DNA FINGERPRINTING AND MINISATELLITE VARIATION OF SWANS

by

Anming Meng, B.Sc.

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, October 1990

Contents Pages

ABSTRACT

Genetic variation in natural populations of four species of swans (*Cygnus bewickii, Cygnus olor, Cygnus buccinator* and *Cygnus cygnus*) has been investigated by examining minisatellite loci using human DNA fingerprinting probes pSPT19.6 and pSPT18.15. It has been found that swan minisatellites are highly variable. However, the degree of variation depends on the population structure and species. Bewick's Swans at Slimbridge have the highest degree of minisatellite variation, Whooper Swans at Caerlaverock come second, then Mute Swans, and Trumpeter Swans in Montana. Comparative study of DNA fingerprints among populations and among species suggested that swan minisatellites are subject to specific as well as population differentiation, although the function of minisatellites remains an unsolved mystery.

Hypervariable minisatellites of swans that are detected by DNA fingerprinting are stably inherited as codominant markers. DNA fingerprinting has been used to study mating behaviour of Mute and Whooper Swans in wild. The results showed that the Whooper swans were almost strictly monogamous and Mute Swans exhibited an adaptable reproductive system.

A genomic library from *Cygnus olor* was constructed and dozens of minisatellites were isolated. Most of the cloned swan minisatellites were variable, some showed specific variation, and one (pcoMS6.1) detected RFLPs in PstI digests of Trumpeter Swans.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. David Parkin, for his support and help throughout. I also give sincere thanks to Royston Carter and Dr. John Wetton for technical assistance, advice and helpful discussions. I am grateful to the following persons who provided blood samples and pedigree data for this study: Dr. Eileen Rees for Whooper and Bewick's Swans, Dr. Philip Bacon for Abbotsbury Mute Swans, Dr. Chris Spray for Scottish Mute Swans and Carl Mitchell for Montana Trumpeter swans. Colleagues in the Avian Genetics Laboratory, Department of Genetics, helped to collect blood samples of Whooper Swans, to whom I would like to express my gratitude. Prof. Alec Jeffreys provided original human minisatellite probes 33.6 and 33.15, I am grateful to him. I acknowledge excellent assistance provided by the departmental staff.

I am very grateful to the Chinese fellows in the University of Nottingham, who helped me to have an enjoyable university life and provided invaluable aid whenever I needed. While I was undertaking this study, my son was born and my wife Hong had looked after him on her own. I could not have completed this study without her support and encouragements.

This study was financially supported by China National Rice Research Institute and NERC grant to D.T. Parkin.

COMMONLY USED ABBREVIATIONS

BPB	Gel loading buffer containing bromophenol blue
BSA	Bovine serum albumin
EDTA	Ethylenediamine-tetra-acetic acid
EPC	Extra-pair copulation
EPF	Extra-pair fertilization
EtBr	Ethidium bromide
HVR	Highly variable region
INP	Intra-specific nest parasitism
IPTG	Isopropropyl-thiogalactoside
LSP	Locus-specific probe
PEG	Polyethylene glycol
RFLP	Restriction fragment length polymorphism
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
sem	Standard error of the mean
SET	A buffer consisting of sodium chloride, EDTA and Tris
SSC	Standard sodium citrate
TAE	Tris acetate
TBE	Tris borate
TE	A buffer consisting of Tris and EDTA
VNTR	Variable number of tandem repeat

CHAPTER 1 INTRODUCTION

1.1 GENETIC VARIATION AND GENETIC MARKERS

It has been universally acknowledged that genetic variation exists in natural populations of various organisms. Genetic variation is the basis of natural selection, and becomes the focus of population genetics, population ecology and evolution. The studies on genetic variation are essentially based on genetic markers. Indeed, any genetic analysis relies on genetic markers. For instance, it was through studies of seven morphological characters of garden pea that Mendel elicited the basic principles of inheritance.

Up to the mid-1960's, most genetic markers had been limited to easily identifiable morphological and physiological traits, such as colour, shape, pattern and red cell blood groups (examples see Ford 1940, Wolda 1969, Mourant 1961). However, not all morphological variants are genetic, many are environmental. For the next ten years or so, the development of such techniques as starch gel electrophoresis (Smithies 1955), isoelectric focusing electrophoresis (Kolin 1955, Leabach and Rutter 1968), two-dimensional electrophoresis (O'Farrell 1975), and so on, allowed the identification of gene products (proteins and enzymes), and, as a result enzymes and other proteins had been found to exist in multi-forms (reviews see Harris 1969, Nevo 1978). The problems in studying such biochemical polymorphisms are that relatively few enzymes and other proteins can be easily identified, and only a small proportion of them are polymorphic.

The development of genetic marker systems started to turn in the late1970's to DNA. In the genomes of higher eukaryotes, only 5-10% of the DNA sequence codes for protein. The remaining could be exploited if the techniques were available. The availability of restriction endonucleases and the advent of DNA cloning have permitted the isolation of specific genes as well as random DNA segments. These cloned segments can be used as probes to look at the level of DNA sequence variation at the locus of a specific probe. Surprisingly, restriction fragment length polymorphisms (RFLPs) are ubiquitous in the genome (Upholt 1977, Jeffreys and Flavell 1979, Wyman and White 1980, Schumm et al. 1985, Bowden et al. 1989). To detect RFLPs, high-molecular-weight DNA, extracted from several individuals, is digested with a restriction enzyme. The resulting restriction fragments are separated by electrophoresis in an agarose gel according to their molecular weight, and then immobilized onto membrane. A specific probe is radiolabelled and hybridized to its homologous DNA fragments on the membrane. Following autoradiography, the variants related to the probe display variation in size among individuals. If the copy number of a particular sequence is high, restriction patterns can even be visualized on the electrophoretic gel following ethidium bromide staining.

The majority of RFLPs result from the loss or creation of a restriction site due to a point mutation, or alternatively they may result from insertion or deletion of blocks of DNA within a segment. Therefore, the detection of RFLPs heavily relies on the use of enzymes. It has been found that the variants of RFLPs are codominantly inherited as Mendelian markers in a simple fashion. The heterozygosity for a given diallelic RFLP would never exceed 50% in a population without selection. Nevertheless, RFLPs can potentially provide an unlimited number of genetic markers.

The RFLPs have proven useful as markers in genetic analysis. By typing a disease pedigree with an RFLP marker, coinheritance of the marker and the disease phenotype would suggest their linkage. In this way, some disease loci in humans have been mapped (reviews see Gusella 1986, Thein and Wainscoat 1986). RFLPs have also been using to construct general genetic linkage maps (White et al. 1985, Donis-keller et al. 1987, Helentjaris 1987, Chang et al. 1988), although it was estimated that 1500 RFLP loci might be needed to cover the whole human genome (Lange and Boehnke 1982). In addition, RFLPs have been applied to parentage analysis (Smouse and Chakraborty 1986, Quinn et al. 1987), and the survey of genetic variation in natural populations.

1.2 MINISATELLITES AND DNA FINGERPRINTING

Wyman and White (1980) isolated a random DNA segment cloned in phage λ Charon 4A from a human genomic library, which has at least 8 variants, and a heterozygosity of over 75%. It was believed that the polymorphism at this locus is the result of DNA rearrangements rather than base-pair substitutions or modifications. Though its structure was not clear, this might be the first report of highly variable regions (HVRs) identified. Thereafter, several other HVRs have been found in the human genome. As a common feature, various HVRs consist of an array of short tandem repeats, and show RFLPs derived from variation in the copy number of repeats. These HVRs include: a region 3' to the human α-globin gene, consisting of 70-450 tandem repeats of oligonucleotides related to GNGGGG(N)ACAG (Higgs *et al.* 1981, Jarman *et al.* 1986); a region 5' to the human insulin gene, consisting of 34 tandem repeats of a family related

to ACAGGGGTGTGGGG (Bell *et al.* 1982); the intervening sequence (IVS) 1 of the pseudo-ζ-globin gene, consisting of 32-58 copies of a 36-bp GC-rich sequence (Goodbourn *et al.* 1983); and a region near the 3' end of intron 1 in the human myoglobin gene, consisting of 4 repeats of a 33-bp sequence (Weller *et al.* 1984). These HVRs are later on referred to as minisatellites (Jeffreys *et al.* 1985a) or VNTR (variable number of tandem repeat) markers (Nakamura *et al.* 1987).

Jeffreys *et al.* (1985) used the myoglobin 33-bp repeat to screen a human genomic library and detected over 40 positive λ clones. A random selection of eight of these positives were picked up and further characterized. Four of them detected RFLPs in HinfI digests of human genomic DNA. The sequence data show that each clone contains a 0.2-2Kb long minisatellite of 3-29 tandem copies of a repeat sequence. The repeat sequence ranges in length from 16bp to 64bp, but all share a core region GGGCAGGA(A/G)G. It was suggested that, if there is no non-core sequence present in the repeat units, the core sequence could cross hybridize to other minisatellites whose repeat units contain the same core sequence. This hypothesis was first tested by using M33.15, subcloned from one of the λ recombinants, which comprises 29 almost identical repeats of an almost perfect 16-bp core sequence. This probe indeed detected a number of HinfI fragments in each individual. The hybridization pattern was extremely variable among individuals, and the heterozygosity for those large fragments detected was almost 100%. Afterwards, another minisatellite clone M33.6, consisting of 18 repeats of a 37-bp sequence (diverged trimer of the most conserved 11-bp 3'end of the core sequence), was also found to detect hypervariable hybridization patterns in humans (Jeffreys et al. 1985b). The probability that two unrelated persons have identical hybridization patterns (i.e. all bands in one person are present in a

second person) for probe 33.15 is $3X10^{-11}$ and this probability is approximately $5X10^{-19}$ if both probes 33.15 and 33.6 are used (Jeffreys *et al.* 1986). Therefore, the profiles of hybridization obtained with the minisatellite probes are unique to individuals, and hence are called DNA 'fingerprints' or 'genetic fingerprints'. The probes are called DNA fingerprinting probes.

Human DNA fingerprints have several properties. Firstly, a DNA fingerprint is usually composed of more than 20 bands (minisatellite fragments). It is estimated that a single DNA fingerprinting probe such as 33.6 can detect some 30 minisatellite loci (Jeffreys *et al.* 1986). Secondly, hypervariable fragments present in parental DNA fingerprints codominantly segregate into offspring following Mendelian inheritance (Jeffreys *et al.* 1986). Most of the resolved parental fragments behave as single heterozygous Mendelian characters and are transmitted on average to half of the offspring . Only very few heterozygous parental fragments show allelism or linkage. It was suggested that the minisatellite fragments detected in a DNA fingerprint are derived from many or all of the human autosomes (Jeffreys *et al.* 1985b). However, Wells *et al.* (1989) argued that the distribution of minisatellites in the human genome is skewed toward chromosome ends, and it is highly clustered in character. Thirdly, DNA fingerprints are individual-specific due to the hypervariability of resolved human minisatellites (Jeffreys *et al.* 1985b). The degree of variation of minisatellite fragments among individuals is higher in the higher molecular region (≥6Kb) of the DNA fingerprints. Lastly, DNA fingerprints show substantial somatic stability between normal tissues or cultured cell lines (Jeffreys *et al.* 1985b). The fingerprinting patterns also have substantial germ-line stability and the mutation rate to new length alleles was estimated at 1/300 (Jeffreys 1987).

The function and formation mechanism of minisatellites in the genome remains largely unknown. It was suggested that the structure of minisatellite could stimulate unequal crossing-over (Smith 1976) and that the core sequence might act as an eukaryotic recombination signal because it is similar in length and in G content to the χ sequence, a signal for homologous recombination in bacterium *E. coli* (Jeffreys *et al.* 1985a). This hypothesis is supported by some evidence (Steinmetz *et al.* 1986, Royle *et al.* 1988, Chandley and Mitchell 1988, Wahls *et al.* 1990), but not by others (Wolff *et al.* 1988, Cox *et al.* 1988, Jeffreys *et al.* 1990). For the formation of minisatellites, Jarman and Wells (1989) proposed an alternative model. They suggested that areas of the genome with a high G+C content have a greater inherent tendency to produce chance duplications. When some duplications have become large enough, unequal crossing-over would be stimulated and result in the formation of minisatellites of various length. According to this model, one can expect that GC-rich minisatellites are most likely to be found in GC-rich regions, and be particularly abundant in regions of high recombination. The slippage-prone, noncoding DNA where minisatellites form can also accommodate tandem repeats of a different composition from other areas of the genome, so that minisatellite composition will be determined by local sequence structure. However, this model has been challenged by the finding of several AT-rich minisatellites that are also variable, although these minisatellites show a narrow variation in size between alleles (Stoker *et al.* 1985, Knott *et al.* 1986, Simmler *et al.* 1987).

1.3 DIVERSIFICATION OF DNA FINGERPRINTING PROBES

Following the pioneering work of Jeffreys and his colleagues, a number

of minisatellites have been isolated from the genome and proved suitable for DNA fingerprinting (see Table 1.1). Most strikingly, Vassart *et al.* (1987) found that wild-type bacteriophage M13 is able to detect hypervariable minisatellites in the human genome and generate individual-specific DNA fingerprints. Most of the DNA fingerprinting probes so far developed are related in sequence to one another, especially in GC-richness. However, they detect substantially different subsets of minisatellites in the genome. As an exception, a AT-rich minisatellite (113I), derived from a human pseudoautosomal locus DXYS15, also detects a number of related minisatellites variable in copy number of tandem repeats, representing a new category of minisatellites (Simmler *et al.* 1987).

In addition, several synthetic oligonucleotides have been used. These can be used as DNA fingerprinting probes to produce DNA fingerprints (Ali and Wallace 1988, Menzel *et al.* 1990, Kashi *et al.* 1990). They are related to the core sequence of some minisatellite fingerprinting probes.

Among the DNA fingerprinting probes available, the human minisatellites 33.6 and 33.15 have been most widely used, including applications in various animals and plants. Second to them is M13, then probe α-globin 3'HVR. The other probes listed in table 1.1 are seldom used by other researchers.

Table 1.1 Multi-locus DNA fingerprinting probes containing genomic sequence

Probes	Nature	Referees
33.6	core-containing,GC-rich human minisatellite	Jeffreys *et al.* 1985a
33.15	core-containing,GC-rich human minisatellite	Jeffreys *et al.* 1985a
113I	AT-rich minisatellite,derived from a human pseudo-autosomal locus DXYS15	Simmler *et al.* 1987
(Unnamed)	two minisatellites in intron B and exon 8 of human factor VII gene	Murray *et al.* 1988
3'HVR	a GC-rich minisatellite 3' to the human α globin locus on chromosome 16	Fowler *et al.* 1988
(Unnamed)	28-bp tandemly reiterated sequence (GC-rich) downstream of human c-Ha-ras-1 oncogene	Washio *et al.* 1989
(Unnamed)	a 200bp long stretch of AG-rich repetitive sequence 5'to the human thyroglobulin gene	Gerard *et al.* 1990
pV47-2	a human minisatellite isolated by hybridizing to M13	Longmire *et al.* 1990
M13	effective sequence is two clusters of 156bp repeats (GC-rich) within the protein III gene of the phage	Vassart *et al.* 1987
pSP64.2.5E	a mouse minisatellite related to Drosophila *"per "* gene and M13 protein III gene	Georges *et al.* 1987
Mo-1 clone	a mouse minisatellite related to Jeffreys' core	Kominami *et al.* 1988
pGB 725	a bovine minisatellite containing poly-TG stretches	Kashi *et al.* 1990
L17	a Willow Warbler minisatellite, isolated by hybridizing to probes 33.6 and 33.15	Gyllensten *et al.*1989
ϕFd103	a bacteriophage in *E. coli* related to M13	Rogaev and Shlensky 1990

1.4 APPLICATION OF DNA FINGERPRINTING

1.4.1 Applications in Humans

DNA fingerprinting came into practical use in humans immediately it was developed. It has been used in forensic tests for positively identifying criminals with a degree of certainty never reached before (Gill *et al.* 1985, Dodd 1985, Connor 1988). It has also helped to resolve immigration cases, where family relationships were disputed in court (Jeffreys *et al.* 1985c, Johnston 1987). The practical applications of DNA fingerprinting outside forensic science have also been demonstrated, for example in determining paternity for general inquiry (Helminen *et al.* 1988), in verifying the pedigree structure of a family under investigation (Wells *et al.* 1988), in determining zygosity in cases of multiple pregnancy (Hill and Jeffreys 1985) and in monitoring the progress of engraftment following allogeneic bone marrow transplantation (Knowlton *et al.* 1986, Min *et al.* 1988).

As a tool for linkage analysis, DNA fingerprinting can be used to search for disease loci that are not linked to any of known biochemical markers (Davies 1985). Indeed Jeffreys *et al.* (1986) observed a hypervariable DNA fragment cosegregating with hereditary persistence of fetal hemoglobin by fingerprinting a large pedigree. More interestingly, it has been found that there is a high rate of somatic mutations at minisatellite loci in human tumours, displaying loss/gain of a given minisatellite or altered minisatellites in size (Thein *et al.* 1987, Armour *et al.* 1989). The minisatellite fragments that have shown linkage to disease loci could be isolated as locus-specific probes for extending the linkage data and mapping the disease loci (Wong *et al.* 1986).

Wells *et al.* (1989) demonstrated that it is possible to directly map the DNA fingerprinting bands using DNA fingerprinting technique in combination with the use of pre-existing markers. However, effectively mapping disease loci or mapping the whole genome requires a large number of informative markers that are locus-specific. Many hypervariable locus-specific minisatellites in the human genome have been isolated by screening a genomic library with pre-existing DNA fingerprinting probes, HVRs or oligonucleotides related in sequence to the core sequence of some fingerprinting probes (Nakamura *et al.* 1987, 1988, Wong *et al.* 1987, Washio *et al.* 1989). The newly isolated minisatellites can in turn be used as probes to isolate other minisatellites (Washio *et al.* 1989).

1.4.2 Applications in Other Organisms

The core sequence present in minisatellites or similar sequences show sufficient interspecific conservation, to allow the detection of minisatellites in the genomes of various organisms. In particular, the most popular DNA fingerprinting probes 33.6, 33.15 and M13, have proven capable of detecting hypervariable minisatellite fragments and generating individual-specific, strain-specific or cultivar (race)-specific hybridization patterns in birds (Burke and Bruford 1987, Parkin 1987, Parkin *et al.* 1988), mammals (Jeffreys and Morton 1987, Weiss *et al.* 1988, Dixson *et al.* 1988, Amos and Dover 1990), livestock (Ryskov *et al.* 1988, Georges *et al.* 1988), fish (Georges *et al.* 1988), plants (Dallas 1988, Ryskov *et al.* 1988, Rogstad *et al.*1988, Nybom *et al.* 1989, 1990), and insects, yeast, fungi and bacteria (Ryskov *et al.* 1988). In fact, DNA fingerprinting has already had a conspicuous impact on the population biology of animals.

To test hypotheses concerning the ecological and evolutionary biology of animals, it is essential to know the genetic relatedness among individuals in the field. Therefore, parentage determination is crucial. Conventional genetic markers such as blood groups and polymorphic proteins can only exclude an individual from parentage with a low degree of probability. By contrast, DNA fingerprinting allows parentage inclusion due to the uniqueness of DNA fingerprints arising from multi-allelism at many minisatellite loci. DNA fingerprinting has been enthusiastically used for studying the mating behaviour of various species of animals, and several reports have shown its power (Wetton *et al.* 1987, Burke *et al.* 1989, Wetton and Parkin 1989, Gyllensten *et al.* 1990, Wellbourn *et al.* 1990). For example, Burke *et al.* (1989) found, using DNA fingerprinting, that in the dunnock *Prunella modularis* (having a flexible mating system) a male was much more likely to feed the brood if he had sired some of the nestlings. Another example is a study on the long-finned pilot whale *Globicephala melaena* (Amos and Dover 1990). The long-finned pilots swim in large groups or pods, usually containing 50-200 individuals, in which one adults leads and the rest often follow. All the attempts in the past to identify individual whales and to assess the relatedness among animals within a pod failed because of the extreme difficulty of access. The researchers turned to DNA fingerprinting and soon obtained astonishing results, suggesting that males move frequently between pods, and some dominate mating within a pod, which is inconsistent with the previous assumption that males stay and females wander.

DNA fingerprinting has been adopted to investigate genetic variability in the genome at population level, promising to transform evolutionary and population biology. In particular, differences or similarities among DNA fingerprints can be used to construct the evolutionary relationships among

closely related populations (Kuhnlein *et al.* 1989, Gilbert *et al.* 1990, Reeve *et al.* 1990).

Conservation biology of animals is another main area in which DNA fingerprinting may be adopted (Parkin 1987). Information on the degree of genetic variation within a population, and its relevant ecology are vital for making a strategic programme to save endangered wild species. DNA fingerprinting could tell what degree of genetic variation there is among individuals and among populations. If there is a very limited amount of intrapopulation variation, this population must have been raised from the same few ancestors. Then the conservation programme should primarily prevent any further inbreeding within this population, possibly by introducing breeding animals from relatively distant populations. Such a study of several endangered species of birds of prey has been initiated some three years ago by a group at the University of Nottingham, England. Based on the same principle, the value of DNA fingerprinting in breeding of farm animals and plants has also been illustrated (Hillel *et al.* 1990). In another aspect of conservation, i.e. protection of rare birds or other animals, DNA fingerprinting could expose the crime of some collectors who had stolen animals under protection of the law but claimed legal ownerships (Parkin *et al.* 1988).

As in humans, linkage analysis in animals could be done by using DNA fingerprint profiles. The only example reported is a linkage study in cattle (Georges *et al.* 1990). This study revealed several cases of genetic linkage between DNA fingerprint bands and classical markers (proteins and sexes) and identified a solid candidate marker for the bovine 'muscular hypertrophy' gene. We can expect that the mapping of animal genes will be easier than that of human genes because large pedigrees can be obtained.

DNA fingerprinting is applicable in yeast, plantsand even in bacteria for giving genetic identities of strains, cultivars or races, especially in bacteria for identifying pathogens or for determining the purity of bacterial cultures.

1.5 ABOUT THIS STUDY

Swans are large birds. Due to their large size and conspicuous plumage, it is easy to watch them without binoculars or telescope. However, swans did not attract the attention of ornithologists until in the early 1960's they were realized to be good subjects for looking at certain aspects of life-history and population biology. Since then, the system of counting and ringing swans has been established, notably in Britain for the Mute Swan, Whooper Swan and Bewick's Swan. Even for the Mute Swan, successful censuses have been regularly ~~token~~ throughout Britain several times (Rawcliffe 1958, Campbell 1960, Ogilvie 1981,1986). However, most of studies on swans so far only concerned migration, age structures and mortality rates within various populations (Scott and Wildfowl Trust 1972, Birkhead and Perrins 1986). Modern genetic technology has not been used for systematically studying the evolution and population biology of swans. The availability of a large number of blood samples, together with field observation data, from various species of swans allows us to carry out this study on various aspects of population and molecular genetics of swans by using the DNA fingerprinting technique.

DNA fingerprinting is a delicate technique. To generate informative DNA fingerprints, the experimental design should be modified according to the species under study. Chapter 2 will present details of technique for

DNA-fingerprinting swans. Then swan DNA fingerprints will be characterized, including their variability, inheritance and stability. In Chapter 4, DNA fingerprinting will be used to study population differentiation in the wild and specific differentiation in four species of swans. The next chapter will present results on parentage analysis and discuss the reproductive biology of swans.

To study individual swan minisatellites, a genomic library from a Mute Swan will be constructed and the minisatellites will be isolated. If possible, the isolated minisatellites will be tested to see whether they act as locus-specific probes, and then be used for a population survey. At the same time, some minisatellites may be sequenced to look at their internal structures. The results in these aspects will be presented in Chapter 6.

CHAPTER 2

BLOOD SAMPLING AND GENERAL METHODS OF DNA FINGERPRINTING

2.1 INTRODUCTION

The basic material for DNA fingerprinting is genomic DNA. Though various tissues are sources of DNA, blood remains the best for fingerprinting non-mammalian vertebrates , especially small-bodied birds. Red blood cells of birds each contain a nucleus. It was reported that the DNA content ranged from 2.81 to 4.97 pg per nucleus in 48 avian species (Venturini *et al* .1986), and that the number of red cells averages about 3 x 10^6 per mm^3 blood in birds (Sturkie 1976). Therefore, 1µl of avian blood could have approximately 11µg of nuclear DNA, which allows a large amount of genomic DNA to be prepared. In addition, it is easy to take blood from a live bird without harm to its health.

DNA fingerprinting is itself a very elaborate technique, which has many distinct components. The process includes the isolation of genomic DNA, digestion of DNA with a restriction enzyme, separation of restriction fragments in agarose gels, immobilization of fragments onto membranes, preparation of radioactive probes, hybridization of the probe to specific fragments on the membrane and autoradiography (see Fig.2.1). A minor mistake or incorrect treatment results in bad DNA fingerprints that are neither reliable nor interpretable.

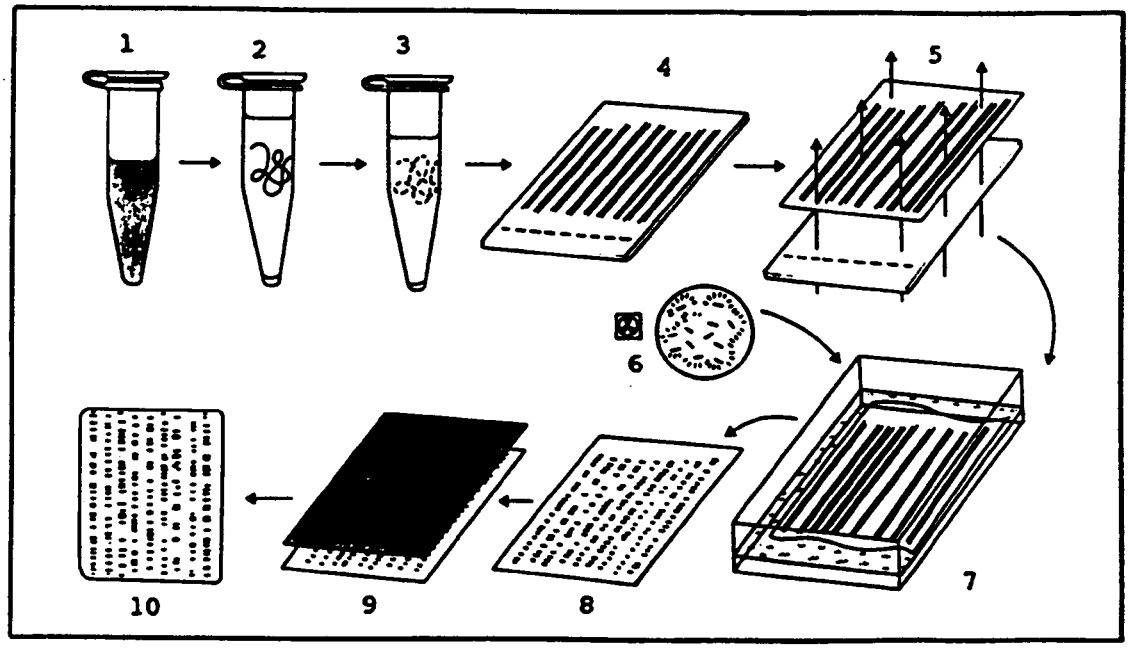


Fig. 2.1 Illustration of DNA fingerprinting. 1. Whole blood; 2. intact genomic DNA extracted from blood; 3. DNA is cut with restriction enzyme; 4. electrophoretic separation of restriction fragments in an agarose gel; 5. the fragments are transferred to a supportive membrane (nitrocellulose or nylon); 6. the fingerprinting probes are radiolabelled; 7. the probes hybridize to the fragments immobilized on the membrane; 8. nonspecifically bound probes have been washed off; 9. the hybridization pattern is visualized by exposing to an X-ray film (autoradiography); 10. DNA fingerprints are obtained after developing the film.

2.2 COLLECTION OF BLOOD SAMPLES AND PEDIGREE DATA

After being captured, each bird was immobilized by wearing a 'jacket'. Blood was collected by bleeding the leg vein using a disposable 2-ml syringe fitted with a 25G hypodermic needle (Fig.2.2). The following is the detailed procedure:

1. Use a sheet of paper tissue wetted with absolute ethanol to rinse an area of skin on the right leg.

2. Flush a syringe and needle with 100μl heparin sodium (5000I.U./ml) to prevent the sample from clotting the syringe. Penetrate the vein and suck gently until approximately 0.5ml of blood is obtained.

3. Remove the needle and syringe, and press a paper tissue on the skin surface for 30 seconds to stop bleeding.

4. Expel the blood sample into a 1.5-ml Eppendorf tube. Write the Darvic Ring Code of the bird on a piece of masking tape and adhere to the tube. Release the bird.

5. Once the whole session is over, immediately transfer all the samples to a freezer (-20°C).

6. For long-distance transport, the samples should be kept in an insulated cold box (4°C)

Only blood samples of Whooper Swans at Caerlaverock, Scotland,were taken by ourselves. The other samples were taken and sent to us by various collaborators. Pedigree data were obtained through direct field observation by the collaborators (see Acknowledgements).

Fig.2.2 Operation of bleeding swan.

2.3 EXTRACTION OF GENOMIC DNA FROM BLOOD SAMPLES

The most common method of DNA isolation is based on phenol extraction (Wallace 1987a). Phenol causes deproteination of solutions containing nucleic acids, so that the nucleic acids can be separated from the proteins by centrifugation. Proteinase can break polypeptides down into smaller units which are more efficiently removed by phenol extraction. Sodium dodecyl sulphate (SDS) is an ionic detergent, promoting the process of cell lysis by removing lipid molecules and causing disruption of the cell membranes. Ethylenediaminetetraacetic acid (EDTA) removes magnesium ions that are essential for the aggregation of nucleic acids to each other and to proteins as well. An extra benefit of EDTA and SDS is their inhibition of nucleases that degrade nucleic acids. Chloroform can denature proteins, and thereby improves the efficiency of nucleic acid extractions by combination with phenol. Also chloroform is ablé to remove the trace of phenol which is contained in the aqueous phase. The presence of isoamyl alcohol added to chloroform prevents foaming of the white coagulated mass which forms at the interface of the aqueous and organic layers. The recovery of nucleic acids from the aqueous solution is achieved by ethanol precipitation in the presence of 0.3M Na^+ (Wallace 1987b).

Protocol of DNA Extraction:

1.Dissolve 25µl of thawed blood into 500µl of 1X SET buffer (all reagents are listed in Appendix) in a 1.5-ml Eppendorf tube.

2.Add 15µl of Proteinase K (10mg/ml) and 8µl of 25% SDS to the solution. Incubate the mixture overnight in a 55°C waterbath after mixing vigorously.

NB: the following handling during DNA extraction should be carried out in a chemical fume hood with a protection screen.

3. Add an equal volume of phenol and mix gently by inverting the tube several times followed by vortexing on a rotary platform for 30 minutes.

4. Centrifuge the mixture at full speed in a microcentrifuge for 10 minutes. The aqueous solution (containing DNA) forms in the upper layer, separated from proteins and cellular debris contained in the lower organic phenol phase. Much of the proteins and debris actually aggregate at the interface to form the flocculent mass.

5. Carefully pipette the upper aqueous solution into a fresh tube without disruption of the interface. The sharp ends of pipette tips are cut off to avoid shearing high-molecular-weight DNA. Keep the volume to 500µl by adding TE buffer.

6. Repeat phenol extraction until the brown colour of the aqueous layer is completely removed. Usually three phenol extractions are sufficient.

7. Extract the aqueous solution once or twice with a mixture of phenol, chloroform and isoamyl alcohol (24:23:1, V/V/V), depending on the sharpness of the interface.

8. Extract the aqueous solution once with the mixture of chloroform and isoamyl alcohol (23:1, V/V).

9. To the aqueous solution add two volumes of cold (-20°C) absolute ethanol. Mix well by rigorously swirling and the DNA aggregates as a white fluffy mass.

10. Place the mixture in a -20°C freezer for 30 minutes and then centrifuge it at full speed for 10 minutes. The DNA pellet is visible at the bottom of the tube.

11. Remove the ethanol supernatant without disturbing the DNA pellet. Wash the pellet with plenty of 75% ethanol and vortex to remove any solute trapped in the precipitate.

12. Briefly centrifuge the tube for 2 minutes to resediment the pellet. Very carefully remove ethanol using a pipette because the pellet in 75% ethanol becomes free and easily flows away.

13. Seal the tube with Parafilm and penetrate with a needle. Then dry the pellet in a vacuum for 10 minutes.

14. Carefully peel off the parafilm and make sure that the pellet is still there. Resuspend the pellet in an appropriate amount (usually 150µl) of TE buffer, depending on the size of the pellet. Leave the tube overnight in a 55°C waterbath this temperature to dissolve, preventing the activity of nucleases that may be present.

Typically about 100µg of DNA can be obtained from 25µl of a blood sample of a swan and up to 72 extractions can be done within a single day by using the above method. The concentration of the DNA solution is measured using TKO 100 Mini-Fluorometer. DNA samples are labelled and stored at 4°C.

A common problem that happens in DNA extraction is the degradation of DNA (Fig.2.3), which could be caused by several factors. Namely, (a) repetitive freeze and thaw of blood samples can degrade DNA, with the result that the yield of intact DNA decreases considerably. To prevent this it is better to extract a large quantity of DNA once the blood sample thaws.

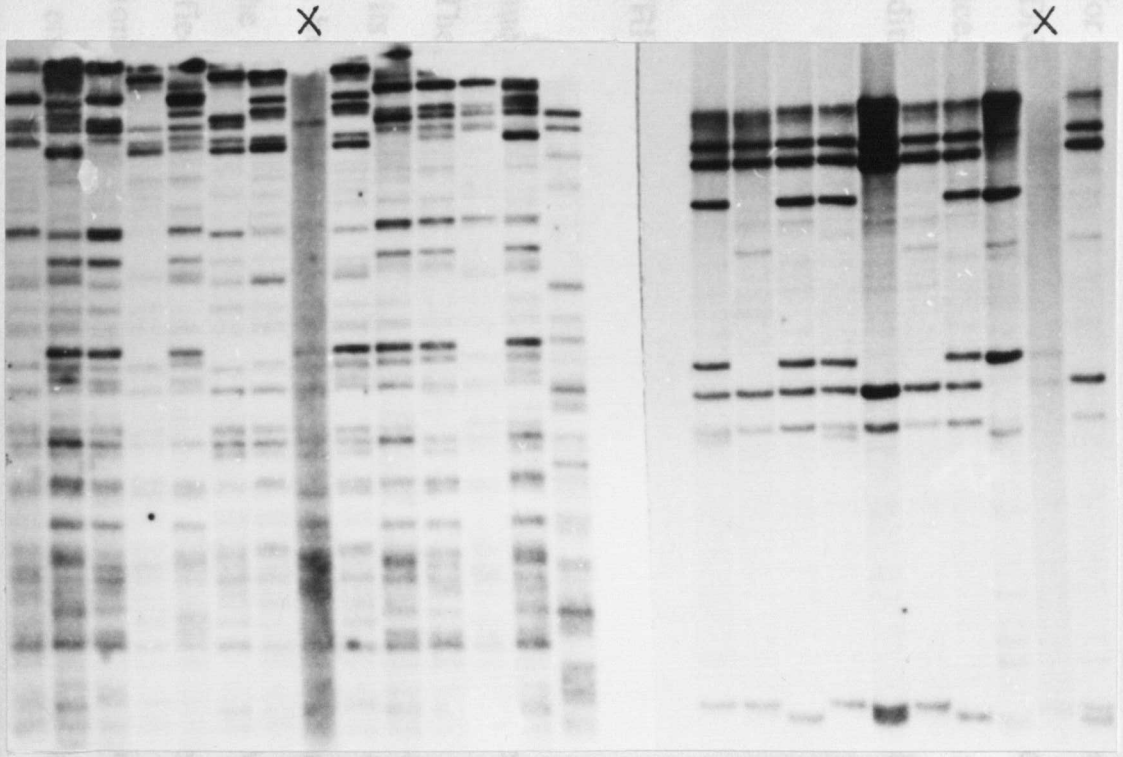


Fig.2.3 Degradation of genomic DNA leads to failure of DNA fingerprinting. The lanes marked with X contain a degraded DNA sample.

(b) Phenolic oxidation, indicated by yellow or pink colouration, produces quinones, diacids and others which cause cleavage of phosphodiester bonds and cross-linking of DNA strands. 0.1% 8-hydroxyquinoline (W/V) (an antioxidant) added to the stock phenol solution can prevent phenol from oxidation for several days. (c) High pH is a very important condition for extracting DNA. At pH5-6 DNA is selectively retained in the organic phase and interface, and depurination of DNA molecules takes place under acidic conditions. Therefore, a pH of 8 or higher is essential for DNA extraction.

2.4 RESTRICTION OF DNA

Endonucleases cleave a DNA molecule at a specific recognition sequence. The activity of such an enzyme is affected by three main factors apart from its own purity. These are the quality of target DNA, the composition of the reaction buffer and the temperature. Phenol-extracted DNA is sufficiently clean for the activities of most enzymes, though some (e.g. MspI) require highly purified substrates. Adequate functioning of an enzyme may require a certain ionic strength (provided by NaCl) and M^{2+} concentration. Nowadays, enzymes of high quality and appropriate buffers can be obtained from a commercial chemical supplier. Most enzymes perform best at 37°C.

Although one unit of enzyme can theoretically digest one microgram of DNA within an hour, in practice an excess amount of enzyme is added to the reaction mixture to ensure that the restriction is complete. The concentration of commercially supplied enzymes usually ranges from 5 to 10 units per microlitre. So one microlitre of enzyme is used to cut 3-10μg of DNA and the incubation lasts overnight. For the best separation and

resolution of restriction fragments 3-5μg DNA is digested per gel track for DNA fingerprinting. It is quite helpful to re-measure the concentration of DNA digests to achieve consistent loading of DNA over all slots in a gel. The presence of 4mM spermidine trichloride helps the restriction to completion.

Efficiency of restriction is improved by reducing the volume of reaction. This should be taken into account when resuspending the DNA pellet in TE buffer at the last stage of DNA extraction. A typical reaction for DNA fingerprinting is carried out in a volume of 20μl or 30μl.

Protocol for DNA Restriction:

1. Pipette the following components into an Eppendorf tube:

3-5μg DNA solution (about 15μl)

1μl enzyme (over 10 units)

2μl 10X reaction buffer

2μl 40mM spermidine trichloride

sterile distilled water (SDW) to 20μl

Briefly spin down. Mix by flicking the tube and spin down again.

When carrying out many digestions, a digestion stock, consisting of all the components except the DNA solution, can be made in advance in a tube and then an aliquot pipetted into each tube containing the DNA solution.

2. Incubate the mixture at the recommended temperature overnight.

3. Assay an aliquot (2μl) of the digest on a minigel (see 2.5) to monitor the progress of the reaction.

The absence of high-molecular-weight fragments indicates the completion of the reaction. The partially digested samples should be incubated for a further

4-6 hours after adding an extra microlitre of the enzyme. The minigel assay can also be used to estimate the concentration of the digests.

4. Measure the concentration using a fluorometer and calculate the amount that will be loaded into the maxigel.

5. Stop the reaction by adding 1/10 volume of 10X BPB and mix well.

If a double digestion is performed, only enzymes that require identical buffer and temperature can be simultaneously added to the reaction. Otherwise it should be done one by one. Two methods are employed in such a case. Restriction with an enzyme requiring lower ionic strength is carried out first, then the salt concentration of the reaction is adjusted to be suitable for the second enzyme by adding NaCl. Alternatively, DNA is precipitated with ethanol from the first digest and resuspended in TE buffer or water, followed by application of another enzyme and appropriate buffer.

2.5 ELECTROPHORETIC SEPARATION OF RESTRICTION FRAGMENTS IN AN AGAROSE GEL

DNA molecules carry negative electric charges. Therefore, DNA restriction fragments move towards the positive electrode in an agarose gel matrix where a current is applied. Gels of different concentrations are used to separate DNA molecules of different sizes (Maniatis *et al.* 1982). Resolving DNA fragments of high-molecular-weight requires a gel of lower concentration. The migration rate of the fragments in the gel is related to their length. The smaller the fragments, the quicker they can migrate through the gel. When stained with the intercalating dye ethidium bromide (EtBr), as little as 0.05µg of DNA in the gel can be visualized under

ultraviolet illumination (Sharp *et al* 1973). There are two types of running gel (maxigel and minigel, based on their capacity) used for DNA fingerprinting.

2.5.1 Maxigel

Horizontal gel electrophoresis apparatus is used for DNA fingerprinting. The gel tank, with electrodes at both ends, can hold 3 litres of electrophoresis buffer.A maxigel is made in a 24 X 20cm plastic plate, the open ends of which are sealed with masking tape. Loading slots are made by inserting a gel comb into molten agarose 2cm away from one end of the plate. 375ml of molten agarose can form a 0.75cm-thick gel through which DNA fragments can be efficiently transferred to a membrane.

Before loading, digested DNA samples are mixed with loading buffer consisting of Ficoll, EDTA, Xylene cyanol and bromophenol blue. EDTA inhibits the activities of endonucleases, Xylene cyanol increases the density of the DNA solution, the use of Ficoll avoids steaming up the samples caused by Xylene cyanol and the dye can indicate the position of DNA in the gel (Southern 1979). Heating the DNA digests just before loading at 65°C for ten minutes prevents DNA fragments from self-ligation of their sticky ends.

The running buffer used for DNA fingerprinting gel is TAE (40mM Tris-acetate, 0.2mM EDTA). When a large volume of buffer is used, it is not necessary to circulate or change the buffer during electrophoresis to cure so-called "buffer exhaustion". EtBr may be added to the buffer and included in the gel so as to locate positions of size markers on a transilluminator. However, the binding of the positively charged dye to DNA fragments will cause the decrease of the mobility and the effects are

differential on DNA fragments of different size classes, which reduces the resolution. In addition, EtBr is a powerful mutagen and thereby dangerous. Only when the visualization of a gel is imperative is EtBr applied.

The width of slots has something to do with resolution. Wider slots do give sharper bands rather than blobs, on which ease of scoring fingerprints relies. 5-mm slots are economical but 10-mm slots give much better resolution.

The voltage gradient has a considerable effect on separation and resolution (Southern 1979). A higher voltage gradient results in fast migration of DNA fragments, but the ratio differs between the fragments of different size classes such that smaller fragments gain more speed than larger fragments. Though a low voltage gradient combined with extension of the running period causes diffusion of small fragments, the resolution of large fragments is greatly improved, which is very important for DNA fingerprinting. For different species, specific conditions may be required. DNA fingerprints of swans contain many large minisatellite fragments (≥10Kb). Therefore, we usually run fingerprinting gels without EtBr for three days at a low voltage gradient (30-35 volts), which can be standardized as 2200-2500 volts.hours (i.e. V.H.).

Preparation of Maxigel and Electrophoresis:

1. Measure 60ml of 50XTAE and dilute to three litres with distilled water as running buffer. 150µl of 10mg/ml EtBr may be added (final concentration is 0.5µg/ml).

2. Weigh the correct amount of LE agarose and transfer to a 500-ml bottle.

3. Add 375ml of the running buffer to the bottle. Pour the remaining buffer into the gel tank.

4.Melt agarose in a microwave oven. Then place the bottle in a 55°C waterbath to cool down.

5.Seal both ends of a gel plate with masking tape and insert a comb in position.

6.Place the plate on an even bench. Shake the bottle containing the gel and pour it into the plate. Make sure no air bubble forms. The presence of air bubbles has a bad effect on the migration of DNA fragments during electrophoresis and on the transfer of DNA fragments during gel blotting.

7. When the gel has cooled to room temperature, remove the tape and place the plate into the gel tank. Remove the comb gently.

8. Heat DNA samples mixed with loading buffer at 65°C for 10 minutes and then quench on ice.

9. Carefully pipette the samples into loading wells. Load the appropriate λ restriction digest into a well as size markers.

10. Put the lid on. Do not apply a current until DNA solutions have settled down to the bottom of wells and are evenly distributed.

11. Carry out electrophoresis at an appropriate v.h.. The gel is then viewed and photographed on a UV transilluminator using a Polaroid camera, if EtBr is used.

2.5.2 Minigel

Minigels are used for rapidly analysing small quantities of DNA, for example, checking DNA extractions, monitoring the restriction progress and estimating DNA concentration. For most purposes a 0.7% agarose gel is used.

When assaying DNA molecules of high-molecular-weight (over 25Kb), gels containing less than 0.5% agarose are used. The minigel apparatus is effectively a maxigel apparatus on a much reduced scale. Running buffer is Tris-borate (TBE) instead of TAE, and 30ml of gel solution is directly poured into the minigel tank. Minigels usually run at 60-80 volts for one hour.

2.6 IMMOBILIZATION OF RESTRICTION FRAGMENTS

Preserving the relative positions of restriction fragments in the gel is the precondition for subsequent detection of particular fragments. This is conveniently achieved by the capillary transfer technique (Southern 1975), called "Southern blotting". DNA fragments that have been separated by electrophoresis in agarose gel can be capillary-transferred to membranes that efficiently bind nucleic acids, and permanently immobilized onto the membrane surface.

Nitrocellulose membrane, traditionally used for Southern blotting, binds single-stranded DNA molecules under high ionic strength conditions. Therefore, the capillary transfer of DNA molecules requires pretreatments of the gels. Two strands of double helix DNA can be separated by disrupting covalent bonds under alkaline conditions, and maintained separate by high salt concentration. Southern blotting makes use of this property by denaturing DNA in alkali solution followed by neutralizing the gel in a solution of high salt concentration. However, neutralization and transfer at high ionic strength allow partial renaturation of DNA molecules and consequently reduce the amount of DNA available for binding to nitrocellulose filter. In addition, the nitrocellulose has low mechanical strength,

requiring great precautions when handling it and making reprobing almost impossible.

Nylon membrane has been extensively exploited and has tended to replace the nitrocellulose filter in most cases, since it was first applied to Southern blotting in 1981. The main advantage of nylon is its high physical strength which makes multiple reprobing of blots possible. Furthermore, double-stranded (native) as well as single-stranded (denatured) DNA can bind to nylon filter in low ionic strength buffers (e.g. 0.4M NaOH) so that rapid blotting of the gels is possible and DNA fragments have little chance to diffuse before transfer (Reed and Mann 1985).

As large DNA fragments (≥10Kb) are transferred with low efficiency, whichever filter is used, it is necessary to break them into smaller pieces before transfer. Acid depurination (0.2M HCl) is the method of choice for this purpose (Wahl *et al.* 1979).

Both nitrocellulose and nylon filters have been successfully used for DNA fingerprinting, but the latter has dominated in this study since 1988. One big problem of the nylon filter Hybond-N (Amersham) is that its quality is not very constant. Two blotting methods are described here. Southern blotting is suitable for both types of filters, whereas alkali transfer is designed only for nylon filters.

Southern Blotting:

A.Pretreatments of gel

1. Following electrophoresis, the gel is inverted/placed into a tray and free-floated with 0.2M HCl. Leave for 25 minutes or until the dye in the gel has changed colour (from blue to yellow) with gentle agitation.

2. Remove acid solution. Wash the gel once with distilled water and then cover it with denaturing solution (1.5M NaCl, 0.5M NaOH). Leave for 40 minutes (30 minutes if using nylon) with shaking.

3.Pour off denaturing solution and wash with distilled water. Rinse the gel with neutralizing solution (3M NaCl, 0.5M Tris, pH 8.0) for 40 minutes (30 minutes if using nylon).

B. Capillary transfer to filter

1. Fill a tray with 200ml of 20X SSC (3M NaCl, 0.3M sodium citrate). The tray is crossed by a plexiglass plate which is slightly bigger than the gel. Place a long sheet of Whatman 3MM filter paper, saturated with 20X SSC, onto the plate. Both ends of the filter paper dip into the solution. Use a glass pipette to smooth out air bubbles trapped between paper and plate. Air bubbles hinder the passage of the transfer buffer and give hollow-patches in DNA fingerprints (Fig.2.4).

2. Place the gel straight on the paper. Avoid distorting the gel, otherwise non-interpretable fingerprints will be produced (Fig.2.4). Remove any air bubbles between the gel and paper.

3. Cut a sheet of membrane to the size of the gel (may be shorter). Place it on top of the gel and remove air bubbles. The nitrocellulose membrane is prewetted in 2X SSC or distilled water prior to contacting the gel. By contrast, most types of nylon membrane do not require the prewet step. Wear gloves to avoid greasing the membrane. Label the membrane with a graphic pencil.

4. Trim off the gel not covered by the membrane and surround the gel with cling film (Saran Wrap) to prevent the transfer buffer from being absorbed directly into the tissue paper stack above.

5. Place two sheets of 3MM paper, cut to the size of the gel or bigger and saturated with 2X SSC, onto the membrane. Remove air bubbles.

6. Place a stack of absorbent paper towels on top of the 3MM paper. Put a plate on it and then a 500-gm weight on the centre of the plate.

7. Allow the transfer to proceed overnight.

8. Dismantle the blotting apparatus. Wash the membrane in 2X SSC for 5 minutes or so to remove any adhering agarose.

If a nylon membrane is used, post-treatment by rinsing it in 0.4M NaCl for 20 minutes prior to equilibration in 2X SSC can improve the resolution of the hybridization pattern and increase hybridization efficiency.

When duplicate membranes from a single gel are required, the bidirectional transfer method is employed (Smith and Summers 1980). The procedure is similar to the above, except that the gel is sandwiched between two sheets of membrane and one stack of paper towels is placed at the bottom of the "sandwich" and one on its top. Tray and plate are not required. The transfer buffer is supplied only by the liquid in the agarose gel itself so that the diffusion of DNA fragments during transfer period is minimized. Equal transfer of DNA fragments in the gel to both filters is achieved by increasing the as much volume of samples as possible to fully fill each loading well in the gel.

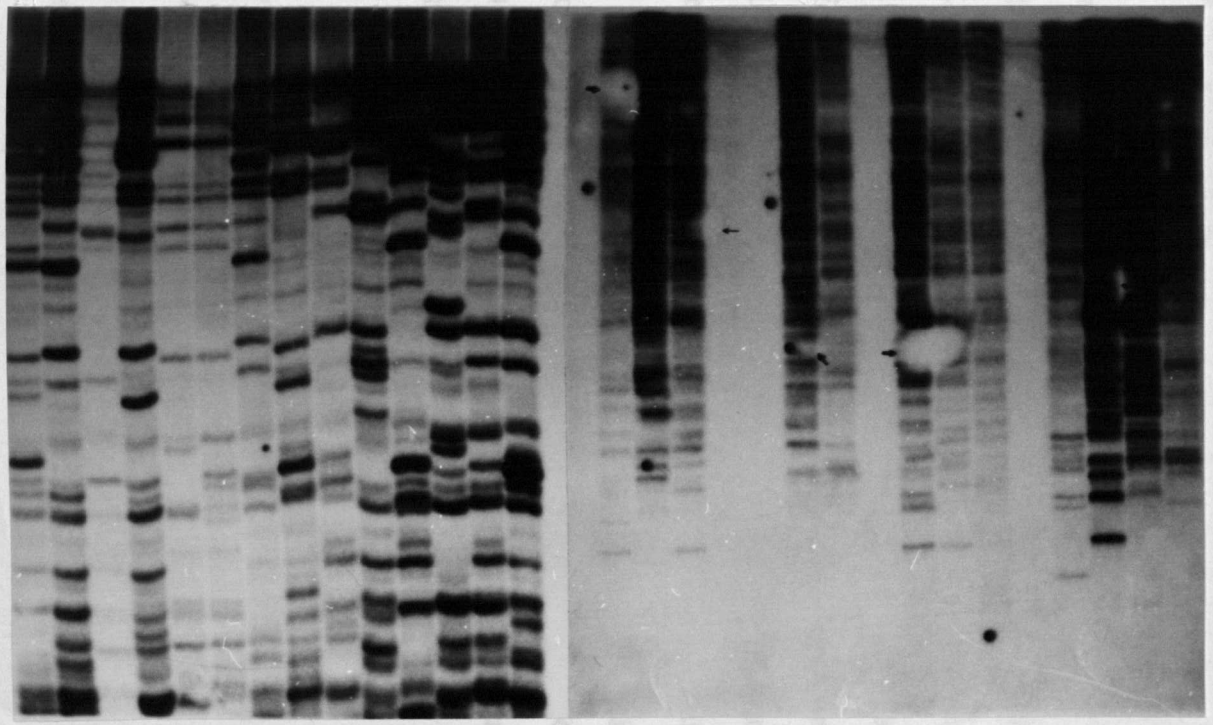


Fig.2.4 Unscorable DNA fingerprints. The left photo shows the effect of gel distortion during blotting, and the right shows hollow-patches (indicated by arrows) in an autoradiograph.

C. Fixation of DNA blots

Membranes are placed between two sheets of 3MM paper and baked at 80°C for two hours in a vacuum oven.

Alkali Transfer:

DNA fragments in the gel are first acid-depurinated followed by alkali denaturation, as by Southern blotting. Next, the gel is rinsed in alkali transfer buffer (1.5M NaCl, 0.25M NaOH) for 15 minutes. Then a transfer assembly is set up as for the Southern blotting using alkali transfer buffer instead of 20X SSC. Alkali conditions during the transfer promote the covalent fixation of transferred DNA to nylon membrane, in addition to maintaining denaturation status of DNA molecules. It has been reported that baking had no effect on binding and hybridization of DNA transferred in NaOH (Rigaud *et al.* 1987). So oven baking is only needed to dehydrate the membrane after alkali transfer. Usually a 30 minute baking is sufficient.

2.7 DETECTION OF FIXED DNA FRAGMENTS WITH RADIOACTIVE PROBES

2.7.1 Introduction

A restriction enzyme digests genomic DNA of higher organisms into hundreds of thousands of fragments of various sizes. After electrophoretic separation of the digest, particular fragments cannot be visualized by using the EtBr staining method. Therefore, it is necessary to develop a sensitive and reliable technique to identify specific sequences among DNA fragments immobilized on membranes. Autoradiography combined with DNA-DNA

or DNA-RNA hybridization provides an answer (Meinkoth and Wahl 1984). DNA molecules, labelled by incorporating nucleotides carrying a radioactive isotope, can hybridize to complementary sequences fixed on a membrane, and subsequent autoradiography visualizes the positions of the sequences on the membrane (blot). It involves two main steps: probe preparation and molecular hybridization.

2.7.2 Preparation of Probes

A. DNA probes prepared by nick translation

There are several method available to make "hot" probes, radiolabelled DNA molecules. In the first year of this study nick translation was used to radiolabel the human minisatellite probes contained in M13 RF. In a typical reaction of nick translation, a mixture of DNase and DNA polymerase I is added. The activity of DNase introduces nicks along a duplex DNA molecule randomly. At such nicks, DNA polymerase I of *E. coli* will successively incorporate nucleotides to replace the previous ones in the duplex (Kelly *et al.* 1970). If any of four nucleotides is radiolabelled prior to incorporation, the duplex will get labelled as well. Alpha-^{32}P labelled dCTP had been used to obtain a product of high specific activity since the minisatellites involved are GC-rich. By nick translation, double-stranded DNA can be labelled to a specific activity of approximately 10^8cpm/μg, and over 60% of radioactive precursor nucleotides are incorporated into the products (Rigby *et al.* 1977). An advantage of nick translation is that the sequences of the substrate are uniformly labelled. However, the hybridization signal from the nick-translated probes is weak because of probe/probe renaturation

during hybridization and the presence of a large proportion of non-insert sequences.

Protocol for Probe Preparation by Nick Translation:

1. Extract minisatellite-containing M13 RF DNA as described in Chapter 6.

2. Thaw 400Ci/mmol [α-^{32}P] dCTP at room temperature.

3. Set up reaction mixture in a 1.5-ml Eppendorf tube using the Nick Translation Reagent Kit supplied by BRL:

5µl solution A2 (0.2mM each of dATP, dGTP and dTTP)

5µl DNA polymeraseI (0.4 Units/µl)/DNaseI (40 pg/µl)

1µg probe DNA

4µl [α-^{32}P] dCTP

Increase the volume to 50µl with SDW

4. Mix gently and then incubate at 15°C for one hour.

5. Add 50µl of stop dye buffer. Take out 2µl and mix with 2ml of scintillation solvent (Escoscin or Emulsifier-Safe™) in a cuvette.

6. Make a chromatography column by filling a 1-ml syringe, plugged with glass wool, with TE-equilibrated Biogel P-60. Using a 15-ml plastic tube as an adaptor, spin down the column at 2,000rpm for a few seconds to drain water in the gel away.

7. Pipette the reaction solution into the column. Centrifuge at 2,000rpm for 30 seconds. The labelled DNA molecules are filtered into the plastic tube and unincorporated nucleotides are detained in the column.

8. Wash the column with 20µl of TE several times until the blue dye in the column has vanished.

9. Collect the probe solution into a fresh Eppendorf tube. Take out 2μl to dissolve in scintillation solvent as before.

10. Use the aliquots taken before and after the probe separation to count their radioactive intensity in a Scintillation Counter. Calculate the incorporation rate of radioactive nucleotides as follows:

$$R = 100\, V_2C_2 / V_1C_1$$

where V_1 and V_2 are the total volume of probe solution before and after separation, respectively, and C_1 and C_2 are the corresponding counts.

If R is less than 20%, the probe is not good and every component in the reaction should be tested before setting up a repeat reaction.

11. Denature the probe by boiling it for 5-10 minutes just before adding it to the hybridization solution.

B. RNA probes (riboprobes) prepared by *in vitro* transcription

Bacteriophage-encoded RNA polymerases can only recognize specific promoters contained in the phage DNA and initiate the transcription (Butler and Chamberlin 1982, Chamberlin *et al.* 1983). In the past few years many vectors containing phage promoters have been developed. The promoters flank the polylinker region into which an insert can be cloned. The insert DNA can subsequently be transcribed *in vitro* into single-stranded RNA in the presence of Mg^{2+} and ribonucleoside triphosphates by using relevant phage-encoded RNA polymerase (Tabor and Richardson 1985, Krieg and Melton 1987, Little and Jackson 1987). In order to obtain radioactive RNA probes of high specific activity, the vector is first digested with a restriction

enzyme to generate a run-off fragment only containing the promoter and insert. So only the insert will be transcribed into RNA.

Riboprobes have several advantages over nick-translated DNA probes. Firstly, the specific activity of an RNA probe can be ten times that of a DNA probe. Secondly, RNA probes are single-stranded , eliminating the denaturation step required for DNA probes, and preventing probe/probe hybridization. Another advantage is that RNA-DNA duplexes are more stable than DNA-DNA duplexes (Casey and Davidson 1977), allowing a high stringency wash to be performed. All these together give a higher signal:noise ratio, in other words, lower background. However, *in vitro* transcription requires highly purified templates, and the products are more sensitive to degradation. The incorporation rate of α–^{32}P ribonucleotides depends on the ratio of RNA polymerase to the amount of DNA templates. The incorporation rate is normally around 30-80%.

The human minisatellites 33.6 and 33.15 were released from M13 RF and subcloned into in vitro transcription vectors pSPT19 and pSPT18 respectively (Carter *et al.* 1989). Only the minisatellites can be transcribed into RNA from either promoter by digesting the vectors with appropriate enzymes (see Fig.2.5). The subcloned minisatellites are referred to as pSPT19.6 and pSPT19.15, respectively, or pSPT18.6 and pSPT18.15.

Protocol for RNA probe Preparation:

SP6/T7 Transcription Kit supplied by Boehringer Co. or Promega had been used. In this study, the T7 promoter was used.

1. Digest pSPT19.6 with EcoRI or pSPT18.15 with HindIII.

2. Set up reaction mixture in an Eppendorf tube:

1µg cut DNA

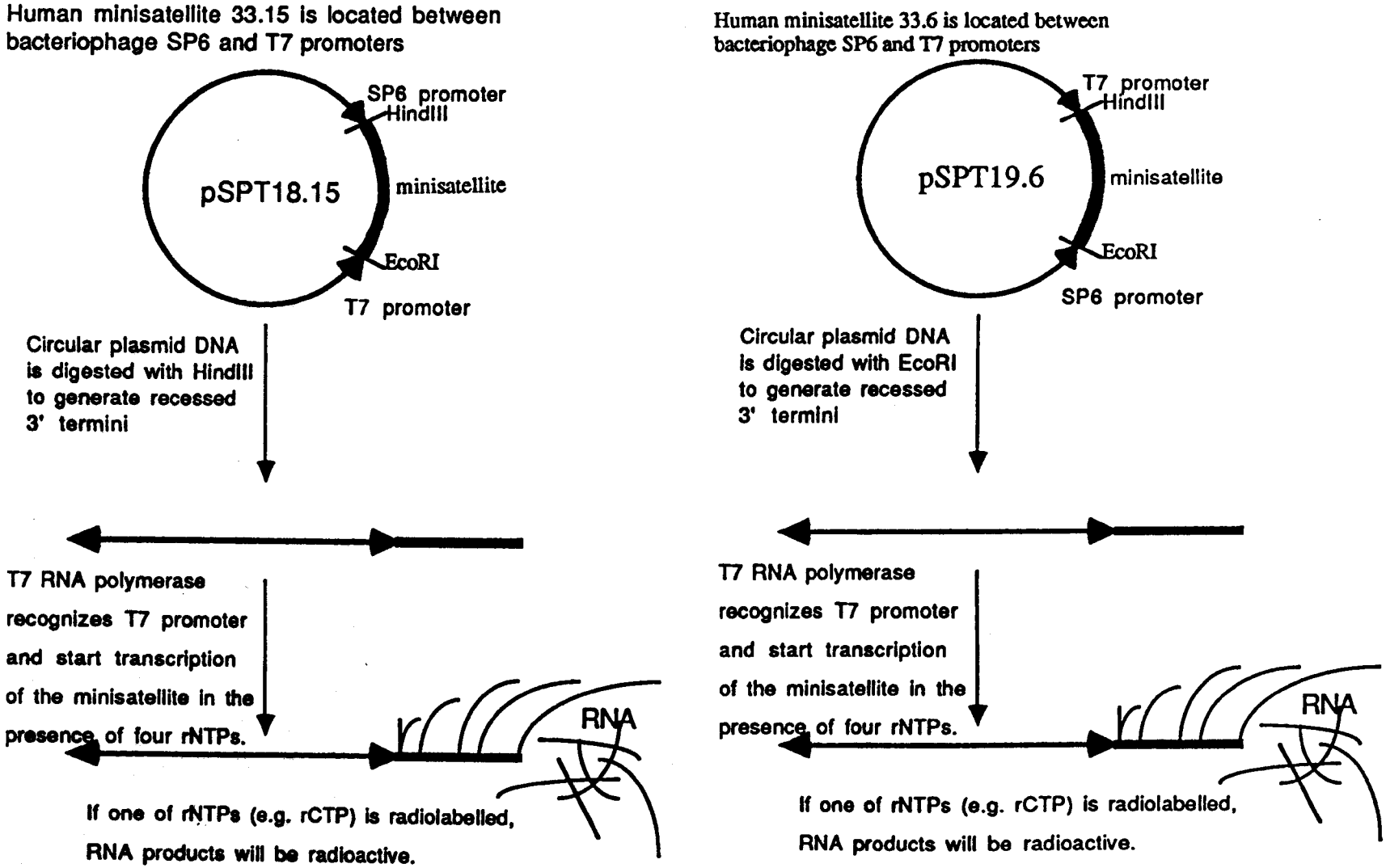


Fig. 2.5 Labelling of DNA fingerprinting probes pSPT19.6 and pSPT18.15 by *in vitro* transcription.

1µl each of ATP, GTP and UTP used
2µl DTT (dithiothreitol, an antioxidant to stabilize enzymes)
1µl RNasin
1µl T7 polymerase
5µl 3000Ci/mmol [α-^{32}P]CTP
SDW to 20µl

3. Mix gently and then incubate in a 38.5°C waterbath for one hour.

4. Stop the reaction by adding 20µl of stop dye buffer.

5.Separate labelled RNA molecules from unincorporated ribonucleotides as described for DNA probe preparation.

6. Measure the radioactive intensity of products and calculate R as described previously.

2.7.3 Filter Hybridization

Radiolabelled probe DNA or RNA can bind to nonspecific nucleic acid binding sites on filters containing immobilized DNA, causing much "noise" and reducing the hybridization signal. Denhardt (1966) first designed a mixture of substances to eliminate nonspecific filter-binding of probes. The mixture, referred to as " Denhardt's solution", consists of 0.2% each of Ficoll, polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA). These substances are able to effectively bind to the areas where no DNA molecule was bound to on the filter. Two-hour incubation of the filters in Denhardt's solution prior to adding probes significantly reduces the

background. Recently it has been proved that nonfat milk powder also effectively blocks the nonspecific binding of probes (Johnson *et al.* 1984).

When hybridizing, an excess amount of probes is added to the hybridization solution. The rate of hybridization between the probe molecules and DNA molecules retained on the filters should follow first-order kinetics. In all cases, overnight (12-16 hours) hybridization is sufficiently long for hybrid formation. The formation of hybrids is a reversible process. The stability of hybrids is affected by many factors, such as base composition of the probe, ionic strength of solution, probe length and so on. Where hybridization is to detect sequences identical to the probe, it must be performed under the most stringent conditions,for instance, low salt concentration plus high temperature. Since the aim of DNA fingerprinting is to obtain as many informative bands as possible, less stringent conditions are used to allow hybrids between the probe minisatellites and related sequences to form. However, the number of bands in a DNA fingerprint must be controlled to maximize the resolution of the fingerprint. For example, it is impossible to accurately score a panel of DNA fingerprints, each containing more than 30 bands in a length of 20cm. The degree of homology between the human minisatellites and minisatellites of other species is variable, so much so that the stringency has to be modified accordingly. The most convenient approach is to perform hybridization under low stringency (e.g.1X SSC)and then wash at increasing stringencies. SDS included in wash solution acts to remove nonspecifically bound probes. It has been shown that hybridization and wash in 1X SSC at 65°C is appropriate for most avian species in the case of DNA fingerprinting. For fingerprinting swans, a higher stringency (0.5X SSC) is used.

The probe concentration in the hybridization solution is crucial to the hybridization signal, and to the ratio of signal to background. Too much

probe can cause high background. On the other hand, an insufficient amount of probe results in weak signal and consequently autoradiography takes a long time. 10^6cpm/ml of nick-translated probe or $1.2X10^5$ cpm/ml of RNA probe is used for DNA fingerprinting.

Hybridizations were done initially in Hybaid hybridization bags (Amersham) and then in a sandwich box instead. Here only the latter is described since the supplier's instructions can be followed when using the Hybaid bags.

Hybridization Protocol:

1. Prepare alternative hybridization solutions in a bottle as follows:

a. Denhardt's hybridization solution

5 X Denhardt's solution	using 50 X stock
5 X SSC	using 20 X stock
1% SDS	

b. Blotto hybridization solution

1 X Blotto	using 10 X stock
1 X SSC	using 20 X stock
1% SDS	

NB: Denhardt's solution was abandoned when blotto was introduced.

2. Warm the solution in a 65°C waterbath until SDS completely dissolves. Then pour it into a sandwich box.

3. Immerse filters in the solution one by one. The volume of solution should allow the filters to free-float.

4. Place the box in a 65°C shaking waterbath. Place a weight on the box. Incubate for at least 2 hours when using the Denhardt's, but 6 hours for the Blotto solution. This process is called prehybridization.

5. Pipette the required amount of RNA probe or denatured nick-translated DNA probe into the solution. Incubate it overnight at the same temperature with constant shaking.

6. Pour off the hybridization solution and replace it with a large volume of wash solution (1X SSC, 0.1% SDS) prewarmed to 65°C. Leave for 10 minutes at 65°C with shaking.

7. Pour off the wash solution and replace with a fresh one. This time leave for 40 minutes at 65°C with shaking.

8. Repeat step 7 once.

9. Wash the filters with 0.5X SSC, 0.1% SDS solution for 40 minutes at the same temperature. Repeat once (optional).

10. Briefly rinse the filters in 0.5X SSC and then place them on tissue towels.

11. Wrap the filters with Saran Wrap while they are still damp.

Using the sandwich box hybridization, as many as 30 blots can be probed simultaneously provided that the amount of the probe is sufficient. In such a case, blots should be turned over when adding the probe and when washing, to avoid sticking and causing some 'patchy' background.

2.7.4 Autoradiography

β-radiation emitted from ^{32}P-labelled nucleotides incorporated in the probes is able to expose X-ray film. Consequently, restriction fragments hybridized to the probe will show up on the autoradiograph. The blots are

placed at the bottom of a cassette. then two intensifying screens and a preflashed X-ray film are placed against the blots. Exposure is carried out at -80°C. Damp screens or cassette and water drops on the blots generate artefactual local blackening. The best resolution comes about by autoradiographying without screens at room temperature, but the exposure period required is much longer.

The exposure period of film is inversely proportion to the radioactive intensity (counts per second, cps) of blots. The relationship listed in Table 2.1 is only an empirical estimate.

2.7.5 Deprobing of Filters

Nylon filters can be probed several times without significant loss of fixed genomic DNA. To remove probes bound to a filter, incubate the filter at 45°C for 30 minutes in 0.4M NaOH followed by incubation in 0.1X SSC, 0.1% SDS, 0.2M Tris-HCl (pH 7.5) at 45°C for another 30 minutes. Alternatively, boil 0.1% SDS solution and then immerse the filter . Allow the solution to cool down to room temperature.

Table 2.1 Relationship between radioactive intensity and exposure length

Radioactive intensity of blot (cps)	Exposure length	
	Double screens	Without screens
≤2	one week	
2-5	3-5 days	
5-15	1-3 days	10 days
15-50	6-16 hours	one week
>50	1-6 hours	2-5 days

CHAPTER 3
CHARACTERIZATION OF SWAN DNA FINGERPRINTS

3.1 HYPERVARIABILITY OF DNA FINGERPRINTS OF SWANS

3.1.1 Optimal Restriction Enzymes for DNA Fingerprinting

It has been revealed that human and bird minisatellites consist of a number of conservative repeat units arranged in tandem (Jeffreys *et al.* 1985a, Gyllensten *et al.* 1989). The repeat units constituting minisatellites of the same family share a consensus sequence, referred to as 'core sequence'. Therefore, a segment of DNA as a probe containing multimers of core sequence is able to cross-hybridize with many minisatellites in the genome under low stringency of hybridization. To separate the minisatellites from the other flanking sequences, restriction enzymes are used. Two important aspects should be considered in the choice of enzymes for DNA fingerprinting. The first consideration is the recognition sequence of the enzymes. Digestion of genomic DNA with an enzyme that has recognition sites within the core sequence will result in the destruction of the minisatellites. So a precondition is the absence of recognition sites within the core sequence. Another consideration is the cutting frequency of the enzymes. Theoretically, enzymes recognizing longer sequences (e.g. hexanucleotides) cleave a long random DNA sequence less frequently and produce longer restriction fragments on average, provided that all bases are equally frequent. The minisatellite-containing fragments produced by such enzymes might include a long flanking sequence which obscures real variation of minisatellites. By contrast, a tetranucleotide target occurs more

frequently in the region immediately adjacent to a minisatellite, and could reveal the true variation of minisatellites.

Fig. 3.1 shows DNA fingerprint patterns of 5 individuals randomly chosen from a population of Mute Swans (*Cygnus olor*) at Abbotsbury, England. Six restriction enzymes were used: AluI, HaeIII, HinfI, TaqI, EcoRI and HindIII. The first four are 4-bp enzymes and the remaining are 6-bp enzymes. The human minisatellite probe pSPT19.6 hybridized to a large number of restriction fragments, whichever enzyme was used, under low stringent condition (0.5X SSC). It suggests that swan minisatellites are to a certain degree similar in sequence to the human minisatellites. However, different enzymes give different DNA fingerprint patterns, resulting from variation in the flanking sequences of minisatellites.

As demonstrated in humans (Jeffreys *et al.* 1985a). and in the Willow Warbler (Gyllensten *et al.* 1989), point mutations do occur within repeat units of minisatellites. Thus restriction sites could be created or destroyed within the minisatellites and thereby affect the DNA fingerprint pattern. It is to be expected that the hexanucleotide-recognition enzymes EcoRI and HindIII will release entire minisatellites with long tail sequences (possibly including structural genes), and indeed the resultant minisatellite-containing fragments are larger and less variable compared with those produced by the tetranucleotide enzymes AluI, HaeIII and HinfI. However, DNA fingerprints generated by the hexanucleotide enzymes are not scorable because of the presence of too many bands. TaqI-DNA fingerprints are similar to EcoRI- and HindIII-DNA fingerprints, suggesting that the TaqI recognition sequence (A/GTC) is less frequent in the genome of swans than the other tetranucleotide enzymes or that the digestion was only partially completed. All in all, it is better to use enzymes that cut frequently for DNA

fingerprinting. For this reason and in consideration of cost, HaeIII and HinfI are extensively used in this study.

3.1.2 Measurement of Variation in DNA Fingerprints

A DNA fingerprint consists of a number of bands, which brings about a lot of statistical problems. The positions of bands in the autoradiographs reflect the sizes of the corresponding minisatellite fragments. So the resolution of bands, on which the scoring of DNA fingerprints relies, is most important. All the DNA fingerprints were scored from original autoradiographs taken at various exposures. The autoradiographic intensity of the bands depends on the size and copy number of the minisatellites as well as the degree of homology between probe and minisatellite. Therefore, a band found in two individuals is scored as identical only when its electrophoretic mobility and intensity in both individuals are indistinguishable. One band may represent an allele at a heterozygous locus or two alleles at a homozygous locus. Segregation analysis of minisatellite fragments in human and canine pedigrees has disclosed that most of the parental fragments are derived from heterozygous loci (Jeffreys *et al.* 1986, Jeffreys and Morton 1987). In the absence of family data, each band will be regarded as an allele to facilitate the following formulations.

Similarity Coefficient:

Lansman and co-workers (1981) used the proportion of fragments shared in mtDNA digestion profiles (F) as an index of relative genetic similarity between populations of rodents and other mammals. It has been shown that F can be used to estimate similarity of DNA fingerprints between

two individuals (Wetton and Parkin 1989). The similarity coefficient is given by

$$F = 2 N_{AB}/(N_A+N_B)$$

where N_A and N_B are the numbers of bands present in individuals A and B, respectively; and N_{AB} is the number of bands shared by both. F varies from zero to one. As the minisatellite fragments are inherited in a Mendelian fashion and the heterozygosity at minisatellite loci is generally high, F is expected to be around 0.5 for first-order relationships and 0.25 for second-order relationships, and so on. However, the true values of F are usually higher than expected due to chance comigration of unrelated minisatellites and the presence of homozygous minisatellite loci in the genome.

Probability of Band Sharing:

The mean probability that individual A shares a band with another individual B can be expressed as

$$x = (N_{AB}/N_A+N_{AB}/N_B)/2$$

Assuming that bands shared by A and B always represent identical alleles of the same minisatellite locus, and the population has reached Hardy-Weinberg equilibrium, then x equals the sum of frequencies of 'genotypes' ++ (q^2) and +- ($2q-2q^2$), that is, $x=2q-q^2$. So the mean allele frequency can be estimated as follows:

$$q = 1-(1-x)^{1/2}$$

The mean heterozygosity (Ht) at minisatellite loci is given by $1-\alpha q^2$, where α is the number of alleles. If the variation of allele frequencies is small, $\alpha=1/q$. So

$$Ht = 1-q = (1-x)^{1/2}$$

Identity Probability of DNA Fingerprints:

Obviously, the probability that all the fragments (n) detected in one individual are present in another individual is x^n or F^n. However, the scored

fragments only account for a proportion of the minisatellites present in the population. The total number of minisatellite fragments from which a particular DNA fingerprint is derived can be estimated as n/x. Thus, the eoretical probability that two individuals randomly chosen have identical DNA fingerprints is $x^{n/x}$. This probability is maximized since the heterogeneity of band sharing for different size classes of bands will reduce it.

Jeffreys *et al.* (1985c) deduced that the chance of band sharing between sibs is $(4+5q-6q^2+q^3)/4(2-q)$. The probability that two sibs have identical DNA fingerprints could be given by $[(4+5q-6q^2+q^3)/4(2-q)]^{n/x}$.

3.1.3 Individual-specific DNA Fingerprints

As shown in Fig.3.1, there is no band present in all five birds randomly selected when the 4-bp enzymes are used, suggesting that the minisatellite fragments of swans are also polymorphic. The invariable bands detected in digests of less frequent enzymes might represent conserved minisatellite or result from chance cosegregation of the fragments.

The number of bands detected per individual varies, and is,on average, approximately 33 for the AluI-digests, 32 for the HaeIII-digests and 31 for the HinfI-digests in the size range greater than 3Kb. The difference in band number is not significant ($P>0.05$) between enzyme treatments. The autoradiographic intensities of bands are heterogeneous.

Using the measurements discussed in 3.1.2, the mean probability of band sharing, heterozygosity and similarity coefficient were computerized and are listed in Tables 3.1a and 3.1b.

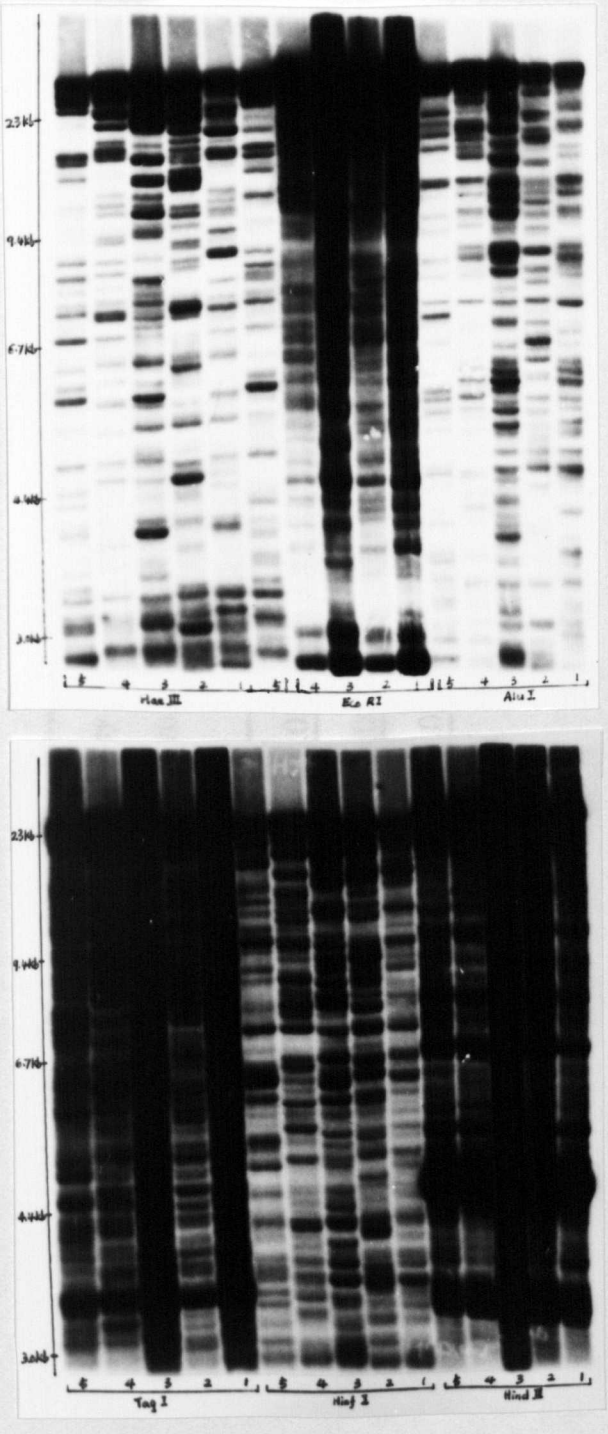


Fig.3.1 DNA fingerprints generated with different enzymes. Approximately 4 μg of genomic DNA digests from 5 random birds of the Mute Swan were separated by electrophoresis and blotted onto nylon membranes. The blots were then probed with pSPT19.6 under a stringency of 0.5XSSC. Size markers are indicated as Kilobases (Kb).

Table 3.1a Variation of DNA fingerprints among random birds of the Mute Swan *C. olor*, based on Fig.3.1

Enzymes	No. bands per bird (sem)	Probability of band sharing (sem)	Maximum mean allelic frequency	Minimum heterozygosity (%)
AluI	33.2(1.1)	0.372(0.038)	0.208	79.25
HaeIII	32.2(2.5)	0.373(0.059)	0.208	79.16
HinfI	31.2(1.3)	0.290(0.037)	0.157	84.26

a: sem= standard error of the mean.

Table 3.1b Similarity coefficients between pairs of birds, based on Fig.3.1

Pairs	Enzymes used		
	AluI	HaeIII	HinfI
1-2	0.333	0.133	0.419
1-3	0.514	0.406	0.355
1-4	0.485	0.491	0.305
1-5	0.349	0.545	0.351
2-3	0.314	0.351	0.353
2-4	0.364	0.358	0.215
2-5	0.444	0.215	0.159
3-4	0.257	0.394	0.277
3-5	0.269	0.464	0.222
4-5	0.381	0.323	0.233
Average	0.371	0.368	0.289
(sem)	(0.027)	(0.039)	(0.026)

Similarity coefficients are variable among different pairwise comparisons, but are low on average. Surprisingly, the similarity coefficients for some pairs vary a lot between different enzymes. For example, F between birds 1 and 2 is 0.419 using HinfI, and reduces to 0.133 using HaeIII. The same holds for pairs 1-3, 1-4, 1-5, 2-5 and 3-5. This could be explained if a minisatellite present in the genomes of some birds contains internal restriction sites of a given enzyme, so that it is cleaved into several fragments that will be shared by these birds, giving values of F that are superficially high. On the other hand, another enzyme cannot cut this minisatellite and results in relatively low values of F. Such enzyme-dependent variability of minisatellites makes it more difficult to study population genetics by using the DNA fingerprinting technique.

Nevertheless, the minisatellites of swans are highly variable. The enzymes used reveal a minimum heterozygosity of around 80% at the minisatellite loci of the Mute Swans. The probability that two random birds have identical DNA fingerprints (x^n) is $5.52X10^{-15}$ with AluI, $1.67X10^{-14}$ with HaeIII and $1.7X10^{-17}$ with HinfI. Obviously, no one could find two swans in the world that have identical DNA fingerprints, considering the current population. Even the chance that two sibs share a DNA fingerprint is very small. By using HaeIII, for instance, this chance is only $2.33X10^{-6}$. Thus it can be seen that swan DNA fingerprints are of individual specificity and can be used to positively recognize individual birds.

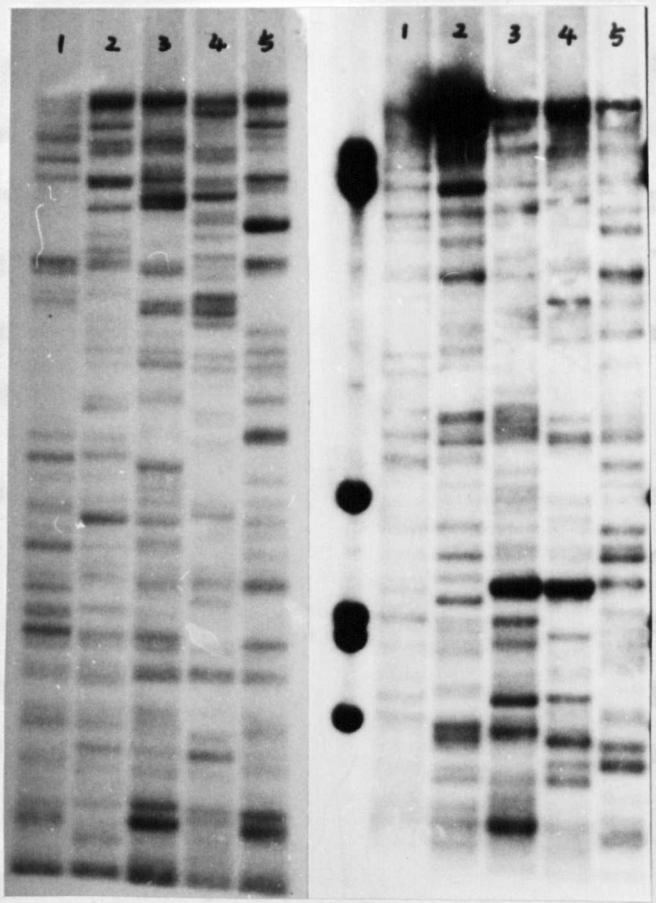
3.1.4 Additional Polymorphic Bands Detected by pSPT18.15

Another human polycore minisatellite probe pSPT18.15 also detects

hypervariable bands in swans (Fig.3.2). The average number of bands detected by pSPT18.15 is 35.4, more than that (31.2) detected by pSPT19.6; but the difference is not statistically significant ($P>0.05$). Approximately 60% of the resolved bands in pSPT19.6 DNA fingerprints are also present in those of pSPT18.15. The high proportion of codetection is likely to result from the chance codetection of different fragments that have similar sizes. Nevertheless, the two probes together detected 48 distinct scorable bands per individual. So the simultaneous use of two or more probes can greatly reduce the chance that two individuals have identical DNA fingerprints.

3.2 INHERITANCE OF DNA FINGERPRINT BANDS

The term 'DNA fingerprint' uses the word 'fingerprint' to express its great variation. Unlike the loops and whorls on a human fingerprint in the usual meaning, each band in an individual DNA fingerprint, except for the occasional mutant, can be found in either or both parents' DNA fingerprints, suggesting that DNA fingerprint bands descend from one generation to the next. Other studies have shown that DNA fingerprint bands are inherited as simple Mendelian characters (Jeffreys *et al.* 1986, Jeffreys and Morton 1987, Burke and Bruford 1987, Meng *et al.* 1990). However, the segregation characteristics of DNA fingerprint bands may change depending on pedigrees or species. Here the inheritance of DNA fingerprints of Whooper and Mute Swans are examined.



pSPT19.6 pSPT18.15

Fig.3.2 Comparison of DNA fingerprints generated by probe pSPT19.6and pSPT18.15. Approximately 6 μg of HaeIII digests from 5 random birds of the Mute Swan were electrophoresed on a 0.8% agarose gel for 3 days and then bidirectionally blotted to two sheets of nitrocellulose membrane. One blot was probed with pSPT19.6, and the other with pSPT18.15, all of which performed under a stringency of 0.5X SSC. The λ EcoRI fragments, shown up in the middle lane after hybridization with radiolabelled whole λ DNA, were used as size markers.

3.2.1 DNA Fingerprint Analyses of Two Large Sibships of Whooper Swan *Cygnus cygnus*

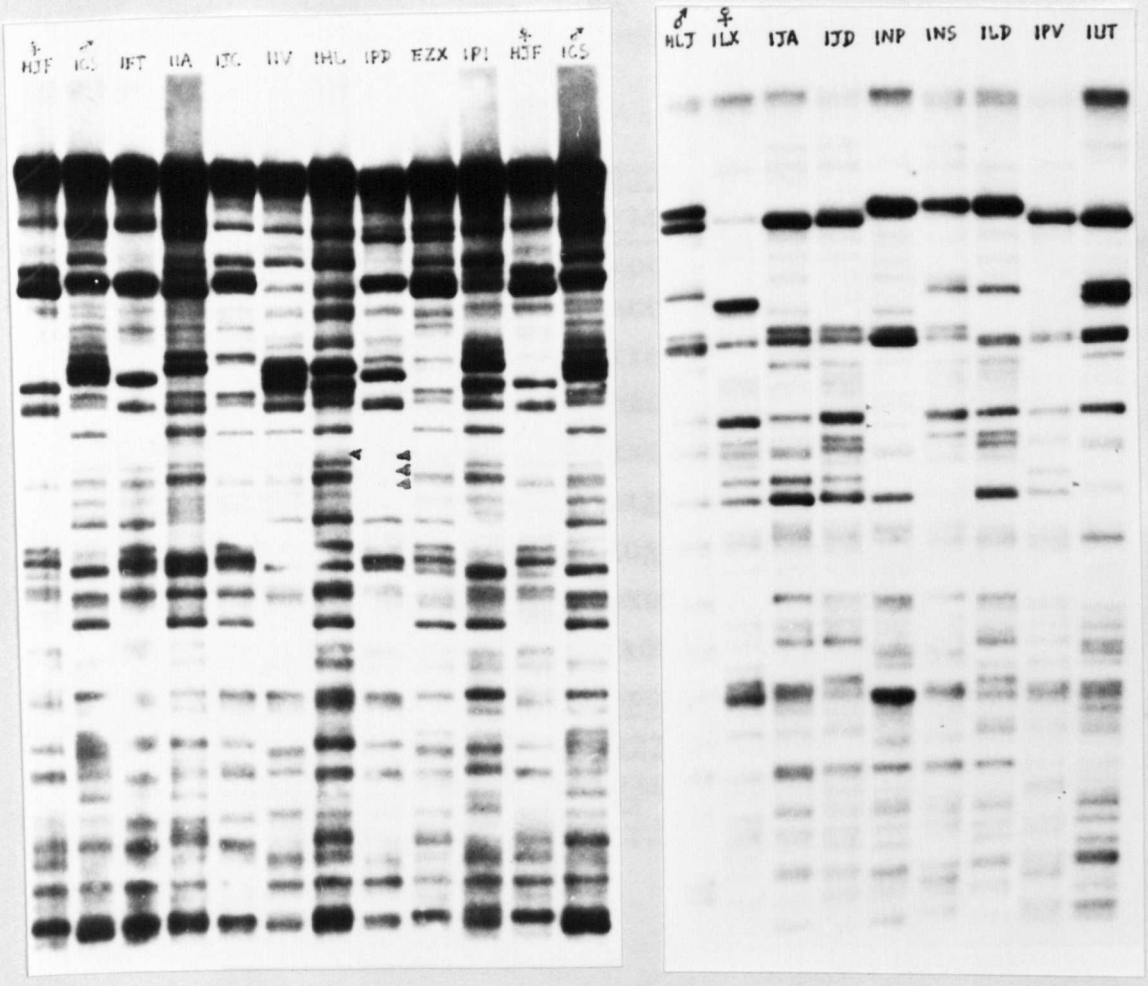
To study the inheritance of swan minisatellites, two large families of Whooper Swan at Caerlaverock, Scotland, were chosen. Male adult ICS (Darvic ring code) and his mate HJF had 5 cygnets (young) in 1986 and 3 in the following year, constituting Family A. The other pair of adults HLJ (male) and ILX (female) had 5 young in 1986 and two more in 1987, and this is Family B.

Fig.3.3 shows DNA fingerprints of the two families, generated by probing HinfI digests with the probe pSPT19.6. Resolved bands were diagramed in Figs.3.4 and 3.5. The Similarity coefficient (F) is 0.19 between ICS and HJF, and 0.218 between HLJ and ILX, suggesting that the parents in each family are not closely related. F values between the father or mother and cygnets as well as between cygnets are all around 0.5 (see Table 3.2), consistent with expectations.

As expected, most of the bands present in the cygnets can be traced back to one or both parents' DNA fingerprints except that a few novel bands (marked as arrowhead in Fig. 3.3 and underlined in Figs. 3.4 and 3.5) appeared. The shared bands between father and mother are excluded from the analyses. Only one paternal band in Family A and one maternal band in Family B (marked with '£') are transmitted to all the offspring, which thus represent homozygous loci. All the other bands represent an allele at a heterozygous locus. The average transmission rate of paternal or maternal bands (exclusive of linked bands) is approximately 50% (Table 3.3), consistent with 1:1 segregation. The number (r) of cygnets receiving each heterozygous band in a sibship of n should follow the binomial distribution

with the probability $P_r={}^nC_r/2^n$. Statistical tests (Table 3.3) confirm that the segregation of heterozygous parental bands is compatible with the binomial distribution ($P<0.01$). Obviously, these heterozygous minisatellites could not be derived from one chromosome, but are scattered over the whole genome, supported by detailed studies of minisatellite locations in human and mouse genomes (Jeffreys *et al.* 1987, Royle *et al.* 1988).

Only one allele was resolved at most of minisatellite loci in these two families, suggesting that the two alleles may be extremely different in size. A study in humans, using the locus-specific minisatellite probe pλg3, indeed showed that HinfI alleles at this locus varied from 1.7 to 20.4Kb in length and only 40% of individuals could have both alleles resolved in their hybridization patterns (Wong *et al.* 1986). Few allelic or linked bands have been found through all possible pairwise comparisons of parental heterozygous bands (Figs. 3.4 and 3.5). For example in Family A, the 10th and 17th bands (paternal) cosegregated into the same offspring, showing tight linkage; on the other hand, both bands are allelic to the 7th band. It is unknown whether the linked bands represent separate minisatellites on the same chromosome or are derived from a single minisatellite containing internal HinfI recognition sites. By pooling the data, the total number of minisatellite loci from which a DNA fingerprint is sampled is approximately 70 (Table 3.4). Since a large number of hypervariable minisatellite loci can be potentially detected by a single polycore probe, DNA fingerprinting may greatly facilitate linkage analysis and genomic mapping. In fact, it has been successfully used for searching for genetic markers linked to some disease loci (Jeffreys *et al.* 1986).



Family A

Family B

Fig.3.3 DNA fingerprints of the Whooper Swan families A and B. HinfI digests of genomic DNA were probed with pSPT19.6 under a stringency of 0.5XSSC. Individuals are identified with the Darvic Ring Codes. Father and Mother are indicated as ♂ and ♀, respectively. Mutant bands are marked with arrowheads.

Fig.3.4 Diagram of segregation of parental bands in Family A,based on Fig.3.3 . The presence/absence of bands are indicated as x/o. Linked pairs of parental bands that segregate xx/oo into offspring are connected by a continuous line; allelic bands that segregate xo/ox are connected by a dotted line; and homozygous band(s) that transmits to all offspring are marked with '£'. Bands underlined are mutant bands that are present in neither of the parental DNA fingerprints.

Band no.	00000000011111111112222222222333333333	
Birds	12345678901234567890123456789012345678	
HJF	xxoooxooxoxoooooxooxooxooxooxoxoxxoxxxoxx	(mother)
IFT	xoxoooxxooxoxoooooxoooxooxooxxoxxxoooxoxo	
IIA	oxoxxxooxxoxxxooxooxoxxoxoxoxxooxxxoxxxx	
IJC	xoxoxxxxooxoxxoxoooxxoxoxooxxxoxxxoxxxxo	
IIV	xoxoxooxoxoxooxoxxxxoxxoxooxxxxooxoxxoox	
IHL	xoxoxooxoxoxooxoxoxxxoxxxoxoxoxxoxooxxxx	
IPD	oxoxooxoooxoooooxoxoooxoxoxoxxoxoooxoxxoxx	
EZX	oxoxxxxoxooxxooxooooxxoxoxoxxxoxxxoxooxxx	
IPI	ooxoxxoxoxoooxxoxoxxoxxoxoxoxxooxxxoxxxx	
ICS	oxxxxoxxoxoxxxxoxxoxxoxoxoxoxoxooxooxxox	(father)
	00000000011111111112222222222333333333334	
	12345678901234567890123456789012345678901	

Fig.3.5 Diagram of segregation of parental bands in Family B,based on Fig.3.3 . All the symbols are the same as used in Fig.3.4.

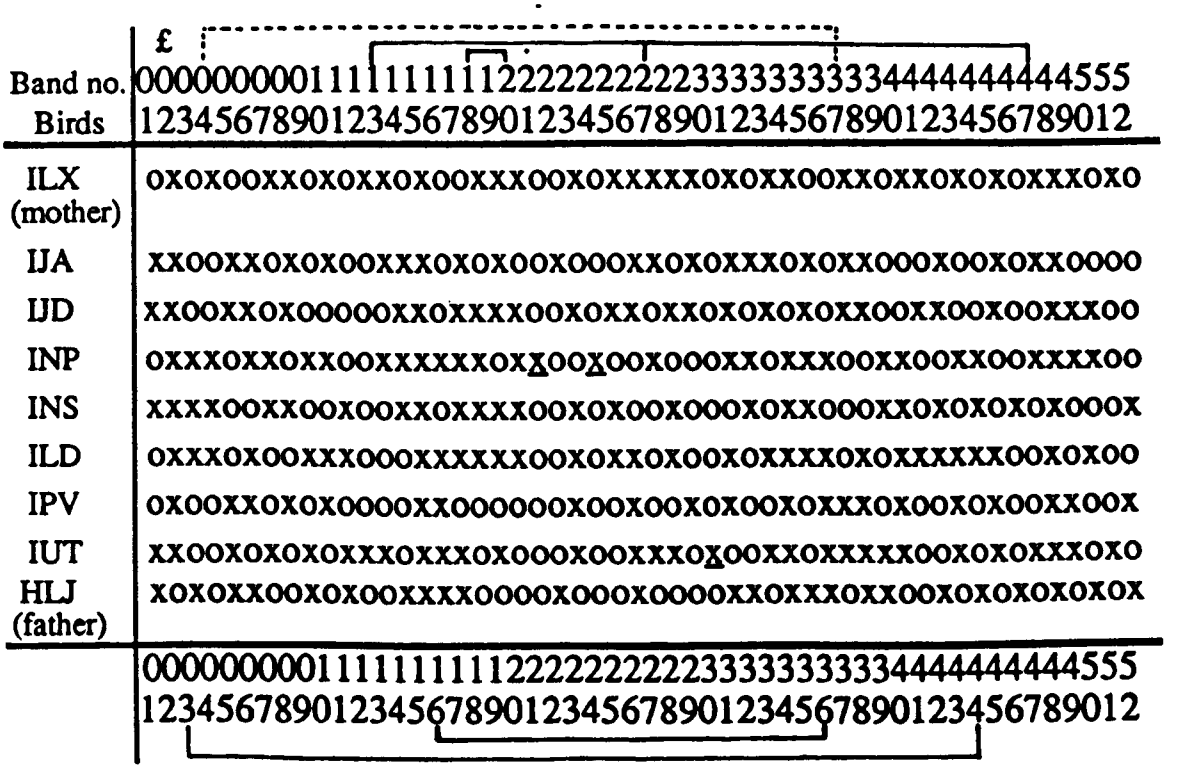


Table 3.2 Similarity coefficients (F) for different relationships in the Whooper Swan families

Families	Relationships	Range	mean	No. comp	SD
	Mother/father	0.190	0.190	1	
	Father/cygnets	0.450-0.766	0.621	8	0.124
	Mother/cygnets	0.341-0.571	0.495	8	0.076
A	Cygnets/cygnets	0.300-0.783	0.544	28	0.134
	Mother/father	0.218	0.218	1	
	Father/cygnets	0.478-0.607	0.541	7	0.056
	Mother/cygnets	0.500-0.700	0.609	7	0.074
B	Cygnets/cygnets	0.340-0.731	0.546	21	0.093

Table 3.3 Segregation of heterozygous parental bands in the Whooper Swan families

Transmission to no. Cygnets (r)	Family A				Family B			
	Paternal		Maternal		Paternal		Maternal	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
0	0	0.06	0	0.06	0	0.13	0	0.16
1	0	0.50	0	0.44	2	0.93	2	1.09
2	0	1.75	1	1.53	1	2.79	1	3.28
3	5	3.50	5	3.06	6	4.65	5	5.47
4	5	4.38	4	3.83	5	4.65	8	5.47
5	3	3.50	3	3.06	2	2.79	4	3.28
6	3	1.75	0	1.53	1	0.93	0	1.09
7	0	0.50	1	0.44	0	0.13	(0)*	0.16
8	(0)*	0.06	0	0.06				
Transmission rate % (sem)	53.1 (3.5)		49.1 (4.2)		48.7 (4.6)		50.7 (3.8)	
Statistical test χ^2	4.56 N.S.		4.22 N.S.		3.29 N.S.		5.12 N.S.	

Assuming that each parental band is transmitted to a cygnet with a probability of 0.5, then it is transmitted to r cygnets in a sibship of n with a probability of ${}^nC_r/2^n$ following the binomial distribution. The observed number of parental bands transmitted to precisely r cygnets is shown for both parents and agrees with the expected segregation patterns. If a pair of heterozygous bands is linked, only one of them is included in the analysis.

*: The band transmitted to all the cygnets is treated as homozygous and hence is ignored in the analysis.

Table 3.4 Summary of DNA fingerprints in the two Whooper Swan families

Families	Parents	No. bands scored n	No. linked pairs b	No. allelic pairs a	No. loci scored L	Total no. loci estim. N
A	Father	24	3	4	17	
	Mother	18	0	1	17	
B	Father	25	2	1	22	
	Mother	30	2	1	26	
Mean		24.25	1.75	1.75		70

Assuming an entire DNA fingerprint, including unresolved (unscored) bands, is derived from N heterozygous loci (2N bands), then the probability that one band is allelic to a band x is 1/(2N-1). Furthermore, provided that (n-b) bands resolved are a random sample of the 2N bands, the probability that a resolved band is allelic to band x is (n-b-1)/(2N-1). So the total number of bands that could be allelic to the others among the resolved bands is (n-b)(n-b-1)/(2N-1), that is, the number of allelic pairs a in the scored DNA fingerprint is (n-b)(n-b-1)/2(2N-1). Thus the number of loci N can be given as

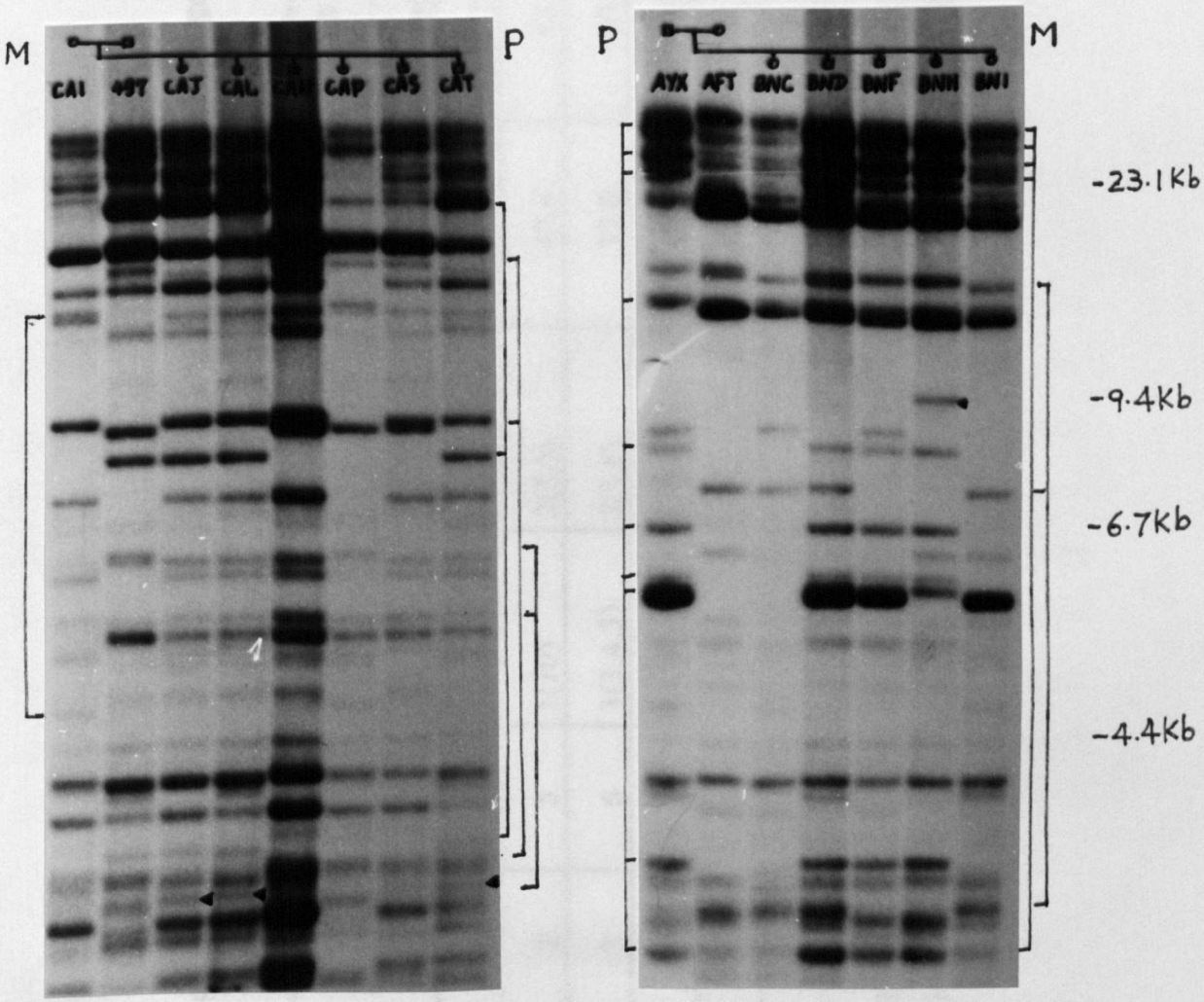
$$N=[1+(n-b)(n-b-1)/2a]/2$$ (J.F.Y.Brookfield, pers.comm.)

Note that the presence of homozygous bands in a DNA fingerprint causes underestimation of N.

3.2.2 Abundant Linkage in HaeIII-DNA Fingerprints of Mute Swans

During DNA-fingerprinting of 16 families of the Mute Swans sampled at Lothian, Scotland, using enzyme HaeIII and probe PSPT19.6, it has been found that linkage groups of parental bands are extensively present in all of five large families which contain both parents and 5-8 cygnets (Table 3.5). Each linkage group consists of 2-10 bands. On average, the linked bands account for about 32% or 38.7% of total paternal or maternal bands, respectively. Also, when HaeIII-digests of DNA from all members of the Whooper Swan Family A were probed with pSPT19.6, ~~fewer~~ bands and slightly more linkage were detected in their subsequent DNA fingerprints compared to their HinfI-DNA fingerprints. It seems that swan minisatellites contain more internal HaeIII sites than HinfI sites. However, small sizes of the sibships might have led to an overestimation of the level of apparent linkage.

Fig.3.6 presents DNA fingerprints of two Mute Swan families as examples. Interestingly, 10 paternal bands in Family 2 cosegregated into the same offspring and hence showed apparent linkage. If all of these bands arise by the cleavage of a single minisatellite, then this minisatellite could be over 120Kb long, being out of the scope of minisatellites. It is more likely that they are derived from a minisatellite cluster or a satellite. Its true nature could only be seen when its structure and organization become clear by cloning and sequencing or internal mapping (Jeffreys *et al.* 1990). Nevertheless, the presence of large linkage groups will reduce the informativeness of DNA fingerprints.



Family 1 Family 2

Fig.3.6 Large linkage groups in the Mute Swans. The birds from two families of the Mute Swans at Lothian, Scotland, were DNA-fingerprinted with HaeIII/pSPT19.6. A number of parental bands in both families are cosegregated into the offspring and hence show linkage. Linked bands are connected by a continuous line. 'M' indicates a mother, 'P' indicates a father. Mutant bands are marked with arrowheads.

Table 3.5 Linkage data from HaeIII-DNA fingerprints of five large Mute Swan families

Families	No. sibs	No. of linkage groups		% of linked bands	
		Paternal	Maternal	Paternal	Maternal
1	7	2(2,2)	5(2,2,2,2,2)	16.7	45.5
2	5	1(10)	2(5,3)	47.6	42.1
3	6	3(3,4,3)	2(2,6)	37.0	40.0
4	8	2(2,3)	2(2,2)	29.4	19.0
5	7	2(4,3)	2(2,6)	29.2	47.1
Average		2	2.6	32.0	38.7

All birds were fingerprinted with pSPT19.6 under the stringency of 0.5X SSC. The numbers in parentheses indicate the number of bands involved in each linkage group.

3.3 STABILITY OF DNA FINGERPRINTS

Minisatellites consist of repeat units arranged in tandem. The presence of the core sequence in the repeat units gives a good opportunity for sister chromatids or homologous chromosomes to form misalignments in pairs at mitosis or at meiosis. As a result, novel minisatellites with changed repeat number might arise through chance crossing-over (Smith 1976, Jeffreys *et al.* 1985a). This speculation has been verified by the finding that hypervariable regions related to minisatellite probe 33.15 are clustered at or around autosomal chiasmata and within the pairing region of XY bivalent at meiosis in man (Chandley and Mitchell 1988). Similarity between the minisatellite core sequence and χ sequence of *E. coli* also suggests that the core sequence acts as an eukaryotic recombination signal for homologous recombination (Jeffreys *et al.* 1985a). Wolff *et al.* (1988) argued that not only unequal crossing-over but also replication slippage or deletion and gene conversion could play a role in the maintenance of minisatellite hypervariability. In all events, the multi-allelism of minisatellites must be associated with a high frequency of spontaneous mutation to new length alleles.

3.3.1 Somatic Stability

To investigate possible somatic changes of DNA fingerprints, genomic DNAs were extracted from blood and various tissues of a Whooper swan killed by foxes, and digested with AluI and HaeIII. The subsequent blot was probed with pSPT19.6. As shown in Fig.3.7, DNA fingerprints from different sources of DNA are almost indistinguishable, except that a single extra band is present in the DNA fingerprints of blood and lung. It is

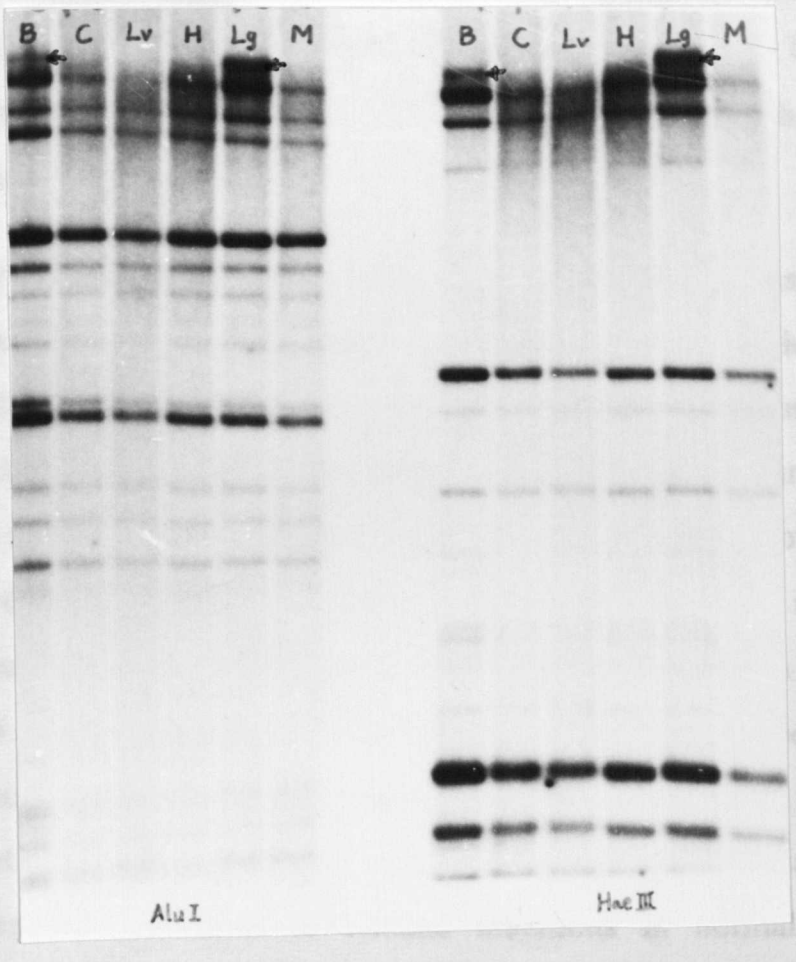


Fig.3.7 DNA fingerprints from blood and various tissues. In winter 1988, a Whooper Swan was found dead in the winter site at Caerlaverock, Scotland, which was probably killed by foxes. Some extravasated blood (B), whole heart (H) and crop (C), a piece of lung (Lg) and liver (Lv) as well as muscle (M) were collected. For extraction of genomic DNA from various tissues, approximately 2g of deep frozen tissues in liquid nitrogen were ground to a powder and then resuspended in 0.5ml of TE buffer followed by normal extractions as from blood samples. About 3µg of each DNA digest was separated on a 0.8% agarose gel for three days and then blotted onto nylon membrane. DNA fingerprinting was done with pSPT19.6 as usual.

unlikely that the presence of this band is due to partial digestion of genomic DNA or tissue-specific methylation of DNA, since both enzymes gave this band and they are non-sensitive to CpG methylation. It is not clear whether this band is somehow related to pathogenesis. As the blood sample was taken from extravasated blood in close vicinity to the lung, this novel band may be only associated with abnormalities within the lung. The progenitor of the novel band, if there is any, is absent in the resolved size range; but the new band probably arose by expansion of the presumptive progenitor minisatellite in the number of repeat units.

Other researchers have found that somatic changes at minisatellite loci are common in DNA fingerprints of human tumours, which include intensity alterations of bands, appearance of novel bands and size alterations arising by contraction or expansion of pre-existing bands compared to those in normal tissues (Thein *et al.* 1987, Armour *et al.* 1989). However, no changes were observed between DNA fingerprints from blood and normal tissue adjacent to the tumour (Thein *et al.* 1987); somatic mutation was rarely detected in lymphoblastoid cell lines (Armour *et al.* 1989). It is believed that somatic mutations could be detected only if mutant cells have made up a significant proportion of the cell population under examination. Nevertheless, the incidence of somatic mutations in normal tissues, if they really occur, could be very low, presenting no problem for reproducibility of DNA fingerprints and linkage analysis.

3.3.2 Germ-line Stability

The application of DNA fingerprinting to establishing relatedness between individuals relies on the stability of the bands representing

minisatellite alleles from one generation to the next. Therefore, it is necessary to know the mutation rate of DNA fingerprint bands in the species of interest.

A large number of full families are essential for the investigation of the mutation frequency. In our study, only two species of swans, *C.cygnus* and *C. olor*, have met this. In the Mute Swan, members of 15 families containing 57 cygnets were fingerprinted using the enzyme HaeIII and the probe pSPT19.6. A total of 10 novel bands were detected in 8 sibships (for examples see Fig.3.6), giving a mutation frequency of 0.007. In the Whooper Swan, 19 novel bands (for examples see Fig.3.3) in HinfI digests were detected by pSPT19.6 in 11 sibships out of 17 families (54 cygnets) screened, giving a mutation frequency of 0.0126. The mutation frequency of Whooper Swan minisatellites is higher than that of Mute Swan minisatellites. Since the detection of mutations to new length alleles is independent of enzymes used (Armour *et al.* 1989), the results are comparable. It has been noted in man that the mutation rate of different minisatellite loci increases with their variability (Jeffreys *et al.* 1988a, Armour *et al.* 1989). So we can predict that the level of variation of Whooper Swan DNA fingerprints is higher than that of Mute Swan, which will be justified by the data presented in the next chapter.

3.4 CONCLUSION

The human minisatellite polycore probes pSPT19.6 and pSPT18.15 can cross-hybridize to a large number of fragments in genomic DNA digests of swans. These swan minisatellites are extremely polymorphic, showing heterozygosities of 80% or so and constituting individual-specific genetic

fingerprints. As predicted, the best DNA fingerprints result from restriction digests with such tetranucleotide recognition enzymes as AluI, HaeIII and HinfI. Pedigree analyses show that the minisatellite fragments of swans are distributed over numerous autosomes and inherited as simple Mendelian characters, although some linkage and allelism exist. Somatic changes reflected in DNA fingerprints may occur with low frequency; however, DNA fingerprint bands have high mutation rates, which is important for the maintenance of the great variation of minisatellites. The incidence of mutation of the fingerprint bands varies depending on species, implying that species with higher frequencies of novel bands could possess more variable minisatellites. The features of DNA fingerprint suggest its potential for the unambiguous recognition of individual birds and establishment of lineage relationships between individuals, which could bring about a revolution in the areas of behavioural, ecological and evolutionary genetic studies in animals. Such applications will be discussed in the following two chapters.

CHAPTER 4

GENETIC VARIATION IN NATURAL POPULATIONS OF SWANS

4.1 INTRODUCTION

It was found some decades ago that genetic variation exists in natural populations of various organisms. The early studies, concentrating upon easily identifiable morphological or physiological variants, failed to estimate the overall genetic variation in populations because of limited variable characteristics. The technique of protein electrophoresis developed in the middle 1960's opened up a new approach for the estimation of genetic variation from many more loci defined as the structural genes encoding enzymes. Unfortunately, the number of proteins that can be easily examined is small. Ten years later, the progress in the techniques of genetic engineering allowed genetists to directly study the genetic material, genomic DNA, and to disclose large amounts of DNA polymorphisms that might provide a more unbiased estimation of genetic variation in the genome of the natural populations. For instance, restriction fragment length polymorphisms (RFLPs) have been detected in the genomes of various organisms. RFLPs provide an unlimited source of genetic markers for identifying the individual variants in a population, to define the populations within a species, and to quantify interspecific variation. However, such use of RFLPs is curbed by their low heterozygosity in a population. By contrast, the variation revealed by DNA fingerprinting is so great that the DNA fingerprints are unique to individuals in most (if not all) species that have studied so far (Jeffreys *et al.* 1985b, Jeffreys and Morton 1987, Wetton *et al.* 1987, Burke *et al.* 1989,

Georges *et al.* 1988). DNA fingerprinting has proven very powerful for identifying individuals and for assigning parentages. As a new technique, however, its full advantages have not yet been exploited.

In the previous chapter, the general features of Swan DNA fingerprints have been discussed based on the Mute and Whooper Swans. This chapter will concentrate on genetic variation at minisatellite loci in natural populations of several species of swans. The polycore minisatellite probes are able to detect repeat-sequence length variants at a large number of loci dispersed throughout the genome, offering an unbiased estimation of genetic variation for this type of sequences in natural populations. Therefore, the genetic variation between populations, as well as between species, will be studied by means of DNA fingerprinting.

4.2 INTRAPOPULATION VARIATION OF DNA FINGERPRINTS

4.2.1 Experimental and Analytical Considerations

To estimate the variation of DNA fingerprints in natural populations, a large number of samples should be taken. The results presented here were obtained by fingerprinting 15 birds from each population of swans. This number just fits the maxigel size (16 slots). If they were on separate gels, pairwise comparisons between samples would be too difficult to conduct. The populations studied are as follows: Bewick's Swans (*C. bewickii*) at Slimbrige, England; Mute Swans (*C. olor*) at Abbotsbury, England; Trumpeter Swans (*C. buccinator*) in Montana, the United States; and Whooper Swans (*C. cygnus*) at Caerlaverock, Scotland. According to the field observations, the birds that were chosen are not closely related to one

another. Since the number of bands present in the DNA fingerprints varies slightly with batches of hybridization, probably due to inconsistent quality of the probe and minor changes of wash conditions, all the samples from the same population, after restriction with an appropriate enzyme, were electrophoresed on a single gel and blotted to a sheet of nylon membrane. The conditions for restriction, electrophoresis and blotting held the same for all the populations. The subsequent hybridization and wash of the blots were carried out simultaneously. Through these measures, the experimental errors could be minimized and the results should be comparable.

Those measures discussed in the third chapter will be used to quantify the degree of variation of DNA fingerprints among individuals within the same population. As mentioned previously, the scoring of DNA fingerprints relies greatly on the quality of the autoradiographs. The faint bands may be obscured due to very dark neighbouring bands or degradation of DNA. Therefore, only clearly visible bands were scored. Identical bands are defined as those that are indistinguishable in electrophoretic mobility and are of similar autoradiographic intensity. Because of the difficulty of comparing two distant gel tracks, only ten adjacent tracks out of fifteen were scored. The accuracy of the scoring might compensate for the decrease of the sample size.

4.2.2 Results and Discussion

Figs. 4.1-4.5 present DNA fingerprints for the four species of Swan. The Jeffreys' polycore probes detect many restriction fragments on each individual (Table 4.1). The variation in autoradiographic intensity implies that the degree of homology between the detected minisatellites and the probe sequence is heterogeneous. The mean number of bands detected with

pSPT19.6, in HaeIII-DNA fingerprints of Bewick's, Mute, Trumpeter and Whooper Swans is 22.3, 27.5, 22.2 and 21.6, respectively. When the same probe hybridizes to HinfI digests, the number of bands is not changed in all the species, suggesting that the minisatellite variants in swans mainly arise from variation in copy number of repetitive sequence. However, the other probe pSPT18.15, detects many more minisatellites on HinfI digests than does pSPT19.6. Family analyses have revealed that most of these minisatellites independently segregate in the pedigrees (Chapter 3, Meng *et al.* 1990). The minimum number of loci under investigation could exceed the number of resolved bands in a given DNA fingerprint, since only one of alleles at most of minisatellite loci is resolved and the resolved alleles are derived from a pool of minisatellite loci in the genome. Thus, the multilocus minisatellite probes give population geneticists an opportunity to look at genetic variation in the whole genome in natural populations.

DNA fingerprints show great variation among individuals within a population,so that each bird has a unique pattern of banding. There is no band shared by all of the 15 birds either in Bewick's, Mute or Trumpeter Swans. The Whooper Swans show similar variation, except for a single band present in all the fingerprints (marked with arrowhead in Fig.4.4). It is suggested that almost all of the minisatellite loci detected by pSPT19.6 are polymorphic. By contrast, using protein techniques surveys of dozens of loci among 103 avian species revealed that the proportion of loci polymorphic (frequency of most common allele $\leq$ 0.99) averaged 0.240, ranging from zero to 0.714 (Evans 1987). Clearly, DNA fingerprinting probes are extremely powerful for identifying a vast number of polymorphic loci in the genome at the population level.

The mean similarity coefficient (F) between individuals is 0.248 in the Bewick's, 0.404 in the Mute, 0.442 in the Trumpeter and 0.276 in the

Whooper, when DNA fingerprints are generated with the combination of pSPT19.6 and HaeIII. These values further show that swan minisatellites are highly variable. Similar results are obtained from pSPT19.6/HinfI fingerprints. Table 4.2 lists the similarity coefficients or band sharing probabilities (x) within populations of various organisms studied by other workers. Apart from those species with high inbreeding coefficients such as Naked Mole-rats and Foxes, all the other species exhibit hypervariable DNA fingerprints within populations, supporting the view that the minisatellites are the most variable sequences in the genomes of higher organisms.

However, similarity coefficients differ in the different populations of swans studied here. For instance, F for the Trumpeter Swans is almost twice as high as that for the Bewick's Swans. What forces explain the evolution of the minisatelites at the population level?

Mayr (1963) classified sources of genetic variation for a population as follows: a, particulate inheritance; b, mutation; c, gene flow from other populations; d, occurrence of new genotypes through recombination. The particulate inheritance means that the frequency of genes in a population remains constant in the absence of selection, of nonrandom mating, and of random genetic drift (Hardy-Weinberg Law). Mutations, generated through external inducers or through internal recombinations, provide the ultimate source of genetic novelties, which can be maintained in the population through inheritance. Migration of individuals among populations results in movements and incorporaton of new alleles. On the other hand, selective mating, natural selection and genetic drift each operate against the unlimited accumulation of genetic diversity in a given population. We shall take into account these aspects when comparing the populations of swans.

Table 4.1 Summary of DNA fingerprints in four species of swan

Probes	Enzymes	Species	No. bands (sem)	F (sem)	x (sem)	q
pSPT19.6	HaeIII	Bewick's	22.3(0.96)	0.25(0.012)	0.25(0.009)	0.134
		Mute	27.5(1.00)	0.40(0.014)	0.41(0.010)	0.230
		Trumpeter	22.2(0.49)	0.44(0.017)	0.44(0.012)	0.254
		Whooper	21.6(0.76)	0.28(0.016)	0.28(0.011)	0.150
pSPT19.6	HinfI	Bewick's	17.6(0.833)	0.19(0.013)	0.19(0.010)	0.102
		Mute	24.6(0.777)	0.29(0.043)	0.29(0.033)	0.165
		Trumpeter	19.0(0.843)	0.32(0.047)	0.32(0.033)	0.174
		Whooper	18.1(1.37)	0.27(0.041)	0.28(0.029)	0.151
pSPT18.15	HinfI	Bewick's	32.0(0.577)			
		Mute	31.9(0.857)			
		Trumpeter	30.0(0.751)			
		Whooper	32.4(1.000)			

F=mean similarity coefficient, x=mean probability of band sharing, q=maximum mean allelic frequency, sem=standard error of the mean. All the values are based on analysis of 10 individuals.

Table 4.2 Intrapopulation variability of DNA fingerprints in eukaryotes

Organisms	Probes[a]	Enzymes	F[b]	x[c]	Referees
Humans	.6	HinfI		0.21	Jeffreys *et al.* (1985b)
	.15	HinfI		0.21	Jeffreys *et al.* (1985b)
	3'HVR	HinfI		0.22	Fowler *et al.* (1988)
Dog	.6/.15	HinfI		0.46	Jeffreys and Morton (1987)
	M13	HaeIII		0.43	Georges *et al.* (1988)
H. sparrow	.6	HaeIII	0.185		Wetton (*pers. comm.*)
Sparrowhawk	.6	HaeIII	0.350		Carter (*pers. comm.*)
Swallow	.6	HaeIII	0.116		Wellbourn et al. (*in prep.*)
Dunnock s	.15	AluI		0.24	Burke et al. (1989)
Cat	.6/.15	HinfI		0.47	Jeffreys and Morton (1987)
Cattle	M13	HinfI		0.35	Georges et al. (1988)
	3'HVR	HinfI		0.33	" " " "
Horse	M13	HaeIII		0.46	" " " "
Pig	M13	HaeIII		0.56	" " " "
Naked mole-rat	.6	HaeIII		0.94	Reeve et al. (1990)
	.15	HaeIII		0.88	" "
	M13	HaeIII		0.99	" "
Fox	.6	HinfI	0.75-1.00[d]		Gilbert et al. (1990)
Pilot Whales	.15	DdeI		0.60	Amos and Dover (1990)

a: probes .6 and .15 are Jeffreys' polycore probes 33.6 and 33.15, respectively; 3'HVR is probe
a-globin 3'HVR.

b: F=similarity coefficient.

c: x=probability of band sharing.

d: the range of x based on several island populations.

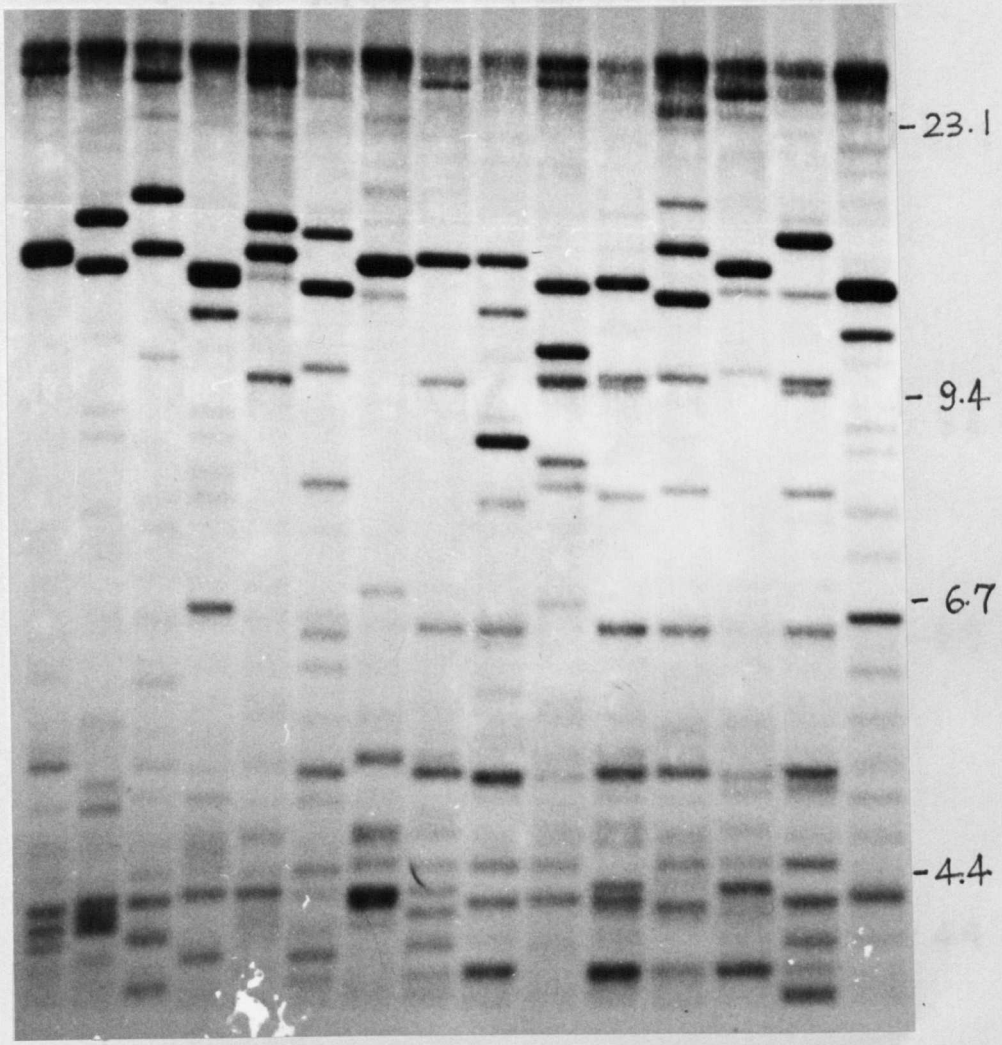


Fig. 4.1 DNA fingerprints of Bewick's Swans. Fifteen birds,randomly chosen from the Slimbridge population,were fingerprinted with the combination of pSPT19.6 and HaeIII. Size markers are in Kb. Only first ten lanes from the right were scored.

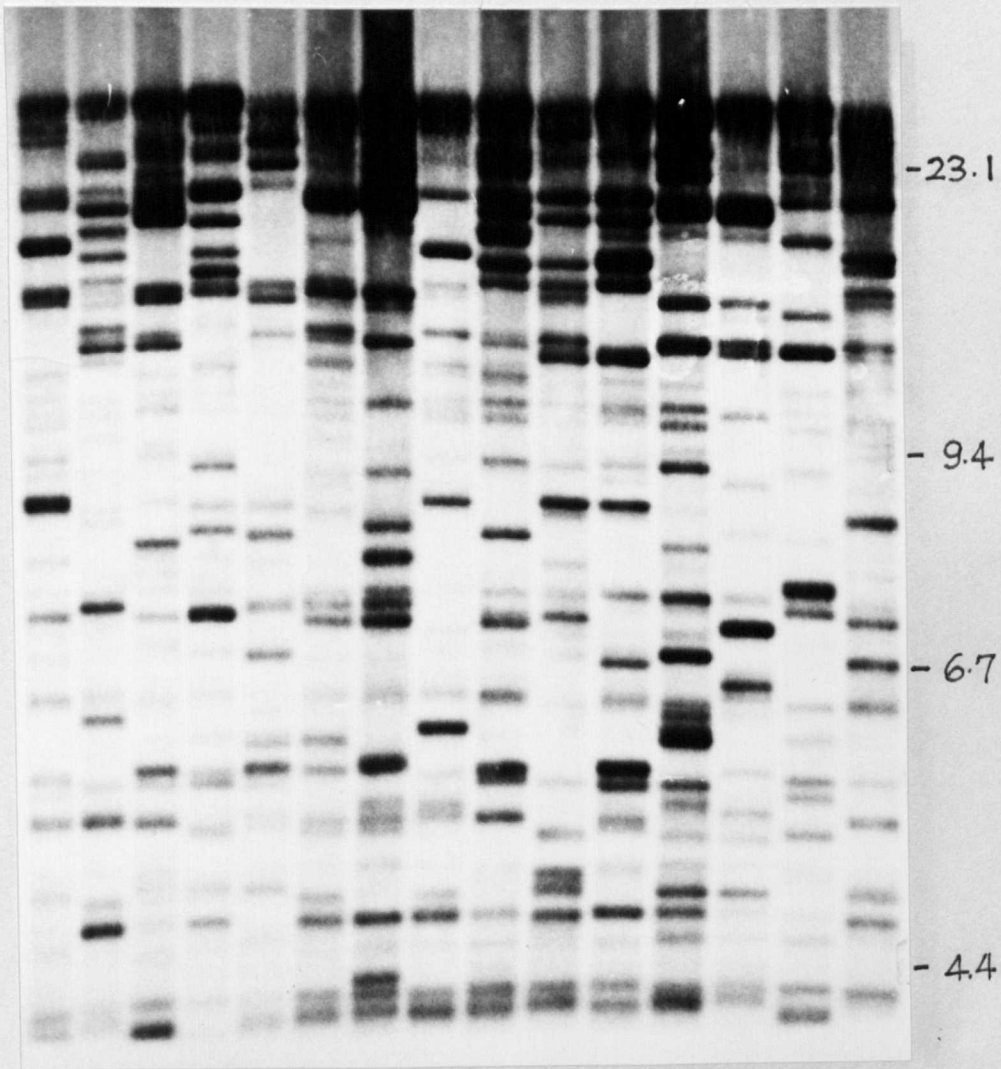


Fig.4.2 DNA fingerprints of Mute Swans. Fifteen birds,randomly chosen from the Abbotsbury population,were fingerprinted with the combination of pSPT19.6 and HaeIII. Size markers are in Kb. Only first ten lanes from the right were scored.

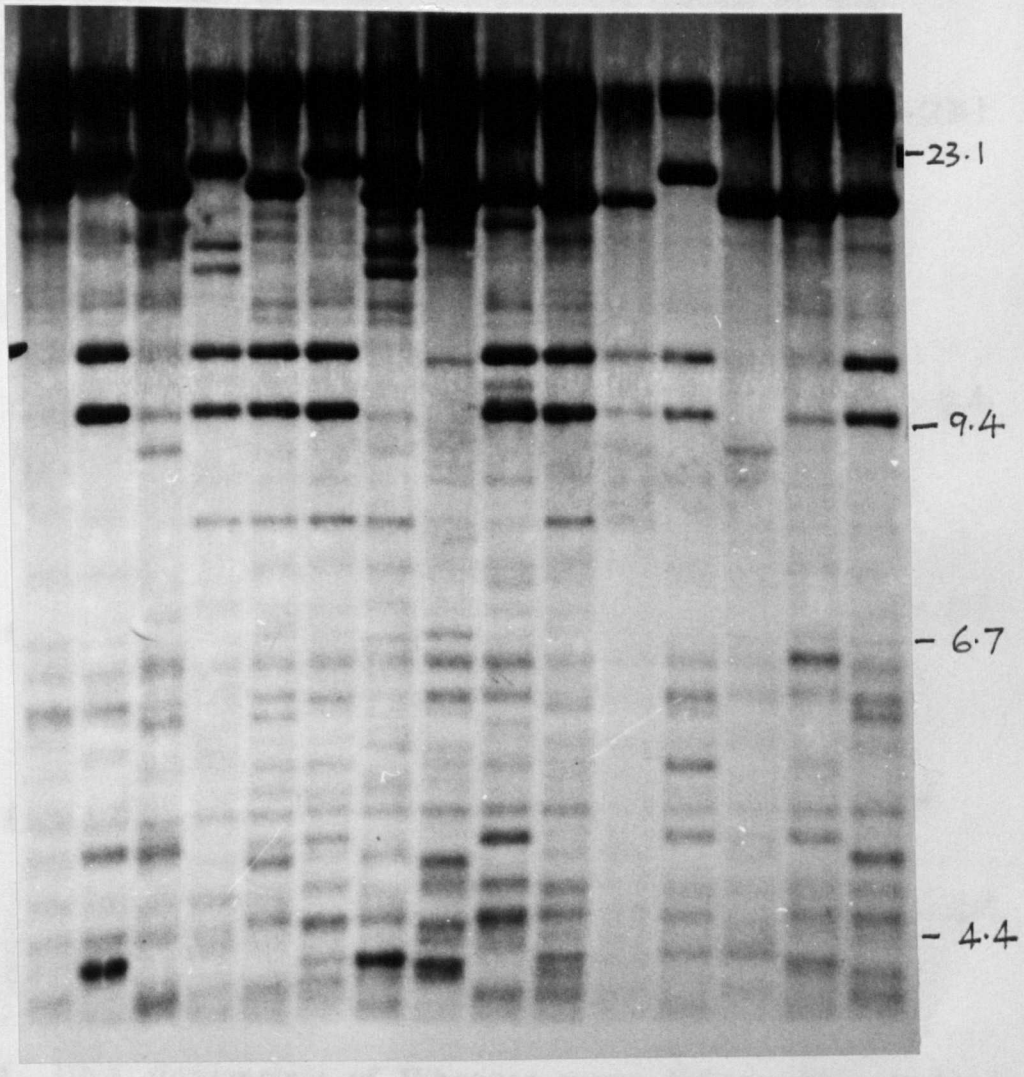


Fig.4.3 DNA fingerprints of Trumpeter Swans. Fifteen birds,randomly chosen from the Montana population,were fingerprinted with the combination of pSPT19.6 and HaeIII. Size markers are in Kb. Only first ten lanes from the right were scored.

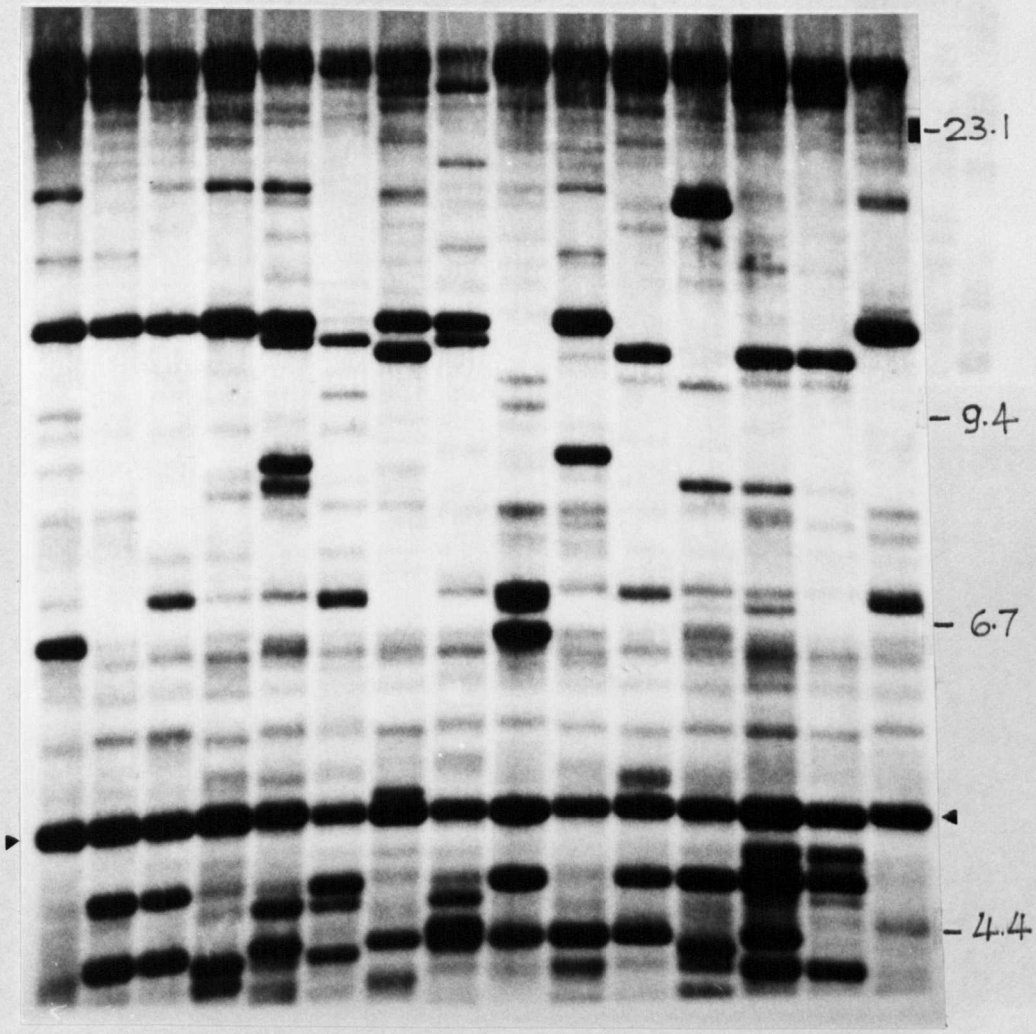


Fig.4.4 DNA fingerprints of Whooper swans. Fifteen birds,randomly chosen from the Caerlaverock population,were fingerprinted with the combination of pSPT19.6 and HaeIII. Size markers are in Kb. Only first ten lanes from the right were scored. A common band, present in all the fingerprints, is marked with arrowhead.

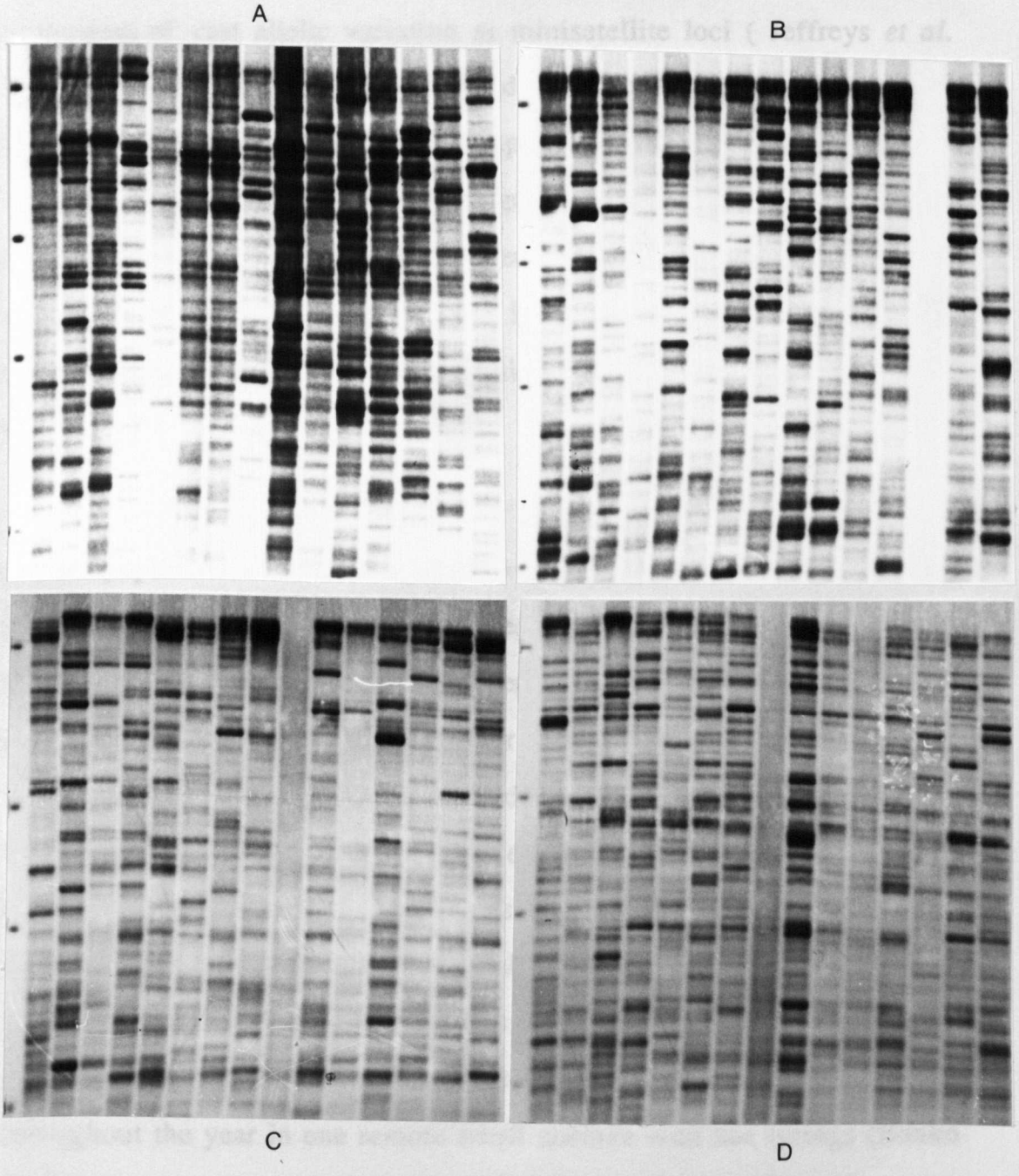


Fig.4.5 DNA fingerprints generated with HinfI/pSPT18.15. Blot A is for the Bewick's Swans, blot B for the Mute Swans, blot C for the Trumpeter Swans and blot D for the Whooper Swans.The samples are the same as those in Figs. 4.1-4.4 and are in the same order. The very left lane on each photo contains λHindIII fragments whose sizes are 23.1, 9.4, 6.7 and 4.4 in Kb sequentially from the top, respectively.

The core sequence, spreading over all of repeat units in a family of minisatellites is believed to be a recombination hotspot, explaining the generation of vast allelic variation at minisatellite loci (Jeffreys *et al.* 1985a, Jeffreys *et al.* 1988, Chandley and Mitchell 1988, Wahls *et al.* 1990). It has been shown in the previous chapter that the Whooper Swan has a higher mutation frequency of DNA fingerprint bands than the Mute Swan, consistent with the fact that the former has DNA fingerprints of higher variability. This explanation however is not satisfactory. Other elements must be involved as well in the differentiation of populations at minisatellite loci.

Let us first look at the Trumpeter Swan population in Montana. The present populations of the Trumpeter Swan in North America descend from only a small number of survivors that escaped from excessive shooting in the nineteenth century, and which become almost non-migratory. For instance, the total number of Trumpeter Swans in Montana and Wyoming was only 69 in 1932, which multiplied to some 600 in 1955 (Scott and Wildfowl Trust 1972). After that bottleneck, the genetic variation in the subsequent populations may have been sharply reduced, compared to that in the ancestral population, due to the elimination of some alleles of low frequency and fixation of some alleles through genetic drift. It was also reported that the Trumpeter Swans at Montana and Wyoming remain throughout the year in one remote small enclave with hot springs (Banko 1960). This kind of self-isolation impedes the possible gene flow in and out of the population, eventually resulting in the decrease of genetic variation.

Bewick's Swans are wholly migratory. They regularly travel over 2,000 miles to find a suitable wintering resort. Although Bewick's Swans prefer returning to their previous winter grounds each year, they may cut

off their journeys and stop at a suitable site where earlier birds may have established a wintering ground (Scott 1967). Except for a few residents, the majority of Bewick's Swans at Slimbridge are wintering birds, emigrating from various breeding grounds in Arctic Russia. Thus, this seasonal population would usually contain a pool of genes established in several or many parental populations in Russia, and so exhibit the great amount of genetic variation which should largely be attributed to geographic variations. This speculation is verified by the fact that the DNA fingerprints of the Bewick's Swans at Slimbridge are most variable. It might be proved that the degree of variation of DNA fingerprints is not so high within a single Russia population.

To a large extent, the Caerlaverock population of Whooper Swan is similar to the Slimbridge population of Bewick's Swan. It is believed that Icelandic and Scandinavian Whooper Swans emigrate into Scotland each year for wintering. In addition, a considerable interchange of wintering Whooper Swans takes place between Scotland and Ireland each year (Boyd and Eltringham 1962). This frequent gene flow might have contributed much to the maintenance of minisatellite variation in this population.

Mute Swans are largely non-migratory. Unlike Bewick's and Whooper Swans, Mutes tend to breed in the general area where they were raised. It was reported that as many as 70.2% of Mute Swans in Britain travel less than 10 miles and even more distant movements only occurred along watercourses (Ogilvie 1967). Also, Birkhead and Perrins (1986) stated ".....the number of birds moving in and out of any one of Britain's main river systems is sufficiently small that this has, at most, very trivial effects on population changes". The Mute Swans at Abbotsbury are colonially-breeding birds. The relatively small effective population size and possible incestuous mating may have brought about the low genetic variation in this

population (see next section). On the other hand, the mutation frequency of fingerprint bands in the Mute Swans is also relatively low, which may have an impact on the amount of genetic variation in this species.

4.3 INTER-POPULATION VARIATION

4.3.1 Application of Genetic Identity Measures of Nei

Because of the complexity of DNA fingerprints, the methods for statistical analysis developed so far remain incomplete, which may mislead fingerprinters into wrong conclusions. To reliably measure genetic variation between populations, it is useful to introduce more indices of genetic identity. We will therefore try the genetic measure of Nei to compare DNA fingerprint profiles between populations.

Nei (1972) proposed the genetic identity at a locus between two populations X and Y, in which the frequencies of the ith alleles are x_i in X and y_i in Y, as

$$I_i = \Sigma x_i y_i / (\Sigma x_i^2 y_i^2)^{1/2} = j_{XY}/(j_X j_Y)^{1/2}$$

The identity of genes between X and Y with respect to all loci is:

$$I = J_{XY}/(J_X J_Y)^{1/2}$$

where J_X, J_Y and J_{XY} are the arithmetic means of j_X, j_Y and j_{XY}, respectively, over all loci.

Now assuming that a, only one allele at a minisatellite locus is resolved in a DNA fingerprint; b, this allele is represented by one fingerprint band; c, the allele frequencies are homogeneous, then we get

$$J_X = \Sigma X_k/N \quad \text{and} \quad J_Y = \Sigma Y_k/N$$

where X_k and Y_k are the frequencies of a band representing the κth locus in populations X and Y, respectively, and N is the total number of loci resolved in DNA fingerprints; and

$$J_{XY} = \Sigma P_k/N.$$

where P_k is the frequency of a given band, which is lower, either X_k or Y_k at the κth locus.

The genetic identity of DNA fingerprints between populations X and Y is

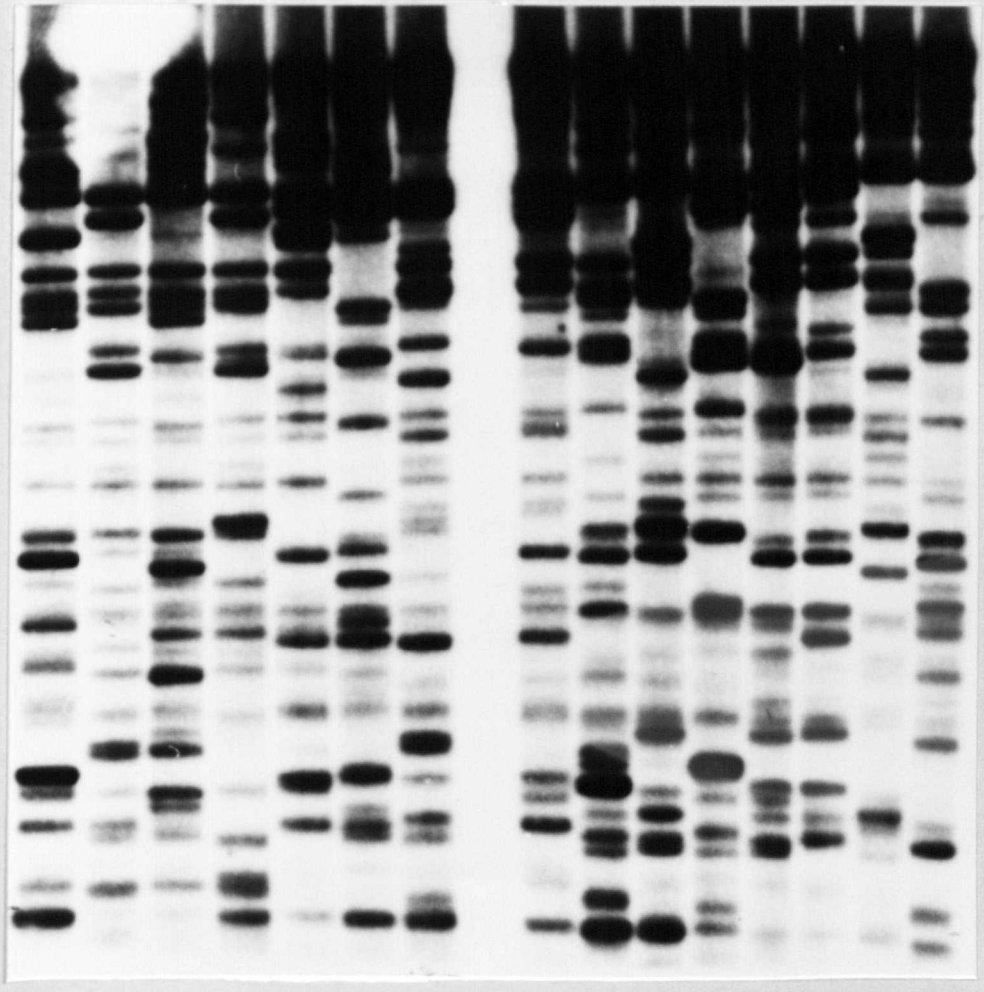
$$I_{FP} = J_{XY}/(J_X J_Y)^{1/2} = \Sigma P_k/(X_k Y_k)^{1/2}$$

This quantity is unity when the two populations have the same fingerprint bands in identical frequencies, while it is zero when they have no identical band.

4.3.2 Genetic Similarity between Lothian and Abbotsbury Populations of Mute Swan

Eight birds chosen from the Lothian population and seven from the Abbotsbury population were fingerprinted with a combination of pSPT19.6 and HaeIII (Fig. 4.6). All the birds from each population are adults, supposedly not closely related. While scoring the fingerprints, only seven out of eight from the Lothian population were taken into account so that the relative frequencies of a given band in the two populations are comparable.

The mean similarity coefficients (F) and genetic identity (I_{FP}) within and between the populations were calculated , and listed in Table 4.3. The mean number of bands per fingerprint is not significantly different ($P>0.05$) between the populations. The mean similarity coefficient in the Lothian population is 0.327, lower than that (0.364) in the Abbotsbury population.



Abbotsbury Lothian

Fig.4.6 Comparison of DNA fingerprints between two Mute Swan populations. Eight and seven birds were randomly chosen from the Lothian and Caerlaverock populations, respectively. The birds from each population were presumably unrelated. All the birds were fingerprinted with HaeIII/pSPT19.6 under the stringency of 0.5X SSC. While scoring, only seven Lothian swans were done.

Table 4.3 Genetic variation within and between two populations of Mute Swan

Populations	No. bands per bird + SD	Similarity coefficients +SD (No. comp.)		Genetic identities + SD (No. comp.)
		Abbotsbury	Lothian	Abbotsbury
Abbotsbury	25.3+1.7	0.364+0.091 (21)		0.685+0.035* (4)
Lothian	27.1+3.3	0.294+0.076 (49)	0.327+0.113) (21)	0.574 (1)

*: This value was calculated based on DNA fingerprints presented in Fig.4.2. 14 out of 15 birds were randomly and repetitively divided into two groups, then genetic identities between the two groups were calculated to obtain the mean genetic identity within the population. All the other values were calculated based on Fig.4.6.

However, this difference is not statistically significant (P>0.05). It is likely that the differentiation of minisatellites in these two populations is a recent event which is not readily detected.

The mean similarity coefficient between the two populations is 0.294. This value is remarkably lower than that within the Abbotsbury population (P<0.05), but not different from that within the Lothian population. The genetic identity between the two populations is lower than that between random groups of the Abbotsbury birds (P<0.01), suggesting that the allelic frequencies at minisatellite loci are different in the two populations. This might imply that these two populations are undergoing differentiation in their genomic structures. It is not clear whether this differentiation has been followed by morphological or physiological changes. The decrease of genetic identity between conspecific populations may be due to differential selection under different environments and geographic isolation.

In some aspects, the Abbotsbury population of Mute Swan is very different from the other British populations. It is a semi-domesticated population, which was established by monks about 900 years ago (Scott and Wildfowl Trust 1972). All the birds there breed in a dense colony, and nest only a few metres apart from one another. The breeding colony itself regularly contains only 30-50 pairs. Because of shortage of breeding territories, some birds of breeding age fail to breed. Immigrants who enter there for plenty of food supplied at Abbotsbury will return to their own territories for breeding so that immigration does not change the structure of the population. We speculate that the higher degree of genetic uniformity within the Abbotsbury population might result from the early founder effect and constantly smaller effective population size. It remains unknown whether incestuous mating could be favoured due to the habit of

colony breeding.The Mute Swans at Lothians, Scotland, are distributed over a large area, including East Lothian, Midlothian and West Lothian. The frequent free-interchange of the birds in these small areas should result in effective exchange of genes, maintaining the substantial genetic variation in this population.

4.4 INTERSPECIFIC VARIATION

DNA fingerprints of swans are subject to specific differentiation. As shown in Figs. 4.1-4.4, DNA fingerprints of different species, using the probe pSPT19.6 and enzyme HaeIII, have their own recognizable features in addition to a great deal of intraspecific variation. The details are discussed below with respect to the four species of Swan: *Cygnus bewickii, Cygnus olor, Cygnus buccinator* and *Cygnus cygnus*.

4.4.1 Variation in Band Number and in Distribution Pattern of Banding

The Jeffreys' polycore probe pSPT19.6 detects a number of minisatellites in the genomes of various species. The mean number of fingerprint bands is, in the size range 30-4Kb, 27.5 for the Mute Swans, 22.3 for the Bewick's Swans, 22.2 for the Trumpeter Swans and 21.6 for the Whooper Swans (Table 4.4). The difference is significant ($P<0.01$) between the Mute Swans and any of the other three, while the difference among the latter three is negligible. Looking at the distribution of bands, we note that Mute Swans indeed have a higher number of large bands ($\geq$10Kb) in their DNA fingerprints than have the other species, whereas the number

Table 4.4 Distribution pattern of fingerprint bands in different species of swans, based on Figs 4.1-4.4

Species	Size range	No. bands per bird + sem	Similarity Coefficient +sem	Band sharing +sem
	>10Kb	7.1+0.41	0.178+0.021	0.181+0.015
Bewick's	10-5Kb	10.5+0.73	0.209+0.019	0.215+0.015
C. bewickii	5-4Kb	4.7+0.40	0.416+0.024	0.429+0.019
	Overall	22.3+0.96	0.248+0.012	0.250+0.009
	>10Kb	11.1+0.0.55	0.326+0.019	0.330+0.014
Mute	10-5Kb	11.1+0.30	0.349+0.014	0.351+0.021
C. olor	5-4Kb	5.5+0.45	0.694+0.021	0.719+0.020
	Overall	27.5+1.00	0.404+0.014	0.407+0.010
	>10Kb	6.9+0.41	0.381+0.027	0.387+0.020
Trumpeter	10-5Kb	11.7+0.30	0.509+0.016	0.510+0.012
C. buccinator	5-4Kb	3.6+0.43	0.314+0.033	0.334+0.026
	Overall	22.2+0.49	0.442+0.017	0.443+0.012
	>10Kb	7.9+0.48	0.228+0.026	0.232+0.019
Whooper	10-5Kb	9.9+0.57	0.284+0.022	0.289+0.016
C. cygnus	5-4Kb	3.8+0.29	0.346+0.033	0.356+0.025
	Overall	21.6+0.76	0.276+0.016	0.277+0.011

of small bands from 10 to 4Kb in size is not different from one another among the four species. Moreover, bands present in DNA fingerprints of Mute Swans are relatively evenly spaced, and have similar autoradiographic intensities, unlike those in the other species that are unevenly scattered and vary a great deal in intensity. These results suggest that there may be closer taxonomic affinity among the Bewick's, Trumpeter and Whooper Swans.

To test whether the probe pSPT18.15 is able to generate species-specific DNA fingerprints, the same birds as used for the pSPT19.6 were fingerprinted with this probe. Interestingly this probe detects many more bands, but the fingerprint banding patterns in different species are so similar to one another that the species are indistinguishable (Fig. 4.5). It is not clear whether the two polycore probes are different in an evolutionary sense.

4.4.2 A Possible Diagnostic Minisatellite Locus

The Mute Swans are readily distinguished from the northern swans: Bewick's, Trumpeter and Whooper Swans, based on the total number of bands per individual in the HaeIII/pSPT19.6 fingerprints. But we cannot discriminate the northern swans in this way. Fortunately, the presence of some diagnostic bands offers an access. These bands have the strongest autoradiographic intensity, even if hybridizations performed under higher stringent conditions (0.3 X SSC, data not shown here). The number and size distribution of these bands (hereafter referred to as 'strong bands') are unique to species.

The Whooper Swan's DNA fingerprints all have a strong band of approximately 5Kb in size (marked with arrowhead in Fig.4.4). In the size

range 4.7-4.0Kb, each fingerprint has 1-4 strong bands. In addition, every one has one or two strong bands mostly located at the position of 12.2-10.8Kb. However, the number of such strong bands reduces to only one or two that have a length of approximately 21.2-18.2Kb, when HinfI-digests are hybridized to the same probe (data not shown here). The pedigree analysis showed that two strong bands present in any one of parents are segregated into offspring as two alleles at a heterozygous locus (Fig.3.3). It is suggested that these strong bands are derived from the alleles at a single minisatellite locus. If so, six alleles have been detected at this locus among the fifteen birds. The alleles at this locus contain different numbers of internal HaeIII sites (CC/GG), and that common HaeIII fragment might mean its conservation in the evolutionary process.

The Trumpeter Swans have one to three strong bands in their DNA fingerprints. The size range of these bands is from 21.2 to 9.4Kb. The band of 9.4Kb in size and its companion band of 11.0Kb in size may be derived from the same allele. The diagnostic locus in this species has no more than three alleles resolved here among 15 birds, supporting the observation that minisatellites of the Trumpeter Swans are less variable.

Similarly, DNA fingerprints of Bewick's Swan have only one or two strong bands in the size range 17.0-10.5Kb. These bands differ markedly from one another in size. Provided that they are derived from the same diagnostic locus, 11 alleles have been resolved among 15 birds and the frequencies vary from 0.067 to 0.400.

Unlike the northern swans discussed above, the Mute Swan's fingerprints possess many strong bands that cannot be defined as alleles at a single locus.

4.4.3 Discussion

Swan species are identified, like most other higher organisms, based on morphology and behaviour. With a little controversy, swans are arranged in seven species: *Cygnus bewickii* (Bewick's Swan), *Cygnus columbianus* (Whistling Swan), *Cygnus cygnus* (Whooper Swan), *Cygnus buccinator* (Trumpeter Swan), *Cygnus olor* (Mute Swan), *Cygnus atratus* (Black Swan) and *Cygnus melanocoryphus* (Black-necked Swan) (Delacour and Mayr 1945), while the species *Coscoroba coscoroba* is also called Swan by some ornithologists (Scott and Wildfowl Trust 1972). *Cygnus columbianus* and *Cygnus bewickii* are treated by most ornithologists as subspecies of the same species because of insufficient difference between the two. It is likely that the speciation of swans takes the allopatric mode, meaning that genetic change takes place during the period of geographic isolation and eventually leads to the reproductive isolation.

Taxonomists are mostly concerned with the uniformity of the populations rather than the differences. Although they are highly variable among individuals within the same species, the minisatellites in swans show a certain degree of conspecific uniformity, and could separate the species. For example, DNA fingerprinting reveals that the Mute Swan is relatively distant from all the three northern species studied here, and that all the Whooper Swans have a conservative HaeIII minisatellite fragment, consistent with the outcome of comparisons based on the other typological characters. This does not mean that DNA fingerprinting is a proper method for studying the typology of swans. Speciation is a process of adaption of integrated gene complexity. Whatever function of minisatellites could be, it seems that some minisatellites of swans are coadapted with other characters. The differentiation of minisatellites may be the result of

adaption to different environments or genetic drift. It is unlikely that new variants of minisatellites directly respond to the selective forces that lead to fixation or elimination, since there is no evidence that the minisatellites have an impact on any phenotypic characters. More likely, the minisatellites evolve merely through genetic drift or through the evolution of flanking or closely linked sequences.

4.5 CONCLUSION

DNA fingerprinting using the polycore minisatellite probes discloses a great amount of genetic variation in natural populations of swans. The quantity of variation in the Slimbridge Bewick's Swans is highest, second in the Caerlaverock Whooper Swans, third in the Abbotsbury Mute Swans and lowest in the Montana Trumpeter Swans. It is suggested that gene flow and genetic drift play an important role in maintaining the intrapopulation variation. The study in the two Mute Swan populations shows that they differ to a certain degree in the variability of their minisatellites, and that the interpopulation variation is greater than the intrapopulation variation. The habit of colony-breeding of Mute Swans at Abbotsbury may be the reason that this population has a lower amount of minisatellite variation. Moreover, some minisatellites of swans are undergoing differentiation among the species, though the function of minisatellites remain mysterious. It is concluded from DNA fingerprints that Bewick's, Trumpeter and Whooper Swans are relatively closely related to one another, yet distant from the Mute Swan. DNA fingerprinting proves to be powerful for revealing the genetic variation in natural populations, and also useful for studying evolutionary biology of animals.

CHAPTER 5

PARENTAGE TESTS BY DNA FINGERPRINTING

5.1 INTRODUCTION

Studies of evolutionary biology very often require the identification of individuals and the determination of genetic relationships between them. For example, when studying reproductive success, one must know whether a male is the true father of his attendant young. The recognition of individuality and the establishment of relationships between birds are usually achieved by field-watching the activities of the birds wearing a numbered ring on one leg through their life. Because of discontinuities in field observations, or failure to identify the parents, pedigree data really need to be confirmed by means of genetic analysis. Such analyses rely on the availability of genetic markers that should be polymorphic and inherited in a simple fashion. The use of traditional genetic markers such as morphological characters, blood groups, chromosome inversions and multi-forms of enzymes are restricted due to their insufficient polymorphisms. The lack of a large number of polymorphic genetic markers became a serious problem for determining the true biological relationships and thereby confirming hypotheses on reproductive biology of animals. Although the finding of locus-specific restriction fragment length polymorphisms (RFLPs) in genomic and mitochondrial DNA provides an unlimited source of genetic markers, the exclusion probability of parentage using these markers is low due to their low variability. Usually many RFLP probes have to be used in combination to obtain a precise conclusion (Quinn

et al. 1987). Therefore there has been a need for methods that are more powerful and easy to use.

DNA fingerprinting is the best of such methods. A DNA fingerprinting probe can simultaneously detect dozens of minisatellite loci in the genome, with very low allelic frequencies. Its power could be equivalent to the sum of dozens of locus-specific RFLP probes. The usefulness of DNA fingerprinting in determining true genetic relationships was at once recognized (Jeffreys *et al.* 1985b), and first came into use for resolving an immigration dispute in humans(Jeffreys *et al.* 1985c). Furthermore, biologists have been pleased that the available DNA fingerprinting probes can be applied to a wide spectrum of species. In particular, DNA fingerprinting has been successfully used to test paternities in the house sparrow *Passer domesticus* (Wetton *et al.* 1987), dunnock *Prunella modularis* (Burke *et al.* 1989, long-finned pilot whale *Globicephala banaena* (Amos and Dover 1990) and swallow *Hirundo rustica* (Wellbourn *et al.* 1990).

Swans are believed to be strictly monogamous. A male and a female form a pair bond and this may last for a lifetime. If the pair breeds, both cooperatively participate in raising the young. However, there are exceptions from this simple mating behaviour. For example, Minton (1968) reported several cases of divorce and change of mates in the Mute Swans in central England. Polygamous breeding (extra-pair copulation) has also been noted among captive as well as among wild Mute Swans (Scott and Wildfowl Trust 1972). There have probably been more such cases, which have not been seen because of the lack of methods for confirming the genetic relationships among individuals. This study is intended to assess genealogical relationships between cygnets and their putative parents in

swans by using the DNA fingerprinting technique, and thereby study the reproductive biology of swans.

5.2 PARENTAGE ANALYSIS IN WILD POPULATIONS

5.2.1 Materials and Methods

The parentage analyses in this study were primarily done on two wild populations of swans. The first one is the Whooper Swan population at Caerlaverock, Scotland, and the other is the Mute Swan population at Lothians, Scotland.

The Whooper Swans were captured and blood-sampled in 1987 and 1988. According to field observations, a total of 109 birds were grouped into 25 broods, 20 families (some families consist of two broods hatched in the two successive years). All the families except one include both putative father and mother. The birds were fingerprinted with pSPT19.6 on HaeIII digests as well as on HinfI digests.

The Mute Swans were sampled in 1987, among which there are 16 families, including 93 birds. However, three are partial families with one of the parents unsampled. All these swans were fingerprinted with pSPT19.6/HaeIII.

Some other families, independently sampled at various locations, were also analysed by DNA fingerprinting. The results obtained from these families can embody some aspects of mating behaviour of the species that they represent, and hence will be presented.

When analysing DNA fingerprint profiles, mismatches between putative parents and their attendant cygnets can be readily ascertained by the following criteria:

.the proportion of novel bands go beyond the mutation rate;

.similarity coefficients between the putative parents and cygnets are strikingly low;

.the probability of parentage is small based on the analysis method described by Brookfield (1989).

5.2.2 Examples of Parentage Analyses

5.2.2.1 MULTIPLE PATERNITY AND MATERNITY IN A SINGLE BROOD OF THE MUTE SWAN

A Mute Swan 'family', sampled at Abbotsbury in 1987, consisted of two adults and six cygnets. They were watched living together as a family at the time of capture. These birds were DNA-fingerprinted with pSPT19.6/HaeIII and their DNA fingerprints were shown in Fig. 5.1.

When comparing the DNA fingerprints of the cygnets with those of the two adults, all the bands in cygnet A can be found in either of the adults; however, the remaining cygnets all have 5-8 bands that are absent from the adults (Table 5.1). If these novel bands all arise through mutation, then the mutation rate is much higher than the estimated in the Mute Swans (see Chapter 3). It is highly unlikely that a single cygnet has so many independent mutations ($P<10^{-4}$). Inclusive of all the cygnets, the analysis also shows that the segregation of heterozygous paternal or maternal bands

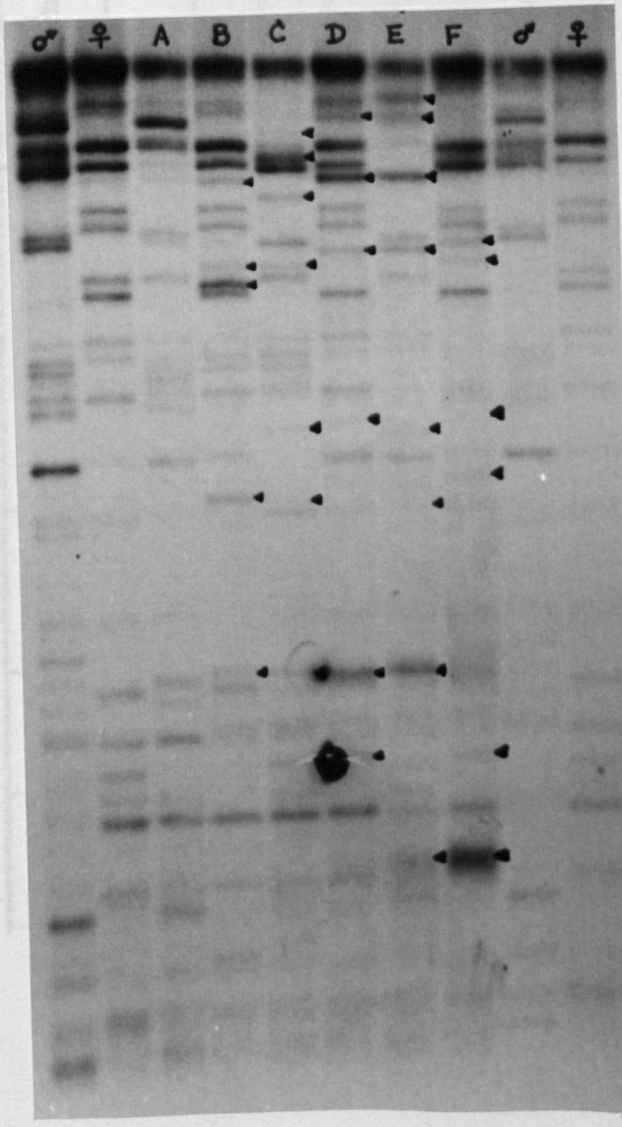


Fig.5.1 DNA fingerprints of a mismatching Mute Swan family. All the birds were fingerprinted with pSPT19.6/HaeIII. Mismatched bands in the cygnets were indicated with an arrowhead.

Table 5.1 Establishment of parentage in the mismatching Mute Swan family

Cygnets	DRC[a]	No. mism. bands	Adult male (Y494)		Adult female (W150)	
			F[b]	Father[c]	F	Mother[c]
A	WAKS	0	0.711	Yes	0.455	Yes
B	WAKT	5	0.423	No	0.745	Yes
C	WAKU	8	0.490	Likely	0.458	No
D	WALA	7	0.353	No	0.680	Yes
E	WALB	8	0.531	Likely	0.458	No
F	WALC	6	0.490	No	0.542	Yes

a: DRC=Darvic Ring Codes;

b: F=similarity coefficients;

c: parentage revealed by the probability test (Table 5.2). 'Likely' means that the adult may or may not be the parent.

Table 5.2 Probabilities of parentages in the mismatching Mute Swan family

Cygnets	Parentage Models			
	1	2	3	4
A	**4.6X10^{-12}**	6.5X10^{-13}	1.7X10^{-17}	1.3X10^{-16}
B	1.1X10^{-21}	1.1X10^{-20}	**9.5X10^{-14}**	9.5X10^{-18}
C	1.9X10^{-29}	*2.7X10^{-18}*	9.4X10^{-19}	**2.9X10^{-17}**
D	6.4X10^{-27}	8.3X10^{-22}	**7.2X10^{-15}**	1.4X10^{-17}
E	6.3X10^{-29}	*1.8X10^{-17}*	9.4X10^{-19}	**2.9X10^{-17}**
F	7.4X10^{-25}	2.7X10^{-18}	**4.1X10^{-17}**	*2.9X10^{-17}*

Parentage test is conducted under four models: Model 1, both adults are the parents; Model 2, the male is the parent and the female is not; Model 3, the female is the parent and the male is not; Model 4, the two adults are not the parents. The most likely model (MLM) is the one with the highest probability, and shown in bold. The models with a probability that is not significantly (P>0.05) different from that of the MLM are shown in *italic*.

significantly deviates from the binomial distribution ($P<0.05$). It is certain that the true parentage in this family is not as observed in the field.

Because the two adults share a high proportion of bands ($F=0.408$), in this case similarity coefficients between the adults and cygnets become less important as indicators of relatedness. Here we use Brookfield's method (1989) to compute the probabilities of four models for each cygnet (Table 5.2). The outcome indicates that the female is likely to be the mother of cygnets A, B, D and F, and that the male is likely to be the father of cygnet A. For cygnets C and E, both Models 1 and 4 are possible, but Model 4 is more likely. So it is likely that cygnets C and E have genetic parents other than the fostering adults. The analysis of relationships among the cygnets indicates that cygnets B, D and F probably have the same father, and that cygnets C and E may probably be derived from the same brood.

Thus in this group of swans, the two adults only have one shared offspring (cygnet A), the remaining cygnets presumably have at least one extra mother or father. The establishment of this community must have involved extra-pair copulation and intraspecific nest parasitism (INP), or adoption of an outsider female's young.

5.2.2.2 PARENTAGE ANALYSIS IN A MULTI-BROOD FAMILY

According to the field observations, an adult male IFP was very active. He changed his mate three times within three breeding seasons. In 1986/87, he and his mate HAU guarded two cygnets IHY and IIP. In the following year he was paired with another female ILD, and accompanied three cygnets IUC, ITU and IUU. In the third year (1988/89), he paired with female ISV and no cygnets were found with them.

Table 5.3 Similarity coefficients between adults and cygnets in the multi-brood Whooper Swan family

Adults	Cygnets				
	IHY[a]	IIP[a]	IUC[b]	ITU[b]	IUU[b]
IFP[abc]	o.655	0.300	0.441	0.426	0.406
HAU[a]	0.440	0.679	0.164	0.111	0.140
ILD[b]	0.172	0.200	0.559	0.492	0.449
ISV[c]	0.281	0.305	0.299	0.300	0.349

a: The birds united in the breeding season 1986/87; b: the birds in 1987/88; c: the birds in 1988/89.

Table 5.4 Result of probability test of parentage for the multi-brood Whooper Swan family

Adults	Parentage	Cygnets				
		IHY	IIP	IUC	ITU	IUU
IFP	Father	Yes	Prob. not	Yes	Yes	Yes
HAU	Mother	Yes	Yes	No	No	No
ILD	Mother	No	No	Yes	Yes	Yes
ISV	Mother	No	No	No	No	No

All the birds were fingerprinted with pSPT19.6/HinfI. The similarity coefficients between the pairs of adults range from 0.190 to 0.254 with a mean of 0.233 (SD=0.026), suggesting that they are not closely related so that the values of F for the parents/offsprings should be approximately 0.5. The actual values of F between the adults and cygnets are listed in Table 5.3. The figures convince us that the female adult HAU was the mother of cygnets IHY and IIP, that ILD was the the mother of IUC, ITU and IUU, and that ISV was related to none of the cygnets. However, this is not the the case for the male adult IFP. The similarity coefficients between IFP and cygnets IHY, IUC, ITU and IUU are all reasonably close to 0.5 (the mean=0.482, SD=0.116), supporting the view that he is the father of these cygnets. His paternity for cygnet IIP is under suspicion because the F between them is 0.300, well below 0.5. In addition, the mean of Fs for the full-sibs IUC/ITU/IUU is 0.689 (SD=0.087), whereas F between IHY and IIP is only 0.333, further suggesting that the cygnet IIP had an uncertain father instead of IFP.

The probabilities of parentages in this family were calculated as previously. The results (Table 5.4) show that all the cygnets had unambiguous maternity, and that IFP is unlikely to be the father of cygnet IIP. The female HAU must have an unidentified mate who fertilized HAU to produce the young IIP.

5.2.2.3 ALLOPARENTAL BEHAVIOUR IN MUTE SWANS (*Cygnus olor*), DETECTED BY DNA FINGERPRINTING

(This is an independent paper that has been submitted for publication in the Proceedings of the Third International Swan Symposium.)

PAGES
NOT SCANNED
AT THE REQUEST OF
THE UNIVERSITY

SEE ORIGINAL COPY
OF THE THESIS FOR
THIS MATERIAL

5.2.3 Proportion of Broods with Multiple parentage

As demonstrated in the above section, parental care of cygnets by adults does not necessarily manifest the true paternity nor maternity in swans. Parentage analysis over a population could allow the estimation of the proportion of broods with multiple parentage.

In the Lothian population of the Mute Swans, 3 out of 16 families have one or two mismatching cygnets, giving a rate of 18.75%. Of the four mismatching cygnets, three have correct paternity but incorrect maternity, and one has correct maternity and incorrect paternity, indicating the involvement of extra-pair fertilizations (EPFs). The incidence of EPFs for the pair male and female is 10.34% and 3.4%, respectively. The extra-pair fertilization of a putative father must be followed by the intraspecific nest parasitism (INP). The result suggests that an outsider female engaging in extra-pair copulation usually produces eggs in the nest of her extra mate, or gives up the duty of rearing the young following hatching. The EPF rate among the cygnets is 4.3%, much lower than that in the house sparrow *Passer domesticus* (Wetton *et al.* in prep.) and in swallows *Hirundo rustica* (Wellbourn *et al.* 1990).

The examination of parentage among 25 broods contained in 20 complete families from the Caerlaverock Whooper Swan population reveals only one mismatching brood, which was described in section 5.2.2.2. It is a case of extra-pair fertilization. The incidence of EPFs is extremely low in the Whooper Swan.

EPFs or INP also takes place in the Trumpeter Swan. However, the insufficient number of families from the Trumpeter and Bewick's Swans

provides no opportunity to investigate reproductive behaviour in these species by DNA fingerprinting.

5.3 DISCUSSION AND CONCLUSION

DNA fingerprinting has become a useful tool for parentage ascertainment in wild populations of birds. Using this technique, parentage exclusion is quite straightforward. Particularly when maternity is certain, paternity can be simply excluded if the offspring has some minisatellite markers that cannot be attributed to the mother nor to the putative father. It was estimated that using a single minisatellite probe (33.15) the probability of non-detection of an incorrectly assigned father in house sparrow would be only about $3x10^{-6}$ if the putative father was unrelated to the genetic father (Burke and Bruford 1987) and about 0.01 in man if it was a close relative (brother or father) (Jeffreys *et al.* 1985b). By contrast, using biochemical markers the probability of non-detection in dogs was 0.3-0.6 (Gundel and Reetz 1981). In house sparrows again but by examining seven enzyme loci, as high as some 50% of mismatching progeny could not be detected (Wetton and Parkin 1989). In fact, DNA fingerprinting has been extensively used for paternity-testing human beings (Jeffreys *et al.* 1985c, Wells *et al.* 1988, Helminen *et al.* 1988), and for demographic study of wild birds (Wetton *et al.* 1987, Burke and Bruford 1987, Burke *et al.* 1989, Wellbourn *et al.* 1990). It has been demonstrated above that the use of a single polycore probe and one enzyme is usually sufficient to identify cases of incorrectly assigned parentage in swans.

This study reveals that extra-pair fertilization, intraspecific nest parasitism and alloparental care all occur in swans. These reproductive

behaviours indeed have been reported in a large and growing number of species of monogamous birds (for review see Ford 1983, Riedman 1982 and Rohwer 1989), due to increasing interest and applications of new techniques for identifying individuals. In wild swans, however, very few cases of extra-pair copulation and egg dumping had been reported (Dewer 1936, Huxley 1947, Miers and Williams 1969). The reason for this is the difficulty of routinely observing the mating behaviour, and the lack of appropriate methods to verify pedigree records. DNA fingerprinting provides an alternative approach for studying the mating biology of swans.

We have found that the EPF rate in swans is very low, supporting the view that swans are faithful to their mates. However, it is notable that the EPF rate in the Whooper Swan is less than in the Mute Swan. This is not surprising if we consider the cost of EPFs and accompanying alloparental care. Whooper Swans are migratory. The young birds remain with their parents during most of their first year of life and the whole family may move from place to place (Scott and Wildfowl Trust 1972). Extra-pair fertilizations or adoption of another female's young will lead to the increase of brood size, and consequently give the pair a heavy burden for looking after the young. The pair is unwilling or unable to invest too much energy in intensive care of the young, and hence copulations are restricted to within the pair-bond. By contrast, Mute Swans are largely non-migratory. The investment of a pair in raising their young is considerably less. The adopted young would not give the pair much trouble. Therefore, extra-pair fertilization and alloparental behaviour are relatively common in the Mute Swan .

It is also noted that the cygnets resulting from the EPFs were raised by the pair male in most cases in the Mute Swan. It is probable that the extra mate of the pair male is a non-breeder who has no experience in hatching

and rearing the young, so that she lays eggs in her rival's nest or leaves the newborn behind.

CHAPTER 6

ISOLATION OF SWAN MINISATELLITES

6.1 INTRODUCTION

DNA fingerprinting has proved to be potent for individual recognition. However, its usefulness for assignment of paternity has been challenged by statistical problems and poor resolution of fingerprinting gels, so that not all conclusions are unambiguous (Lewin 1989, Cohn 1990, also see Chapter 5). Furthermore, screening a population of adults to identify one or both missing parents using DNA fingerprinting is costly and time-consuming and might be inefficient.Therefore, the application of locus-specific probes (LSPs) that are capable of detecting multialleles, combined with the use of multilocus DNA fingerprinting probes, may greatly facilitate the establishment of kinships in a population due to the simplicity of SLP analysis.

However, it is inefficient to obtain the polymorphic LSPs by screening single-copy genomic sequences, because these sequences are relatively conservative (Schumm *et al.* 1988, Bowden *et al.* 1989). The hypervariability of minisatellites makes it ideal to develop polymorphic SLPs by cloning individual minisatellites. The availability of single minisatellites will also allow the mapping of their locations and the study of the evolution of minisatellite sequences. Wong *et al.* (1986) demonstrated in humans that hypervariable minisatellites are clonable. Indeed large-scale isolation of human minisatellites have been done (Wong *et al.* 1987, Nakamura *et al.* 1987, 1988). Some polymorphic minisatellites of animals

have also been isolated (Kelly *et al.* 1989, Gyllensten *et al.* 1989, Hanotte *et al.* 1990).

Two approaches have been used to isolate the minisatellites in the genome. One is by directly cloning specific minisatellites selected from a DNA fingerprint (Wong *et al.* 1986); the other is by screening random minisatellites from a genomic library using either pre-existing minisatellite probes (Wong *et al.* 1987, Gyllensten *et al.* 1989, Kelly *et al.* 1989, Washio *et al.* 1989, Hanotte *et al.* 1990) or synthetic oligonucleotides similar to the consensus sequence of VNTR markers (Nakamura *et al.* 1987, 1988). Although both approaches are equally efficient, the latter approach was adopted in this study since a genomic library could be repetitively used for various purposes.

6.2 CHOICE OF VECTORS AND BACTERIAL STRAINS FOR CONSTRUCTION OF GENOMIC LIBRARY

6.2.1 Choice of Vectors

Plasmids, bacteriophages (λ) and cosmids can all be used as vectors to construct a genomic library. The choice is determined by the length of the sequences under consideration. Most plasmid vectors can accept fragments of foreign DNA up to 10Kb in size, which are shorter than most genes of higher eukaryotes and therefore are not satisfactory for constructing a representative genomic library. At the other extreme, a cosmid is a vector that requires large pieces of foreign DNA, the recombinants containing donor fragments of less than 33Kb are unlikely to be packaged into phage λ particles (Feiss *et al.* 1977, Collins 1979). As most minisatellites are less than 23 Kb, it is not worth using cosmids since these cause more technical

problems than cloning in phage λ. Charomid vectors eliminate the capacity limit of common cosmid vectors, allowing a wide size range (5.3-52Kb) of genomic DNA fragments to be cloned (Saito and Stark 1986); however, they are more difficult to use than the usual cosmid vectors. The remaining choice is phage λ, which has a satisfactory capacity for our purpose as well as a wealth of detailed information about its genomic organization and function. In fact, the phage λ has become the vector of choice for the routine construction of genomic libraries since it was first used as a vector to clone bacterial genes in 1974 (Murray and Murray 1974, Rambach and Tiollais 1974, Thomas *et al.* 1974).

DNA of phage λ is a linear duplex molecule of approximately 49Kb in length. The genome is packaged into the head of the mature phage particle as a linear double-stranded molecule with single-stranded 12-bp 5'-protruding termini. The middle 'stuffer' of the genome, in which no essential gene for lytic growth and plaque formation resides, can be replaced with foreign DNA for cloning. Because the phage DNA remains packageable when its length is 78-105% of the wild type genome length (Weil *et al.* 1973), lambda replacement vectors can usually accept foreign DNA fragments of 9-25Kb in size.

Nowadays, there are a variety of lambda vectors (Sambrook *et al.* 1989). For construction of a genomic library, several basic criteria to be considered are: large vector capacity, multiple cloning sites, high cloning efficiency, minimum non-recombinant background, and ability to propagate in recombination-deficient hosts. In this study, a replacement vector Lambda GEM™-11 (Promega) was chosen.

Lambda GEM™-11 is a multi-functional genomic vector (Fig.6.1), and has been constructed to maximize the size range (9-23Kb) of inserts.

The presence of BamHI sites offers easy ligation of Sau3AI-created genomic fragments to BamHI-cleaved λ arms, while the inserts can be released from the chimeric phages by using the other unique restriction sites in the polylinker region. The ligation products of the left arms and right arms are too small to be packaged. The dual opposed bacteriophage T7 and SP6 RNA polymerase promoters enable RNA probes specific to either ends of the cloned insert to be generated *in vitro*, simplifying chromosome walking.

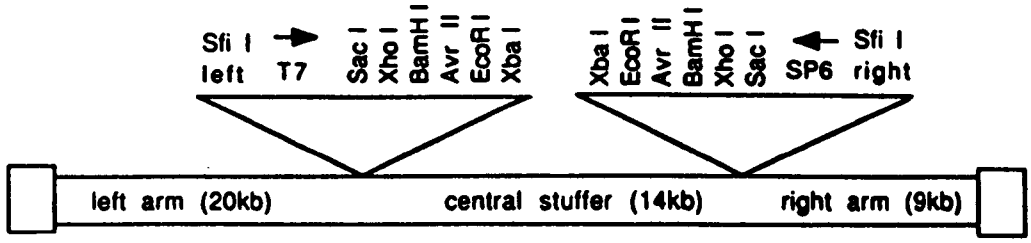


Fig. 6.1 Diagram of vector Lambda GEM™-11. From Figure 1 in Promega Protocols and Applications Guide, photocopied with permission of Promega Co.

Using Lambda GEM™-11, the *Spi*⁻ phenotypic selection against non-recombinants is available. After the replacement of the central stuffer fragment, the recombinants lack the *red* and *gam* genes involved in recombination and can grow well when plated on a RecBC host strain containing a resident P2 lysogen (Kaiser and Murray 1985). However, the growth of non-recombinants ligated between the central stuffer to arms is restricted on *E. coli* P2 lysogen strains. Inasmuch as the molecules ligated

from arms cannot be packaged, *Spi*⁻ genetic selection is unnecessary when the central stuffer has been removed prior to ligation.

6.2.2 Choice of Host Strains

The choice of host strains is very important for successful propagation, amplification and screening of a genomic library that embraces all the sequences of the genome. The host of choice should ensure that all recombinant λ clones are able to grow with equal efficiency, and that any cloned sequence is able to remain unchanged.

The growth of phage depends on viral replication and packaging, which is the outcome of interactions between host and vector genes. The product of the *gam* gene, which usually resides in the middle stuffer of the λ chromosome, inactivates Exonuclease V encoded by the recBCD genes of *E. coli*, so that concatameric λ DNA produced via rolling-circle replication is protected (Enquist and Skalka 1973, Amundsen *et al.* 1986). However, the recombinants arisen by cloning in most λ replacement vectors are *gam*⁻ *red*⁻, and unable to produce the concatamers on rec⁺ hosts that are efficient packageable substrates. Then the generation of packageable substrates (dimers) relies on homologous recombination between monomeric circles produced by θ-form replication in rec⁺ cells. A host containing mutations in recBCD is otherwise required to propagate *gam*⁻ phage, which could restore the rolling-circle replication and concatamer formation.

It has been found that some sequences of genomic DNA are lethal to the vector, or undergo rearrangment when recombinant phage are plated on wild-type *E. coli* hosts (Leach and Stahl 1983, Wyman *et al.* 1985, Wong *et al.* 1986), and these events were believed to be associated with host

recombination systems. These sequences contain palindromes (i.e. inverted repetitions) or direct repetitions (e.g. minisatellites), which are ubiquitous in eukaryotic genomes (Wyman and Wortman 1987). The rec$^-$ hosts (recombination deficient) have been used to propagate λ phages containing such sequences. Leach and Stahl (1983) reported that the palindrome-containing λ phages can efficiently generate plaques only on strains carrying the recBC and sbcB mutations. However, the palindrome showed instability. The viability and stability of the palindromes could be improved by using the sbcC recD or sbcC recD recA strains (Chalker *et al.* 1988).

The main aim of this study was to isolate hypervariable minisatellites. Since other researchers previously found that the minisatellite-containing λ clones showed abnormal growth on rec$^+$ hosts (Wong *et al.* 1986), we used *E. coli* strain DL538 (hsdR, mcrAB, recD1009, sbcC201, SupE44, .leu, pro, Hri-1, F$^-$) to propagate the library and the isolated minisatellite clones. This strain is rec$^-$ and carries the sbcC mutation to enhance the stability of recombinant phages. Another advantage is its tolerance to cytosine methylation in phage recombinants to a certain degree, since eukaryotic DNA is usually methylated (Woodcock *et al.* 1989).

6.3 METHODS FOR CONSTRUCTING GENOMIC LIBRARY

6.3.1 Isolation of high-molecular-weight genomic DNA

With regard to exogenous DNA, the major requirement is that genomic DNA should remain as intact as possible before being exposed to the restriction enzyme that has been chosen for cloning. DNA breakage during the isolation process mainly results from mechanical shearing. The

fragments generated by mechanical shearing have flush ends, which cannot ligate to the vector arms to form packageable recombinants. Since such fragments cannot be removed from the restriction digest, their presence in the donor DNA preparation will lower the cloning efficiency. To obtain DNA of high quality, the procedure for DNA extraction should minimize the number of manipulation steps.

Blood samples were available from several species of swans. The Mute Swan Cygnus olor was selected for the construction of the genomic library because it is a well-studied species and hundreds of blood samples had been collected from different populations. The latter fact made it possible to study population genetics using SLPs. Genomic DNA was extracted from a bird (YLXI), sampled at Abbotsbury, England.

The method of DNA extraction was a scale-up of the one described in Chapter 2, but with more precautions. Approximately 2.2mg of high-molecular-weight (>100Kb) DNA was obtained from 0.3ml of blood.

6.3.2 Size Fractionation of Genomic DNA Digest

In order that a genomic library covers as complete the genome as possible and consists of as few clones as possible, random fragments from the entire genome should be used for cloning. Random fragmentation of genomic DNA can be achieved by controlled mechanical shearing (Maniatis *et al.* 1978), or more conveniently by digestion with restriction enzymes. The most common enzymes used for cloning, recognizing either hexanucleotides or tetranucleotides, cleave genomic DNA into pieces that are smaller than clonable size if the digestion has gone to completion. This can be circumvented by partial digestion, which leaves some of target sites

uncut so as to produce a set of overlapping fragments in the desired size range.

Less frequently cutting enzymes, such as EcoRI (on average once every 4096bp in random sequence DNA), may release long stretches due to lack of cleavage sites. Large fragments cannot be cloned in certain vectors, thereby resulting in loss of some regions. By contrast, 4-bp enzymes have higher density of target sites in the genome and provide even cutting of the DNA. So for most cloning strategies, frequent cut enzymes are used, for example, Sau3AI or MboI.

The vector Lambda GEM™-11 has BamHI sites, so Sau3AI is used in this study. Sau3AI cuts the DNA at /GATC sites, leaving a 5'-overhanging terminus CTAG that is compatible with the single-stranded projection generated by BamHI cleavage (G/GATCC). Sau3AI on average cleaves a long random DNA molecule once every 256 base pairs, provided that all bases are equally frequent. To generate fragments in the size range 10-23KB, the enzyme is only required to cut at 1/40-1/90 of the available recognition sites, which can be performed by controlling enzyme concentration or reaction duration.

The partial genomic digest will certainly contain a proportion of fragments that go beyond the limits in size for a particular vector. Such fragments ligate to the vector arms to form unpackageable recombinants. Furthermore, smaller fragments might self-ligate to form multiple inserts. Therefore it is necessary to prevent the random association of genomic DNA fragments during cloning. One of methods is the physical separation of fragments of the desired sizes, which gives rise to higher cloning efficiency.

The vector Lambda GEM™-11 accepts donor DNA fragments in the size range 9-23Kb. Fragments in that range can be physically separated from the rest either by velocity centrifugation through sodium chloride or sucrose gradients or by electroelution following electrophoresis in agarose gel (Maniatis *et al.* 1982). The latter was used in this study.

Protocol For Preparation of Donor DNA fragments:

1. Digest 250µl genomic DNA with Sau3AI (0.0334 units/µg DNA)

250µg DNA
1.0µl Sau3AI (8units/µl)
500µl 10X reaction buffer 4
100µl BSA (5mg/ml)
SDW to 1ml

Mix by gently inverting, then dispense the mixture into ten 1.5-ml Eppendorf tubes. Incubate at 37°C for 30 minutes. Cool the reaction mixture on ice. Take out an aliquot (0.5µg) of digest to check the progress of the digestion on a 0.4% agarose gel. When the fluorescence shows the correct size distribution, stop the digestion reaction by adding 1/10 volume of 10XBPB.

2. Following the separation of the Sau3AI genomic DNA digest on a 22-cm long, 0.4% agarose gel overnight at 40 volts, the gel slices containing fragments in the desired size range (9-23Kb) are cut out free from the other parts under a transilluminator.

3. Heat a piece of dialysis tubing in boiling water containing 1mM EDTA for 10 minutes. Wash the tubing thoroughly with distilled water.

4. Seal one end of the tubing with a dialysis clip. Fill the bag with plenty of 0.5X TAE, and place the gel slices into the bag. Remove most of buffer and any air bubbles. Then clip the other end of the bag just above the gel slices.

5. Place the bag into a minigel tank holding plenty of 0.5X TAE. After running at 100 volts for 3 hours, reverse the polarity of the current for 2 minutes so as to release the DNA from the inner wall of the bag.

6. Open the bag and collect all of the buffer surrounding the gel slices into Eppendorf tubes. Wash the inner wall of the bag with 0.5X TAE and collect the buffer into the tubes.

7. Pass the collected DNA solution through a column of packed siliconized glass wool made in a 1-ml syringe. Purify the DNA by extracting sequentially once with phenol, once with phenol /chloroform and once with chloroform as described in Chapter 2.

8. Recover the DNA by ethanol precipitation. Resuspend the DNA pellet in an appropriate volume of TE.

Using the above method, approximately 15µg (in 65µl TE) DNA fragments in the size range 9-23Kb were obtained from 250µg of λ Sau3AI genomic DNA partial digest. It was noted that a mere trace of fragments less than 9.0Kb in size was present in the preparation.

6.3.3 Ligation and *in vitro* Packaging

The joining of DNA fragments through phosphodiester bonds is catalyzed by DNA ligase that promotes the covalent linkage of the 3'-hydroxyl terminus of one strand of DNA to the 5'-phosphate residue of a second if both strands are paired on the same molecule (Lehman 1974). In a ligation mixture, DNA fragments are first joined between complementary sticky ends through hydrogen bonds, then the ligase covalently seals the

nicks present in the joined molecules. The first process has a low melting temperature of 5° to 6°C, while the ligase performs best at 37°C. Consequently, a ligation reaction is usually carried out at 10-16°C as a compromise. Since T4 DNA ligase can join sticky ends as well as flush ends, it has been extensively used for cloning. A higher concentration of the substrates will favour intermolecular joining over intra-molecular joining.

To become infectious phage particles, λ recombinant DNA must be packaged into phage heads. Inasmuch as concatenated molecules and multimers are the most efficient substrates for packaging, the ligation conditions should favour the formation of concatenated molecules or multimers, which depends on the molar ratio of arms to the inserts. The optimum ratio for a particular experiment can only be determined by trial reactions.

The λ recombinant DNA can be efficiently packaged *in vitro*. Packaging extracts are prepared from either one or two bacterial *E. coli* strains containing lysogenic phage λ and are commercially available. Two-strain extracts are the mixtures of extracts prepared from two strains that have complementary defects in λ packaging protein genes (Hohn and Hohn 1974), and usually cause a high background of plaques generated from packaged endogenous phages. One-strain extract is prepared from a single bacterial strain whose λ prophage is deleted for *cos* sites and has much lower endogenous phage background (Rosenberg *et al.* 1985). An extra advantage of a one-strain extract is that it is free from Eco K that can cleave some genomic DNA cloned during packaging, since it is prepared from a lysogenic bacterial strain of *E. coli* C rather than from *E. coli* K-12 derived strains. The efficiency of packaging recombinant DNA (whichever system is used) can be over 10^7pfu/μg of vector (Promega 1988), which is sufficiently high for successful construction of a representative genomic

library. However, the packaging efficiency may vary between different batches of packaging extracts.

In this study, a cloning kit was purchased from Promega. The kit consisted of vector Lambda GEM™-11 BamHI arms and one-strain packaging extracts. The central stuffer of the vector had been removed by the manufacturer, which simplified the cloning procedure.

The method for ligation and *in vitro* packaging is adopted from the manufacturer's recommendations (Promega 1987):

1. Set up ligation mixture in a 0.5-ml Eppendorf tube

   0.5µg Lambda GEM™-11 BamHI arms (1mg/ml)
   0.5µg preparation of swan Sau3AI DNA fragments
   0.5µl 10X ligase buffer
   1.0µl T4 ligase
   H_2O to 5µl

2. Incubate the mixture at 14°C overnight.

3. Thaw the packaging extract (50µl) on ice. Add the ligation mixture to the extract and mix by gently flicking the tube. Incubate at room temperature for 2 hours.

4. Add 445µl of phage buffer and 25µl chloroform to the packaging mixture. Gently vortex to mix and allow the chloroform to settle to the bottom of the tube. Chloroform will help to kill any viable bacterial cell.

5. Store the packaged phage at 4°C.

6.3.4 Plating Bacteriophage λ

The packaged recombinants have to be introduced into *E. coli* cells to

propagate via lytic growth. At the final stage of the phage infection, a infected bacterial cell is lysed and the neighbouring bacteria become infected by the progeny virus particles. If the infected cells are spread onto a solid agar or agarose medium, then cell lysis can be visualized on a lawn of bacteria as a clear area, called a 'plaque'. The number of plaques is equivalent to the number of viable packaged λ recombinants in the absence of non-recombinant background, because each plaque contains the progeny of a single phage particle.

Protocol for Plating Bacteriophage λ

The following procedure is based on the method described by Sambrook et al. (1989).

1. Preparation of plating bacterial cells: Grow an overnight culture of bacterial strain DL538 by inoculating a single colony into 5 ml of LB (or TB) medium and incubate at 37°C overnight. The following day, inoculate 50ml of LB (or TB) medium, supplemented with 0.5ml of 20% maltose, with 1ml of the overnight culture and incubate with agitation at 37°C until O.D.$_{600}$ has reached 0.6. Centrifuge the cells at 4,000g for 10 minutes. Resuspend the cell pellet in 10ml of 10mM $MgSO_4$, followed by incubating at 37°C for 30 minutes. Store the cell preparation at 4°C.

2. Prepare tenfold serial dilutions of phage stock (packaged phage or phage elution). Mix 0.1ml of each dilution with 0.2ml of plating bacterial cells, and incubate at 37°C for 30 minutes to allow the phage particles to absorb to the bacteria.

3. Add 3ml of molten (45°C) top agarose (0.6%) LB to the mixture. Vortex briefly and immediately pour onto LB plates containing approximately 35ml of hardened bottom agarose (1%) LB. Allow the top agarose to harden

and incubate inverted at 37°C overnight. Plaques will start to appear after 7 hours of incubation.

6.4 IDENTIFICATION AND ISOLATION OF MINISATELLITE-CONTAINING PLAQUES

A genomic library may contain as many as 10^6 (or more) independent clones. It is essential to identify and isolate particular clones that contain a sequence of interest from the library. The most commonly used method is *in situ* hybridization (Benton and Davis 1977) if there is a suitable probe. The phages are plated and the pattern of plaques is determined by imprinting individual plaques from the agarose plate onto a membrane filter. Then the filter is rinsed with alkali solution to denature the phage DNA so that the phage DNA will be irreversibly bound to the filter by baking. After that, the filter hybridization is carried out in the same way as Southern blot hybridization. Following autoradiography , hybridizing plaques will show their locations on the autoradiograph.

6.4.1 Procedure for Identification and Isolation of Positive Clones

1. Mix 0.4ml of the packaged phage with 0.6ml of plating bacterial cells. Incubate at 37°C for 30 minutes.

2. Add 25ml of molten top agarose LB. Vortex and immediately spread onto a 22.5X22.5cm plastic dish holding 300ml of hardened bottom agarose LB.

3. Leave for 30 minutes at room temperature and then incubate inverted at 37°C overnight.

4. Place the dish at 4°C for at least one hour . This will prevent the top layer from being removed when the filter is lifted.

5. Lay a sheet of 20X22cm dry Hybond-N (Amersham) filter on the surface of the top layer, and allow to absorb for 30 seconds. Mark the filter and the plate by stabbing through both with an hypodermic syringe needle containing Indian ink. Lift the first filter and lay another dry filter following the same procedure.

6. Place the filters plaque-side up for 5 minutes on Whatman paper presoaked in 2X SSC, 5% SDS. Transfer the paper with filters to a microwave oven and heat for 3 minutes at full setting. These treatments result in lysis of cells, denaturation of DNA and fixation of DNA to the filter (Buluwela *et al.* 1989).

7. Carry out filter hybridization as usual except that post-hybridization wash is done in 1X SSC and 0.1% SDS. One filter is probed with pSPT19.6 and the other with pSPT18.15.

8. Align the film with the plate following autoradiography . Pick the positive plaques by invertedly plunging a 5-ml test tube to the bottom through the agarose surface inside which there is at least one positive plaque.

9. Expel the plaque-containing agarose into 2ml of phage buffer in a 25-ml test tube. Add 50µl of chloroform and vortex to kill the bacteria. Allow the phage particles to diffuse out of the agarose at room temperature for at least two hours or at 4°C overnight.

10. For storage of non-positive plaques, overlay the plate with 100ml of phage buffer and place at 4°C overnight. Remove the phage suspension to two 50-ml sterile polypropylene tubes containing 2.5ml chloroform. Centrifuge at 4,000g for 5 minutes to remove cell and agarose debris. Transfer the supernatant to fresh tubes and add 0.3% chloroform. The phage suspension is stored at 4°C.

Because the plaque density for plating the library is high, each isolate contains more than one plaque. Therefore it is necessary to further purify the isolates to obtain the pure progeny of individual phage particles. This has been achieved by three rounds of successive rescreening, during which only well-separate positive plaques are isolated.

6.4.2 Preparation of Phage λ DNA

The method used for extraction of phage λ DNA is based on that described by Maniatis *et al.* (1982).

1. After the last round purification, plate 25ml of eluted phage on a 90cm petri dish as previously described. To obtain a high yield of phage DNA, plaque density should be very high (visualized as confluent).

2. Following incubation overnight, add 5ml of phage buffer onto the plate and elute the phage for 2 hours at room temperature with constant shaking or overnight at 4°C.

3. Remove the buffer to a 15-ml polypropylene tube. Centrifuge at 8,000g for 10 minutes to remove bacterial debris.

4. Recover the supernatant and add RNase A and DNase I to a final concentration of 1μg/μl each. Incubate at 37°C for 30 minutes.

5. Add an equal volume of 20% polyethylene glycol(PEG), 2M NaCl in phage buffer and incubate for at least one hour at 0°C (in ice-water). The PEG absorbs water in the presence of salt, thereby causing phage particle assemblies to precipitate as a white mass.

6. Recover the precipitated phage by centrifugation at 10,000g for 20 minutes at 4°C.

7. Discard the supernatant and resuspend the phage pellet in 0.5ml phage buffer. Transfer the phage suspension to an Eppendorf tube and centrifuge for 2 minutes to remove debris.

8. Remove the supernatant to a fresh tube, followed by adding 5μl of 10% SDS and 5μl of EDTA (pH 8.0). Incubate at 68°C for 15 minutes.

9. Extract once with phenol, once with phenol/chloroform and once with chloroform sequentially as described before.

10. To the final aqueous phase add an equal volume of absolute ethanol. Freeze at -70°C for one hour. Centrifuge for 15 minutes at 4°C.

11. Resuspend the phage DNA pellet in 50μl of TE buffer.

Using the above method, only six to ten micrograms of λ DNA are routinely obtained.

6.4.3 Results

Using the Lambda GEM™-11 BamHI arms, the recombinant

efficiency was $3.5X10^5$ pfu/μg DNA for our library construction. The genomic library of the Mute Swan consisted of approximately $1.8X10^5$ recombinants. By *in situ* hybridization, the polycore probes pSPT19.6 and pSPT18.15 under low stringency (1X SSC) each revealed in the non-amplified library several hundred positive recombinants, which showed variation in autoradiographic intensity (Fig. 6.2). By using the two-dimensional DNA fingerprinting system, Uitterlinden *et al.* (1989) indeed resolved as many as 372 minisatellite fragments per individual for probe 33.15 and 625 for probe 33.6 in humans.

In the first round of screening, 40 plaques that positively hybridized to pSPT19.6 and 25 to pSPT18.15 were isolated from the genomic library. Only 12 of them, half hybridized to pSPT19.6 and half to pSPT18.15, were chosen to enter the next round of screening. This time only one well-separated positive plaque was isolated from each replating isolate, and was subjected to another round of purification. At the last stage, 15 positives hybridized to pSPT19.6 and 13 to pSPT18.15 were isolated. These isolates were numbered sequentially starting with λcoMS6.1 and λcoMS15.1, in which λcoMS6 and λcoMS15 referred to minisatellite-containing λ phage isolated from *Cygnus olor* by hybridizing to the human multi-locus probes pSPT19.6 and pSPT18.15, respectively.

To confirm whether the isolated clones contain minisatellites, λ DNAs were extracted , and analysed by digestion with restriction enzymes, gel electrophoresis and hybridization. The λcoMS were first analysed using the enzyme XhoI. The restriction patterns showed that every clone contained at least one fragment apart from the λ arms (data not shown here). When digested with EcoRI, most of the recombinants exhibited a large number of smaller fragments that were derived from the inserts. However, some clones showed identical or similar restriction patterns, suggesting that they may

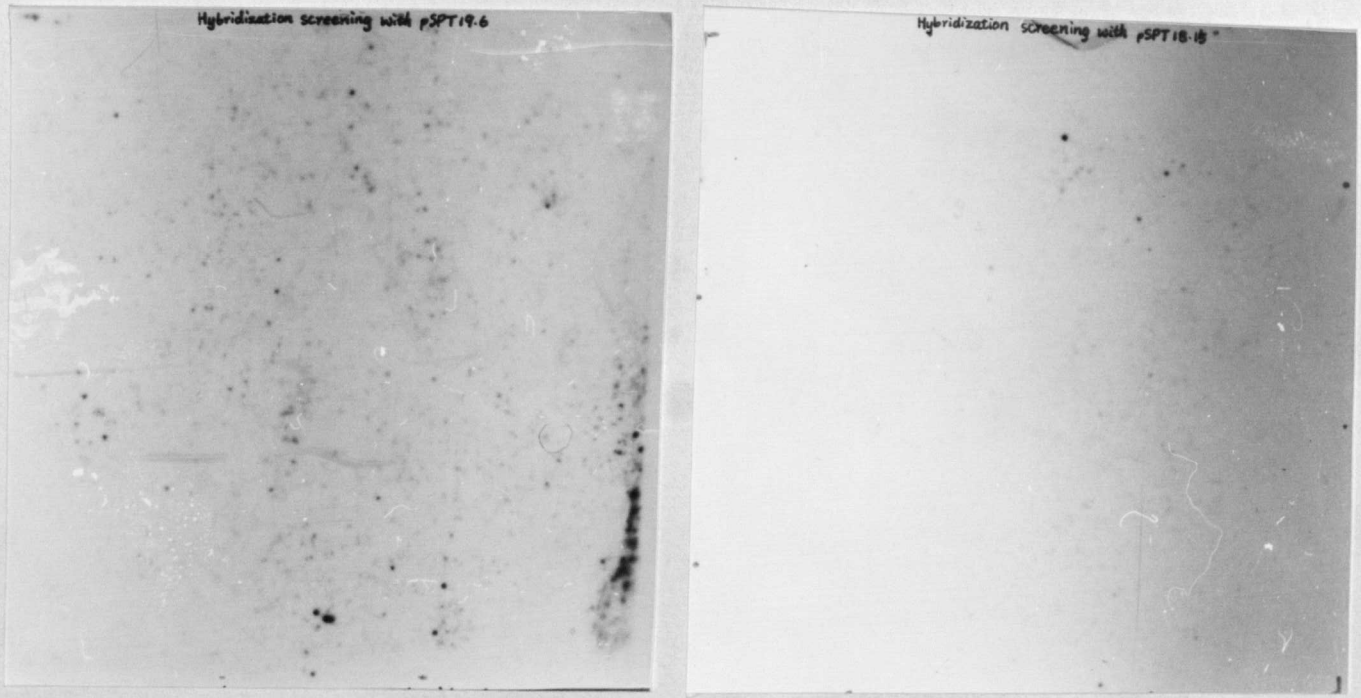


Fig.6.2 Screening of swan minisatellite clones from the λ genomic library. The dark spots are positive clones that may contain a minisatellite related in sequence to the corresponding probe . Procedure for identification of positives was described in the text.

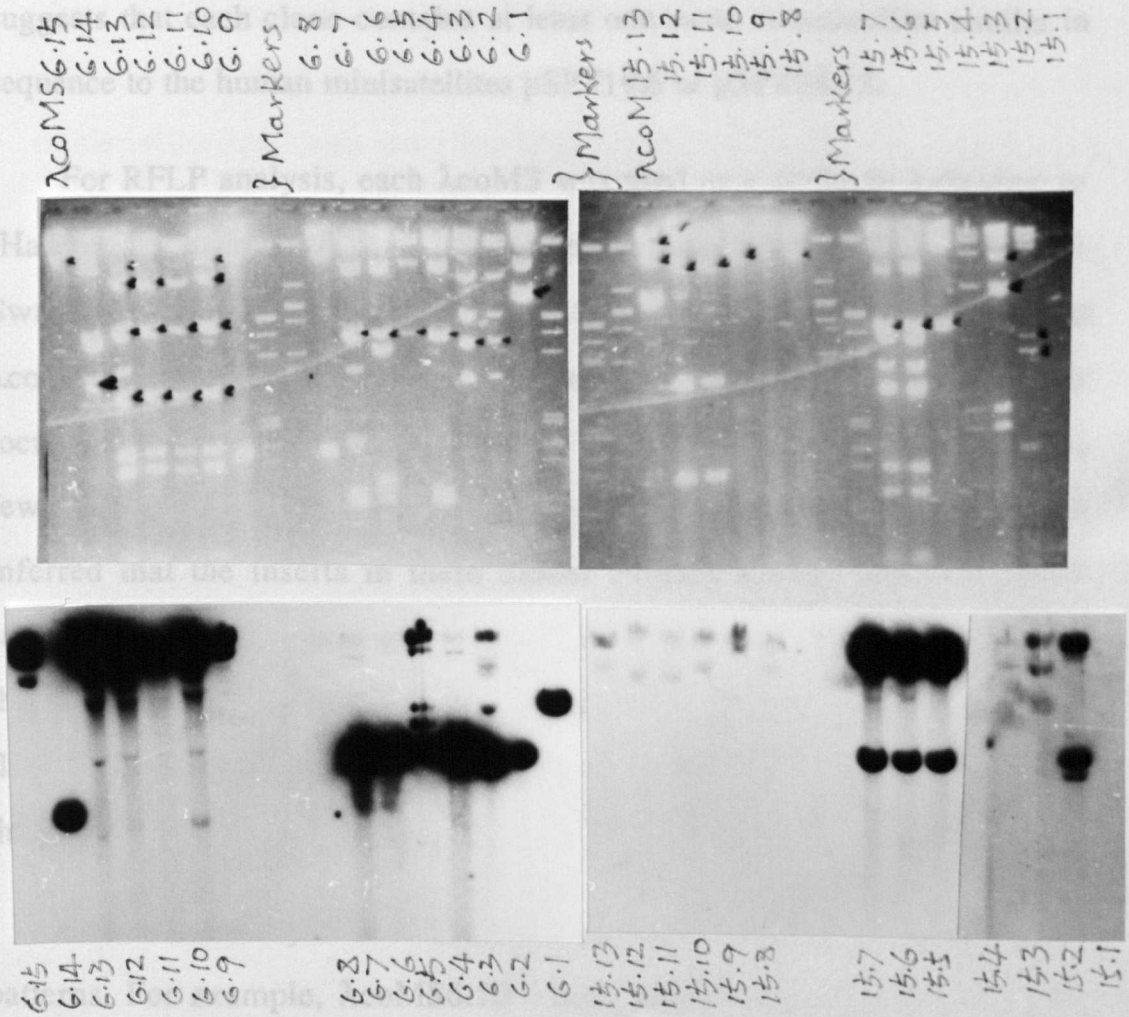


Fig.6.3 EcoRI restriction patterns of λcoMS. Approximately 2μg of λ DNA was diges
EcoRI and the fragments were electrophoretically separated on 1% agarose gels. The gels were
under a transilluminator after EtBr staining (top photos). Then the gels were blotted and the
blots were hybridized to a corresponding probe (pSPT19.6 or pSPT18.15) under a stringency of
The autoradiographs were obtained after 24 hour exposure.

have been derived from the same locus (Fig.6.3). Hybridization revealed that all the clones have at least one EcoRI fragment strongly or weakly hybridizing to the appropriate human multi-locus probes (Fig.6.3).This suggests that each clone contains at least one swan minisatellite similar in sequence to the human minisatellites pSPT19.6 or pSPT18.15.

For RFLP analysis, each λcoMS was used as a probe to hybridize to (HaeIII, EcoRI and PstI) restriction digests of a panel of six random Mute Swans including the one used for constructing the genomic library. Most λcoMS detected many restriction fragments in the genome, rather than few locus-specific fragments even at a high stringency (0.1X SSC), whereas a few detected two to three monomorphic fragments (data not shown). It was inferred that the inserts in these clones contain a long stretch of DNA sequence, which might include non-minisatellite sequences that intervene in the detection of polymorphisms. Therefore, it was decided to remove the flanking sequence by subcloning specific restriction fragments derived from the λcoMS.

Restriction analysis showed that some have almost identical restriction patterns. For example, λcoMS6.10 - 6.13, which were derived from the same positive clone of the second-round screening, showed only one different fragment in EcoRI restriction pattern (Fig.6.3). This difference may have resulted from recombination during purification.

6.5 SUBCLONING OF SWAN MINISATELLITES

6.5.1 Strategy for Subcloning

As mentioned above, the inserts contained in λcoMS are a mixture of DNA fragments, including minisatellite(s) and flanking sequences. The

flanking sequences may interfere in the detection of minisatellite variation. Through subcloning of minisatellites to remove as much flanking sequence as possible, it is possible to study individual minisatellites in detail. During characterization of λcoMS, EcoRI digestion revealed some small minisatellite fragments contained in the lcoMS. The sizes of these minisatellite fragments were less than 10Kb, falling into the capacity range of plasmid vectors. Hence a plasmid vector pGEM-3zf(+) (Promega) was used for subcloning.

The pGEM-3zf(+) is a multi-purpose plasmid vector (Fig.6.4), derived from pUC plasmids. It has a polycloning site flanked by SP6 and T7 RNA polymerase promoters, allowing easy cloning and *in vitro* transcription of the cloned insert. The presence of the origin of replication of the filamentous phage f1 in the vector allows production of single-stranded plasmid DNA, suitable for sequencing, mutagenesis and other applications

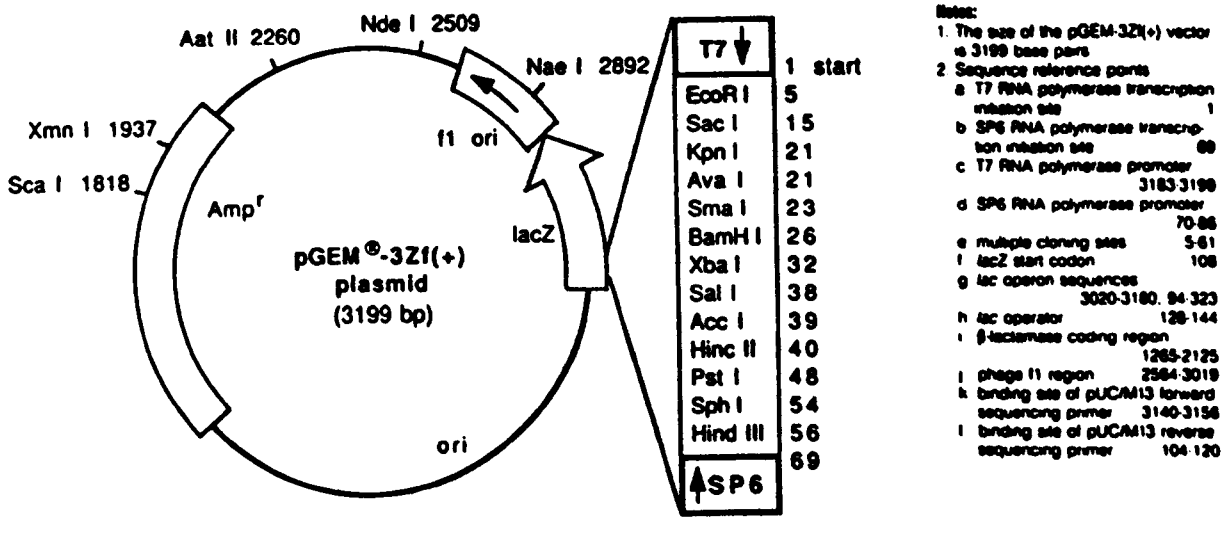


Fig. 6.4 Diagram of Plasmid vector pGEM-3zf(+). From Figure 15 in Promega Protocols and Applications Guide, photocopied with permission of Promega Co.

When cloning, the EcoRI minisatellites of interest contained in the λcoMS are first purified, then ligated to EcoRI digested and dephosphorylated vector. Treatment of vector with calf intestinal alkaline phosphatase (CIAP) prior to ligation removes 5' phosphate groups and thus prevents recircularization of the vector during ligation.

The ligation mixture is used to transform *E. coli* strain NM522 cells. This strain carries the *lac* ZΔM15 and *lac* I^Q on an F' episome, allowing identification of recombinants. When X-gal and IPTG (isopropyl-thiogalactoside) are added to agar along with ampicillin, recombinant colonies, the cells of which cannot synthesize β-galactosidase, are white, distinguished from blue-coloured non-recombinant colonies (Vieira and Messing 1982).

6.5.2 Procedure for Subcloning

I. PREPARATION OF VECTOR DNA [The vector pGEM-3zf(+) DNA was purchased from Promega.]

1. Digest 10μg of the plasmid DNA to completion with EcoRI.

2. To dephosphorylate the linear plasmid DNA, add the following components to the digested vector DNA:

5.0μl CIAP buffer
0.8 unit CIAP (0.01 unit/Mole of ends)
H_2O to 50μl

Incubate for 30 minutes at 37°C.

3. Stop the reaction by adding 1µl of 0.5M EDTA and heat to 65°C for 20 minutes.

4. Following purification of DNA by extracting with phenol/chloroform and chloroform, precipitate the DNA with ethanol and resuspend the DNA pellet in 20µl of TE (final concentration: 0.3µg/µl).

II. PREPARATION OF INSERT DNA

The λcoMS are first digested to completion with EcoRI and the fragments are separated by electrophoresis in 1% agarose gel. The fragments of interest are recovered by using the liquid nitrogen method (Koenen 1989) as follows.

1. Following electrophoresis, cut the band out of the gel on a transilluminator that has been stained. Place the gel slice into a yellow pipette tip plugged with cotton and submerge it for 5 minutes in liquid nitrogen.

2. Puncture a 0.5-ml Eppendorf tube at the bottom and stick the tip through the hole and then put the tube into a 1.5-ml Eppendorf tube.

3. Centrifuge for 5 minutes at full speed. The extracted aqueous solution containing the DNA is collected in the 1.5-ml tube.

4. Add 1/10 volume of 4M LiCl and extract once with one volume of phenol.

5. To the aqueous solution add 3X volumes of absolute ethanol and leave at -80°C for 30 minutes.

6. Centrifuge for 10 minutes and wash the DNA pellet with 75% ethanol. Resuspend in an appropriate quantity (10-15µl) of SDW for use.

III. LIGATION

Because of the difficulty of estimating the concentration of insert DNA, I simply used as much insert DNA as possible within the limit of the volume of ligation reaction (10μl). The low yield of recombinants would not affect the efficiency of the identification of recombinants since only one recombinant colony (white) is required.

Set up the 10μl ligation reaction as follows:

6μl	insert DNA
1μl (0.3μg)	dephosphorylated vector DNA
2μl	5X ligation buffer
1μl (1 Weiss unit)	T4 DNA ligase

Incubate overnight at 4°C.

IV. PREPARATION AND TRANSFORMATION OF COMPETENT CELLS

The following procedure is a modification of that described by Kushner (1978).

1. Inoculate 20ml of L-broth with 0.5ml of an overnight culture. Grow cells at 37°C for 1-2 hours until the $O.D._{600}$ is between 0.13-0.15.

2. Centrifuge the cells for 5 minutes at 5,000g in a 30ml Corex tube.

3. Remove the supernatant and resuspend the cells in 1ml of solution A (10mM MOPS, pH7.0, 10mM rubidium chloride). Then bring the volume up to 10ml and pellet the cells as described above.

4. Remove the supernatant and resuspend the cells in 10ml of solution B (10mM MOPS, pH6.5, 10mM rubidium chloride, 50mM $CaCl_2$). Incubate on ice for 30 minutes, followed by centrifugation.

5. Remove the supernatant and drain the tube thoroughly. Gently resuspend the cells in 1ml of solution B.

NB: at this stage, 10% glycerol may be added to solution for long-term storage. The competent cells remain stable for 5-6 weeks when stored at -70°C.

6. Add 3µl of dimethylsulfoxide (DMSO) to 0.2ml of competent cells. Then add the ligation mixture and incubate on ice for 30 minutes.

7. Heat shock the cells at 42°C for 2 minutes. Chill the cells on ice for 1-2 minutes.

8. Add 4ml of L-broth and incubate for 60 minutes at 37°C. This allows the transformed bacterial cells to recover and to express the antibiotic resistance encoded by the plasmid.

9. Centrifuge at 5,000g for 5 minutes. Gently resuspend the cells in 200µl of L-broth.

10. Transfer the cells onto the centre of a 90-mm LB plate containing 100µg/ml ampicillin, 0.5mM IPTG and 40µg/ml X-gal. Using a sterile bent glass rod, gently spread the cells over the surface of the agar plate.

11. Leave the plate at room temperature until the liquid has been absorbed. Then incubate inverted at 37°C overnight.

V. SELECTION AND DNA EXTRACTION OF RECOMBINANT PLAS~MIDS

As mentioned previously, a colour screening for recombinants is available for pGEM-3zf(+). The bacterial colonies harboring recombinant plasmids are white, while the remaining colonies are blue. Therefore, three

independent white colonies were picked from each plate and then overnight cultures were grown. Plasmid DNA was extracted from the cultures and analysed by restriction and gel electrophoresis. Only one selected colony (containing recombinant plasmid with the right size) from each subcloning was stored for subsequent use.

The following procedure for preparation of plasmid DNA from a small-scale culture ("miniprep") is adapted from that described by Ish-Horowicz and Burke (1981).

1. Grow fresh overnight culture by inoculating a single colony into 5ml of LB containing 0.5mg ampicillin.

2. Spin down 1.5ml of the culture for 2 minutes in a microcentrifuge in a 1.5ml Eppendorf tube.

3. Remove supernatant and resuspend the cells in 100µl of miniprep buffer. Incubate at room temperature for 5 minutes.

4. Add 200µl of freshly prepared solution of 0.2M NaOH, 1%SDS and incubate at room temperature for 5 minutes.

5. Add 150µl of precooled (4°C) 5M potassium acetate. Mix gently and leave on ice for 5 minutes. SDS, protein and chromosomal DNA will precipitate as white mass.

6. Following centrifugation for 5 minutes, transfer the aqueous solution to a fresh tube.

7. Extract the solution with phenol and chloroform and precipitate DNA with ethanol as described before.Resuspend the DNA pellet in 50µl of TE buffer.

When extracting DNA from a large-scale culture (e.g.500ml), the addition of solutions is just scaled up. However, chloramphenicol may be added at a concentration of 170μg/ml to the culture that has grown to the middle log phase prior to harvesting of the cells. This treatment will result in the increase of copy number of plasmids without the increase of cells, facilitating the purification of plasmid DNA.

6.5.3 Minisatellite-containing Plasmids

Restriction of λcoMS with EcoRI revealed some small minisatellite fragments. By subcloning 9 λcoMS, we obtained nine independent recombinant plasmids. These plasmids were simply named by replacing the symbol λ in λcoMS with p (plasmid) as pcoMS). For instance, pcoMS6.1 means a recombinant plasmid containing a specific minisatellite fragment derived from λcoMS6.1. Two EcoRI fragments from the λcoMS6.11 were subcloned, and the resulting two clones were names as pcoMS6.11B and pcoMS6.11S. The derivation and size of the inserts in pcoMS are listed in Table 6.1.

The pcoMS were digested with EcoRI to release the inserts. The digests were probed with pSPT19.6 and pSPT18.15. The insert in pcoMS6.11B and pcoMS6.11S hybridized very weakly to pSPT19.6, suggesting that they had little homology in sequence with pSPT19.6 (Fig.6.5).The remaining pcoMS all hybridized strongly to the corresponding human multi-locus probes.

6.6 VARIATION OF SWAN MINISATELLITES

All the pcoMS were first used to probe random birds of the Mute

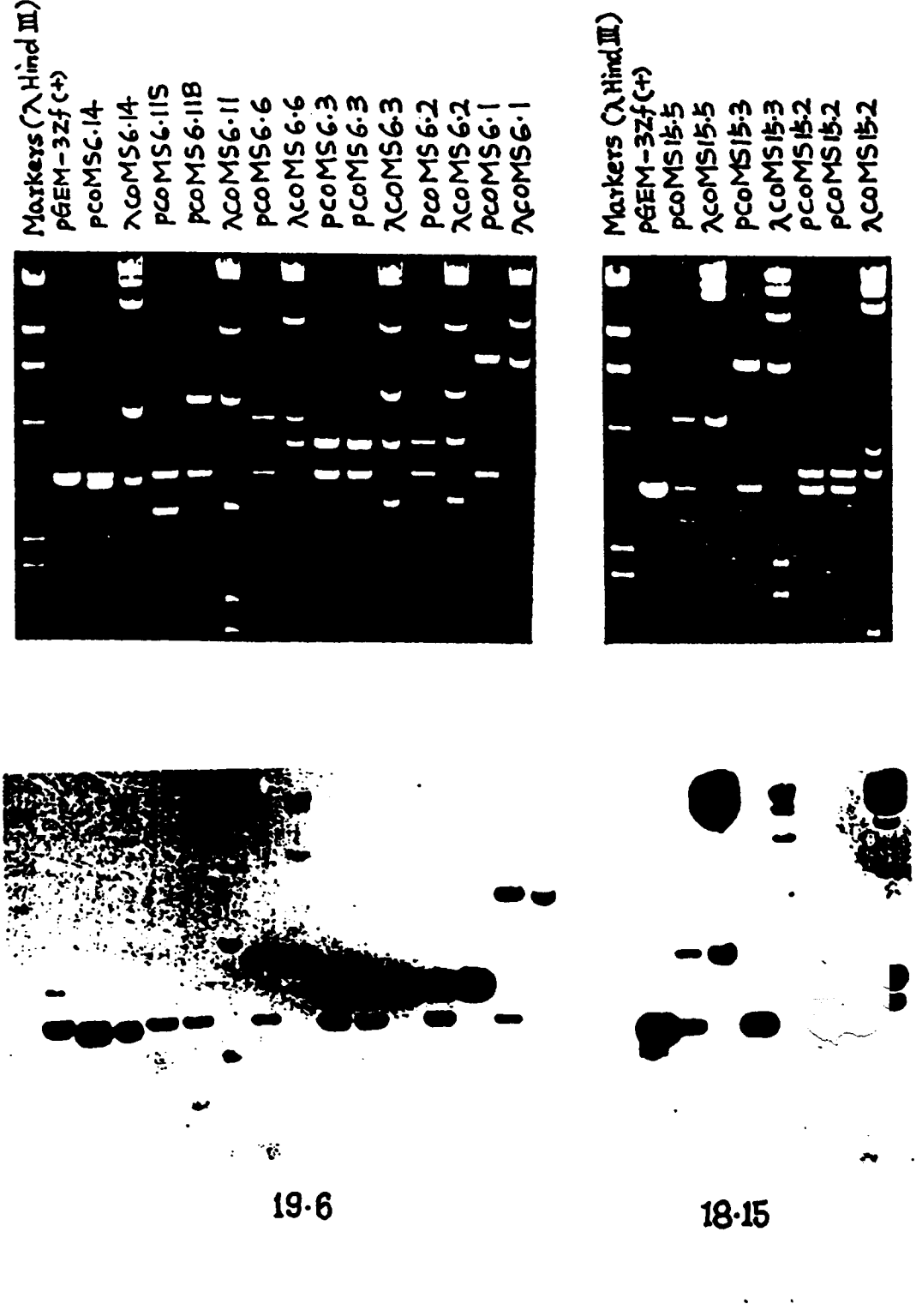


Fig.6.5 Comparison of λcoMS with their derivatives pcoMS. Top: EtBr-stained gels show EcoRI restriction patterns; bottom: hybridization patterns obtained by hybridizing to a corresponding probe. The methods were described in the legend of Fig.6.3.

Table 6.1 Swan minisatellites cloned in plasmid pGEM-3zf(+)

Recombinant plasmids	Size Kb	Hybridization to	Origin
pcoMS6.1	6.9	pSPT19.6	λcoMS6.1
pcoMS6.2	3.9	pSPT19.6	λcoMS6.2
pcoMS6.3	3.9	pSPT19.6	λcoMS6.3
pcoMS6.6	4.6	pSPT19.6	λcoMS6.6
pcoMS6.11B	5.0	pSPT19.6	λcoMS6.11
pcoMS6.11S	2.8	pSPT19.6	λcoMS6.11
pcoMS6.14	3.1	pSPT19.6	λcoMS6.14
pcoMS15.2	3.4	pSPT18.15	λcoMS15.2
pcoMS15.3	7.2	pSPT18.15	λcoMS15.3
pcoMS15.5	5.1	pSPT18.15	λcoMS15.5

Swans. A set of restriction enzymes were used, such as EcoRI, HaeIII, HpaII and PstI. Apart from pcoMS15.2 and pcoMS15.3, the other minisatellites show invariable hybridization profiles on EcoRI digests. It was noted that PstI was the best enzyme for the detection of RFLPs. In addition, each swan minisatellite was used to hybridize to PstI digests from a panel of birds of various swan species (i.e. *C. olor, C. bewickii, C. buccinator* and *c. cygnus*), to identify species-specific minisatellite probes.

During hybridization analysis, I suffered from some technical impediments in the identification of locus-specific variation. All the swan minisatellites failed to detect a specific locus in the genome in the absence of competitor DNA. Although adding competitor DNA (i.e. sonicated Herring sperm DNA) to the hybridization solution significantly reduced the background, it also resulted in a decrease of the hybridization signal. Particularly, use of salmon sperm DNA as competitor led to a heavy loss of the hybridization signal. Another problem was the instability of hybrids. Despite a low stringency post-hybridization wash (i.e. 1x SSC), all of radioactive probes were almost washed off when the wash lasted more than half an hour. There so far is no explanation of these phenomena, and the difficulties need to be overcome. Therefore, the results presented here are preliminary.

1. pcoMS6.1. It detects two monomorphic bands in the genomes of the Mute, Bewick's and Whooper Swans (Fig.6.6A). These two bands may each represent a homologous locus. However, it detects an RFLP in PstI digests of the Trumpeters. Almost every Trumpeter has a common PstI fragment, suggesting that it may represent a homologous locus. Apart from that fragment, each Trumpeter has one or two fragments that could be derived from another locus. A total of five alleles have been detected at the second (polymorphic) locus among 21 individuals (Fig.6.6A-C). Restriction with other enzymes such as EcoRI, HaeIII and HinfI generated invariable

patterns in the same samples (Fig.6.6B), suggesting that the polymorphism at this locus result from loss/gain of PstI sites rather than from variation in the number of repeats. The lack of pedigrees from the Trumpeter Swan disallowed further study of the inheritance of this RFLP.

2. pcoMS6.2 and pcoMS6.3. They detect the same pattern of restriction fragments, suggesting that they are essentially the same sequence. Under low stringency (1X SSC) in the absence of competitor DNA, they hybridize to many PstI fragments in all of species (Fig.6.7), the majority of which are invariable. But specific variation is identifiable in the fingerprint-like hybridization patterns.

3. pcoMS6.6. It detects a number of variable PstI fragments in all four species of swans under low stringency (Fig.6.8). In the presence of competitor DNA, however, invariable but species-specific LSP patterns were obtained under high stringency (appearing as dark bands in Fig.6.8). This swan minisatellite can be of use only as a multi-locus probe.

4. pcoMS6.11B and pcoMS6.11S. Although they are derived from the same λ recombinant, they essentially hybridize to different loci. Under high stringency, pcoMS6.11B detects two monomorphic PstI fragments (3.6Kb and 1.9Kb in size, respectively) in all species, while pcoMS6.11S detects one monomorphic PstI fragment with a length of approximately 4.0Kb (Fig.6.9). It seems that these two loci are very conservative among species of swans. However, pcoMS6.11S is able to hybridize in 1X SSC to a set of variable fragments in PstI digests of Mute Swans.

5. pcoMS6.14. This minisatellite hybridizes very strongly to the human minisatellite pSPT19.6, but shows substantial intra-specific conservation (Fig.6.10). It detects two PstI fragments in each individual.

The bigger fragment has an autoradiographic intensity threefold stronger than the small one, suggesting that each fragment represents a separate homologous locus. Interestingly, pcoMS6.14 is conservative among the northern swans - Bewick's, Trumpeter and Whooper Swans, consistent with their taxonomic relationships.

6. pcoMS15.2. It hybridizes to many restriction (PstI or HpaII) fragments even under high stringency conditions (1x SSC, 65°C). The hybridization patterns are highly variable among individuals (Fig.6.11), similar to those generated with human polycore probes. Further characterization shows that the hypervariable fragments are inherited in a Mendelian fashion. It can be used as a DNA fingerprinting probe.

7. pcoMS15.3. It detects three monomorphic fragments as well as many hypervariable fragments in PstI digests of birds from various species of swans (Fig.6.12). This is another DNA fingerprinting probe.

8.pcoMS15.5. This minisatellite invariably detects two PstI fragments among individual swans from all species under study (data not shown), being thus of little useful value.

6.7 DISCUSSION AND CONCLUSION

A genomic library based on a single bird of the Mute Swan was constructed using the λ vector Lambda™-11. By screening with the human polycore probes pSPT19.6 and pSPT18.15, 28 minisatellite-containing λ recombinants were isolated. However, these λ clones do not act as locus-specific probes like those in humans (Wong *et al.* 1986, 1987). The same problem also applied during cloning of minisatellites in the genomes of

sparrowhawk, falcon peregrine and merlin (R.E. Carter, A. Meng and Hutchinson, unpublished data). The reason for this may be the interference of flanking sequences of minisatellites or the inadequacy of cloning and detection techniques.

The minisatellites in the λ clones were refined by subcloning EcoRI minisatellite fragments into the plasmid pGEM-3zf(+). The minisatellites in plasmids can readily propagate in large quantities and be easily analysed. Under high stringency hybridization conditions, in the presence of competitor DNA (Herring sperm DNA), most of the subclones did identify a single locus, but the probe loci are monomorphic. However, six probes, i.e. pcoMS6.2, pcoMS6.6, pcoMS6.11B, pcoMS6.11S, pcoMS15.2 and pcoMS15.3, detect other minisatellites related to the probe sequence under low stringency. pcoMS15.2 and pcoMS15.3 are able to hybridize to a number of highly variable fragments to produce fingerprint-like hybridization profiles. Some swan minisatellite probes (e.g. pcoMS6.1, pcoMS6.2 and pcoMS6.14) detect minisatellite variation among species, supporting the view that minisatellites are subject to differentiation among populations or species (see Chapter 4). Unfortunately, only one minisatellite, pcoMS6.1, detects locus-specific variation in PstI digests of the Trumpeter Swans.

This study was largely unsuccessful in isolating locus-specific swan minisatellites that are polymorphic. Although several groups of workers have also been engaging in the same adventure in other avian species, encouraging results have been rarely reported. Hanotte *et al.* (1990) isolated some locus-specific minisatellites in the genome of peafowl *Pavo christatus*, none of which has more than five alleles in a population of 23 supposedly unrelated Indian peafowls. The heterozygosity at the peafowl minisatellite loci range from 22% to 78%, also less than that at the human minisatellite

loci (Wong *et al.* 1987). It is probable that cloning of individual hypervariable minisatellites in avian species is more difficult, even impossible.

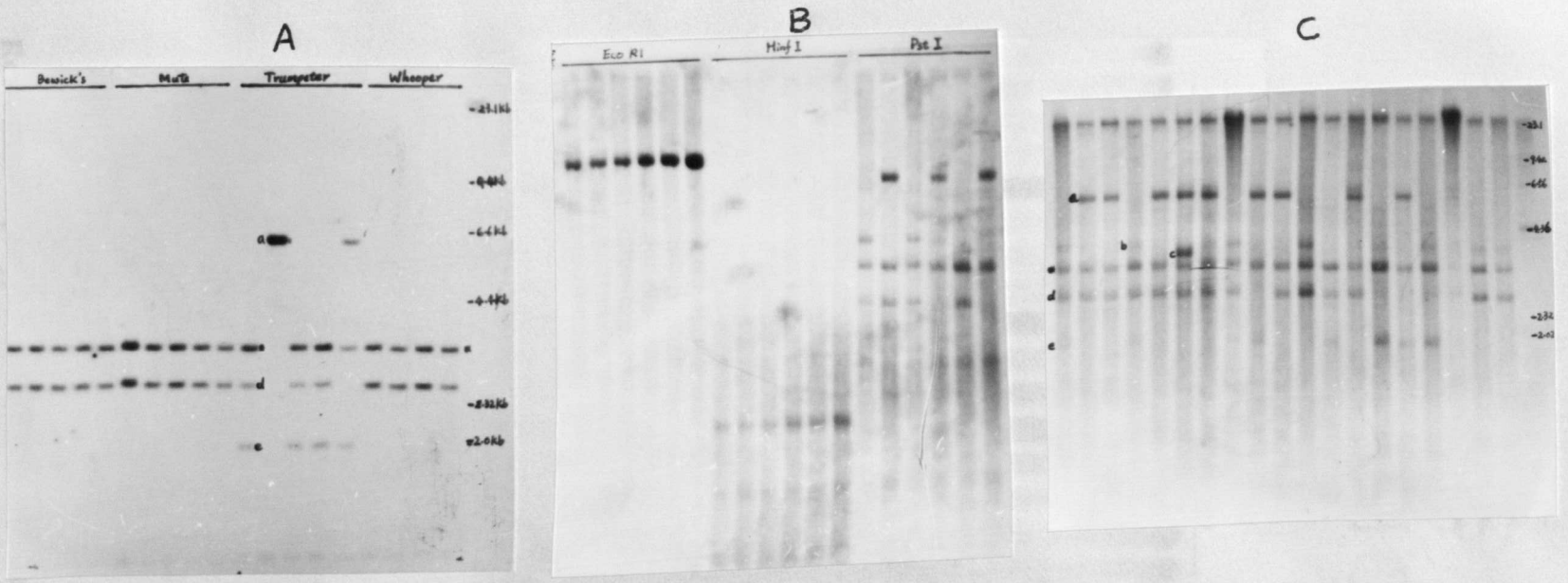


Fig.6.6 RFLP detected by pcoMS6.1 in the Trumpeters. Genomic DNAs from four species of swans were digested with appropriate enzymes. The digests were electrophoresed overnight on 1% agarose gels, followed by blotting to Zeta-Probe® membrane (Bio-Rad) using alkali transfer method. Plasmid DNA was radiolabelled by random priming using Random Primers DNA Labelling System (BRL). Prehybridization and hybridization were carried out in a solution of 5X SSC,1% SDS and 1X Blotto [or in one case (photo A),with addition of 100µg/ml sonicated Herring sperm DNA]. Posthybridization wash was performed first in 2X SSC, 0.1% SDS for 15 minutes, and then in 1X SSC /0.1% SDS for 15 minutes (otherwise in 0.1X SSC/0.1% SDS for 15 minutes if high stringency was required).Following a 3-day exposure with double intensifying screens at -80°C, autoradiographs were developed. λHindIII fragments were used as size markers. The invariable band marked with '*' in Trumpeter Swans may be derived from a conservative homozygous locus, while the other bands indicated by a letter (a-e) are polymorphic. B: Bewick's; M: Mutes; T: Trumpeters; W: Whoopers.

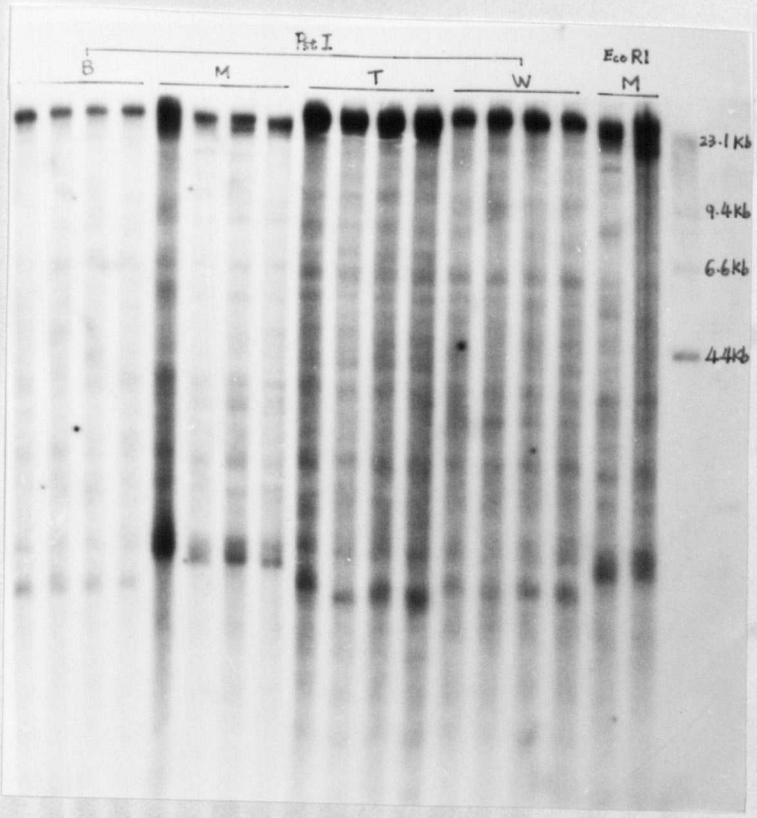


Fig. 6.7 Restriction patterns detected by pcoMS6.2. Hybridization performed under a stringency of 1X SSC without competitor DNA. (see legend in Fig.6.6)

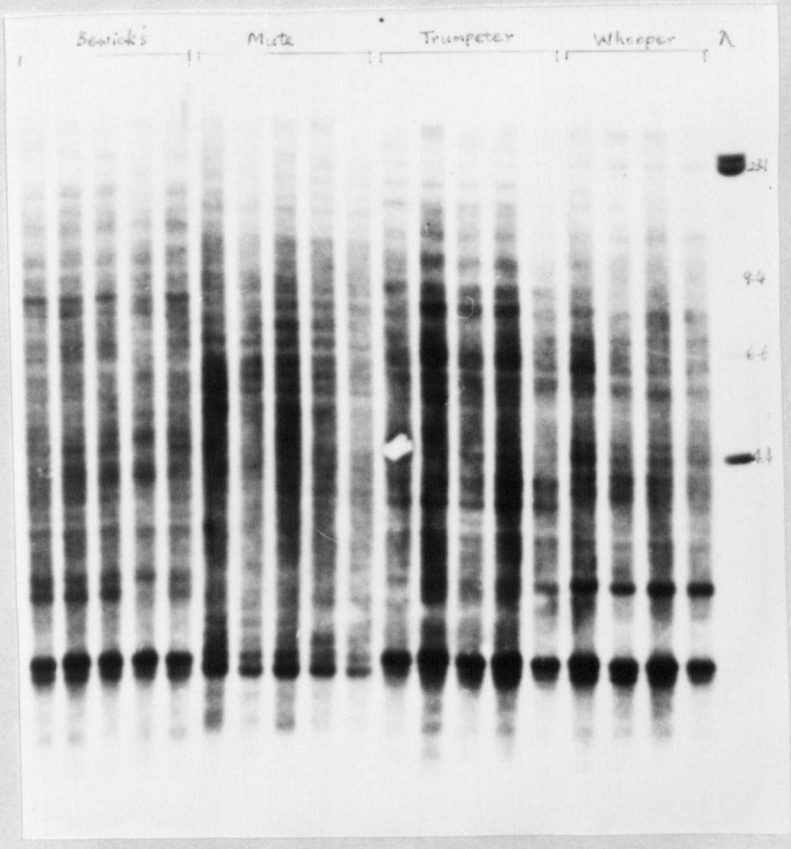
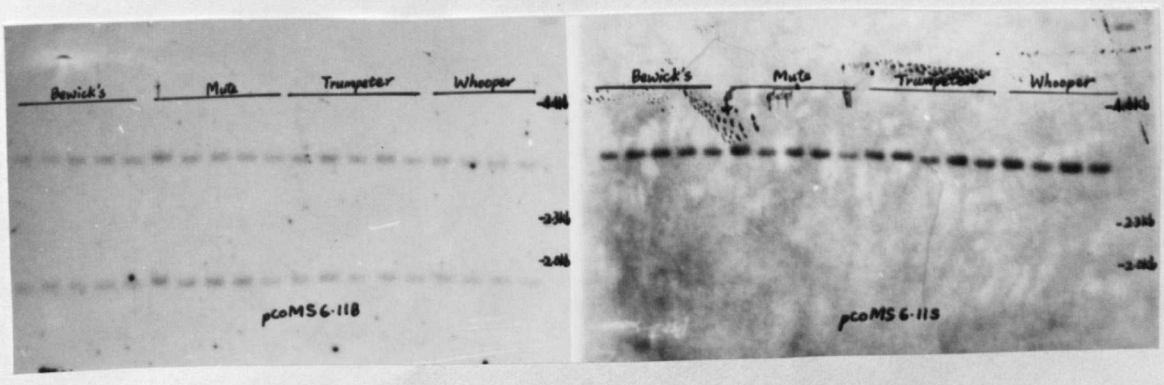
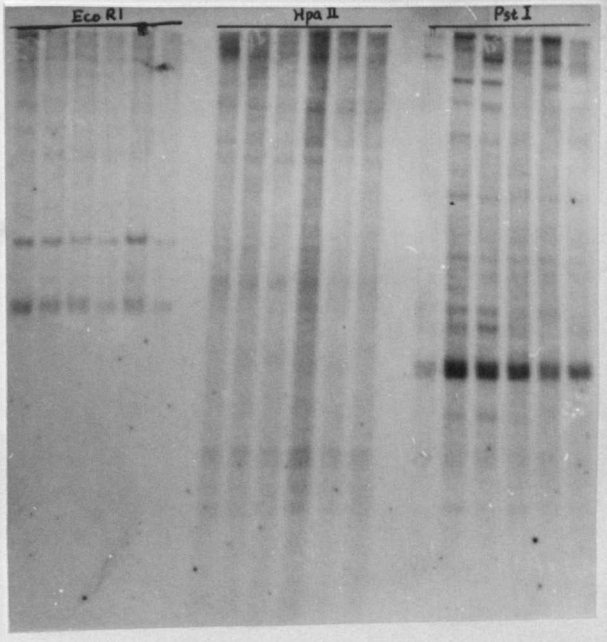


Fig.6.8 Restriction patterns detected by pcoMS6.6. Hybridization performed under a stringency of λ 1X SSC without competitor DNA. (see legend in Fig.6.6)





pcoMS6·11S

Fig.6.9 Restriction patterns detected by pcoMS6.11B and pcoMS6.11S. Hybridization performed under a stringency of 0.1X SSC with competitor DNA (top) or under a stringency of 1XSSC without competitor DNA (bottom). (see legend in Fig.6.6)

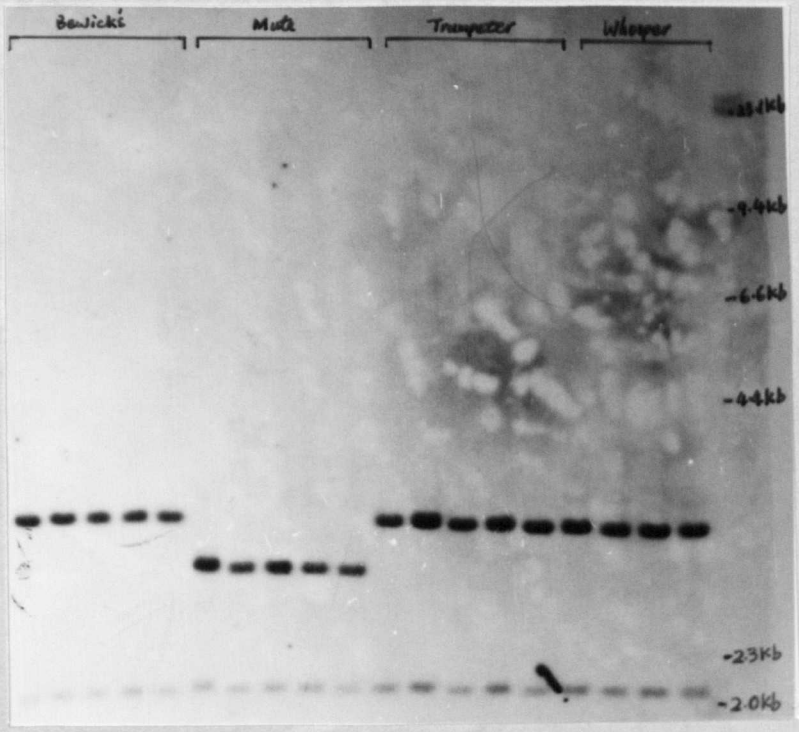


Fig. 6.10 Restriction patterns detected by pcoMS6.14. Hybridization performed under a stringency of 0.1X SSC with competitor DNA. (see legend in Fig.6.6)

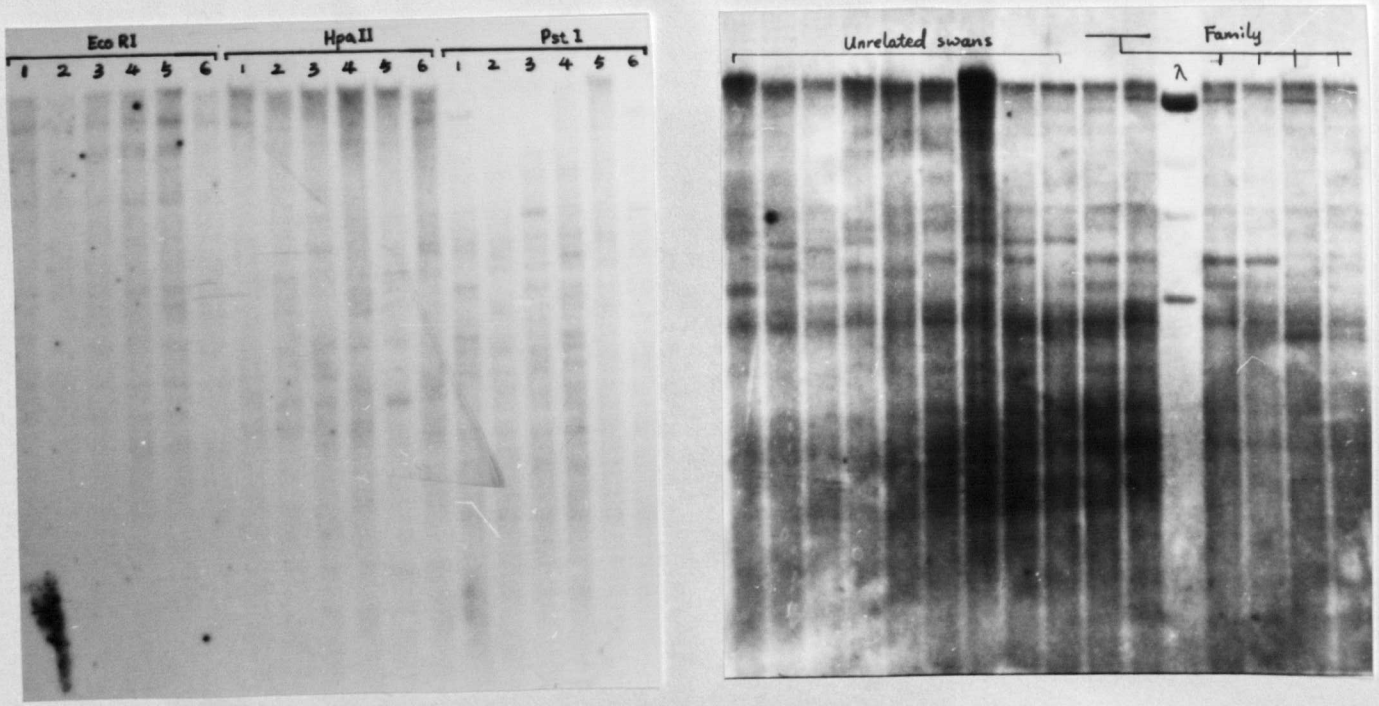


Fig. 6.11 Restriction patterns detected by pcoMS15.2. Hybridization performed under a stringency of 1X SSC without competitor DNA. (see legend in Fig.6.6).Left: restriction patterns of six unrelated Mutes restricted with different enzymes; right: unrelated Mutes and a pedigree restricted with PstI.

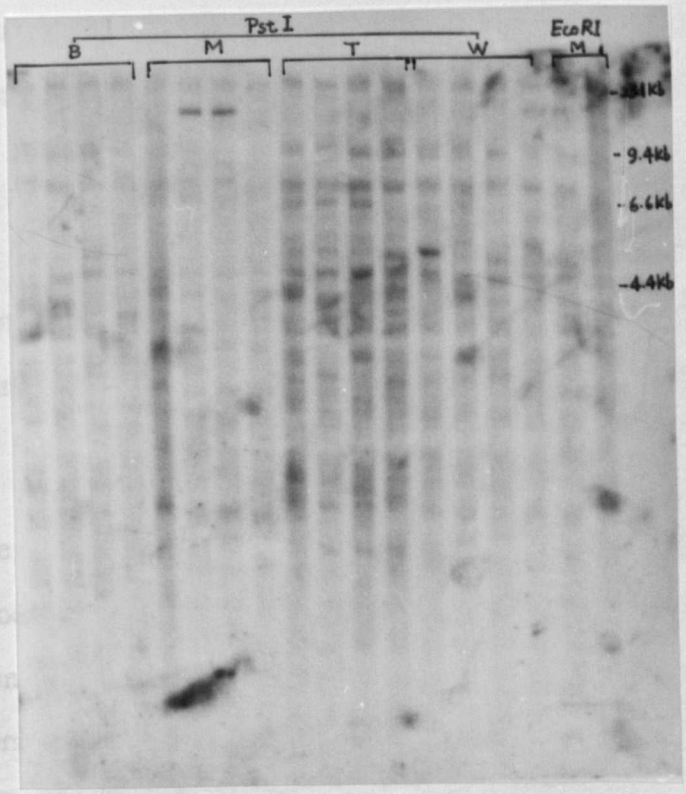


Fig. 6.12 Restriction patterns detected by pcoMS15.3. Hybridization performed under a stringency of 1X SSC without competitor DNA. (see legend in Fig.6.6)

CHAPTER 7
GENERAL CONCLUSIONS AND PROSPECTS

7.1 GENERAL CONCLUSIONS

Through this study, the DNA fingerprinting technique on swans has been established based on the human polycore probes. This study concerned three aspects: characterization of DNA fingerprints of swans, application of DNA fingerprinting to studies on population and behavioral biology of swans, and the isolation of swan minisatellites. Some of the main conclusions are drawn as follows:

i). The human minisatellite probes pSPT19.6 (i.e. 33.6) and pSPT18.15 (i.e. 33.15) are able to detect a large number of highly variable minisatellite fragments in the genome of swans and to generate individual-specific DNA fingerprints.(see Chapter 3)

ii). Most of the resolved swan minisatellite loci are in the heterozygous status, and the alleles are codominantly inherited as simple Mendelian characters. However, linked bands account for a considerable proportion in HaeIII- DNA fingerprints, suggesting that internal HaeIII recognition sites are relatively common in the swan minisatellites. (see Chapter 3)

iii). DNA fingerprint bands have substantial germ-line stability and the mutation rate is species-dependent. The mutation frequency to novel bands in the Whooper Swan is almost twice as high as in the Mute Swan. (see Chapter 3)

iv). A given species of swans has DNA fingerprinting patterns with a certain degree of uniformity, suggesting that DNA fingerprints are subject to specific differentiation. Interpopulation comparison of DNA fingerprints in the Mute Swan

showed that minisatellites might be undergoing population differentiation because of geographic isolation and genetic drift. (see Chapter 4)

v). Parentage analysis using DNA fingerprinting revealed that extra-pair fertilization, intraspecific nest parasitism or alloparental behaviour occur in swans. Such events are rare in migratory species such as the Whooper Swan as compared with the non-migratory Mute Swan, suggesting that the migratory swans are unable to afford the cost of these events because they need a strong pair-bond and contribute more energy to raising the young. (see Chapter 5)

vi). It is difficult to clone individual hypervariable minisatellites in swans, although most of the isolated swan minisatellites can cross-hybridize to other polymorphic minisatellites in the genome of swans under low stringency conditions. Only one of nine cloned minisatellites from the Mute Swan detected locus-specific PstI polymorphism. (see Chapter 6).

7.2 LIMITATION OF DNA FINGERPRINTING

Since DNA fingerprinting is still in the development stage, some limitations restrict its applications and even confound the genetic analysis. We here discuss some major constraints.

i). Control markers. The central part of DNA fingerprinting analysis is the scoring of DNA fingerprints, which mainly deals with band matching. When comparing two adjacent gel tracks, the task is easy and interpretation is relatively precise. However, it is far more difficult to compare two distant gel tracks in the same blot, and even impossible to compare samples in separate blots. The use of adequate size markers, e.g. a standard DNA fingerprint consisting of well-resolved bands, can improve the scoring on the basis of single blots, but not the scoring between blots

since the high density of banding disallows the discrimination of bands with slight changes in mobility or in intensity due to inconsistent electrophoretic or hybridization conditions. It is expected that progress in the development of a control marker system would stimulate rigorous studies on the comparative biology of animals using the DNA fingerprinting technique.

ii). Heterogeneity of band intensity and banding distance. DNA fingerprints usually consist of a set of bands whose autoradiographic intensities are variable. For instance, the intensity of some bands may be tenfold that of others. The distance between two bands ranges from invisible (≤ 1mm) to a few centimetres. In areas of high density, strong (very dark) bands may blur neighboring faint ones. Hence, DNA fingerprints consisting of bands of high density would lead to the increase of probability of chance comigration. This problem may be circumvented by reducing the number of bands and exposing the X-ray film without intensifying screens.

iii). Contradiction between the number of informative bands and resolution of gels. The mobility of restriction fragments in the gel matrix depends on their length as well as gel concentration and electrophoretic conditions (e.g. voltage gradient and temperature). Gel concentration has a dramatic impact on the resolution. A given gel concentration can only maximize the resolution of bands of a certain size class. For example, in this study 0.8% agarose gels were run for 3 days for DNA fingerprinting, and the resolution in the size range 15-30Kb was poor. Prolonged electrophoresis can improve the resolution in that size range, but many polymorphic fragments of less than 6Kb could run off the gel and the number of informative bands could reduce to 15 or so for the Mute Swans, and to about 10 for the other three species. Otherwise, using gels of lower concentration (e.g. 0.6%) could sharply reduce the resolution of smaller bands while large bands are well separated. Therefore, a compromise has to be adopted according to the purpose of study.

iv). Divergence of repeat sequence. Repeat elements constituting minisatellites are not identical (Jeffreys *et al.* 1990), so the distribution of restriction sites is uneven among the repeats. As a result, similarity coefficient between two random individuals may change from category of unrelatedness to the category of relatedness depending on the enzyme. Consequently, DNA fingerprinting at present has substantial shortcomings for studying population differentiation, and in establishing relatedness among individuals within a population, because the proportion of shared bands not necessarily represent the proportion of shared genes.

v). Statistical problems. The limitations of DNA fingerprinting technique stated above raise several statistical problems concerning the estimation of relatedness. Under various statistical models (Jeffreys *et al.* 1986, Lynch 1988, Brookfield 1989, Honma and Ishiyama 1989), some assumptions are questionable (Cohen 1990). For example, they all ignore the comigration of unrelated bands (alleles), the presence of allelism and linkage, and heterogeneity of allelic frequencies over the loci detected. It is believed that such statistical problems will remain, and so several locus-specific minisatellite probes will have to be used in combination to avoid inadequate assumptions.

7.3 RECOMMENDATIONS

As a continuation of this study, further studies are suggested as follows:

i). Collect blood samples from all eight species of swans to study the evolution of swans with respect to the minisatellites in the genome. To ease the analyses of DNA fingerprints, a higher hybridization stringency (e.g. 0.3X SSC) can be used to obtain well-resolved banding patterns (consisting of 5 bands or so).

ii). Study different populations of swans such as Bewick's and Whooper Swans. This might lead to a better understanding of population differentiation, and to what degree geographic isolation and migration have an impact.

iii). Detailed study of mating behaviour using DNA fingerprinting technique is a interesting area. However, this requires a large number of blood samples plus recorded pedigree data.

iv). Sequence some of swan minisatellites that have been cloned in plasmids. This will reveal the organization and structure of swan minisatellites, and some regions of cloned minisatellites might perform as hypervariable LSPs.

APPENDIX

PREPARATION OF REAGENTS AND SOLUTIONS

A: Reagents and solutions for DNA fingerprinting .

Alkali transfer buffer: 0.25M NaOH

1.5M NaCl in H_2O.

10X Blotto: For 100ml dissolve

10g nonfat dried milk

0.2g sodium azide in H_2O.

Add 10µl DEPC (diethyl pyrocarbonate) and stir overnight at room temperature to evaporate, or evaporate at 42°C for 4 hours.

10X BPB: 20% Ficoll
0.2M EDTA
0.25% Bromophenol blue
0.25% Xylene cyanol FF in H_2O.

Store at room temperature.

Chloroform: Chloroform used for DNA extraction is always the mixture of chloroform and isoamyl alcohol (23:1, V/V). The mixture is stable and can be stored at room temperature.

Denaturing solution: 1.5M Tris

0.5M NaOH in H_2O.

50X Denhardt's solution: 1% Ficoll

1% Polyvinylpyrrolidone

1% BSA (Pentax Fraction V)

in H_2O.

DNase solution: 1mg/ml DNase in H_2O. Store at -20°C.

0.5M EDTA (pH 8.0): Add 186.1g of EDTA to 800ml of H_2O. Stir vigorously on a magnetic stirrer and adjust the pH to 8.0 with NaOH (about 20g of NaOH pellets).

Ethidium bromide (10mg/ml): Dissolve 1g of EtBr in 100ml Of H_2O by stirring on a magnetic stirrer for several hours. Wrap the container in aluminium foil and store at 4°C.

Fluorometer dye solution: 1mg/ml Hoechst 33258 in H_2O. Store in foil-wrapped tube at 4°C. Working concentration is 0.1µg/ml.

Los Almos buffer: 0.5% SDS
100mM Tris
100mM EDTA pH8.0
100mM NaCl
in H_2O.

Neutralizing solution: 1M Tris
1.5M NaCl in H_2O.

(Labelling) stop dye buffer: 0.9% Blue dextran
0.03% Bromocresol purple
20mM EDTA
in TE buffer.

Phenol: Equilibrate cystaline phenol with 1M Tris (pH 8.0) (10:3, V/V), and add 0.1% 8-hydroxyquinoline (W/V). Melt in a 65°C waterbath. The yellow-coloured phenol retains in the lower phase. The pH of the aqueous phase should be over 7.6. The phenol solution can be stored at room temperature up to 10 days.

Phenol/chloroform: A mixture of phenol, chloroform and isoamyl alcohol (24:23:1, V/V/V).

Proteinase K: 10mg/ml stock solution in H_2O. Working concentration is 0.30mg/ml. Store at -20°C.

RNase solution: dissolve pancreatic RNase at a concentration of 10mg/ml in 10mM Tris (pH 7.5) and 15mM NaCl. Heat to 100°C for 15 minutes and allow to cool slowly to room temperature. Store at -20°C.

25% SDS: Dissolve SDS in H_2O in a 55°C waterbath. Adjust pH to 7.2 by adding a few drops of concentrated HCl. Store in a 37°C oven.

20X SET: 3M NaCl
1M Tris
20mM EDTA in H_2O.
Adjust pH to 8.0 with HCl.

3M Sodium acetate: Dissolve sodium acetate in H_2O. Adjust pH to 5.2 with glacial acetic acid.Store at 4°C.

20X SSC: 3M NaCl
0.3M sodium citrate in H_2O.

Adjust pH to 7.0 with NaOH.

1X TE buffer: 10mM Tris
1mM EDTA (pH 8.0) in H_2O.

10X TEN (i.e.TNE): 100mM Tris
10mM EDTA
1M NaCl in H_2O.
Adjust pH to 7.4 with HCl. Store at 4°C.

1M Tris: Dissolve 121.1g Tris base in 800ml of H_2O. Adjust pH to the desired value with concentrated HCl. Make up the volume to one litre.

50X Tris-acetate (TAE): 2M Tris
50mM EDTA (pH 8.0) in H_2O.
Adjust pH to 8.0 by adding glacial acetic acid (~57.1ml per litre).

1X Tris-borate (TBE): 0.089mM Tris-borate
0.089mM Boric acid
0.200mM EDTA in H_2O.
Adjust pH to 7.8-7.8 with HCl.

B. Reagents and solutions for molecular cloning

Ampicillin stock (4mg/ml): Dissolve 400mg ampicillin in 100ml H_2O.Store at 4°C.Working concentration ranges from 50-100µg/ml.

Chloramphenicol stock:Add 3.4g chloramphenicol to 100ml of 100% ethanol.Store at -20°C.Working concentration is 170µg/ml.

IPTG stock (0.1M): Dissolve 1.2g IPTG in H_2O. Store at 4°C. concentration is 0.5mM.

LB (Luria Bertani) medium: per litre

10g Bacto-tryptone

5g Yeast extract

0.5g NaCl in H_2O.

Adjust to pH7.5 with NaOH and supplemented with 10mM $MgSO_4$ for the growth of λ and its derivatives.

LB agar: As LB medium with addition of 15g of Bacto-agar and 10mM $MgSO_4$ per litre.

LB bottom agarose: As LB medium with addition of 4.5g NaCl,10g agarose and10mM $MgSO_4$ per litre.

LB top agarose: As LB medium with addition of 4.5g NaCl, 6g agarose and10mM $MgSO_4$ per litre.

Miniprep buffer: 50mM glucose

25mM Tris (pH 8.0)

10mM EDTA in H_2O.

PEG/NaCl: 20% PEG 6000

2.5M NaCl in phage buffer.

Autoclave and store at 4°C.

Phage buffer: 20mM Tris.HCl, pH 7.4

100mM NaCl

10mM $MgSO_4$ in H_2O.

Autoclave and store at 4°C.

5M KoAc: 60ml 5M potassium acetate

11.5ml glacial acetic acid

H_2O to 100ml

The mixture is 3M with respect to potassium and 5M with respect to acetate. Store at 4°C.

Phage buffer: 50mM Tris.HCl, pH 7.5

100mM NaCl

8mM $MgSO_4$

0.01% gelatine in H_2O.

Autoclave and store at 4°C.

X-Gal stock (50mg/ml): Dissolve in N,N'dimethylformamide. Store at -20°C. Working concentration is 40µg/ml.

REFERENCES

Ali, S. and Wallace, R. B. 1988. Intrinsic polymorphism of variable number tandem repeat loci in the human genome. Nucleic Acids Res., 16:8487-8489

Amos,W. and Dover,G.A. 1990. DNA fingerprinting and the uniqueness of whales. Mammal Rev., 20:23-30.

Amundsen, S.K., Taylor, A.F., Chaudhury, A.M. and Smith, G.R. 1986. recD: the gene for an essential third subunit of exonuclease V. Proc. Natl. Acad. Sci. USA, 83:5558-5562.

Armour,J.A.L, Patel,I., Thein,S.L., Fey, M. F. and Jeffreys, A.J. 1989. Analysis of somatic mutation at human minisatellite loci in tumours and cell lines. Genomics, 4:328-334.

Armour,J.A.L., Wong, Z., Wilson, V., Royle, N.J. and Jeffreys, A.J. 1989. Sequences flanking the repeat arrays of human minisatellites: association with tandem and dispersed repeat elements. Nucleic Acids Res.,17:4925-4935.

Banko, W.E. 1960. The Trumpeter Swan. North American Fauna, No. 63: W.S.F.W.S., Washington.

Bell, G.I., Selby, M.J. & Rutter, W.J. 1982. The highly polymorphic region near the human insulin gene is composed of simple tandemly repeating sequences. Nature, 295:31-35.

Benton, W.D. and Davis, R.W. 1977. Screening λgt recombinant clones by hybridization to single plaques *in situ*. Science, 196:180-182.

Birkhead, M. and Perrins, C. 1986. The Mute Swan. Croom Helm, London.

Bostein, D., White, R.L., Skolnick, M. and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet., 32:314-331.

Bowden, D.W., Muller-Kahle, H.M., Gravius, T.C., Helms, C., Watt-Morgan, D., Green, P. and Donis-Keller, H.1989. Identification and characterization of 23 RFLP loci by screening random cosmid genomic clones. Am. J. Hum. Genet., 44:671-678.

Boyd, H. and El-tringham, S.K. 1962. The Whooper Swan in Great Britain. Bird Study, 9:217-241.

Braithwaite, K.S. and Manners, J.M. 1989. Human hypervariable minisatellite probes detect DNA polymorphisms in the fungus *Colletotrichum gloeoasorioides*. Curr. Genet., 16:473-475.

Brookfield, J.F.Y. 1989. Analysis of DNA fingerprinting data in cases of disputed paternity. IMA J. Math. Appl. in Med. and Biol., 6:111-132.

Buluwela, L., Forster, A., Boehm, T. and Rabbits, T.H. 1989. A rapid procedure for colony screening using nylon filters. Nucleic Acids Res., 17:452.

Burke, T. and Bruford, M.W. 1987. DNA fingerprinting in birds. Nature, 327:149-152.

Burke,T., Davies, N.B., Bruford, M.W. & Hatchwell, B.J. 1989. Parental care and mating behaviour of polyandrous dunnocks *Prunella*

modularis related to paternity by DNA fingerprinting. Nature, 338:249-251.

Burke,T. 1989. DNA fingerprinting and other methods for the study of mating success. Trends in Ecol. & Evol.,4:139-144.

Butler, E.T. and Chamberlin, M.J. 1982. Bacteriophage SP6-specific RNA polymerase. I. Isolation and characterization of the enzyme. J. Biol. Chem., 257:5772-5778.

Campbell, B. 1960. The Mute Swan census in England and Wales, 1955-56. Bird Study, 7:208-223.

Carrano, A.V., Lamerdin, W., Ashworth, L.K., Watkins, B., Branscomb, E., SSlezak, T., Raff, M., De Jong, P.J., Keith, D., McBridge, L., Meister, S. and Kronic, M. 1989. A high-resolution, fluoresence-based, semiautomated method for DNA fingerprinting. Genomics, 4:129-136.

Carter, R. E., Wetton, J.H. and Parkin, D.T. 1989. Improved genetic fingerprinting using RNA probes. Nucleic Acids Res., 17:5867.

Casey, J. and Davidson, N. 1977. Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. Nucleic Acids Res., 4:1539-1552.

Chalker, A.F., Leach, D.R.F. and Lloyd, R.G. 1988. *Escherichia coli* sbc C mutations permit stable propagation of DNA replicons containing a long palindrome. Gene, 71:201-205.

Chamberlin, M., Kingston, R., Gilman, M., Wiggs, J. and De Vera, A. 1983. Isolation of bacterial and bacteriophage RNA-polymerases and their use in synthesis of RNA *in vitro* . In: Methods in Enzymology, Vol. 101

(eds. Wu, R., Grosman, L. and Moldave, K.). pp.540-568. Academic Press, New York.

Chandley, A.C. and Mitchell, A.R. 1988. Hypervariable minisatellite regions are sites for crossing-over at meiosis in man. Cytogenet. cell Genet., 48:152-155.

Chang, C., Bowman, J.L., De John, A.W., Lander, E.S., Meyerowitz, E.M. 1988. restriction fragment length polymorphism linkage map for *Arabidopsis thaliana* . Proc. Natl. Acad. Sci. USA, 85:6856-6860.

Chen, P., Hayward, N., Kidson, C. and Ellem, A.O. 1990. Conditions for generating well-resolved human DNA fingerprints using M13 phage DNA. Nucleic Acids Res., 18:1065.

Cohen, J.E. 1990. DNA fingerprinting for forensic identification: potential effects on data interpretation of subpopulation heterogeneity and band number variability. Am. J. Hum. Genet., 46:358-368.

Collins, J. 1979. *Escherichia coli* plasmids packageable *in vitro* in λ bacteriophage particles. In: Methods in Enzymology, Vol. 68 (eds. Wu, R.). pp.309-326. Academic Press, New York.

Connor, S. 1988. Genetic fingers in the forensic pie. New Sci., No.1597:31-32.

Cox, N.J., Bell, G.I. and Xiang, K. 1988. Linkage disequilibrium in human insulin/insulin-like growth factor II region of human chromosome 11. Am. J. Hum. Genet., 43:495-501.

Dallas, J.F. 1988. Detection of DNA "fingerprints" of cultivated rice by hybridization with a human minisatellite DNA probe. Proc. Natl. Acad. Sci. USA, 85:6831-6835.

Davies, K.E. 1985. New hypervariable DNA markers for mapping human genetic disease. Trends in Genet., 1:97.

Delacour, J. and Mayr, E. 1945. The family Anatidae. The Wilson Bulletin, 57:3-55.

Denhardt, D. 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophs. Res. Comm., 23:641-646.

Dewer, J.M. 1936. Menage a trois in The Mute Swan. Brit. Birds, 30:178.

Dixson, A.F., Hastie, N., Patel, I. and Jeffreys A.J. 1988. DNA 'fingerprinting' of captive common marmosets (*Callithrix jacchus*). Folia Primatol., 51:52-55.

Dodd, B.E. 1985. DNA fingerprinting in matters of family and crime. Nature, 318:506-507.

Donis-Keller, H. and 32 co-authors. 1987. A genetic linkage map of the human genome. Cell, 51:319-337.

Enquist, L.W. and Skalka, A. 1973. Replication of bacteriophage λ DNA dependson the function of host and viral genes. I. Interaction of *red*, *gam* and *rec*. J. Mol. Biol., 75:185-212.

Evans, P.G.H. 1987. Electrophoretic variability of gene products. In: Avian Genetics - A Population and Ecological Approach (eds. Buckley, P. and Cooke, F.). pp.105-162. Academic Press. New York.

Feiss, M., Fishier, R. A., Crayton, M.A. and Enger, C. 1977. Packaging of the bacteriophage λ chromosome: effect of chromosome length. Virology, 77:281-293.

Ford, E.B. 1940. Polymorphism and taxonomy. In: The New Systematics (ed. Huxley, H.). pp.493-513. Clarendon Press, Oxford.

Ford, N.L. 1983. Variation in mate fidelity in monogamous birds. In: Current Ornithology, Vol. I (ed. Johnston, R.F.). pp.329-356. Plenum Press. New York.

Ford, S. and Thompson, W.S. 1990. A question of identity - some reasonable doubts about DNA "fingerprints". The Sciences, 30:37-43.

Fowler, S.J., Gill, P., Werrett, D.J. and Higgs, D.R. 1988. Individual-specific DNA fingerprints from a hypervariable region probe: alpha-globin 3'HVR. Hum. Genet., 79:142-146.

Georges, M., Cochaux, P., Lequarre, A-S., Young, M.W. and Vassart, G. 1987. DNA fingerprinting in man using a mouse probe related to part of the *Drosophila* ' per' gene. Nucleic Acids Res., 15:7193.

Georges, M., Lequarre, A-S., Castelli, M., Hanset, R. and Vassart, G. 1988. DNA fingerprinting in domestic animals using four different minisatellite probes. Cytogenet. Cell Genet., 47:127-131.

Georges, M., Lathrop, M., Hilbert, P., Marcotte, A., Schwers, A., Swillens, S., Vassart, G. and Hanset, R. 1990. On the use of DNA fingerprints for linkage studies in cattle. Genomics, 6:461-471.

Gerard, C., Christophe, D., Compere, T. and Vassart, G. 1990. The poly (purine) poly (pyrimidine) sequence in the 5' end of the thyroglobulin gene used as a probe, identifies a DNA fingerprint in man. Nucleic Acids Res., 18:4297.

Gilbert, D.A., Lehman, N., O'Brien, S.J. & Wayne, R.K. 1990. Genetic fingerprinting reflects population differentiation in the California Channel Island fox. Nature, 344:764-767.

Gill, P., Jeffreys, A.J. & Werrett, D.J. 1985. Forensic application of DNA 'fingerprints'. Nature, 318:577-579.

Goodbourn, S.E.Y., Higgs, D.R., Clegg, J.B. and Weatherall, D.J. 1983. Molecular basis of length polymorphism in the human ζ-globin gene complex. Proc. Natl. Acad. Sci. USA, 80:5022-5026.

Gundel, H. and Reetz, I. 1981. Exclusion probabilities obtained by biochemical polymorphisms in dogs. Anim. Blood Groups and Biochem. Genet., 12:123-132.

Gusella, J.F. 1986. DNA polymorphism and human disease. Ann. Rev. Biochem., 55:831-854.

Gyllensten, U.B., Jakobsson, S., Temrin, H. and Wilson, A.C. 1989. Nucleotide sequence and genomic organization of bird minisatellites. Nucleic Acids Res., 17:2203-2214.

Gyllensten, U.B., Jakobsson, S. & Temrin, H. 1990. No evidence for illegitimate young in monogamous and polygamous warblers. Nature, 343:168-170.

Hanotte, O., Burke, T., Armour, J., Miller, A.O. and Jeffreys, A. J. 1990. Hypervariable minisatellite DNA sequences in the peafowl *Pavo christatus*. (in press)

Harris, H. 1969. Enzyme and protein polymorphism in human populations. Brit. Med. Bulletin, 25:5.

Helentjaris, T. 1987. A genetic linkage map for maize based on RFLPs. Trends in Genet., 3:217-221.

Helminen, P., Ehnholm, C., Lokki, M.L., Jeffreys, A.J. and Peltonen, L. 1988. Application of DNA "fingerprints" to paternity determinations. The Lancet, i:574-576.

Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg. 1983. Lambda II. Cold Spring Harbor Laboratory, New York.

Herrmann, B.G. and Frischauf, A.M. 1987. Isolation of genomic DNA. In: Methods in Enzymology, Vol. 152 (eds. Berger, S.L. and Kimmel, A.R.). pp.180-183. Academic Press, New York.

Higgs, D.R., Goodbourn, S.E.Y., Wainscoat, J.S., Cleggs, J.B. and Weatherall, D.J. 1981. Highly variable regions of DNA flank the human α globin genes. Nucleic Acids Res., 9:4213-4224.

Higgs, D.R., Wainscoat, J.S., Flint, J., Hill, A.V.S., Thein, S.L., Nicholls, R.D., Teal, H., Ayyub, H., Peto, T.E.A., Falusi, A.G., Jarman, A.P., Clegg, J.B. and Weatherall, D.J. 1986. Analysis of the human α-globin gene cluster reveals a highly informative genetic locus. Proc. Natl. Acad. Sci. USA, 83:5165-5169.

Hill, A.V.S. and Jeffreys, A.J. 1985. Use of minisatellite DNA probes for determination of twin zygosity at birth. The lancet, ii:1394-1395.

Hillel, J., Schaap, T., Haberfeld, A., Jeffreys, A.J., Plotzky, Y., Cahaner, A. and Lavi, U. 1990. DNA fingerprints applied to gene introgression in breeding programs. Genetics, 124:783-789.

Hohn, B. and Hohn, T. 1974. Activity of empty headlike particles for packaging of DNA of bacteriophage λ *in vitro*. Proc. Natl. Acad. Sci. USA, 83:5165-5169.

Honma, M., and Ishiyama, I. 1989. Probability of Paternity in paternity testing using the DNA fingerprint procedure. Hum. Hered., 39:165-169.

Huxley, J.S. 1947. Display of the Mute Swan. Brit. Birds, 40:130-134.

Inglehearn, C.F., Papiha, S. S., Jay, M., Wright, A.F., Moore, A.T., and Bhattacharya, S.S. 1990. Linkage of internal minisatellite loci on chromosome 1 and exclusion of autosomal dominant retinitis pigmentosa proximal to rhesus. J. Med. Genet., 27:14-16.

Jarman, A.P., Nicholls, R.D., Weatherall, D.J., Cleggs, J.B., Higgs, D.R. 1986. Molecular characterization of a hypervariable region downstream of the human α-globin gene cluster. EMBO J., 5:1857-1863.

Jarman, A.P. and Higgs, D.R. 1988. A new hypervariable marker for the human α-globin gene cluster. Am. J. Hum. Genet., 43:249-256.

Jarman, A.P. and Wells, R.A. 1989. Hypervariable minisatellites: recombinators or innocent bystanders?. Trends in Genet., 5:367-371.

Jeffreys, A.J., Flavell, R. 1977. A physical map of the DNA regions flanking the rabbit β-globin gene. Cell, 12:429-439.

Jeffreys, A.J., Wilson, V. &. Thein, S.L. 1985a. Hypervariable 'minisatellite' regions in human DNA. Nature, 314:67-73.

Jeffreys, A.J., Wilson, V. &. Thein, S.L. 1985b. Individual-specific 'fingerprints' of human DNA. Nature, 316:76-79.

Jeffreys, A.J., Brookfield, J.F.Y. &. Semeonoff, R. 1985c. Positive identification of an immigration test-case using human DNA fingerprints. Nature, 317:818-819.

Jeffreys, A.J., Wilson, V., Thein, S.L., Weatherall, D.J. and Ponder, B.A.J. 1986. DNA "fingerprints" and segregation analysis of multiple markers in human pedigrees. Am. J. Hum. Genet., 39:11-24.

Jeffreys, A.J. and Morton, D.B. 1987. DNA fingerprints of dogs and cats. Animal Genet.,18:1-15.

Jeffreys, A.J., Wilson, V., Kelly, R., Taylor, B.A. and Bulfield, G. 1987. Mouse DNA 'fingerprints': analysis of chromosome localization and germ-line stability of hypervariable loci in recombinant inbred strains. Nucleic Acids Res., 15:2823-2836.

Jeffreys, A.J. 1987. Highly variable minisatellites and DNA fingerprints. Biochem. Soc. Trans., 15:309-317.

Jeffreys, A.J., Royle, N.J., Wilson, V. &. Wong, Z. 1988a. Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. Nature, 332:278-281.

Jeffreys, A.J., Wilson, V., Neumann, R. and Keyte, J. 1988b. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic Acids Res., 16:10953-10971.

Jeffreys, A.J., Neumann, R. and Wilson, V. 1990. Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. Cell, 60:473-485.

Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene. Anal. Techn., 1:3-8.

Johnston, K. 1987. UK immigration authorities may use DNA fingerprinting. Nature, 329:5.

Kaiser, K. and Murray, N.E. 1985. The use of phage lambda replacement vectors in the construction of representative genomic libraries. In: DNA Cloning, Vol. I - A Practical Approach (ed. Glover, D.M.). pp.1-47. IRL Press, Oxford.

Kashi, Y., Tikochinsky, Y., Genislav, E., Iraqi, F.,Nave, A., Beekmann, J.S., Grucenbaum, Y. and Soller, M. 1990. Large restriction fragments containing poly-TG are highly polymorphic in a variety of vertebrates. Nucleic Acids Res.,18:1129-1132.

Kelly, R., Bulfield, G., Collick, A., Gibbs, M. and Jeffreys, A.J. 1989. Characterization of a highly unstable minisatellite locus: evidence for somatic mutation during early development. Genomics, 5:844-856.

Kelly, R.B., Cozzarelli, N.R., Deutscher, M.P., Lehman, I.R. and Kornberg, A. 1970. Enzymatic synthesis of deoxyribonucleic acid. XXXII, Replication of duplex deoxyribonucleic acid by polymerase at a single strand break. J. Biol. Chem., 245:39-45.

Knott, T.J., Wallis, S.C., Pease, R.J., Powell, L.M. and Scott, J. 1986. A highly hypervariable region 3' to human apolipoprotein B gene. Nucleic Acids Res., 14:9215-9216.

Knowlton, R.G., Brown, V.A., Braman, J.C., Barter, D., Schumm, J. W., Murray, C., Takvorian, T., Ritz, J. &. Donis-Keller, H. 1986. Use of

highly polymorphic DNA probes for genotypic analysis following bone marrow transplantation. Blood, 68:378-385.

Koenen, M. 1989. Recovery of DNA from agarose gels using liquid nitrogen. Trends in Genet., 5:137.

Kolin, A. 1955. Isoelectric spectra and mobility spectra: a new approach of electrophoretic separation. Proc. Natl. Acad. Sci. USA, 41:101-110.

Krieg, P.A. and Melton, D.A. 1987. *in vitro* RNA synthesis with SP6 RNA polymerase. In: Methods in Enzymology, Vol. 155 (ed. Wu, R.). pp.397-415. Academic Press, New York.

Krowczynska, A.M., Rudders, R.A. and Krontiris, T. G. 1990. The human minisatellite consensus at breakpoints of oncogene translocations. Nucleic Acids Res., 18:1121-1127.

Kuhnlein, U., Dawe, Y., Zadworny, D. and Gavora, J.S. 1989. DNA fingerprinting: a tool for determining genetic distances between strains of poultry. Theor. Appl. Genet., 77:669-672.

Kushner, S.R. 1978. An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids. In: Genetic Engineering: Proceeding of the International Symposium on Genetic Engineering, Milan, March 1978 (eds. Boyer, H.W. and Nicosia, S.N.). pp.17. Elsevier, Amsterdam.

Lange, K. and Boehnke, M. 1982. How many polymorphic genes will it take to span the human genome?. Am. J. Hum. Genet., 34:842-845.

Lansman, R.A., Shade, R.D., Shapira, J.F. and Avise, J.C. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. J. Mol. Evol., 17:214-226.

Leabach, D.H. &. Rutter, A.C. 1968. Polyacrylamide isoelectric focusing: a new technique for the electrophoresis of proteins. Biochem. Biophys. Res. Comm., 32:447-453.

Leach, D.R.F. and Stahl, F.W. 1983. Viability of λ phages carrying a perfect palindrome in the absence of recombination nucleases. Nature, 305:448-451.

Lehman, I.R. 1974. DNA ligase: structure, mechanism and function. Science, 186:790-797.

Lewin, R. 1986. DNA fingerprints in health and disease. Science, 233:521-52.

Lewin, R. 1989. Limits to DNA fingerprinting. Science, 243:1549-1551.

Litt, M. and Luty, J.A. 1989. A highly hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within cardiac muscle actin gene. Am. J. Hum. Genet., 44:397-401.

Little, P.F.R. and Jackson, I.J. 1987. Application of plasmids containing promoters specific for phage-encoded RNA polymerases. In: DNA Cloning, Vol. III - A Practical Approach (ed. Glover, D.M.). pp.1-18. IRL press, Oxford.

Maniatis, T., Hardison, R.C., Lacy. E., Lamer, J., O'Connel, C., Quorn, D., Sim, G.K. and Efstratiadis, A. 1978. Isolation of Structural genes from libraries of eukaryotic DNA. Cell, 15:687-701.

Longmire, J.L., Kraemer, P.M., Brown, N.C., Hardekopf, L.C. and Deaven, L.L. 1990. A new multi-locus DNA fingerprinting probe: pV47-2. Nucleic Acids Res., 18:1658.

Lynch, M. 1988. Estimation of relatedness by DNA fingerprinting. Mol. Biol. Evol., 5:584-599.

Macedo, A.M., Medeiros, A.C. and Pena, S.D.J. 1989. A general method for efficient non-isotopic labelling of DNA probes cloned in M13 vectors: application to DNA fingerprinting. Nucleic Acids Res., 17:4414.

Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, New YorK.

Mayr, E. 1963. Animal Species and Evolution. pp.136-262. The Belknap Press of Harvard University Press. Cambridge, Massachusetts.

Meinkoth, J. and Wahl, G. 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem., 138:267-284.

Meng, A., Carter, R.E. and Parkin, D.T. 1990. The variability of DNA fingerprints in three species of swan. Heredity, 64:73-80.

Menzel, A., Lagoda, P.J.L. and Issinger, O-S. 1990. Hypervariable individual-specific DNA band patterns revealed by a 22mer promoter-specific oligonucleotide probe containing an SP1 site. Nucleic Acids Res., 18:4287.

Miers, K.H. and Williams, H. 1969. Nesting of Black Swans at Lake Ellesmere. New Zealand Wildfowl, 20:23-32.

Min, G.L., Hibbin, J., Arthur, C., Apperley, J., Jeffreys, A.J. and Goldman, J. 1988. Use of minisatellite DNA probes for recognition and

Minton, C.D.T. 1968. Pairing and Breeding of Mute Swans. Wildfowl, 19:41-60.

characterization of relapse after allogeneic bone marrow transplantation. Brit. J. Haematol., 68:195-201.

Morton, D.B., Yaxley, R.E., Patel, I., Jeffreys, A.J., Howes, S.J. and Debenham, P.G. 1987. Use of DNA fingerprint analysis in identification of the sire. The Veterinary Record, 121:592-594.

Mourant, A.E. 1961. Blood groups. In: Genetic Variation in Human Populations (ed. Harrison, G.A.). pp.1-15. Pergamon Press, Oxford.

Moyzis, R.K., Buckingham, J,M., Cram, L.S., Dani, M., Deaven, L.L., Jones, M.D., Meyne, J., Ratliff, R.L. and Wu, J.R. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc. Natl. Acad. Sci. USA, 85:6622-6626.

Murray, M.J., Haldeman, B.A., Grant, F.J. and O'Hara, P.J. 1988. Probing the human genome with minisatellite-like sequences from the human coagulation factor VII gene. Nucleic Acids Res., 16:4166.

Murray, N.E. and Murray, K. 1974. Manipulation of restriction targets in phage λ to form receptor chromosomes for DNA fragments. Nature, 251:476-481.

Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Fumlin, E., White, R. 1987. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science, 235:1616-1622.

Nakamura, Y., Carlson, M., Krapcho, K., Kanamori, m. and White, R. 1988. New approach for isolation of VNTR marker. Am. J. Hum. Genet., 43:854-859.

Nei, M. 1972. Genetic distance between populations. The American Naturalist, 106:283-292.

Nevo, E. 1978. Genetic variation in natural populations: Patterns and theory. Theor. Pop. Biol., 13:121-177.

Nybom, H., Schaal, B.A. and Roystad, S.H. 1989. DNA "fingerprints" can distinguish cultivars of blackberries and raspberries. Acat. Hortic., 262:310-315.

Nybom, H., Rogstad, S.H. and Schaal, B.A. 1990. Genetic Variation detected by use of the M13 " DNA fingerprint" probe in *Malus, Prunus, and Rubus* (Rosaceae). Theor. Appl. Genet., 79:153-156.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem., 250:4007-4021.

Ogilvie, M.A. 1967. Population changes and mortality of the Mute Swan in Britain. Wildfowl Trust Ann. rep., 18:64-73.

–. 1981. The Mute Swan in Britain, 1978. Bird Study, 33:87-106.

–. 1986. The Mute Swan *Cygnus olor* in Britain, 1983. Bird Study, 33:121-137.

Parkin, D.T. 1987. The value of genetic fingerprinting to the breeding and conservation of birds of prey. In: Breeding and Management of Birds of Prey (ed. Hill, D.J.). University of Bristol.

Parkin,D.T.,Hutchinson,I. and Wetton,J.H.1988.Genetic fingerprinting and its role in bird research and law enforcement. RSPB Cons. Rev., No.2:22-24.

Promega. 1987. Technique Bulletin, No. 055.

– 1988. Promega Notes, No. 13.

Quinn, T.W. and White, B.N. 1987. Identification of restriction fragment length polymorphisms in genomic DNA of the lesser snow goose. Molec. Biol. Evol., 4:126-143.

Quinn, T.W., Quinn, J.S., Cooke, F. and White, B.N. 1987. DNA marker analysis detects multiple maternity and paternity in single broods of the lesser snow goose. Nature, 326:392-394.

Rambach, A. and Tiollais, P. 1974. Bacteriophage λ having EcoRI endonuclease sites only in the non-essential region of the genome. Proc. Natl. Acad. Sci. USA, 71:3927-3930.

Rawcliffe, C.P. 1958. The Scottish Mute Swan census 1955-56. Bird Study, 5:45-55.

Reed, K.C. and Mann, D.A. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res., 13:7207-7221.

Reeve, H.K., Westneat, D.F., Noon, W.A., Sherman, P.W. and Aquadro, C.F. 1990. DNA "fingerprinting" reveals high level of inbreeding in colonies of the eusocial naked mole-rat. Proc. Natl. Acad. Sci. USA, 87:2496-2500.

Riedman, M.L. 1982. The evolution of alloparental care and adoption in mammals and birds. The Quart. Rev. Biol., 57:405-435.

Rigaud, G., Grange. T. and Pictet, R. 1987. The use of NaOH as transfer solution of DNA onto nylon membrane decreases the hybridization efficiency. Nucleic Acids Res., 15:857.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, C. 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol., 113:237-251.

Rogaev, E.I. and Shlensky, A.B. 1990. The genomic DNA ϕFd103 probe is sensitive marker for detection of human hypervariable genomic regions. Nucleic Acids Res., 18:1081.

Rogstad, S.H., Pattern II, J.C. and Schaal, B.A. 1988. M13 repeat probe detects DNA minisatellite-like sequences in gymnosperms and angiosperms. Proc. Natl. Acad. Sci. USA, 85:9176-9178.

Rohwer, F.C. 1989. The distribution of conspecific nest parasitism in birds. Can. J. Zool., 67:239-253.

Roseberg, S.M., Stahl, M.M., Kobayashi, I. and Stahl, F.W. 1985. Improved *in vitro* packaging of coliphage lambda DNA: a one-strain system free from endogenous phage. Gene, 38:165-175.

Royle, N.J., Clarkson, R.E., Wong, Z. and Jeffreys, A.J. 1988. Clustering of hypervariable minisatellites in the proterminal regions of human autosomes. Genomics, 3:352-360.

Ryskov, A.P., Jincharadze, A.G., Prosnyak, M.I., Ivanov, P.L. and Limborska, S.A. 1988. M13 phage DNA as a universal marker for DNA fingerprinting of animals, plants and microoganisms. FEB, 233:399-392.

Saito, I. and Stark, G.R. 1986. Charomids: cosmid vectors for efficient cloning and mapping of large or small restriction fragments. Proc. Natl. Acad. Sci. USA, 83:8664-8668.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual (second edition). Cold Spring Harbor Laboratory, New York.

Schafer, R., Zischler, H. and Eppenlen, J.T. 1988. DNA fingerprinting using non-radioactive oligonucleotide probes specific for simple repeats. Nucleic Acids Res., 16:9344.

Schumm, J.W., Knowlton, R.G., Braman, J.C., Barker, D., Vovis, G., Akots, G., Brown, V., Gravius, T., Helms, C., Hsiao, K., Rediker, K., Thurston, J., Bostein, D., Donis-Keller, H. 1985. Detection of more than 500 single copy RFLP's by random screening. Cytogenet. Cell. Genet., 40:739.

Schuum, J.W., Knowlton, R.G., Braman, J.C., Barker, D.F., Bostein, D., Akots, G., Brown, V., Gravius, T., Helms, C., Hsiao, K., Rediker, K., Thurston, J. and Donis-Keller, H. 1988. Identification of more than 500 restriction fragment length polymorphisms by screening random genomic clones. Am. J. Hum. Genet., 42:143-159.

Scott, D. 1967. The Bewick's Swan at Slimbridge 1966-67. Wildfowl Trust Ann. Rep., 18:24-27.

Scott, P. and Wildfowl Trust. 1972. The Swans. Michael J seph, London.

Sharp, P.A., Sugolen, B. and Sambrook, J. 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry, 12:3055-3063.

Signer, E., Kuenzle, C., Thomann, P.E. and Hubscher, U. 1988. Modified gel electrophoresis for higher resolution of DNA fingerprints. Nucleic Acids Res., 16:7739.

Simmler, M.C., Johnsson, C., Petit, C., Rouyer, F., Vergraud, G. and Weissenbach, J. 1987. Two highly polymorphic minisatellites from the pseudoautosomal region of the human sex chromosomes. EMBO J., 6:963-969.

Smith, G.E. and Summers, M.D. 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem., 109:123-129.

Smith, G.P. 1976. Evolution of repeated DNA sequences by unequal crossover. Science, 191:528-534.

Smithies, O. 1955. Zone electrophoresis in starch gels: group variation in the serum proteins of normal human adults. Biochem. J., 61:629.

Smouse, P.E. and Chakraborty, R. 1986. The use of restriction fragment length polymorphisms in paternity analysis. Am. J. Hum. Genet., 38:918-939.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., 98:503-517.

Southern, E. 1979. Gel electrophoresis of restriction fragments.In: Methods in Enzymology, Vol. 68 (ed. Wu, R.). pp.152-176. Academic Press, New York.

Steinmetz, M., Stephan, D. and Lindahl, K.F. 1986. Gene organization and recombinational hotspots in the murine major histocompatibility complex. Cell, 44:895-904.

Stoker, N.G., Cheah, K.S.E., Griffin, J.R., Pope, F.M. and Solomon, E. 1985. A highly polymorphic region 3' to the human Type II collagen gene. Nucleic Acids Res., 13:4613-4622.

Sturkie, P.D. 1976. Avian Physiology. Goltringen, New York.

Tabor, S. and Richardson, C.C. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA, 82:1074-1078.

Tautz, D. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res., 17: 6463-6471.

Thein, S.L., Jeffreys, A.J., Gooi, H.C., Cotter, F., Flint, J., O'Conner, N.T.J., Weatherall, D.I. and Wainscoat, J.S. 1987. Detection of somatic changes in human cancer DNA by DNA fingerprint analysis. Bri. J. Cancer, 55:353-356.

Thein, S.L. and Wainscoat, J.S. 1986. The clinical applications of DNA polymorphisms. Disease Markers, 4:203-217.

Thomas, M., Cameron, J.R. and Davis, R.W. 1974. Viable molecular hybrids of bacteriophage λ and eukaryotic DNA. Proc. Natl. Acad. Sci. USA, 86:2742-2746.

Uitterlinden, A.G., Slagboom, P.E., Knook, D.L. and Vijg, J. 1989. Two-dimensional DNA fingerprinting of human individuals. Proc. Natl. Acad. Sci. USA, 86:2742-2746.

Upholt, W.B. 1977. Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. Nucleic Acids Res., 4:1257-1265.

Vassart, G., Georges, M., Monsieur, R., Brocas, H., Lequarre, A.S. and Christophe, D. 1987. A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. Science, 235:683-684.

Venturini, G.R., D'Ambrogl, R. and Capanna, E. 1986. Size and structure of the bird genome - I. DNA content of 48 species of Neognathae. Comp. Biochem. Physiol., 85B:61-65.

Vieira, J. and Messing, J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene, 19:259-268.

Wahl, G.M., Stern, M. and Stark, G.R. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA, 76:3683-3687.

Wahls, W.P., Wallace, L.J. and Moore, P.D. 1990. Hypervariable minisatellite DNA is a hotspot for homologous recombination in human cells. Cell, 60:95-103.

Wallace, D.M. 1987a. Large- and small-scale phenol extractions. In: Methods in Enzymology, Vol. 152 (eds. Berger, S.L. and Kimmel, A.R.). pp.33-41. Academic Press, New York.

_. 1987b. Precipitation of nucleic acids. In: Methods in Enzymology, Vol. 152 (eds. Berger, S.L. and Kimmel, A.R.). pp.41-48. Academic Press, New York.

Washio,K.,Misawa, S. and Ueda, S. 1989. Probe walking: development of novel probes for DNA fingerprinting. Hum. Genet., 83:223-226.

Weil, J., Cunningham, R., Martin, R.III, Mitchell, E. and Bolling, B. 1973. Characteristics of λp4, a λ derivative containing 9% excess DNA. Virology, 50:373-380.

Weiss, M.L., Wilson, V., Chan, C., Turner, T. and Jeffreys, A.J. 1988. Application of DNA fingerprinting probes to old world monkeys. American J. Primatol., 16:73-79.

Wellbourn, M., Wetton, J.H., Davies, C.E. and Parkin, D.T. 1990. Determination of parentage in the swallow (*Hirundo rustica*) by DNA fingerprinting. (in press)

Weller, P., Jeffreys, A.J., Wilson, V. and Blanchetot, A. 1984. Organization of the human myoglobin gene. EMBO J., 3:439-446.

Wells, R.A., Wonke, B. and Thein, S.L. 1988. Prediction of consanguinity using human DNA fingerprints. J. Med. Genet., 25:660-662.

Wells, R.A., Green, P. and Reeders, S.T. 1989. Simultaneous genetic mapping of multiple human minisatellite sequences using DNA fingerprinting. Genomics, 5:761-772.

Westneat, D.F., Noon, W.A., Reeve, H.K. and Aquadro, C. 1988. Improved hybridization conditions for DNA 'fingerprints' probed with M13. Nucleic Acids Res., 16:4161.

Wetton, J.H., Carter, R.E., Parkin, D.T. &. Walter, D. 1987. Demographic study of a wild house sparrow population by DNA fingerprinting. Nature, 327:147-149.

Wetton, J.H. and Parkin, D.T. 1989. Genetic fingerprinting of house sparrow *Passer domesticus* . In: Electrophoretic Studies on Agricultural Pests (eds. Loxdale, H.D. and Hollander, J.D.). pp.375-391. Clarendon Press. Oxford.

White, R., Leppert, M., Bishop, D.T., Barker, D., Berkowitz, J., Brown, C., Callahan, P., Holm, T. and Jerominski, L. 1985. Construction of linkage maps with DNA markers for human chromosomes. Nature, 313:101-105.

Wolda, H. 1969. Stability of a steep cline in morph frequencies of the snail Cepaea nemoralis (L.). J. Anim. Ecol., 38:623-625.

Wolff, R.K., Nakamura, Y. and White, R. 1988. Molecular characterization of a spontaneously generated new allele at a VNTR locus: no exchange of flanking DNA sequence. Genomics, 3:347-351.

Wong, Z., Wilson, V., Jeffreys, A.J. and Thein, S.L. 1986. Cloning a selected fragment from a human DNA 'fingerprint': isolation of an extremely polymorphic minisatellite. Nucleic Acids Res., 14:4605-4616.

Wong, Z., Wilson,V., Patel, I., Povey, S. and Jeffreys, A.J. 1987. Characterization of a panel of highly variable minisatellites cloned from human DNA. Ann. Hum. Genet., 51:269-288.

Woodcock, D.M., Growther, P.J., Doherty, J., Jefferson, S., DeCruz, E., Noyor-Weidner, M., Smith, S.S., Michael, M.Z. and Graham, M.W. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res., 17:3469-3478.

Wyman, A.R. and White, R. 1980. A highly polymorphic locus in human DNA. Proc. Natl. Acad. Sci. USA, 77:6754-6758.

Wyman, A.R., Wolfe, L.B. and Bostein, D. 1985. Propagation of some human DNA sequences in bacteriophage λ vectors requires mutant *Escherichia coli* hosts. Proc. Natl. Acad. Sci. USA, 82:2880-2884.

Wyman, A.R. and Wortman, K.F. 1987. Host strains that alleviate underrepresentation of specific sequences: overview. In: Methods in Enzymology, Vol. 152 (eds. Berger, S.L. and Kimmel, A.R.). pp.173-180.

Zischler, H., Schafer, R. and Epplen, J.T. 1989. Non-radioactive oligonucleotide fingerprinting in the gel. Nucleic Acids Res., 17:4411.