.088	-2.7338	106.1658
	CB	100.63000*	14.62472	.000	46.1802	155.0798
	CB6	79.50000*	14.62472	.000	25.0502	133.9498
3A6	1A	-38.58600	14.62472	.607	-93.0358	15.8638
	1B	-49.02600	14.62472	.147	-103.4758	5.4238
	1C	-57.69400*	14.62472	.024	-112.1438	-3.2442
	1D	-47.88300	14.62472	.180	-102.3328	6.5668
	1A6	-20.45300	14.62472	1.000	-74.9028	33.9968
	1B6	-14.97700	14.62472	1.000	-69.4268	39.4728
	1C6	-45.30600	14.62472	.272	-99.7558	9.1438
	1D6	-29.26300	14.62472	.957	-83.7128	25.1868
	2A	21.32500	14.62472	.999	-33.1248	75.7748
	2B	27.86800	14.62472	.975	-26.5818	82.3178
	2C	36.21700	14.62472	.730	-18.2328	90.6668
	2D	38.23600	14.62472	.626	-16.2138	92.6858
	2A6	14.95400	14.62472	1.000	-39.4958	69.4038
	2B6	18.22400	14.62472	1.000	-36.2258	72.6738
	2C6	41.52500	14.62472	.450	-12.9248	95.9748
	2D6	18.53300	14.62472	1.000	-35.9168	72.9828
	3A	-77.87100*	14.62472	.000	-132.3208	-23.4212
	3B	-76.98100*	14.62472	.000	-131.4308	-22.5312
	3C	-71.75500*	14.62472	.001	-126.2048	-17.3052
	3D	-68.91900*	14.62472	.001	-123.3688	-14.4692
	3B6	-7.04400	14.62472	1.000	-61.4938	47.4058
	3C6	-.00200	14.62472	1.000	-54.4518	54.4478
	3D6	-17.20300	14.62472	1.000	-71.6528	37.2468
	CB	31.71100	14.62472	.905	-22.7388	86.1608
	CB6	10.58100	14.62472	1.000	-43.8688	65.0308
3B6	1A	-31.54200	14.62472	.909	-85.9918	22.9078
	1B	-41.98200	14.62472	.426	-96.4318	12.4678
	1C	-50.65000	14.62472	.109	-105.0998	3.7998
	1D	-40.83900	14.62472	.486	-95.2888	13.6108
	1A6	-13.40900	14.62472	1.000	-67.8588	41.0408
	1B6	-7.93300	14.62472	1.000	-62.3828	46.5168
	1C6	-38.26200	14.62472	.625	-92.7118	16.1878
	1D6	-22.21900	14.62472	.999	-76.6688	32.2308
	2A	28.36900	14.62472	.970	-26.0808	82.8188
	2B	34.91200	14.62472	.790	-19.5378	89.3618
	2C	43.26100	14.62472	.363	-11.1888	97.7108
	2D	45.28000	14.62472	.273	-9.1698	99.7298
	2A6	21.99800	14.62472	.999	-32.4518	76.4478

	2B6	25.26800	14.62472	.993	-29.1818	79.7178
	2C6	48.56900	14.62472	.160	-5.8808	103.0188
	2D6	25.57700	14.62472	.992	-28.8728	80.0268
	3A	-70.82700*	14.62472	.001	-125.2768	-16.3772
	3B	-69.93700*	14.62472	.001	-124.3868	-15.4872
	3C	-64.71100*	14.62472	.004	-119.1608	-10.2612
	3D	-61.87500*	14.62472	.008	-116.3248	-7.4252
	3A6	7.04400	14.62472	1.000	-47.4058	61.4938
	3C6	7.04200	14.62472	1.000	-47.4078	61.4918
	3D6	-10.15900	14.62472	1.000	-64.6088	44.2908
	CB	38.75500	14.62472	.598	-15.6948	93.2048
	CB6	17.62500	14.62472	1.000	-36.8248	72.0748
3C6	1A	-38.58400	14.62472	.607	-93.0338	15.8658
	1B	-49.02400	14.62472	.147	-103.4738	5.4258
	1C	-57.69200*	14.62472	.024	-112.1418	-3.2422
	1D	-47.88100	14.62472	.180	-102.3308	6.5688
	1A6	-20.45100	14.62472	1.000	-74.9008	33.9988
	1B6	-14.97500	14.62472	1.000	-69.4248	39.4748
	1C6	-45.30400	14.62472	.272	-99.7538	9.1458
	1D6	-29.26100	14.62472	.957	-83.7108	25.1888
	2A	21.32700	14.62472	.999	-33.1228	75.7768
	2B	27.87000	14.62472	.975	-26.5798	82.3198
	2C	36.21900	14.62472	.730	-18.2308	90.6688
	2D	38.23800	14.62472	.626	-16.2118	92.6878
	2A6	14.95600	14.62472	1.000	-39.4938	69.4058
	2B6	18.22600	14.62472	1.000	-36.2238	72.6758
	2C6	41.52700	14.62472	.449	-12.9228	95.9768
	2D6	18.53500	14.62472	1.000	-35.9148	72.9848
	3A	-77.86900*	14.62472	.000	-132.3188	-23.4192
	3B	-76.97900*	14.62472	.000	-131.4288	-22.5292
	3C	-71.75300*	14.62472	.001	-126.2028	-17.3032
	3D	-68.91700*	14.62472	.001	-123.3668	-14.4672
	3A6	.00200	14.62472	1.000	-54.4478	54.4518
	3B6	-7.04200	14.62472	1.000	-61.4918	47.4078
	3D6	-17.20100	14.62472	1.000	-71.6508	37.2488
	CB	31.71300	14.62472	.905	-22.7368	86.1628
	CB6	10.58300	14.62472	1.000	-43.8668	65.0328
3D6	1A	-21.38300	14.62472	.999	-75.8328	33.0668
	1B	-31.82300	14.62472	.902	-86.2728	22.6268
	1C	-40.49100	14.62472	.504	-94.9408	13.9588
	1D	-30.68000	14.62472	.931	-85.1298	23.7698
	1A6	-3.25000	14.62472	1.000	-57.6998	51.1998
	1B6	2.22600	14.62472	1.000	-52.2238	56.6758
	1C6	-28.10300	14.62472	.973	-82.5528	26.3468
	1D6	-12.06000	14.62472	1.000	-66.5098	42.3898
	2A	38.52800	14.62472	.610	-15.9218	92.9778
	2B	45.07100	14.62472	.282	-9.3788	99.5208
	2C	53.42000	14.62472	.062	-1.0298	107.8698
	2D	55.43900*	14.62472	.040	.9892	109.8888
	2A6	32.15700	14.62472	.892	-22.2928	86.6068
	2B6	35.42700	14.62472	.767	-19.0228	89.8768
	2C6	58.72800*	14.62472	.019	4.2782	113.1778
	2D6	35.73600	14.62472	.753	-18.7138	90.1858
	3A	-60.66800*	14.62472	.011	-115.1178	-6.2182
	3B	-59.77800*	14.62472	.014	-114.2278	-5.3282

	3C	-54.55200*	14.62472	.049	-109.0018	-.1022
	3D	-51.71600	14.62472	.088	-106.1658	2.7338
	3A6	17.20300	14.62472	1.000	-37.2468	71.6528
	3B6	10.15900	14.62472	1.000	-44.2908	64.6088
	3C6	17.20100	14.62472	1.000	-37.2488	71.6508
	CB	48.91400	14.62472	.150	-5.5358	103.3638
	CB6	27.78400	14.62472	.976	-26.6658	82.2338
CB	1A	-70.29700*	14.62472	.001	-124.7468	-15.8472
	1B	-80.73700*	14.62472	.000	-135.1868	-26.2872
	1C	-89.40500*	14.62472	.000	-143.8548	-34.9552
	1D	-79.59400*	14.62472	.000	-134.0438	-25.1442
	1A6	-52.16400	14.62472	.081	-106.6138	2.2858
	1B6	-46.68800	14.62472	.219	-101.1378	7.7618
	1C6	-77.01700*	14.62472	.000	-131.4668	-22.5672
	1D6	-60.97400*	14.62472	.011	-115.4238	-6.5242
	2A	-10.38600	14.62472	1.000	-64.8358	44.0638
	2B	-3.84300	14.62472	1.000	-58.2928	50.6068
	2C	4.50600	14.62472	1.000	-49.9438	58.9558
	2D	6.52500	14.62472	1.000	-47.9248	60.9748
	2A6	-16.75700	14.62472	1.000	-71.2068	37.6928
	2B6	-13.48700	14.62472	1.000	-67.9368	40.9628
	2C6	9.81400	14.62472	1.000	-44.6358	64.2638
	2D6	-13.17800	14.62472	1.000	-67.6278	41.2718
	3A	-109.58200*	14.62472	.000	-164.0318	-55.1322
	3B	-108.69200*	14.62472	.000	-163.1418	-54.2422
	3C	-103.46600*	14.62472	.000	-157.9158	-49.0162
	3D	-100.63000*	14.62472	.000	-155.0798	-46.1802
	3A6	-31.71100	14.62472	.905	-86.1608	22.7388
	3B6	-38.75500	14.62472	.598	-93.2048	15.6948
	3C6	-31.71300	14.62472	.905	-86.1628	22.7368
	3D6	-48.91400	14.62472	.150	-103.3638	5.5358
	CB6	-21.13000	14.62472	1.000	-75.5798	33.3198
CB6	1A	-49.16700	14.62472	.144	-103.6168	5.2828
	1B	-59.60700*	14.62472	.015	-114.0568	-5.1572
	1C	-68.27500*	14.62472	.001	-122.7248	-13.8252
	1D	-58.46400*	14.62472	.020	-112.9138	-4.0142
	1A6	-31.03400	14.62472	.922	-85.4838	23.4158
	1B6	-25.55800	14.62472	.992	-80.0078	28.8918
	1C6	-55.88700*	14.62472	.036	-110.3368	-1.4372
	1D6	-39.84400	14.62472	.539	-94.2938	14.6058
	2A	10.74400	14.62472	1.000	-43.7058	65.1938
	2B	17.28700	14.62472	1.000	-37.1628	71.7368
	2C	25.63600	14.62472	.991	-28.8138	80.0858
	2D	27.65500	14.62472	.978	-26.7948	82.1048
	2A6	4.37300	14.62472	1.000	-50.0768	58.8228
	2B6	7.64300	14.62472	1.000	-46.8068	62.0928
	2C6	30.94400	14.62472	.924	-23.5058	85.3938
	2D6	7.95200	14.62472	1.000	-46.4978	62.4018
	3A	-88.45200*	14.62472	.000	-142.9018	-34.0022
	3B	-87.56200*	14.62472	.000	-142.0118	-33.1122
	3C	-82.33600*	14.62472	.000	-136.7858	-27.8862
	3D	-79.50000*	14.62472	.000	-133.9498	-25.0502
	3A6	-10.58100	14.62472	1.000	-65.0308	43.8688
	3B6	-17.62500	14.62472	1.000	-72.0748	36.8248
	3C6	-10.58300	14.62472	1.000	-65.0328	43.8668

	3D6	-27.78400	14.62472	.976	-82.2338	26.6658
	CB	21.13000	14.62472	1.000	-33.3198	75.5798

Tukey HSD post hoc test (Hsu, 1996) performed using the SPSS v17.0 package (SPSS, 2009). *The mean difference is significant at the 0.05 level (shown on a grey background). The 12 buffers being compared (1A, 1B, 1C, 1D, 2A, 2B, 2C, 2D, 3A, 3B, 3C, 3D) are named as in chapter 7. CB = The control lysis buffer of Holzmann *et al*., 1996. A number 6 after the buffer name indicates that samples were stored in the buffers for 6 weeks after incubation, and prior to chloroform extraction/ ethanol precipitation.

9.1 References

Bé, A.W.H., & Tolderlund, D. S. 1971. Distribution and ecology of planktonic foraminifera. In: *The Micropaleontology of Oceans* (eds Funnell BM, Riedel WR), Cambridge University Press, London, pp.105-150

Cohen, J. 1988. *Statistical power analysis for the behavioural sciences*. Hillsdale, NJ. Erlbaum

Darling, K. F, Kucera, M., & Wade, C. M. 2007. Global molecular phylogeography reveals persistent Arctic circumpolar isolation in a marine planktonic protist. *Proc. Natl. Acad. Sci. USA,* **104**, 5002-5007

Darling, K. F., & Wade, C. M. 2008. The genetic diversity of planktic foraminifera and the global distribution of ribosomal RNA genotypes. *Marine Micropaleontology,* **67**, 216 – 238

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996a. The isolation and amplification of the 18s ribosomal RNA gene from planktonic foraminifers using gametogenic specimens. ***In:*** *Microfossils and oceanic environments* (eds. Moguilevsky, A & Whatley, R.): University of Wales, Aberystwyth Press, 3.1, 249-259

Darling, K. F., Kroon, D., Wade, C. M., & Leigh Brown, A. J. 1996b. Molecular phylogeny of the planktonic foraminifera. *Journal of Foraminiferal Research*, **26** (4), 324-330

Darling, K. F., Kucera, M., Pudsey, C. J., & Wade, C. M. 2004. Molecular evidence links cryptic diversification in polar plankton to Quaternary climate dynamics. *Proc. Natl. Acad. Sci. USA*, **101**, 7657-7662

Darling, K. F., Kucera, M., Wade, C. M., von Langen, P., & Pak, D. 2003. Seasonal distribution of genetic types of planktonic foraminifer morphospecies in the Santa Barbara Channel and its paleoceanographic implications. *Paleoceanography,* **18**, (2) 1032-1042

Darling, K. F., Wade, C. M., Kroon, D., & Leigh Brown, A. J. 1997. Planktic foraminiferal molecular evolution and their polyphyletic origins from benthic taxa. *Marine Micropaleonto*logy, **30**, 251-266

Darling, K. F., Wade, C. M., Kroon, D., Leigh Brown, A. J., & Bijma, J. 1999. The diversity and distribution of modern planktic foraminiferal small subunit ribosomal RNA genotypes and their potential as tracers of present and past ocean circulation. *Paleoceanography,* **14**, 3-12

Darling, K. F., Wade, C. M., Steward, I. A., Kroon, D, Dingle, R, & Leigh Brown, A. J. 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. *Nature,* **405**, 43-47

de Vargas, C., Bonzon, M., Rees, N. W., Pawlowski, J., Zaninetti, L. 2002. A molecular approach to diversity and biogeography in the planktonic foraminifer *Globigerinella siphonifera* (d'Orbigny). *Marine Micropaleontology* **870**, 1-16

de Vargas, C., Norris, R., Zaninetti, L., Gibb, S. W., & Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proceedings of the National Academy of Science* USA **96**, 2864-2868

de Vargas, C., Sáez, A. G., Medlin, L. K., Thierstein, H. R. 2004. Super-species in the calcareous plankton. In: *Coccolithophores-from molecular processes to global impact* (eds. Thierstein H. R., Young, J. R.). Springer. pp 271-298

de Vargas, C., Zaninetti, L., Hilbrecht, H. and Pawlowski, J. 1997. Phylogeny and rates of molecular evolution of planktonic foraminifera: SSU rDNA sequences compared to the fossil record. *J. Mol. Evol.* **45** (3), 285-294

Dopazo, J. 1994. Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. *J. Mol. Evol*. **38**, 300-304

Efron, B. 1982. The Jackknife, Bootstrap, and other resampling plans. *CBMS-NSF Regional conference series in applied mathematics*, Society for industrial and applied mathematics, Philadelphia

Felsenstein, J. 1985. Phylogenies and the comparative method. *American Naturalist* **125**, 1-15

Goldman, N. 1993. Statistical tests of models of DNA substitution. *Journal of Molecular Evolution*, **36**, 182-198

Harris, R. J. 1994. *ANOVA: An analysis of variance primer*. Itasca, I11: Peacock

Hsu, J. C. 1996. *Multiple Comparisons: Theory and Methods*. Chapman & Hall/CRC, Boca Raton, London, New York, Washington, D. C. pp. 119-121

Kishino, H., & Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution*, **29**, 170-179

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics,* **14**, 817-818

Rodriguez, F., Oliver, J.L., Marin, A. and Medina, J.R. 1990. The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology*, **142**, 485-501

Sarich, V. M. & Wilson, A. C. 1967. Immunological time scale for hominid evolution. *Science,* **158**, 1200-1203

Schmidt, H. A. 2009. Testing tree topologies, theory. In: *The Phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing*, 2nd ed. (eds. Lemey, P., Salemi, M. and Vandamme, A-M.). Cambridge University Press, New York, pp. 381-404

SPSS for Windows, v. 17.0. 2009. Chicago: SPSS Inc

Stewart, I. A. 2000. The molecular evolution of planktonic foraminifera and its implications for the fossil record. Ph.D. thesis, University of Edinburgh

Stewart, I. A., Darling, K. F., Kroon, D., Wade, C. M., & Troelstra, S. R. 2001. Genotypic variability on subarctic Atlantic planktic foraminifera. *Marine Micropaleontology*, **43**, 143-153

Swofford D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Swofford, D. L., Olsen, G.J., Waddell, P.J. and Hillis, D.M. 1996. Phylogenetic Inference. In: *Molecular Systematics*, 2nd ed. (eds. Hillis, D.M., Moritz, C. and Mable, B.K.). Sinauer Associates, Inc., USA, pp. 495-496

Yang, Z. 1993. Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates vary over sites. *Molecular Biology and Evolution,* **10**, 1396-1401