Sexual Development and Sclerotial Formation in the Black Aspergilli

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Abstract

The black aspergilli are a group of species from the genus *Aspergillus* that are typically characterised by the production of dark brown or black conidia. This group is formally referred to as section *Nigri* and includes the species *Aspergillus niger* and *Aspergillus tubingensis*, which are of biotechnological importance due to their production of organic acids such as citric acid, enzymes such as amylases, and their emerging utility in bioremediation. The group was formally believed to be comprised of solely asexual species. However, there have been recent successes in elucidating sexual cycles in *A. tubingensis* and *A. sclerotiicarbonarius*. These studies demonstrated that the production of sclerotia is a necessary prerequisite to sexual reproduction in the black aspergilli with cleistothecia (ascomata) developing within the interior of the sclerotia, linked to a sexual *Aspergillus* sexual morph known as saitoa (superseding the use of the sexual morph petromyces). Sclerotia are hardened, melanised and metabolically active structures that are resistant to adverse environmental conditions.

The reporting of sexual reproduction in the black aspergilli have encouraged efforts to try and induce sex in *A. niger*, as this would allow strain improvement via sexual breeding and also provide a valuable genetic tool for this economically important species. Prior to the present study, conditions had been described to produce sclerotia in *A. niger* under laboratory conditions. In addition, analyses of the MAT locus of *A. niger* had revealed a proto-heterothallic nature, with isolates of two separate mating types found in global collections, which were defined by the presence of either a *MAT1-1-1* α-box- or *MAT1-1-2* HMG-encoding gene at the MAT locus, HMG and α-box proteins are key transcriptional regulators for sex.

The present study aimed to gain knowledge about processes governing sclerotial formation and mating in the black aspergilli, with the ultimate goal of inducing sexual reproduction *in A. niger.* Work was first completed to reproduce and optimise the conditions used to produce sclerotia in the black aspergilli with a focus on *A. niger*. Media and environmental conditions were identified which could be used to reliably induce formation of sclerotia, although the conditions were species specific and there was much variation in numbers of sclerotia depending on the strain used. Secondly, genetic characterisation of two transcription factors, termed SclR and SclB, thought to be involved in sclerotia production in the aspergilli was also completed. SclR was shown to be required, but not essential, for sclerotia formation in *A. niger* whilst deletion of *sclB* resulted in enhanced production of sclerotia in the majority but not all strains of *A. niger*, Investigations into production of sclerotia were complemented by studies examining whether any chemical factor(s) with a role in sclerotia formation

could be uncovered. No lipid morphogen was found despite extracting from mated cultures over a wide period of maturation of sclerotia.

Attempts were then made to induce sexual reproduction in *A. niger*, together with a series of other *Aspergillus* species as controls. A mating-type diagnostic was applied to allow expedient PCR determination of mating type for isolates of *A. niger* to assist with crossing efforts by identifying putative compatible MAT1-1 and MAT1-2 strains. The accuracy of the diagnostic was confirmed through later genome sequencing of representative isolates. These studies were complemented with the use of scanning electron microscopy (SEM) to examine sclerotia for the possible presence of ascomata, confocal microscopy to examine changes within sclerotia during 'mating', and the development of flow cytometry techniques to apply cell sorting to separate different spore types (i.e. conidia and ascospores). Sexual reproduction was indeed induced in certain of the control *Aspergillus* species. However, all studies failed to yield direct observation of the expected products of sexual recombination, namely cleistothecia or ascospores, in any crosses involving *A. niger* despite extensive efforts involving the crossing of multiple MAT1-1 and MAT1-2 isolates under a range of conditions and using a variety of crossing techniques. However, there were observations of formation a distinct red structure within the sclerotia of certain 'mated' cultures that were absent from control unmated axenic cultures of *A. niger*. This was complemented by the identification via SEM of a rare and distinct tissue type at the centre of sclerotia, as well as distinct channels within certain sclerotia. Fluorescent strains of MAT1-1 and MAT1-2 mating type of *A. niger* were created and confocal microscopy used to reveal that germinating conidia of one mating type could produce hyphae with the potential for ingrowth into sclerotia of the opposite mating type. Finally, studies were completed to analyse whether a suite of meiosis-specific genes were transcriptionally upregulated during this 'mating'. This confirmed that during the 'mating' process, certain meiosis genes (*dmc1*, *rec8*, *hop2* and *msh4*) as well as *sclR* (a regulator of sclerotia formation) were indeed upregulated.

Taken as whole, results from the present study add to increasing evidence of an extant but cryptic sexual cycle in *Aspergillus niger*.

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Abbreviations

˚C	**degrees Celsius**
ACM	***Aspergillus* complete media**
AMF	**arbuscular mycorrhizal fungi**
AMM	***Aspergillus* minimal media**
ANOVA	**analysis of variance**
AP	**alkaline phosphatase**
aspGD	***Aspergillus* genome database**
BCIP	**5-bromo-4-chloro-3-indolyl phosphate**
BLAST	**basic local alignment search tool**
bp	**base pair**
cDNA	**complementary DNA**
cm	**centimetre**
CTAB	**cetyl trimethylammonium bromide**
CYA	**Czapeks yeast autolysate media**
CYAR	**Czapeks yeast autolysate media supplanted with raisins**
DEPC	**diethyl pyrocarbonate**
DNA	**deoxyribonucleic acid**
dNTP	**deoxyribonucleotide triphosphate**
dsDNA	**double stranded DNA**
EDTA	**ethylenediaminetetra-acetic acid**
G	**gram or gravitations**
GPCR	**G-protein coupled receptor**
H	**hour**
HMG	**high mobility group**
ITS	**internal transcribed spacer**
Kbp	**kilobase pairs**
kV	**kilovolt**
L	**Litre**
LB	**Luria Bertani media**
M	**molar**

MAPK	**mitogen activated protein kinase**
MAT	**mating-type**
MCA	**mixed cereal agar (media)**
MEA	**malt extract agar (media)**
mg	**milligram**
min	**minute**
mM	**millimolar**
mRNA	**messenger RNA**
NBT	**nitro-blue tetrazolium chloride**
NCBI	**national centre for biotechnology information**
NEB	**New England Biolabs**
ng	**nanogram**
OA	**oatmeal agar (media)**
ORF	**open reading frame**
PBS	**phosphate buffered saline**
PCR	**polymerase chain reaction**
PDA	**potato dextrose agar (media)**
PEG	**polyethylene glycol**
RAPD	**randomly amplified polymorphic DNA**
RNA	**ribonucleic acid**
ROUT	**robust regression and outlier removal**
rpm	**revolutions per minute**
rRNA	**ribosomal RNA**
RT-PCR	**reverse transcription- polymerase chain reaction**
s	**second**
SDS	**sodium dodecyl sulfate**
SEM	**scanning electron microscopy**
SNP	**single nucleotide polymorphism**
SOC	**super optimal with catabolite repression (broth media)**
sp.	**species (singular)**
spp.	**species (plural)**
ssDNA	**single stranded DNA**

TAE	**tris-acetate-EDTA buffer**
TBE	**tris-borate-EDTA buffer**
TE	**tris-EDTA buffer**
UV	**ultraviolet**
V	**volt**
v/v	**volume/volume**
w/v	**weight/volume**
YEPD	**yeast extract peptone dextrose**
µg	**microgram**
µl	**microlitre**
µM	**micromolar**

Chapter 1: Introduction

1.1 Background – Ascomycete Fungi

It is thought that fungi first appeared as divergent from plants and animals as early as 1.58 billion years ago (Wang, Kumar and Hedges, 1999). A firmer estimate of the emergence of the Ascomycota is around 500-650 million years ago (Lucking *et al.,* 2009). Ascomycetes are a cosmopolitan group, known to thrive in both terrestrial and aquatic habitats (Maharachchikumbura *et al.,* 2021). Many have adapted to a lichenised lifestyle and are seen as necessary for terrestrialisation (Naranjo-Ortiz and Gabaldon, 2019). Many others are mycorrhizal, mycoparasitic, or plant or human pathogens (Kowal *et al.,* 2018; Berbee, 2001). Compared to other fungi they tend to have an abundance of enzymes that produce secondary metabolites and shape their environment (Chavez *et al.,* 2015).

Around 98 % of all described species of fungi are part of either the Ascomycota or Basidiomycota with the Ascomycota comprising the majority, around two thirds of all known species (Naranjo-Ortiz and Gabaldon, 2019). Ascomycota are divided into three further groups or subphyla: the Pezizomycotina, Taphrinomycotina and Saccharomycotina (James *et al*., 2006). The Pezizomycotina (previously known as Euascomycota) is the largest subphylum and is characterised by mostly consisting of true 'filamentous fungi' as well as forming complex fruit bodies. This subphylum contains numerous examples, including foodstuffs such as Morels (*Morchella esculenta*) and Quorn™ (*Fusarium venenatum*), the opportunistic human pathogens *Aspergillus fumigatus* and *Histoplasma capsulatum,* and crop pathogens such as *Sclerotinia sclerotiorum* and *Pyrenopeziza brassicae*. The Taphrinomycotina contain species that are strictly yeast like (single celled) such as *Pneumocystis spp*., as well as dimorphic species (which can switch between yeast-like and filamentous morphs) such as *Taphrina spp.*. The *Saccharomycotina* are referred to as the 'true yeasts' and contain the widely known *Saccharomyces cerevisiae* and the common human pathogen *Candida albicans*.

Ascomycetes that are known to reproduce sexually are defined by the production of the 'ascus' which is a sac like structure (from the Greek for 'wine bag' or 'skin bag') containing internal sexual spores as opposed to basidiomycetes that produce sexual spores externally on a specialised club like cell called a basidia. In ascomycetes, the sexual cells are produced as a result of a conserved sequence of events, namely, plasmogamy, karyogamy and meiosis (Lee *et al.,* 2010). The morphology of the sexual structures that house asci can be very distinct, even amongst species previously thought to be closely related. The aspergilli for example, are named after the structure by which they produce asexual spores and have been thought to be closely related. However, the variation in morphology of sexual structures within this group has previously led to renaming and regrouping of genera (Butler, 2010).

An example is the commonly studied fungus *Aspergillus nidulans*, which is mainly known after its asexual state (anamorph). However, it also previously had the name *Emericella nidulans* based on the morphology of its sexual structures (teleomorph or sexual morph) (Chen *et al*., 2016; Brakhage, 1997). It has been argued that the use of two names to describe the asexual and sexual states has led to confusion and the practice has been formally discontinued under the "One Fungus, One name" system of nomenclature (Hawksworth *et al.,* 2011). Genera of sexual species can be described by sexual morph, as this provides evidence of inter-relatedness, and allows for subdivision of groups such as the aspergilli (Dyer and O'Gorman, 2012). However, old habits are hard to break, and many fungal researchers outside of research on fungal sex and recombination (and many within it) still cling to old terminology. Additionally, the use of the two terms may be preferable for researchers to distinguish between the two lifecycles (Pitt and Taylor, 2014). For the purposes of this thesis, teleomorphs will be referred to as sexual morphs and will not be named using fungal naming nomenclature, but reference may be given to the sexual morph non-italicised, for example, with *Emericella nidulans*, we will refer to *Aspergillus nidulans* (sexual morph emericella).

This thesis will focus on a group of fungi within the Pezizomycotina within the Ascomycota. This group are members of the aspergilli and are referred to as the 'Black aspergilli' due to their preponderance for producing black conidia. Much of the following background material will therefore focus on this group. The term '*Aspergillus* section *Nigri'* is used to as a collective term for species with particularly close morphological and phylogenetic relationships to the type species *Aspergillus niger sensu stricto* (Vesth *et al.,* 2018; Samson *et al*., 2007b).

1.2 Asexuality in Ascomycete Fungi

Asexuality in ascomycete fungi describes the state whereby certain species are restricted to only asexual reproduction (i.e. lacking the ability to undergo sexual reproduction), which results solely in the production of genetically identical propagules from a parent (Dyer and Kuck, 2017). These propagules are termed conidia and can lead to a rapid production of multiple copies of a parent through mitosis (Geiser, Timberlake and Arnold, 1996; Yu, 2010). The use of mitosis to produce new individuals ensures that successful genetic combinations for a particular environment are maintained (Guinea *et al*., 2006; Butler, 2010). The combination of success within an environment and the low energetic cost of the production of conidia can ensure rapid pioneer spread through a new environment (Baltussen *et al*., 2020).

There are limitations to asexuality as a sole mode of reproduction. Firstly, populations become clonal in nature with the only variation arising because of occasional, rare mutations (Carbone and Kohn, 2004). Clonal populations can have a reduction in ability to respond successfully to changes in their environment, whereas sexual propagation can generate different gene combinations that can give rise to certain progeny suiting new niches (Naranjo-Ortiz and Gabaldon., 2019). In the case of asexual reproduction, the mutations that arise are increasingly likely to be deleterious and may lead to a level of mutational load or 'mutational meltdown' that can lead to the demise of asexual lineages (Lynch *et al*., 1993; Laine, Sackton and Meselson, 2022). This is described by the concept of 'Mullers Ratchet' whereby deleterious mutations can only increase in successive generations of a purely asexual population until they are so pervasive that the overall population declines (Muller, 1964; Felsenstein, 1974; Varga *et al*., 2014).

Despite the supposed detrimental features of solely asexual reproduction, certain asexual lineages have been reported as successful. Bdelloid rotifers and arbuscular mycorrhizal fungi (AMF) are two groups which have been described as purely asexual (Riley and Corradi, 2013; Wilson, Nowell and Barraclough, 2018; Welch and Meselson, 2000). The existence of these groups for millions of years without a described sexual state must mean that they exhibit some way(s) to escape the effect of increasing deleterious mutations, such as through a cryptic sexual state or parasexuality (Taylor *et al*., 2015; Riley and Corradi, 2013; Pontecorvo *et al*., 1953). Indeed, for certain AMF there is genomic evidence for the presence of highly conserved genetic machinery for meiosis (Sanders, 2011; Corradi and Brachmann, 2017). Furthermore, and in quite a paradigm shift, evidence has been put forward for sex in the bdelloid rotifer *Macrotrachella quadricornifera* (Laine, Sackton and Meselson., 2022), requiring evaluation of the success of these asexual lineages as a point of reference.

1.2.1 Conidiation

In the aspergilli, and more generally within the Ascomycota, asexual spores are named conidia and are formed as chains from a specialised structure called a conidiophore. The conidiophore is itself made up of a stalk, vesicle, metulae and phialides which then produce the conidia (Bayram and Braus, 2011) as shown in **Figure 1.1**. However, metulae are not produced in all *Aspergillus* species (Samson *et al*. 2004). The terms uniseriate and biseriate are used to describe species in which metulae are absent or present, respectively (Abarca *et al*., 2004). *Aspergillus niger (sensu stricto)* and *Aspergillus nidulans* are examples of biseriate development and *Aspergillus fumigatus* is uniseriate (Teertstra *et al*., 2017). Beyond Aspergillus niger sensu stricto it seems the majority of species within *Aspergillus* section *nigri* are also biseriate with some recorded exceptions occurring within the *A. aculeatus* clade (Varga *et al*., 2011).

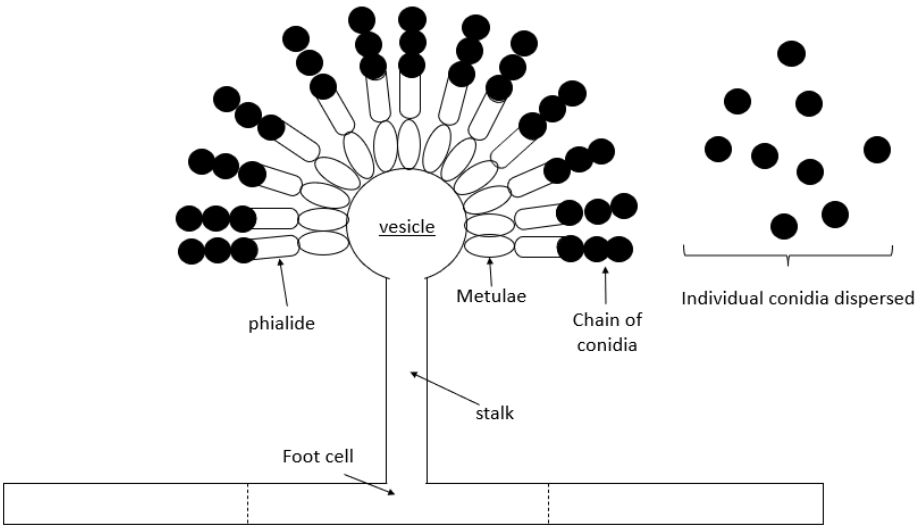

Figure 1.1. Structure of an *Aspergillus* conidiophore. Development begins with a foot cell which develops into a stalk and vesicle. Vesicle then produce either phialides only or metulae and subsequent phalides. Conidia bud from the apices of the phialides.

As can be seen in **Figure 1.1**, chains of conidia branch from the metulae and phialides. Two methods of conidial genesis are described. The first is where within the phialide a directed nuclear division occurs, and a single nucleus remains in the phialides whereas a second (or further) migrates into the forming conidium. Therefore, nuclear division occurs only in the basal cells (phialides) with the oldest conidium in each chain being furthest from the basal cell. This is described as basipetal development or basipetal conidiogenesis. In contrast, metulae can bud in such a way that chains branch out from the centre, with the newest forming at the emerging tip, giving rise to new conidia more distantly

from the centre. This method of conidial development is known as acropetal development (Sewall *et al*., 1990; Son *et al*., 2014).

Conidia can be uninucleate (containing a single nucleus) or multinucleate (more than one nucleus). *A. nidulans*, *A. niger*, *A. fumigatus* and *A. flavus* are all examples of uninucleate species whilst *A. terreus* and *A. oryzae* are multinucleate (Ishi *et al*., 2005; Gomi, 2004; Deak *et al*., 2011).

1.2.2 Central Conidiation Pathway in *A. nidulans*

The genes involved with the central conidiation are well conserved amongst members of the aspergilli and amongst more distantly related fungi, but here we shall discuss some of the main genes involved in the process, in the context of *A. nidulans*. Whilst conserved within the genus *Aspergillus* (as well as *Penicillium* and *Talaromyces*), there is less conservation amongst other members of the *Eurotiomycetes* (Ojeda-Lopez *et al*., 2018; de Vries *et al*., 2017).

Control of conidiation by development of the morphology is characterised by the interactivity of the genes *brlA* (bristle), *abaA* (abacus) and *wetA* (wet, white conidia) and the preceding regulatory genes *fluG* (fluffy) and *flbA-E* (Ojeda-Lopez *et al*., 2018). *fluG* mutants form colonies of undifferentiated, cottony mycelia instead of switching into the conidiogenesis or the asexual cycle and prevent the expression of *brlA* (D'Souza, Lee and Adams, 2001). *brlA* encodes for a C_2H_2 zinc finger transcription factor. Loss of function mutations in *brlA* result in a bristly phenotype lacking the normal characters of conidiophores such as vesicles, metullae and phialades and the conidiophore can reach a length twenty times that of the wildtype. Additionally, overexpression of *brlA* can compensate for the absence of conditions necessary for normal conidiation and allow for expression of the downstream *abaA* and *wetA* (Han and Adams, 2001).

The expression of *abaA* is heightened during the middle stages (10-12 hours) of conidiation when metulae and phialides are being formed. Loss of function mutants have swellings between conidiogenous cells rather than developing into separate chains of conidia. Furthermore, loss of function mutants demonstrated an abnormal expression of downstream developmental genes (Son *et al.,* 2020; Yu, 2010). Metulae are not always present in *Aspergillus sp.* such as *A. fumigatus*, but similar effects are seen in these species (Tao and Yu, 2011). The gene *abaA* acts as a reciprocal activator to *brlA* as well as activating *wetA*. The reciprocal activation of *brlA* means that the once *abaA* is activated, the two are independent of external events and the conidiation pathway is set (Andrianopoulos and Timberlake, 1994).

So called *wetA* mutants form white conidia which autolyze within a few days to produce liquid droplets (hence 'wet') due to the absence of a crucial cell wall component (Yu, 2010). The gene *abaA*, but not *brlA*, directly affects *wetA* expression as overexpression of *abaA* can promote *wetA* expression even in Δ*brlA* background (Mirabito, Adams and Timberlake, 1989; Yu, 2010) as seen in **Figure 1.2**.

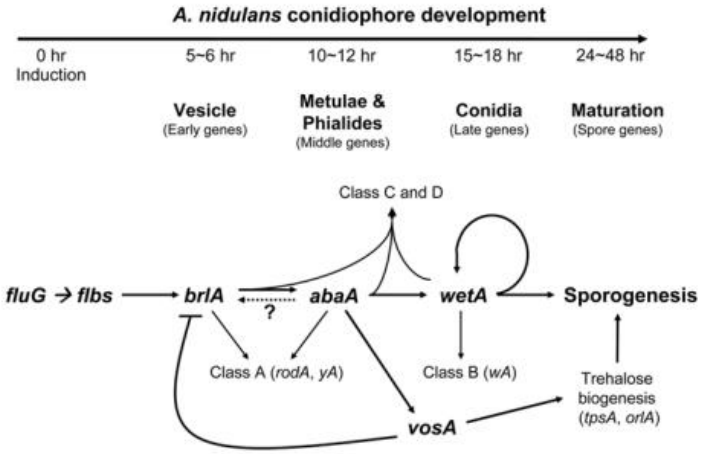

Figure 1.2. Genes involved in the developmental regulation of conidiation in *Aspergillus nidulans* [Taken from Yu, 2010].

1.3 Sexual Reproduction in Ascomycete Fungi

Many of the key stages of sex occur in all sexually reproducing species across many kingdoms of life including fungi, plants, and animals (Ni *et al.,* 2011). These stages include ploidy changes, the formation of sexual gametes through meiosis, mate recognition, cell-cell fusion (fertilisation) and the production of a zygote (Ni *et al*., 2012). Sexual spores formed by the Ascomycota are termed ascospores and are normally formed as one of eight in asci as a product of meiosis (Ainsworth 2008). The phylum Ascomycota is named after these asci, or sacs of ascospores (Bennett and Turgeon 2016). The asci normally develop within fruiting bodies termed ascomata (informally 'ascocarps'), which can contain up to hundreds of thousands of asci. Ascomycete fungi can have sex primarily either through homothallism (self-compatibility) or heterothallism (obligate out-breeding) although nuances and exceptions occur (described in **Section 1.3.1**). Within the filamentous Pezizomycotina sexual spores are produced in one of four main types of sexual fruit body. These are named cleistothecia, perithecia, apothecia and pseudothecia. These fruit bodies differ primarily by shape but also by size and organisation of asci (Poggeler *et al*., 2006; Dyer and O'Gorman, 2012). Cleistothecia are closed fruiting bodies, capable of deliquescence to disperse their internal ascospores (Dyer and O'Gorman, 2012) and may have attached but developmentally distinct tissues such as Hülle cells or sclerotia (Geiser, 2009; Dyer and O'Gorman, 2012; Horn *et al*., 2013). Perithecia are similar, but flask shaped, with a pore that allows ascospore dispersal, and ascospores can be forcibly discharged (Manstretta and Rossi., 2016). Apothecia mature so that the previously internal cavity becomes concave and exposes a layer called a hymenium, usually on the upper surface, where ascospores are generated and from which they are dispersed (Clarkson *et al*., 2003). Pseudothecia are initiated within a stroma and sexual organs are formed within the developing ascocarp (Liang *et al*., 2021). Finally, there is an additional fruit body comparable to the cleistothecium, known as a gymnothecium, which is common in the closely related *Talaromyces*, but not assigned to *Aspergillus* (Metin and Heitman, 2020).

1.3.1 Sexual Breeding Systems

The Pezizomycotina exhibit both homothallic and heterothallic breeding systems. In this section, these systems and closely related systems will be described. In addition, parasexuality will be described as this process can produce recombination independent of meiosis or sexual reproduction.

Heterothallism

Heterothallism refers to obligate out-breeding, and therefore heterothallic species are unable to undergo self-fertilisation. Sexual reproduction therefore occurs between partner isolates of opposite 'mating type' (Vonk and Ohm, 2018). The opposite mating types are designated *MAT1-1* or *MAT1-2* in the aspergilli (sometimes shortened to *MAT1* and *MAT2*), but may be referred to as *MatA* and *Mata*, *Mata* and *Matα*, or *Mat+* and *Mat–* in other species such as *Podospora anserina* (+/-) (Grognet *et al*., 2014) and *Saccharomyces cerevisiae* (a/α) (Butler, 2010). The MAT designations for *MAT*-types refer to the inclusion of either locus containing different DNA binding transcription factors (Wilson *et al*., 2021). Using the *MAT1-1* and *MAT1-2* designations, at a molecular level the *MAT1-1* locus encodes a protein containing a characteristic alpha box domain (gene *MAT1-1-1*), and the *MAT1-2* locus encodes a protein containing an HMG domain (gene *MAT1-2-1*) (Dyer *et al*., 2016). Further descriptions of these loci are in **Section 1.3.5**. Outcrossing occurring between two distinct nuclei and always results in recombination in the offspring (Billiard *et al.,* 2012).

Homothallism

Homothallism or primary homothallism is the ability to self-cross (self-fertility), although sexual outcrossing is also possible in many homothallic species and may even be favoured (Hoffmann *et al*., 2001; Paoletti *et al*., 2007). Modes of secondary homothallism exist, these are termed pseudohomothallism and mating-type switching and are described below.

In homothallic species such as *A. nidulans,* both the alpha box and HMG-domain transcription factors are produced from genes contained within a single nucleus and genome; in the case of *A. nidulans* these are unlinked on separate chromosomes, 6 and 3 (Paoletti *et al*., 2007; Dyer *et al*., 2016). However, the *MAT1-1* and *MAT1-2* genes may also be linked on the same chromosome in other homothallic species. This contrasts with the situation in heterothallic ascomycetes, where a single idiomorph (either *MAT1-1* or *MAT1-2*) is found in each nucleus of opposite mating type (Lu *et al*., 2011). It is hypothesised that homothallism evolved from the fusion of both idiomorphs into a single genome allowing self-fertility (Dyer *et al*., 2016). By contrast, it had also previously been hypothesised that the genes were originally adjacent to each other and became separate later through translocation

or aberrant segregation i.e. that heterothallism evolved from homothallism (Galagan *et al.,* 2005; Paoletti *et al*., 2005). In either case, the genes are the orchestrators of processes involved in mate recognition, cellular differentiation into parental ascogenous hyphae (such as the female trichogyne), fertilisation and meiosis (Dyer and O'Gorman 2011; Yu *et al*., 2017). Other *MAT* genes have been characterised in certain other species (Dyer *et al*., 2016) such as a *MAT1-2-4* gene within the genome of *A. fumigatus* (Yu *et al*., 2017), which when deleted resulted in the inability to mate with *MAT1-1* strains, demonstrating a role as a mating-type factor. Bioinformatic analysis has revealed homologues of *MAT1-2-4* in many other, but not all, *Aspergillus* species. More recently, a *MAT1-2-4* gene has been identified in a *MAT1-2* neotype *A. niger* strain (Ellena *et al*., 2021b).

Other than the role of *MAT* genes in sexual development, ChIP-Seq studies have revealed that the mating-type transcription factors are also involved in processes as diverse as asexual development, iron metabolism, amino acid metabolism and secondary metabolism based on studies of *Penicillium chrysogenum* (Becker *et al*., 2015). That these transcription factors regulate secondary metabolism offers new methods for the optimisation of fungal production strains via manipulation of expression of *MAT* genes. It also provides evidence that *MAT* genes do not solely upregulate transcription levels of genes involved in sex, but also regulate many other developmental and metabolic processes (Becker *et al*., 2015).

Pseudohomothallism

Pseudohomothallism is one of the modes of secondary homothallism (Wilson *et al.,* 2015; Raju and Perkins, 1994). Pseudohomothallic species can produce single spores that contain two distinct nuclei, each of which is either of the two opposite mating types. In this way, the germinating spore generates a mycelium that is heterokaryotic and therefore functionally heterothallic (Lin and Heitman, 2007). The homothallic definition arises from the fact that a single spore can undergo independent sexual reproduction (Wilson *et al*., 2015). The model organism for the study of pseudohomothallism is *Neurospora tetrasperma*. In this species, many of the spores produced are much larger than closely related heterothallic species. In addition, the asci yielding these large spores contain 4 (rather than the typical 8) spores (hence *tetrasperma*) (Jacobsen, 1995). A reduced number of smaller spores are also produced which contain only a single nucleus and are therefore heterothallic. In this way, *N. tetrasperma* can maintain the benefits of both types of reproduction (Raju, 1992).

Mating-type Switching

An additional form of secondary homothallism to pseudohomothallism is mating-type switching (Butler, 2010). Mating-type switching has been well described in *Saccharomyces cerevisiae* as well as other yeast such as *Kluyveromyces lactis* (Hanson and Wolfe, 2017; Barsoum *et al*., 2011) and additionally, the genetic components necessary for mating-type switching have been found in 'asexual' yeast such as *Candida glabrata* (Wong *et al*., 2003; Fabre *et al*., 2005). Within the characterised *S. cerevisiae*, mating identity is defined as either α or **a** in the haploid state and α/**a** in the diploid (Souza *et al*., 2003). Mating-type switching arises by replacing the genetic information expressed at the *MAT* locus. The MAT locus in this species is located towards the centre of chromosome 3 and is flanked towards the end of that chromosome by two 'silent' cassettes termed *HMR* and *HML*. The cassettes are silent because they are maintained in heterochromatin structure (Haber, 1998). A HO endonuclease initiates mating-type switching by introducing a double strand break at a recognition site and replacing the expressed cassette at the MAT locus. The HO endonuclease expression is repressed in diploid cells by the a1/α2 repressor (formed only within diploids) (Butler, 2010). In this way, populations containing only one of the two mating types have a means to generate sexual partners and undergo functional sexual reproduction.

Unisexuality

The most recent type of homothallism to be described is that of unisexual homothallism or unisexuality. Unisexual species can undergo sex, even though they only contain the genes associated with one mating type or another (Heitman, 2015; Wilson *et al.,* 2020). In this sense, they can be described as sexually homothallic in physiological behaviour but genetically heterothallic. It is possible that other described species that seem to undergo homothallic sex are in fact unisexual, and therefore genomic data will be needed to clarify (Wilson *et al*., 2020). Unisexuality has been described in ascomycetes such as *Candida albicans* (Alby and Bennett, 2011) as well as members of the filamentous *Neurospora* genus and *Huntiella moniliformis* (Glass and Smith, 1994; Liu *et al.*, 2018). In these filamentous fungi, only one mating type has been described from all strains isolated so far (Wilson *et al*., 2020).

Parasexuality

Parasexuality, whilst strictly not a sexual breeding system within fungi, does allow for genetic recombination. The parasexual cycle describes a process whereby the hyphae of two fungal isolates fuse by anastomosis (plasmogamy) and subsequent fusion of two haploid nuclei (karyogamy) to form a diploid nucleus. Genetic recombination occurs via mitotic crossing over events at matching chromosomes and is followed by separation of the chromosomes and reformation of the haploid nuclei state. Whereas during meiosis ascospores are formed, parasexuality leads to the formation of haploid nuclei amongst vegetative cells, although they differ genetically from the genome of either of the parent mycelium (Pontecorvo, 1953). Parasexuality has some of the benefits of genetic recombination, although this is limited compared to meiotic recombination, but contrarily does not have the benefits meiosis has such as production of resistant ascospores or the lack of transmission of dsRNA elements in meiotic offspring (Coenen *et al*., 1997). One proposed benefit of parasexuality over meiosis is the lack of spore production, where spores might be highly antigenic. Parasexuality may therefore be beneficial in fungal pathogen-host interactions (Sherwood and Bennett, 2009). Parasexuality was described by Pontecorvo in *A. nidulans* in 1952 and *A. niger* in 1953 (Pontecorvo, 1956) as well as more recently being recorded in *Penicillium roqueforti* (Durand *et al*., 1993), *C. albicans* (Bennett, 2015) and more recently *A. fumigatus* (Engel *et al*., 2020).

Parasexuality can only occur when the two interacting isolates are of compatible vegetative compatibility groups (VCG's), also known as heterokaryotic incompatibility (HET) or vegetative incompatibility groups (VIC). If a potential heterokaryon is formed from two incompatible isolates, the heterokaryon is not viable (Leslie, 1993). Compatibility is determined when isolates are identical at a particular set of loci (which may be termed 'het' genes). Differences at one or more of these loci leads to rejection of the heterokaryon, or compartmentalisation and death (Glass *et al*., 2000). MAT genes have been described as acting as 'Het' genes, with differences in MAT loci leading to heterokaryon rejection unless sexual reproduction is triggered in certain ascomycete species. In *N. tetrasperma*, parents can be vegetatively incompatible, and therefore pseudohomothallic offspring can exhibit growth problems because of differences in 'Het' genes (Glass and Kaneko, 2003).

1.3.2 Mating in Saccharomyces cerevisiae

Sex in *S. cerevisiae* involves two haploid cells fusing and undergoing karyogamy to become diploid. The haploid cells are required to be of opposite mating types known as **a** and **α** and the resulting diploid cells are referred to as **a/α**. Under normal conditions, each of the haploid cells produces a pheromone (called either **a-** and **α**-factor) which diffuses and binds to the respective cognate receptors located on the cell membrane of cells of the opposite mating type. The detection of these pheromones directs growth of a 'shmoo' - a cell polarising projection, utilising the concentration gradient towards the site of pheromone production. Haploid α-cells contain both an α1 and α2 gene at their *MATα* allele. They use the *Mat-α2* polypeptide to repress transcription of the a-factor and *Mat-α1* induces production of α pheromones (MFα1 and MFα2) and the a-pheromone receptor (Ste3) (Ammerer, 1989). The α-factor is produced by *MFα1* and *MFα2* and is a 13-residue hydroxylated peptide (WHWLGLKPGQPMY-OH). The a-factor comes in two forms produced by MFA1 and MFA2. The two forms differ at a single residue and are farnesylated lipopeptides (YIIKG*V/L*FWDPAC-OCH_3) (Kurjan, 1993). The pheromone receptors are called Ste3 and Ste2 (these are for the **a-** and **α**-factor respectively) both of which activate a Gαβγ heterotrimeric G-protein (Merlini *et al*., 2013). The Gα dissociates from Gβγ and associates with a MAPK scaffold Ste50 to activate transcription of downstream mating specific genes such as the transcription factor Ste12 and the conserved Ste5 MAPK scaffold (Whiteway *et al*., 1995; Elion *et al*., 2000). The Ste12 transcription factor increases the expression of over 200 genes (Elion *et al*., 2000). The Gβγ heterodimer subunit interacts with key effectors, Gβ specifically binds to the p21-activated kinase (PAK)-like kinase (Wu *et al*., 1998; Elion *et al*., 2000). The interaction with PAK in turn interacts with the Ste5 MAPK scaffold. The MAPK kinase directs actin formation with Bni1 at the site of shmoo formation. An additional interaction of the Gβγ is with the Far1 protein which provides the switch from bud growth to shmoo formation and arrests the cell cycle, so that all cells in a population are at the same stage (Butty *et al*., 1998; Elion *et al*., 2000). Subsequent growth of the chemotropic shmoos results in cell contact, fusion, plasmogamy and finally karyogamy to form the diploid zygote. The resultant a/α diploids form an a1/α2 polypeptide heterodimer which represses genes for the haploid phase such as *rme1* (*R*egulator of *Me*iosis) which in turns is a repressor of meiosis (Covitz *et al*., 1991).

After formation of the diploid zygote, nitrogen starvation leads to the formation of an ascus (Kassir *et al*., 2003).

1.3.3 Mating in Neurospora crassa

The filamentous fungus *Neurospora crassa* has been used widely for genetic studies and offers a study of the similarities between it and *S. cerevisiae* as well as describing the added complexities of both multicellularity and differentiation for sex. *N. crassa* is heterothallic and its two mating types are referred to as *MAT**A*** and *MAT**a*** (Kim and Borkovich, 2006).

Sex in *N. crassa* involves a female and male element (anisogamy). The male element can be any of a microconidium, macroconidium, or mycelial fragment, as there are not any exclusively antheridial cells. The female element begins as a protoperithecium or ascogonium. Each ascogonium has specialised hyphal extension called a trichogyne which grows outwards into the environment in search of male elements. There are pheromones that allow for chemotropic growth of the trichogyne towards the male elements (Bistis, 1981; Raju, 1980). Once the trichogyne and male element fuse, the nuclei of both parents migrate to the ascogonium. The perithecia then develop and the heterokaryotic cells develop into croziers from ascogenous hyphae. Croziers contain one each of the nuclei from the parents and these develop into asci containing eight ascospores after meiosis I, meiosis II, and a round of post meiotic mitosis (Bowring *et al*., 2006). The ascus is the only stage of the life cycle where the cell is diploid rather than dikaryotic. Perithecia which have developed contain these asci within their structure and ascospores are discharged through a small opening at the top of the perithecium called an ostiole (Bistis *et al*., 2003).

Genetic control of the pheromones is under the control of the *MAT* loci. *MATA* cells exclusively express a pheromone from the gene *ccg-4* (*c*lock-*c*ontrolled *g*ene 4) whilst *MATa* cells exclusively express a pheromone from the gene *mfa-1* (*m*ating *f*actor expressed in *a* strains) (Bobrowicz *et al.*, 2002). The pheromones produced are formed from cleavage of a precursor in a manner like that seen in *S. cerevisiae*. Conidia without the respective gene or without the control of the MAT locus are unable to attract trichogynes (Bobrowicz *et al.*, 2002). MAT**A** cells express a pheromone receptor called Pre-1 which is a G-Protein Coupled Receptor (GPCR) essential for the ability of *MAT**A*** females to recognise and fuse with *MAT**a*** male cells which would express Mfa-1 (Kim and Borkovich, 2004). Similarly, *MATa* cells express a receptor Pre-2 which functions as a receptor for ccg-4 (Kim and Borkovich, 2006). The G protein coupled receptor consists of a Gα and Gβγ dimer again, like that seen in *S. cerevisiae* (Kim and Borkovich, 2006). Further studies of the roles of the G-proteins revealed functions in aerial hyphae formation and asexual sporulation (Li *et al*., 2005; Kim and Borkovich, 2004). Furthermore, studies of the *N. crassa* homologs of *S. cerevisiae* Fus3 (MAP Kinase) and Ste12 (transcription factor) were completed. In *N. crassa*, the Fus3 homolog is called mak2 and the Ste12 homolog is called pp-1. Studies demonstrated their involvement in growth rate, and normal aerial hyphae production, but more

importantly, that they co-regulated the expression of downstream genes that were normally upregulated in protoperithecial development (Li *et al*., 2005).

1.3.4 Mating in Aspergillus nidulans

As seen in *N. crassa*, *A. nidulans* is a filamentous fungus and sex involves differentiation of tissue types. However, *A. nidulans* is homothallic and contains both *MAT1-1* and *MAT1-2* alleles within the same genome, termed *MAT1* and *MAT2*, respectively, as distinct loci within the genome. The species *A. nidulans* is therefore self-fertile as well as having the ability to have sex with genetically dissimilar isolates (Dyer and O'Gorman, 2011). Nuclear fusion occurs within reproductive structures called cleistothecia, which are surrounded by Hülle cells. The diploid zygote undergoes meiosis to form haploid ascospores in asci contained within the cleistothecia (Paoletti *et al*., 2007). The aspergilli have not been described as having exclusively male or female structures (such as trichogynes or ascogonia) other than the genus *Fennellia* and as such there is general undifferentiation in the interacting hyphae (Varga *et al*., 2014).

The basis of self-fertility was used to examine the possible roles of homologs of the same genes identified as pheromones used for mating recognition in *S. cerevisiae* and *N. crassa*. An α-like pheromone gene called *ppgA* as well as both an a-factor like receptor gene called *preA* and an α-factor like receptor gene called *preB* have all been identified (Paoletti *et al*., 2007; Dyer and O'Gorman, 2011). Further study of these genes revealed that their expression is not under the direct or subsequent control of the *MAT* loci as for *S. cerevisiae* or *N. crassa* (Paoletti *et al*., 2007). Many of the other genes responsible for the central MAPK pathway have also had homologs identified. The conserved GPCR heterotrimer has homologs of *fadA*, *sfaD* and *gpgA* (Rosen *et al*., 1999; Seo *et al*., 2005). Similarly, the Ste12 transcription factor from *S. cerevisiae* has a homolog SteA in *A. nidulans* which is of the C_2H_2 zinc finger homeobox domain with functions in cleistothecium formation and ascosporogenesis (Vallim *et al*., 2000; Borneman *et al*., 2001). Furthermore, the MAP kinases that attach to the Ste5 scaffold in *S. cerevisiae* have been identified as SteC, Ste7 and MpkB. MpkB is the homolog of Fus3 MAPK in *S. cerevisiae* (Paoletti *et al*., 2007).

Research using *A. nidulans* as a model organism for sexual reproduction has revealed many more genes involved in a multitude of functions. Many of these genes are intermediates in how *A. nidulans* reacts with its environment and some of these will be listed and described in **Section 1.5.** *Aspergillus nidulans* has been the target of extensive research into the roles of many transcription factors linked with sex or the control between asexual and sexual differentiation. This has been well reviewed (Dyer and O'Gorman, 2012) but a number will be described briefly here. As well as *SteA* (described above) (Vallim *et al*., 2000), *nsdC* and *nsdD* (*N*ever in *S*exual *D*evelopment) have been demonstrated to be necessary for sex and/or negative regulation of asexuality (Han *et al*., 2001; Kim *et al*., 2009). A further transcription factor, *rosA* has been shown to be a negative regulator of sex, with knockouts showing a mild increase in sex under normal conditions, but a greater number of cleistothecia and sexually

associated Hülle cells under both low glucose and high osmolarity, as well as under submerged culture conditions. This indicates that *rosA* has a function in repression of sex under certain environmental signals (Vienken *et al*., 2005). Linked to *rosA* is *nosA* (*N*umber *O*f *S*exual spores), which acts to represses the expression of *rosA* (Vienken *et al*., 2006). The gene *nosA* is required for primordial or cleistothecial maturation, and the balance of *nosA* and *rosA* provides a nice example of how the balance of opposing transcription factors can have a role in determining sexual or asexual pathways.

1.3.5 Biology of Mating Type Factors

As described above, the sexual identity of strains of ascomycete fungi is determined by the presence of mating type factor(s) at the *MAT* locus. The respective genes responsible for this identity are either *MAT1-1-1* or *MAT1-2-1* (determining MAT1-1 or MAT1-2 identity respectively). *MAT1-1-1* and *MAT1-2-1* both encode proteins with HMG (High Mobility Group) domains, the MAT1-1-1 alpha domain having been shown to reside in the overall HMG-protein family as recently as 2010 (Paoletti *et al*., 2007; Martin *et al.,* 2010; Dyer and O'Gorman, 2011). Homothallic ascomycetes contain both *MAT* loci in a single genome whereas heterothallic ascomycetes contain one *MAT* locus or the other (Ojeda-Lopez *et al*., 2018; Dyer and O'Gorman, 2011). The *MAT1-1-1* or *MAT1-2-1* HMG domain encoding genes are very dissimilar and are referred to as idiomorphs, rather than distinct alleles of one gene (Paoletti *et al*., 2005; Butler, 2007). Within the aspergilli these genes, and their respective loci, are commonly flanked by conserved genes *apn2* and *slaB* (Butler, 2010; Ojeda Lopez *et al*., 2018). However, work in *A. niger* has shown that inversions have occurred within the region of the MAT1-1 locus, as compared to other MAT1-1 loci in the aspergilli and the MAT1-2 locus in *A. niger*, such that both *apn2* and *slab* are on the same flank (Darbyshir 2016; Ellena *et al*., 2021b). It may therefore be necessary to state that *adeA* and *slaB* are, more generally, the typical conserved flanking genes. The gene *apn2* encodes for an anaphase promoting complex (Ramirez-Prado *et al*., 2008; Moore *et al.*, 2016). The gene *slaB* encodes for a DNA lyase enzyme (Ramirez-Prado *et al*., 2008; Moore *et al*., 2016). The gene *adeA* encodes for a SAICAR synthetase involved in purine nucleotide biosynthesis (Ellena *et al*., 2021b; Datt and Sharma, 2014).

Mating-type genes have been shown to exist in many of the supposed 'asexual' species, and often as a prelude to the discovery of sex in these species. Such species have been described as 'proto-heterothallic' (Houbraken and Dyer, 2016). This includes *A. fumigatus* (Paoletti *et al.,* 2005; O'Gorman *et al*., 2009) as well as *A. flavus* and *A. parasiticus* (Ramirez-Prado *et al*., 2008; Horn *et al*., 2009). These population studies of 'asexual' fungi revealed insight into a possible hidden or cryptic sexual cycle as they uncovered a near 1:1 population distribution of *MAT* types. When a worldwide selection of *A. fumigatus* strains was subsequently screened for *MAT* type, there was an 43.3%:56.7% ratio of *MAT1-1* to *MAT1-2* types (Paoletti *et al*., 2005), which is approximately even, and the slightly higher level of *MAT1-2* could be explained due to a small and skewed subset of isolates from France. This even distribution of *MAT* types was consistent with sexual reproduction, as for any locus with two segregating alleles, and has been described elsewhere as evidence of meiotic recombination (O'Gorman *et al*., 2009). For example, Horn *et al*., (2013) and Olarte *et al*., (2015) later characterised an uneven *MAT* type distribution (94% *MAT1-1*) in strains of *A. tubingensis* sampled from North Carolina, which was tentatively explained by the *MAT1-1* genotype offering a vegetative growth

advantage over *MAT1-2* strains. Subsequent work showing sexual recombination of these strains resulted in a more even distribution of 37:32 *MAT1-1: MAT1-2* from a total of 67 progeny strains and was used as evidence of sexual recombination. Level or even distributions of this type indicate a single allele segregation concomitant with sexual recombination.

The existence of other *MAT* genes in the *MAT* loci distinct from the HMG domain encoding genes has been described. For example, *MAT1-1-1, MAT1-1-2* and *MAT1-1-3* genes have all been characterised in *Fusarium spp* (Kim *et al.,* 2012). Within the aspergilli, a novel *MAT1-2-4* has been characterised as being necessary for mating and cleistothecium formation in *A. fumigatus* (Yu *et al*., 2017). The *MAT1-2-4* gene has a homolog in the closely related *A. lentulus* (Swilaiman *et al.*, 2013), as well as in both more distantly related *B. cinerea* and *S. sclerotiorum* (Terhem *et al*., 2013; Doughan and Rollins, 2016).

In *A. niger*, *MAT1-1* has been the dominant genotype amongst sequenced strains. However, more recently, a *MAT1-2* strain was sequenced. This uncovered both an inversion event in the *MAT1-1* locus and a *MAT1-2-4* homolog (Ellena *et al*., 2021b).

One very exciting application of knowledge of the *MAT* genes has been the discovery of sexual reproduction in previously considered 'asexual' species (Dyer and O'Gorman 2012; Dyer and Kuck 2018). In pioneering work, many supposedly asexual species were shown to contain MAT loci and MAT genes, and the MAT genes were shown to be functional when expressed in a heterologous sexual host. For example, both mating types could be found in *Bipolaris sacchari* and when the MAT genes were expressed in mat deleted strains of the heterothallic *Cochlioblous heterostrophus*, it was shown that the genes were functional in restoring sexual ability. When the experiment was reversed, the MAT genes of *C. heterostrophus* did not alter the asexual phenotype in *B. sacchari* (Sharon *et al*., 1996). Two conclusions that can be drawn from this were firstly, that asexuality (in *B. sacchari* at least) evolved from sexuality. Secondly, that the asexual nature of *B. sacchari* is not due to a non-functioning or absent MAT but could instead be due to mutations collecting in any of the suite of genes in the mating pathway, or so called 'sex genes', some of which have been discussed in **Section 1.3.4** and more will be discussed in **Section 1.5**. This work on the occurrence of MAT genes in asexual species was later applied to various *Aspergillus* species of economic and medical importance (Dyer and O'Gorman, 2012). The need for compatible mating partners in heterothallic species necessitated the need for creation of diagnostic PCR tool that could identify each *MAT* type. This in turn allowed for directed crosses between sexually compatible strains, rather than unsuccessfully attempting to cross incompatible strains (Paoletti *et al*., 2005) and led in part to the success of revealing the sexual cycle in *A. fumigatus* (O'Gorman *et al*., 2009), similar work is demonstrated in this study in **Chapter 4.**

1.4 Development and morphogenesis

1.4.1 Sclerotia

Much of the research on sclerotia has focussed on those produced by necrotrophic plant pathogens such as the *Sclerotiniaceae*, but the loose definition of sclerotia applying to structures of variable morphology (Smith *et al*., 2015) can be applied to any number of structures of several fungal genera observed sporadically across the Ascomycota and Basidiomycotina (Willetts and Bullock, 1992) as well as being seen in the plasmodium of the non-fungal slime moulds such as *Fuligo septica* (Krzywda *et al*., 2008).

Sclerotia can generally be defined as being dormant resting structures consisting of hardened masses of melanised hyphae, and their main function is to allow survival of adverse environmental biotic and abiotic conditions (Chang *et al*., 2020; Samson *et al*., 2004) such as fungivory (Wicklow *et al*., 1996; Whyte *et al.,* 1996; Schardl *et al*., 2006; Gloer *et al.,* 2007), microbial degradation (Henis and Papavivas, 1983; Mehmood *et al.,* 2020), oxidation (Henson *et al.,* 1999) and UV damage (Liang *et al*., 2018). Sclerotia are even a means for the organism to endure freezing or surviving the absence of a host (Smith *et al*., 2015). After a period of dormancy sclerotia germinate, using reserves accrued during previous vegetative development (Willetts and Bullock 1992).

Sclerotia can play a major role in the life cycle of plant pathogens (such as the crop pathogens of the *Sclerotiniaceae*) as they provide the ability to survive for long periods under dormancy in adverse environmental field conditions (Bolton *et al.,* 2006). Sclerotia of the *Sclerotiniaceae* have been reported to survive in soil for up to eight years (Adams and Ayers, 1979), after a period of dormancy, the sclerotia germinate, completing another stage of the infective cycle.

Sclerotial germination can happen in any of three ways: myceliogenic, carpogenic or sporogenic (Coley-Smith and Cooke, 1971). In myceliogenic germination, the sclerotia produces fresh hyphal growth or myceliogenic plugs. Sporogenic germination is the direct production of asexual spores, and carpogenic germination is the production of fruiting bodies and subsequent production of sexual spores. Examples of carpogenic germination are seen with *S. sclerotiorum* (Bolton *et al*., 2006) where fruiting bodies (apothecia) emerge from the sclerotium as well as with *Aspergillus flavus* (sexual morph petromyces) where ascospores form within the sclerotia under the right conditions and with a suitable mating partner (Horn *et al*., 2009).

Due to the lower energy requirements of myceliogenic germination over carpogenic or sporogenic germination, sclerotia that undergo primarily myceliogenic germination tend to be smaller and are typically associated with root infecting fungi (Willetts and Bullock 1992). The nature of spore

production in sporogenic and carpogenic germination is historically associated with aerial dispersal of crop pathogens, as well as being more energy exhaustive resulting in the need for typically larger sclerotia (Willetts and Bullock, 1992).

Occurrence

Occurrence of sclerotia is broad and sporadic, but the functional and anatomical similarity is probably a result of convergent evolution (Willetts and Bullock, 1992; Willetts, 1997). Furthermore, sclerotia have been discovered that are not clearly linked with previously described or culturable fungus (Smith *et al.,* 2015).

Of those species that produce sclerotia that have been described, they are diverse in both morphology and the host range that they inhabit. One example, and perhaps the most famous, is that of *Claviceps purpurea* (Douhan *et al.,* 2008) commonly found as a pathogen on rye and responsible for crop loss due to the production of ergot alkaloids. Sclerotia production can also been found in the entomopathogenic fungi such as *Ophiocordyceps sinensis* (Xing and Guo, 2008) of which the sclerotia grows from within the tissue of the infected host caterpillars, amongst mycoparasitic basidiomycetes such as *Laetisaria arvalis* (Burdsall *et al.,* 1980) and as a final example, *Trechispora sp.,* which form a symbiotic relationship with termites, morphologically mimicking termite eggs (Matsuura and Yashiro, 2010).

Within the Ascomycota, there are several sclerotia-producing species, and these exhibit sclerotia with diverse sizes which may range from 220-1800 µm in diameter, with *A. costaricaensis* and *A. piperis* the species reported as regularly producing the largest size sclerotia (Abu El-Souod *et al*., 2017; Frisvad *et al.,* 2014).

Aspergillus flavus (TePaske *et al.,* 1992*;* Wicklow, 1987*)*, *A. parasiticus* (Bennett *et al.,* 1986; Wicklow and Donahue, 1984), *A. oryzae* (Geiser *et al*., 2000) as well as *A. nomius* upon its description (Kurtzmann, Horn and Hesseltine, 1987) are all species described to produce sclerotia amongst the *Aspergillus* section *Flavi*.The occurrence of sclerotia within section *Nigri* has been well documented by Samson *et al.* (2004). Species such as *A. costaricaensis*, *A. ellipticus*, *A. piperis* and *A. sclerotioniger* were described as reliably producing sclerotia, as well as species that were known to have produced sclerotia under some conditions albeit not reliably. These latter species included A. *tubingensis*, A. *carbonarius* and *A. aculeatus.* At this stage of discussion*, A. niger sensu stricto* had not been described as producing sclerotia, but this changed upon the addition of raisins to standard media (Frisvad *et al.,* 2014). Noonim *et al.* (2008) described a further species *A. sclerotiicarbonarius* that was found to reliably produce sclerotia and could be differentiated from other species in section *Nigri* from its

inability to grow on CREA agar at 37 ˚C. A species designated UFLA DCA 01 was later described as an additional section *Nigri* species, with similarities to *A. carbonarius* in that it produced abundant sclerotia and had reduced conidiation, but was described as distinct through differences in its calmodulin sequence and differences in conidial ornamentation under SEM (Silva *et al.,* 2011).

Sclerotia have been hypothesised to be either the morphological vestiges of cleistothecia, or to be required as the stromatal precursor to internalised cleistothecial production (Calvo, 2008; Calvo and Cary 2015), such as seen in aspergilli with petromyces sexual morph states. This latter hypothesis is supported by the observation that many of the genes and genetic regulatory pathways that control cleistothecia, also control sclerotial production (Dyer and O'Gorman 2011) as well as secondary metabolite production (Frisvad *et al*., 2014; Calvo and Cary 2015).

Amongst those species described above, some heterothallic *Aspergillus* species such as *A. flavus*, *A. parasiticus*, and *Aspergillus nomius* (petromyces sexual morphs) can produce sclerotia (stromata) which also play an essential role in sexual reproduction by containing the ascospore bearing fruiting bodies, termed cleistothecia, following fertilization by a sexually compatible strain (Horn *et al.,* 2016).

Origin and Similarity

Sclerotia can be informally divided according to the tissue types they contain. Although not a formal classification, the tissue types prosoplectenchyma, paraplectenchyma and pseudoparenchyma have been used to describe the different tissues. Namely the medulla, cortex, and rind, which may or may not be determinable in each given species, and there is likely little determination between the tissue types (Willetts, 1972).

Fungi that can produce sclerotia are both ecologically diverse and genetically disparate. One study of the literature showed sclerotia in 85 genera of 20 different orders of fungi, with the authors proposing that on this evidence, the ability to form sclerotia has evolved within fungi independently more than fourteen times (Smith *et al*., 2015) although the functionality of the sclerotium between these orders are very distinct, giving weight to the idea that the sclerotium is likely a description given to a wide variety of distinctly functioning biological structures. Sclerotia are therefore likely a result of convergent evolution and produce these structures as either resting structures (to survive adverse conditions), sexual structures or a both (Willetts, 1972; Smith *et al*., 2015).

Sclerotia of the common plant pathogen *Sclerotinia sclerotiorum* were found to contain a sclerotium specific protein (Ssp1). Ssp1 is likely a lectin involved in agglutination (Li and Rollins, 2010). Ssp1 proteins amassed in sclerotial tissues but were limited in non sclerotial tissues. During the germination of the sclerotium into apothecia, levels of Ssp1 reduced at the site of apothecial development and was

vastly limited or absent from the apothecia itself (Li and Rollins, 2009). Deletion of Ssp1 resulted in a 50% reduction in sclerotia production (Li and Rollins, 2009). Orthologs of this protein were found in the sclerotia producing *Aspergillus* species *A. flavus* and *A. oryzae* (Li and Rollins, 2009). The presence of an orthologous gene involved in sclerotia produced amongst these diverse fungi, suggests a genetic linkage. Comparative genomics revealed 245 orthologous genes shared between sclerotium producing fungi *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *A. oryzae* and *A. flavus*. These genes were absent in non-sclerotia producing *A. nidulans* and *A. fumigatus* (Amselem *et al*., 2011). Amongst these genes was *ssp1*. These genes may be sclerotium-production specific and may provide insight into the evolution of sclerotia (Amselem *et al*., 2011).

Metabolite Production

It has been hypothesised that the production of unique secondary metabolites might occur concurrent with the production of sclerotia. Indeed, many of the secondary metabolites linked with production only of sclerotia have anti-insectan properties (Gloer, 1995). The morphogenetic production of sclerotia under suitable conditions may allow for the awakening of otherwise silent gene clusters and the study of gene expression allowing for such novel metabolite production may be of commercial interest (Frisvad *et al*., 2014; Calvo and Cary 2015).

The sclerotia of *A. niger* that were formed on a variant of Czapek's Yeast Autolysate Agar supplemented with Raisins (CYAR) produced indoloterpenes of the 10,23-dihydro-24,25-dehydroaflavinine type and the polyketide mycotoxin Ochratoxin A, which is one of the most abundant mycotoxin contaminants of food. These sclerotial metabolites that were detected were markedly absent in cultures that produced no sclerotia (Frisvad *et al*., 2014). These findings provide evidence that sclerotial formation is linked to expression of otherwise 'silent gene clusters' and that particular cell types within sclerotia are expressing these distinct metabolites under inducing conditions.

It has been hypothesised that the ergot alkaloids produced by *Claviceps purpurea* on grasses evolved as a means of preventing herbivory (Gloer, 1995). Additionally, further evidence of the co-evolutionary nature of these interactions is provided through the detoxification ability of certain fungivore species (Niu *et al*., 2008; Kempken and Rohlfs, 2010). Whilst not an example of sclerotia, perhaps the most amusing example of detoxification is that of reindeer seeking out and consuming the common *Amanita muscaria* mushrooms which produce muscimol, ibotenic acid and amatoxins (Michelot *et al*., 2003). Consumption leads to intoxication like that of alcohol intoxication in humans, but never death (Lee *et al.,* 2018). More amusing still is the hypothesised link between these intoxicated mushroom eating reindeer and the idea of reindeer flying as being a core element of ethnomycological western cultural ideas of Christmas (Carboue and Lopez, 2021). Magic indeed!

Morphology of Sclerotia

The study and reporting of morphology and structure of sclerotia have mostly been completed on the species of the *Sclerotiniacae*, including the widely described and studied *Sclerotinia sclerotiorum*. Similarities between these widely described sclerotia and the lesser described sclerotia of other ascomycetes have been reviewed (Willets and Bullock, 1992) and more recently described in the aspergilli, including *A. niger* (Ellena *et al*., 2021a). There will be further discussion of sclerotial morphology in both Section 1.6.3 and Section 3.1.1.

1.5 Sex Related Genes in Fungi

1.5.1 Use of Sex Related Genes to Uncover Covert Sexuality

Evidence for 'covert' or 'cryptic' sexuality can be uncovered before a complete sexual cycle has been described (Dyer and O'Gorman 2011, Dyer and Kuck 2018). In the case of *A. fumigatus*, which was previously thought to be asexual (or *fungi imperfecti*), early evidence for sexuality came from the identification of certain sex-related genes (Pöggeler 2002; Dyer *et al*., 2003), and then the sequencing of the genome of strain *Af293*, which revealed the presence of all known genes necessary for sexual reproduction (Galagan *et al*., 2005). If this species was truly asexual, then it might have been expected that deleterious gene mutations would have accumulated in these genes, which would then become pseudogenes with loss of function. An example of this is that 70% of olfactory receptor genes in humans have gained deleterious mutations and become pseudogenes since the rise of hominids (Schurko and Logsdon Jr, 2008). In comparison, all identified 'sex genes' in *A. fumigatus* appeared to have been conserved in a functional form, and this would imply the presence of an undescribed 'covert' sexual cycle (Galagan *et al*., 2005).

A worldwide selection of *A. fumigatus* strains was screened for *MAT* type, revealing that there was an 43.3%: 56.7% ratio of *MAT1-1* to *MAT1-2* types which is approximately even, and the slightly higher level of *MAT1-2* could be explained due to a small but skewed subset of isolates from France (Paoletti *et al*., 2005). This even distribution of *MAT* types was consistent with sexual reproduction, as for any locus with two segregating alleles, and has been described elsewhere as evidence of meiotic recombination (O'Gorman *et al*., 2009; Horn *et al*., 2013; Olarte *et al*., 2015). Paoletti *et al*., (2005) then used a semi-quantitative RT-PCR approach to investigate the expression of genes linked to sex in *A. fumigatus*, such as the *MAT* genes, and pheromone precursors and receptors, and discovered that, these were all expressed during vegetative growth. From an evolutionary point of view, it was argued that 'the expression of genes involved with sexuality in a truly asexual species is metabolically wasteful' (Dyer and Paoletti, 2005) and again supported the idea that this was evidence of a 'hidden' sexual cycle. Indeed, sexuality is often one of the first traits to be lost when fungal isolates are subjected to mutagenic treatment, given that sex is reliant on so many genes working in concert (Dyer and Paoletti, 2005). O'Gorman *et al*. (2009) then demonstrated the presence of a sexual cycle and recombination in *A. fumigatus*, by crossing known *MAT1-1* and *MAT1-2* isolates under suitable *in vitro* conditions, which was shown to result in the formation of cleistothecia and ascospores characteristic of sex in the aspergilli. Recombination was confirmed using RAPD-PCR with 5 genetic markers and *MAT* genes, which identified novel genotypes of progeny. It is therefore apparent that similar

collections of evidence or 'sex tests' of sexuality can hint at a sexual cycle before it has been elucidated in other supposedly asexual species (Dyer and O'Gorman, 2011).

1.5.2 The Velvet Complex

The *velvet* family of proteins; VeA, VelB, VelC and VosA all contain the velvet domain and are highly conserved in dimorphic and filamentous fungi (Bayram and Braus, 2011; Park *et al*., 2012) as well as between Ascomycota and Basidiomycotina (Ni and Yu, 2007). However, they are absent in both the yeasts *S. cerevisiae* and *C. albicans* (Bayram and Braus 2011). The *velvet* domain consists of a region of around 150 amino acids and forms a novel DNA-binding motif structurally like the Rel homology domain (RHD) of the mammalian transcription factor NF-kB (Park *et al*., 2014). Conserved regions including proline residues are found in the middle of the domain and it is tempting to speculate that these may be responsible for protein-protein interactions that form complexes (Bayram and Braus 2011). **Figure 1.3** demonstrates the interactions between the different proteins which will now be described.

The name *velvet* is derived from the founding member of these proteins: *veA*. This was due to the appearance of velvet-like conidiation mutants in early genetic studies of *A. nidulans* (Käfer 1965). The mutation that was detected was later determined to be an ATG to ATT point mutation in what would have been the start codon of *veA*. This resulted in an N-terminal truncated protein as the transcription would begin from the second ATG codon, this mutant was termed *veA1*. Functional studies of wild type *veA* determined that its cellular localisation was dependent on light; in light it would localise in the cytoplasm, in darkness it would localise in the nucleus, and that this would interact on the KapA importin protein and then an interaction with the red phytochrome FphA (Bayram *et al*., 2008a; Purschwitz *et al*., 2008; Stinnett *et al*., 2007). Localisation of the VeA1 truncated proteins was unaffected by light/dark and resulted in greater cytoplasmic accumulation than the wild type. Furthermore, *A. nidulans* Δ*veA* mutants hyperconidiated and did not form sexual fruiting structures under any conditions known to stimulate sex (Kim *et al*., 2002), nor produce any transcripts of proteins necessary for penicillin or sterigmatocystin production (Kato *et al*., 2003). Likewise, Δ*veA* mutants in *A. parasiticus* did not produce sclerotia or cleistothecia and lost functional aflatoxin synthesis (Calvo *et al*., 2004). In both cases, *veA* was linked with both sexual development and secondary metabolism. It can be inferred that Δ*veA* corresponds to a permanent light state in the wildtype – resulting in perpetual asexual reproduction. In more recent studies of *A. fumigatus* transcriptomics, *veA* was linked to the expression of hundreds of genes (Dhingra *et al*., 2012).

In other fungal genera, knockout mutants of homologs of *veA* showed similar phenotypes to those described above for the aspergilli. In *Fusarium verticilloides ΔFeVE1* resulted in a greater ratio of macroconidia to microconidia as well as the inability to produce the mycotoxins fuminosin and fusarins (Myung *et al*., 2009). *Penicillium chrysogenum Pcvel* was shown to be involved in both conidiation and penicillin biosynthesis (Hoff *et al*., 2010). The homologous nature of these genes was demonstrated as they could complement deletion mutants in other species by heterologous expression, for example a *A. nidulans* Δ*veA* strain could be complemented with the *N. crassa ve-1* gene to restore functionality (Bayram *et al*., 2008b).

The methyltransferase regulatory protein LaeA was first described by Bok and Keller (2004). It has been described as both a member of the velvet family of proteins due to its intimate involvement in the velvet complex, or simply as an associated regulatory protein. It will be described here as the latter due to the absence of a velvet domain, as described in Bayram and Braus (2011).

As a regulator of secondary metabolism, Δ*laeA* mutants of *A. nidulans* lack secondary metabolite synthesis, such as sterigmatocystin, lovastatin and penicillin. Bok *et al*. (2006) showed that *in vivo A. fumigatus* Δl*aeA* mutants had reduced gliotoxin production as well as reduced infection rates in a murine model. Conidial ornamentation was also affected, with less extrusions caused by reductions in transcript levels of genes responsible for rodlet formation, and this could perhaps account for other physical characteristics such as enhanced internalisation by macrophages and substrate adherence compared to wildtype. The reduced production of metabolites such as gliotoxin could account for the reduced ability of Δ*laeA* mutants to kill neutrophils, an important stage in the development of pathogenicity and cause of invasive aspergillosis. LaeA also has an important role in sexual development where it has a light-dependent support role in asexual development, antagonistic to *veA*; it can be inferred that Δ*laeA* corresponds to permanent dark in the wild type – perpetual sexual reproduction (Bayram and Braus, 2012).

The VeA/VelB/LaeA heterotrimeric *velvet* complex forms was first described in 2008 (Bayram *et al*., 2008a) and is one of many complexes formed between this family of proteins, as illustrated in **Figure 1.3**. It forms in the dark where the light controlled VeA is imported into the nucleus with VelB and both can be detected in increased accumulation alongside the constitutively nuclear LaeA. VeA acts as both a bridge protein for LaeA and VelB and is necessary for VelB import into the nucleus, whilst LaeA acts as an epigenetic factor controlling transcript levels. Whilst it was originally thought that the complex controlled both sexual development and secondary metabolism (Bayram *et al*., 2008a), later studies showed that in a Δ*laeA* background, fertile cleistothecia still developed, though they were significantly smaller (20-40µm compared to the wildtype size of 200µm), and there were no apparent Hülle cells. It therefore follows that VelB/VeA was responsible for sexual development through

cleistothecia formation and LaeA for the formation of the Hülle cells which act to nourish the developing sexual structures (Bayram *et al*., 2010). One explanation of this nourishment is that Hülle cells produce α-1,3 glucanase that mobilise carbon sources for formation of the fruiting body (Wei *et al*., 2001), though it has also been suggested that the oxidative reactions in Hülle cells lead to the formation of multiple reactive oxygen species (ROS) and that these are necessary for cleistothecial development. Hülle cells also exhibit increased levels of enzymes involved in coping with ROS such as catalases and peroxidases (Bayram and Braus, 2011) intimating at their role in cellular redox reactions.

Another member of the family of proteins - VosA (Viability Of Spores A), forms a light-controlled complex with VelB that acts as both a repressor of asexual reproduction and controls spore maturation and trehalose biogenesis and accumulation which promote viability (Bayram *et al*., 2010). Light has been shown to reduce protein levels of both VosA and VelB and this correlates with the increased asexual reproduction and conidiation.

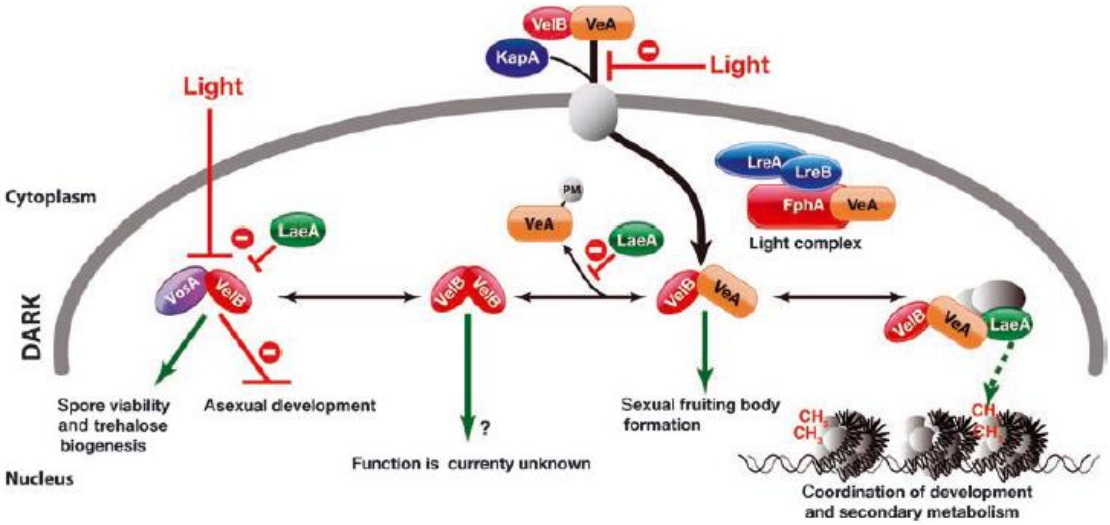

Figure 1.3. Interactions of velvet complexes from *A. nidulans* on cellular processes involved with sexual and asexual reproduction as well as secondary metabolism. Green lines represent activation of a process. Red lines with a flat head represent repression of the process. Black lines represent dynamic movement of proteins between cellular compartments and complexes. PM – Post-translational modifications. Taken from Bayram and Braus (2011).

The final member of the family: VelC, has been much less studied. It was characterised by Park *et al*. (2014), with increased mRNA levels of *velC* observed in the early part of sexual development. ΔvelC mutants showed increased production of conidia and reduced numbers of sexual structures. *In vitro* and yeast model studies showed an interaction between VelC and VosA.

Finally, an interaction between VeA, VelB, LaeA and FluG was shown to control sclerotial development in *A. flavus* (Calvo and Cary 2015). The gene *fluG* contributes to the inactivation of a G-protein signalling pathway, and mutants of the *fluG* gene lacked both sexual and asexual structures and did not express sterigmatocystin (Wieser *et al*., 1994). When compared to studies of *fluG* in *A. flavus* (Chang *et al*., 2012) it was observed that whilst conidiation was decreased, sclerotial production was upregulated, and there was no effect on aflatoxin production. This hints at less conservation of *lox*/*fluG* function between the aspergilli than for the function of the *velvet* proteins, and that the *velvet* complexes are upstream of either *lox* or *fluG* function as they globally affect both secondary metabolism and sexual morphogenesis.

That there are common conserved genes involved in regulation based on external signals should not be surprising, as these would have been selected for early on in fungal evolution. Indeed, in these soil dwelling organisms it will be advantageous for them to sense whether they are in the soil or at ground level as this can be a signal for them to produce environmentally resilient conidia (Dyer and O'Gorman 2012) and disperse these conidia from the surface of a substrate. In *A. nidulans* it is recognised that there was a period of 'developmental competence' in which spores required at least 20 hours of growth before they responded to external stimuli (Bayram *et al*., 2010) or rather that they had a 'light sensitive' period after 20 hours (up to 50h) which would delay the onset of the sexual development pathway (Yager, 1992) or prevent the formation of cleistothecia altogether, as previously discussed.

Whilst studies in this area have investigated *A. nidulans* as a model organism (Kim *et al*., 2002; Kato *et al*., 2003; Park *et al*., 2012) and other aspergilli posing a health risk such as *A. parasiticus*, *A. flavus* and *A. fumigatus* (Calvo *et al*., 2004; Chang *et al*., 2013; Dhingra *et al*., 2012), fewer studies have investigated the effects of Δ*veA* mutants in biotechnologically important *Aspergillus* species. In *A. niger* (Wang *et al*., 2015), a single study has shown that a Δ*veA* mutant exhibited a reduction in the proportions of conidiophores and numbers of conidia.

1.5.3 The STRIPAK Complex

The STRIPAK (Striatin-Interacting Phosphatase And Kinase) complex is a multi-protein assembly that functions as a molecular scaffold for a variety of proteins and external interacting proteins. It is highly conserved amongst eukaryotes, with some authors proposing a link between its control of secondary metabolism in fungi to the control of immunity in higher eukaryotes (Elramli *et al.,* 2019). It physically interacts with a multitude of protein signalling pathways to set up a dynamic protein signalling network (Hwang and Pallas, 2014; Elramli *et al*., 2019). The major component – Striatin, was first discovered in rat brain and found to function as part of the CNS (Kück, Radchenko, Teichert, 2019). The major subunits of the human STRIPAK complex allowed for subsequent characterisation of the homologs in other species. In humans, the subunits are Striatin, PP2AA, PP2Ac, STRIP, Mob, SLMAP, GCKIII, a small-coiled coil protein and finally CCM3 (Kück, Radchenok and Teichert, 2019).

Various fungi have been researched intensively and have had homologs of the STRIPAK complex identified (at least in part). Study species include *Sordaria macrospora*, *Saccharomyces cerevisiae*, *A. nidulans* and *N. crassa* in which STRIPAK components have been named and functionally characterised. The STRIPAK components in these fungi have been shown to have a range of functions, but notably impact on different stages of sexual and fruit body formation. We will know look at the individual component homologs and their functionality in *A. nidulans*. However, as of early 2022 there have been no studies looking into the role of STRIPAK or Striatin in *A. niger* specifically, other than to mention the presence of a striatin homolog (Tanti *et al*., 2014).

Within the context of *A. nidulans*, a mechanistic model was suggested that STRIPAK phosphorylates MAK-2/ERK and shuttles it into the nucleus. MAK-2 is a kinase of the yeast pheromone response pathway (Elramli *et al.,* 2019). Furthermore, the complex was identified as being made of three subcomplexes and placed on the nuclear envelope. At the nuclear envelope it shuttles messages, including interactions with the velvet complex to coordinates fungal metabolism, stress response and secondary metabolism (Kück and Stein, 2021). In that study, the heptameric complex of protein Striatin (StrA) and six interacting proteins (Sips A-F) was characterised. SipA was the Mob 3 type kinase activator (homolog of Mob in humans) and attaches singly to the scaffold striatin. SipB and SipD form a heterodimer as a second subcomplex. The remaining Sip's (SipC, SipE and SipF) form a heterotrimer.

In *A nidulans*, the loss of StrA led to reduced hyphal growth, conidiation and reduction in the production of ascospores (Wang *et al.,* 2010). The scaffold striatin is also necessary for correct nuclear envelope localisation (Elramli *et al.,* 2019). Loss of SipB, SipC, SipD and SipE all shared phenotypes consistent with the loss of StrA, namely reduced conidiation, growth and the absence of fruit bodies. Δ*sipF* was seemingly critical for viability as no mutants were collected (Elramli *et al*., 2019), this is a

finding shared by that in *N. crassa* (Dettmann *et al.*, 2013). Δ*sipA* deletion mutants are opposite in phenotype to the other complex proteins, with increased conidiation, radial growth and fruit body production, although ascospores were absent from these fruiting bodies (Elramli *et al*., 2019). Taken together, these data show that the complex is necessary for correct light response in the pathways that lead to asexual and sexual development. The genes *abaA* and *brlA* (**Section 1.2.2)** were induced by STRIPAK and are necessary for correct asexual development (Elramli *et al*., 2019).

Of interest, it is noted that in the heterothallic, anisogamic species *N. crassa*, deletion of the *strA* homolog affected the female fertility element, but the male fertility element was unaffected (Kück and Stein, 2021).

1.5.4 Genes Involved with Environmental Sensing

As mentioned in **Section 1.3.4**, the sexual cycle of *A. nidulans* is induced only under a restricted set of conditions. These conditions are in stark contrast to those required for sex in *S. cerevisiae*. In the case of *A. nidulans* sexual development is favoured by well-nourished conditions, while stressors including carbon or nitrogen starvation, high osmolarity or light block fruiting body formation (Dos Reis *et al*., 2019; Kim *et al*., 2009). At high carbon or nitrogen concentrations, sexual development is again blocked, but this is remedied by balancing the C:N ratio (Han *et al*., 2003). Many of the genes involved in the complex processes of environmental sensing and its link to sexual morphogenesis are shown in **Figure 1.4**.

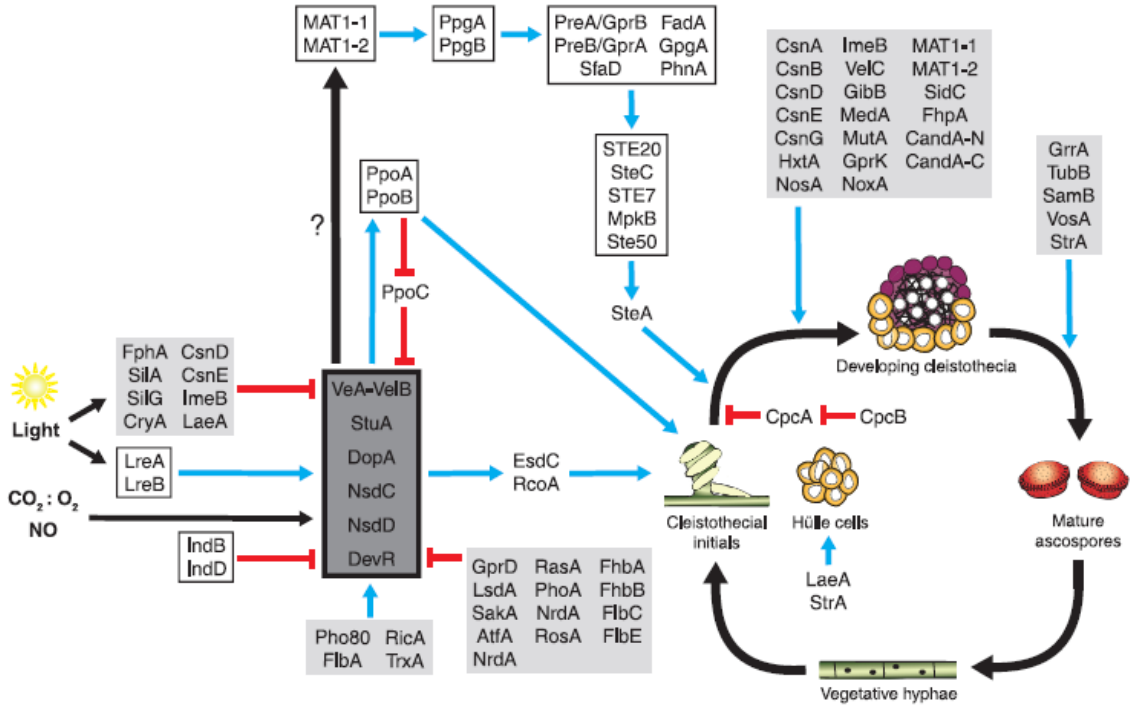

Figure 1.4. A model showing the interactions and relatedness of genes involved in sexual reproduction in the aspergilli. This is principally from research of the model organism *A. nidulans*. Blue lines represent activation of the subsequent gene(s). Red lines with a flat head represent repression of the subsequent gene(s). Taken from Dyer and O'Gorman 2012.

Light

One of the key conditions regulating sexual development is the absence or highly reduced level of light (Han *et al*., 2003), which was also discussed in **Section 1.5.2**. As well as having an impact on the sexual cycle of *A. nidulans*, the absence of light has been documented in the sexual cycles of other described *Aspergillus* species (Horn *et al*., 2009; O'Gorman *et al*., 2009; Olarte *et al*., 2015). Several genes have been shown to have roles in light responsiveness as shown in **Figure 1.4.** A number of these will now be briefly discussed in turn before discussing genes involved in other means of environmental sensing.

A red-light sensing phytochrome called FphA acts to promote conidiation and repress sexual development under red light conditions in *A. nidulans* (Blumenstein *et al.,* 2005). A *fphA* null mutant would be less responsive to light in that it would produce cleistothecia under conditions in which it normally would not (namely red light). However, it was determined that the *fphA* mutant produced only around 10% of the cleistothecia under the conditions of red light than it did when incubated in the dark. This suggests that the *fphA* knockout was not entirely red-light blind and that other red-light sensors were present in *A. nidulans* (Blumenstein *et al*., 2005). The same researchers also used GFP tagging to determine the migration of a fusion protein under conditions of red light. The fusion protein did not migrate to the nucleus, although *fphA* was later shown to activate the HOG MAP kinase pathway to influence nuclear transcriptional response (Yu *et al*., 2021). Furthermore, FphA has been proposed to interact with a multi domain transcription factor termed RlcA in red light (Yu *et al*., 2020).

Two homologues of the white- collar blue-light sensing system in *N. crassa* (WC1 and WC2) were found in *A. nidulans* (Purschwitz *et al*., 2008). Whilst both red and blue light promoted conidiation, both red and blue light were simultaneously necessary to provide levels consistent with those produced under white light. It was determined that there are several interactions between *fphA*, *veA*, and the two blue light sensors. The interactions are those of a large protein complex, responding to both red and blue light (Purschwitz *et al*., 2008). The interaction between *fphA* and *lreA* was shown via the epigenetic acetylation of a third light induced gene *ccgA*. The gene *fphA* is responsible for acetylation in the light via a further acetyl transferase gene *gcnE*, with *lreA* responsible for deacetylation of *ccgA* in darkness via a histone deacetylase *hdaA* (Hedtke *et al*., 2015).

A blue-light sensing cryptochrome termed CryA is a repressor of sexual differentiation in *A. nidulans*. A Δ*cryA* mutant was found to produce Hülle cells under conditions normally prohibitive of sexual differentiation such as in liquid culture (Bayram *et al*., 2008c). It has been predicted to be an upstream negative regulator of sexual development upon integration of the environmental signals of blue and UV light (Bayram *et al*., 2008c).

Six mutants of *A. nidulans* have been generated that are able to produce cleistothecia under light conditions and were termed *silA-silF*, with a further discovered and termed *silG* (Min *et al.,* 2007; Kim *et al*., 2005). Further analysis of *silA* revealed it to be a Zn_2Cys_6 transcription factor. The gene *silA* acts as a repressor of sexual development under conditions of light exposure (Han *et al*., 2008).

A mitogen activated protein kinase termed *imeB* (related to *ime2* in *S. cerevisiae*) was shown to be a repressor of sexual development. *imeB* mutants overproduced cleistothecia on agar under light and additionally produced Hülle cells in liquid culture. A double deletion of both *imeB* and *fphA* resulted in a total loss of light response (Bayram *et al*., 2009).

Osmolarity

In addition to the impact of light on the determination of asexuality over sexuality, osmolarity is an additional key feature. High osmolarity caused by the introduction of salts favours asexual over sexual development (Han *et al*., 2003). A key gene involved in the sensing of high salt conditions and relaying this to inhibit sexual development is *lsdA* (Lee *et al*., 2001). Disruption of functional *lsdA* preferentially causes sex even under salt conditions that normally inhibit sexual development whilst having little affect under normal salt conditions. This implies/suggests that *lsdA* only inhibits sex under the environmental signal of high salt (Lee *et al*., 2001).

Nutrient Levels

G-protein coupled receptors (GCPR's) communicate changes in the extracellular environment to intracellular G-proteins. The signalling then coordinates a transcriptional response to the changes in environment (Dos Reis *et al*., 2019). Several GPCR's have been demonstrated to be involved in the carbon sensing of the environment and negatively regulate sexual development. The gene *gprH* was transcriptionally increased under carbon/glucose starvation and therefore demonstrated nutrient sensing and response. In response to carbon starvation, GprH represses sexual development (Dos Reis *et al*., 2019). Δ*gprH* mutants produced few conidia in the light and more conidia in the dark when compared to wild type. Additionally, under both conditions of light and dark, these mutants produced greater numbers of cleistothecia (Dos Reis *et al.,* 2019). Finally, Δ*gprH* caused accumulation of VeA in the nucleus under carbon starvation in the response to light, thereby mimicking the wildtype effect of dark exposure and demonstrating the interaction between these carbon and light responsive proteins (Bayram *et al*., 2008a; Dos Reis *et al*., 2019).

A key nutrient in growth and development is nitrogen. Involved in nitrogen response is one of the 'fluffy' genes previously discussed in relation to conidia production, namely *flbD*. The gene *flbD* is rather unique in that it is required for both sexual and asexual development. In sexual development *flbD* is essential for peridium development. Under conditions of nitrogen starvation, a *flbD* mutant ($flbD^{C46A}$) did not induce conidiation under nitrogen starvation conditions usual of the 'fluffy' genes, demonstrating a role in nitrogen sensing (Arratia-Quijada *et al*., 2012).

Another gene involved in nutrient sensing and sexual development is *phoA*. PhoA is a cyclin-dependent kinase which functions in cellular response to phosphorus limitation. A *phoA* mutant was shown to consistently undergo sexual reproduction even under (normally) phosphorus limiting conditions. The gene *phoA* is hypothesised to therefore regulate sexual development under phosphorus limited growth (Bussink and Osmani, 1998).

A study considered the unintended consequences of creating riboflavin auxotrophic mutants. The marker *riboB2* is a commonly used to produce mutants. The *riboB2* mutants are typically self-sterile but exogenously added riboflavin can restore the sexual phenotype (Zheng *et al*., 2015). Additionally, a further study identified that exogenous uracil added to *pyrG* mutants produced greater numbers of both cleistothecia and Hülle cells when compared to wild type (Yun *et al.,* 2013). These findings have important ramifications for the impact of using these auxotrophic markers for the analyses of genetic based sex differences.

Finally, the COP9 signalosome is a key developmental regulator in eukaryotes. Members of the COP9 signalosome include *csnD* and *csnE*. In *A. nidulans*, these genes control the development of sexual primordia into functional sexual fruiting bodies (Busch *et al*., 2003). It was found that *csnD* deletion strains were blind to light regulation, with interaction between the CsnD and VeA proteins predicted (Busch *et al*., 2003).

A key to successful sex in fungi described is the use of sexual morphogens and pheromones. These are described in more detail in **Section 3.1.4**. These pheromones can initiate growth towards a sexual partner, as well as induce MAPK pathways leading to transcriptional response to the partner and induction of sex. Even amongst homothallic species such as *A. nidulans* during 'selfing', the pheromones and receptors and associated MAPK pathways are all up regulated. This suggests that the pathways for attracting partners of the opposite mating type are integral, even amongst fungi that may require no such partner attraction due to their homothallic lifestyle (Paoletti *et al*., 2007).

1.6 The Genus Aspergillus

1.6.1 Introduction

The genus *Aspergillus* has been commonly estimated to have around 250-260 species (Dyer and O'Gorman, 2012; Geiser *et al*., 2007; Samson and Varga, 2009), with higher estimates of 837 (Hawksworth *et al*., 2011), and recent taxonomic studies stating 446 (Houbraken *et al.,* 2020). The genus contains several notable species with importance in agriculture, medicine, industry and pharmaceuticals (Taniwaki, Pitt and Magan, 2018; Kwon-Chung and Sugui, 2009; Huang *et al*., 2021). It has been stated that *Aspergillus* diverged from its closest ancestor *Penicillium* 81.7 mya (Steenwyk *et al*., 2019). Of primary interest is the range of secondary metabolites and enzymes that the genus can produce, mirrored by the range of habitats that the genus inhabits (Bennett, 2007). Through modulation of their chemical diversity, they can develop niches in many disparate environments, including human hosts (Abdel-Azzem *et al*., 2016; De Vries *et al*., 2017; Lass-Flörl *et al*., 2021).

The most common way of describing the habitats of *Aspergillus* species is 'ubiquitous' as they are found almost everywhere. These species are one of the dominating groups in terms of biodiversity in soil, compost and decaying vegetation, as well as stored grains and foodstuffs (Klich, 2002; Wilson *et al*., 2002). Furthermore, they can be opportunistic pathogens to a broad range of animal hosts, such as humans to the sea fan corals that can be host to *A. sydowii* (Alker, Smith and Kim, 2001).

Often described as opportunistic pathogens, species of the genus do not depend on a particular host or hosts for survival (Bennett, 2009). In this sense, members of the genus *Aspergillus* are recognised as free living saprobes and have not evolved to take advantage of their hosts immune systems.

Only a small number of Aspergillus species are directly linked to human disease and of these, members of the *Aspergillus fumigatus* section are the most common, followed by members of the *A. flavus*, *A. niger* and *A. terreus* sections respectively (Lass-Flörl., 2018). *A. fumigatus* infection in humans is predominantly of the lung, *A. flavus* of the larger airways and sinuses, and *A. niger* primarily infects wounds and burns (Thompson *et al*., 2021) and in some cases ears – where infection is termed otomycosis (Schuster *et al*., 2002). Members of the *A. terrei* section have emerged as the causal organism in an increasing number of cases of invasive aspergillosis (IA), and bring a unique challenge to treatment, due to increased amphotericin B resistance and mycotoxin production (Lass-Flörl *et al*., 2021; Malani and Kauffman., 2007). As well as the major contributors to disease are several less common pathogenic species including, but not limited to, *A. clavatus*, *A. glaucus*, *A. nidulans*, *A. oryzae*, *A. ustus*, *A. versicolor* and *A. ochracheus* (Gugnani, 2003).

Aspergilli can cause a range of different diseases including invasive aspergillosis (IA) and chronic pulmonary aspergillosis (CPA) including aspergilloma, hyper-sensitivity pneumonitis and allergic bronchopulmonary aspergillosis (ABPA) (Hammond *et al*., 2020; Gugnani, 2003). The number of cases of aspergillosis increases yearly, in large part due to improvements in cancer therapy which increases the length of the aspergillosis risk window (Thompson *et al.,* 2021).

Conidia of pathogenic species are numerous and easily become airborne due to their small size and hydrophobicity. The conidia are also highly stress tolerant, and these factors make them ideal for pathogenicity (Lass-Flörl *et al*., 2021). Additionally, the species causing disease can colonise and damage the host, and furthermore to adapt well to nutritional and biophysical challenges (Lass-Flörl *et al*., 2021). Insights from genomics have failed to make obvious any single such virulence factor responsible for *A. fumigatus* human pathogenicity, and therefore, no single target for drug companies to exploit (Bennett, 2009).

Economic damage can be attributed to several aspergillus species that are crop pathogens and are therefore agricultural threats. *A. flavus* and *A. parasiticus* are members of section *Flavi* and inhabit a broad range of crop hosts*. A. flavus* is a major producer of aflatoxins on its host range of maize, peanuts, corn, cottonseed and spices (Horn *et al*., 2009; Amaike and Keller, 2011) and on stored grains (Amaike and Keller, 2011). Aflatoxin B1 is the principal mycotoxin and is the most toxic of the naturally occurring aflatoxins (Horn *et al*., 2009; Tola and Kebede, 2016). First described in 1809, *A. flavus* then became widely known in 1962 because of Turkey X disease where thousands of poultry were killed as result of feeding on aflatoxin produced by contaminated poultry feed (Amare and Keller, 2014; Horn *et al*., 2009).

Importantly, it's not all bad! Fungi have certainly had a positive impact on society – with impacts in medicine, food, and culture. Several species of the aspergilli have been used for thousands of years in food production. *A. oryzae*, *A. sojae* and *A. tamari* have roles in food fermentation processes, with *A. oryzae* used in the production of soy sauce, miso and sake (Gugnani, 2003). *A. niger* is widely used to produce a range of enzymes including cellulases, proteases, lipases, xylanases and amylases (Andersen *et al*., 2011). Additionally, *A. niger* is widely used to produce citric acid (this is discussed in more detail in **Section 1.6**). An additional factor that makes *A. niger* and *A. oryzae* particularly useful for these purposes is the species efficient secretion systems (Liu et al., 2014; Li *et al*., 2020). These enzymes are widely applicable for use as detergents and cleaning agents (Devi *et al*., 2008).

1.6.2 Asexual Morphology

The genus Aspergillus was named after the apparent asexual structure – the conidiophore. First discovered by Antonio Micheli in 1727, the conidiophore was deemed to appear as a catholic aspergillum (Bennett, 2007). The conidiophores emerge erect from foot cells into rounded or club shaped vesicles. The vesicles then develop phialides and from there chains of conidia (Adams *et al*., 1998). The size, arrangement and colour of the conidia are important identifying characteristics (Bennett, 2007, Peterson, 2008). The arrangement is determined by the phialides and can be either uniseriate or biseriate, with uniseriate species lacking metulae (Teertstra *et al*, 2017). The colour of colonies is conidia dependent and can be used to classify species, with *A. niger* group being black, *A. ochraceus* is yellow to brown, *A. fumigatus* and *A. nidulans* are green and *A. flavus* (with its name derived from the Latin for yellow - Flavo) is yellow to green (Bennett, 2007). The genus can be subdivided into six subgenera named: *Circumdati*, *Nidulantes*, *Fumigati*, *Polypaecilum*, *Cremei* and *Aspergillus* (Peterson, 2008; Frisvad and Larsen, 2015; Lass-Flörl, 2021).

Aspergillus terreus (of *Circumdati*) is unique amongst the aspergilli in that the species produces aleuroconidia, or accessory conidia. Single conidia are formed at the blown-out end of conidiogenous cells or at hyphal branches (Posch *et al*., 2018). The presence of these aleuroconidia can be used as a morphological method of distinguishing members of the *A. terreus* section from other *aspergillus* species (Lass-Flörl, 2018).

1.6.3 Sexual Morphology

sexuality amongst the aspergilli is universal in nature, however sexuality has so far been corroborated in only around 31-36% of species (Ojeda-Lopez *et al.,* 2018). The most widely studied species of the aspergilli is the homothallic *Aspergillus nidulans*. A sexual cycle including the production of cleistothecia and the presence of Hülle cells gives *A. nidulans* the sexual morph emericella (Dyer and O'Gorman, 2011). Indeed, *Aspergillus* is a good example of where the previous system of dual nomenclature, with naming of both the asexual anamorph and sexual teleomorph, have been applied (Pitt and Samson, 2007).

However, there has recently been a proposal termed the 'one fungus = one name' protocol whereby a single organism with pleiomorphic states would have just one Latin name recognised. The Amsterdam declaration proposed the change in a bid to clarify continuing difficulties with the use of two Latin binominal names, but their use is still common due to the benefits of describing distinct differences in life cycles and elucidating taxonomy (Hawksworth *et al*., 2011).

The teleomorph name given to any newly elucidated sexual species of *Aspergillus* has based on the morphological characteristics of its cleistothecia. As of 2021, there were nine teleomorphic genera described with an *Aspergillus* anamorph (Visagie and Houbraken, 2021).

Dyer and O'Gorman (2012) described the colour and composition of cleistothecia for each of the four most common sexual morphs (teleomorphic genera): eurotium*,* emericella*,* neosartorya and petromyces. Eurotium species have 'a single layer of frequently yellow, large, flattened cells that form the cleistothecial wall'. This is a useful diagnostic for naming and has been widely utilised (O'Gorman *et al*., 2009; Horn *et al*., 2011; Horn *et al*., 2013). Each new teleomorphic genus is presented as it is discovered, such as the genus petromyces, which was first used to describe the sexual stage of *Aspergillus alliaceus* (Malloch and Cain, 1972) and has gone on to describe the sexual state of several species of section *Flavi* and *Nigri* (Horn *et al*., 2009; Horn *et al*., 2009; Olarte *et al*., 2015; Darbyshir, 2014). Petromyces was defined by the presence of a hard outer wall (sclerotium) in which the ascomata are housed. Indeed, there is a strong relationship between the teleomorphic genera and the different sections of the *Aspergillus* genus. **Figure 1.5** demonstrates a relationship within the sections of genus *Aspergillus* and the sexual morph states of sexual members of these sections. Of note is the recent discoveries of sex in section *Nigri* revealing the sexual morph of petromyces and thus further demonstrating the phylogenetic relationship with the sister clade of section *Flavi* (Peterson *et al*., 2008).

Although **Figure 1.5** shows a relationship between the apparent and emerging sexual morphs, a more recent analysis of the sections shows that section *nigri* has a sister relationship to sections *nidulantes*, *versicolores*, *usti* and *ochraceorosei* rather than *circumdati* (Steenwyk *et al*., 2019).

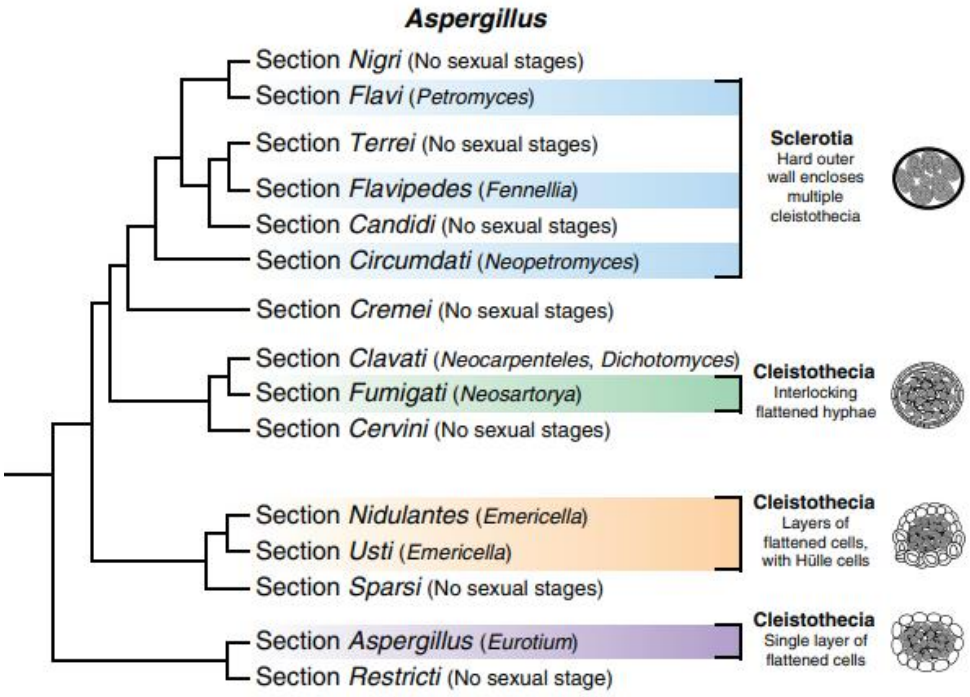

Figure 1.5. Relationship between *Aspergillus* sections and the respective teleomorphic genera that describe their sexual state. Recent discoveries of sex in section *Nigri* reveal a petromyces teleomorph or sexual morph (Olarte *et al*., 2015) and evidence of relationship to section *Flavi*. Taken from Dyer and O'Gorman (2011). Note that there has been changes proposed showing a sister relationship between section *nigri* and *nidulantes* and *usti* as opposed to *circumdati* (Steenwyk *et al*., 2019).

It is noted that ascospore ornamentation has also been used as a useful characteristic in the naming of newly described teleomorphs or sexual morphs (Horn *et al*., 2011; Horn *et al*., 2013; Samson and Varga, 2010; O'Gorman *et al*., 2009; Dyer and O'Gorman, 2012).

1.6.4 Aspergillus section *Nigri*

Of particular importance to the present study are members of *Aspergillus* section *Nigri*, with emphasis on the type species *A. niger*. These species are colloquially known as the 'black aspergilli' and typically form dark or black conidia and colonies (Silva *et al*., 2020; Silva *et al*., 2011; Gugnani, 2003). It has been stated that section *nigri* originated around 49.4 mya. For comparison, the same paper states that *A. flavus* and its domesticated counterpart *A. oryzae* diverged only 3.8 mya (Steenwyk *et al*., 2019). *Aspergillus piperis* and *A. lacticocoffeatus*, whilst producing black conidia, rarely produce conidia on most media, and colonies instead appear pink on white due to the presence of many small sclerotia (Samson *et al*., 2004; Cardoso *et al*., 2017). As well as *A. piperis* (Samson *et al*., 2004) and *A. lacticocoffeatus* (Samson *et al*., 2004) the section includes *A. sclerotiicarbonarius* (Noonim *et al*., 2008) *A. neoniger* (Varga *et al*., 2007a), *A. vadensis* (de Vries *et al*., 2005), *A. tubingensis* (de Vries *et al*., 1997), *A. brasilensis* (Varga *et al*., 2007c) and *A. eucalyptica* (Varga *et al*., 2007a).

A more recent analysis of section *nigri* formalised a further subdivision of five 'series' containing *A. niger* sensu stricto and referred to and compartmentalised as series *nigri*. This series recognised the number of species as six. These species are namely *A. brasiliensis*, *A. eucalypticola*, *A. luchuensis* (syn. *A. piperis*), *A.niger* (syn. *A. vinaceus*, *A. welwitschiae*) *A. tubingensis* (syn. *A. chiangmaiensis*, *A. costariciensis*, *A. neoniger* and *A. pseudopiperis*) and finally *A. vadensis* (Bian *et al*., 2022).

Species belonging to this taxonomic grouping are important in biomedicine, bioenergy, medical mycology and biotechnology (Vesth *et al*., 2018). Enzyme production for industry is dominated by the section with 49 of 260 fungal enzymes used industrially coming from member species, the most notable of which are *A. niger*, *A. tubingensis*, *A. aculeatus*, and *A. luchuensis* (Vesth *et al*., 2018). As discussed previously and as will be further discussed in **Section 1.6**, species of this section also produce organic acids such as citric acid. The species *A. niger* has been granted GRAS (Generally Regarded As Safe) status by the US FDA (Silva *et al*., 2011; Schuster *et al*., 2002; Frisvad *et al*., 2011). Members of this section are generally considered soil dwelling and saprophytic on leaf litter (Samson *et al*., 2002). They have also been isolated from a broad range of sources including dried fruits such as raisins, as well as nuts, grapes, beans and cereals (Samson *et al*., 2004; Nielsen *et al*., 2009).

Species of the black aspergilli can also produce a variety of mycotoxins and contaminate various food stuffs which is of importance to agricultural interests. These mycotoxins include Ochratoxin A (Samson *et al*., 2004) and Fuminosin B_2 (Frisvad *et al*., 2007; Nielsen *et al.*, 2009). Classification of species has proven difficult, and many approaches have been proposed, including microscopic, genetic and chemotaxonomic approaches (Frisvad and Larsen, 2015). Combining these methods can be described as polyphasic and is beneficial as no one approach is flawless in distinguishing species (Varga *et al*.,

2007b; Samson *et al*., 2006; Silva *et al*., 2011; Samson and Varga, 2009). Additionally, MALDI-TOF has been used to distinguish between species of section *Nigri* (D'hooge *et al*., 2019). One notable difference between species of the section is the presence of both uniseriate and biseriate species (Silva *et al*., 2011). Using these approaches, around 26/27 species had been described as belonging to the black aspergilli of section *Nigri* (Varga *et al*., 2007b; Vesth *et al*., 2018), down from an earlier stated number of 35 (Samson *et al*., 2004). At the onset of studies of this PhD, several species of section *Nigri* had emerged as sexual (see below) (Darbyshir, 2014; Olarte *et al.,* 2015). However, the biotechnologically important species *A. niger* has remained elusive in its sexual requirements and might have evolved to asexuality (Varga *et al*., 2014; Dyer and Kück, 2017).

1.6.5 Sexuality in the Black Aspergilli

The recent discoveries of sex in certain members of the black aspergilli have demonstrated the potential for sex in other members of section *Nigri*. *A. niger* stands out as a novel prime target due to its biotechnological significance (Cairns *et al*., 2018). Within the section *Nigri* and the closely related section *Flavi*, species have been shown to produce sexual spores housed in sclerotia, defining the teleomorphic genera as petromyces (Olarte *et al*., 2015; Horn *et al*., 2009). *Aspergillus flavus*, *A. parasiticus*, *A. nomius* and more recently *A. tubingensis* have demonstrated both heterothallism and the necessity for sclerotia production as necessary prerequisites to sex (Horn *et al*., 2009; Horn *et al*., 2009; Horn *et al*., 2011; Olarte *et al*., 2015). The phylogenetic relationship between these sections and demonstrable relationship between section and teleomorph or sexual morph, indicate a similar sexual lifecycle might be possible for *A. niger*. Therefore, to demonstrate sex in *A. niger*, it is likely that sclerotia formation will be a necessary prerequisite, as well as the identification and matching of strains of opposite mating type. Sclerotia formation has already been demonstrated in *A. niger* on media containing raisins (Frisvad *et al*., 2014). Despite increasing genome sequencing, by the onset of this PhD, only *MAT1-1* strains of *A. niger* had been described in the literature. A *MAT1-2* strain was described towards the summation of this PhD (Ellena *et al*., 2021b).

A novel sexual morph described as saitoa was described in 1980 and likened to the sexual morph petromyces (Rajendra and Muthappa, 1980). The sclerotia described in saitoa was similar to that of petromyces but the distinction was drawn at the level of ascospores with saitoa bearing spiny protrusions and two equatorial crests, this was described as distinct from ascospores of petromyces described at the time which were smooth with an equatorial crest. However, the discoveries in petromyces and the description of different ascospores has cast doubt on this being a distinguishing characteristic. Furthermore, the fact that *A. japonicus* is uniseriate suggests that it is likely in the *aculeatus* series rather than the *nigri* series.

1.7 Biotechnological Applications of *A. niger* and other Black Aspergilli

One of the great commercial successes of fungal biotechnology is the production of citric acid (2-hydroxy – 1,2,3 propanetricarboxylic acid, $C_6H_8O_7$). This organic acid is an intermediate in central carbon metabolism within the TCA or Krebs cycle. It is used principally in the food industries as a food stabiliser, acidulant, or for pH adjustment and for its antioxidant properties, which account for around 70-75% of demand. Furthermore, it is in pharmaceuticals, detergents and used for its metal binding properties, particularly with bioremediation (Cairns *et al.,* 2018). *Aspergillus niger* strains are responsible for annual worldwide production of between $1x10^6$ - $9x10^6$ metric tonnes of citric acid (Baker, 2006; Karaffa and Kubicek, 2003) with a value of $3.6 billion in 2020 (Cairns *et al.,* 2018). Additionally, strains of *A. tubingensis* are used for citric acid production (Yoshioka *et al.,* 2020). Strains of both *A. niger* and *A. tubingensis* utilised for citric acid are picked for their metabolite expression, rheology and absence of mycotoxins, amongst other factors (Karaffa and Kubicek, 2003; Sun *et al*., 2018; Schuster *et al*., 2002).

The Currie-Thom fermentation process was commercialised in 1923 utilising *Aspergillus niger* as the fermentative organism grown in an acidified sucrose media supplemented with minimal trace metal salts (Currie, 1917; Soccol *et al.,* 2006). Notable was the application of strain selection to the purpose of citric acid production, as well as the limitation and bioavailability of nitrogen source and the effect of starting pH on the development of citric acid. It has been stated elsewhere that low a starting pH of 2 favours Citric acid production over Gluconic acid and Oxalic acid (Niu *et al.,* 2016; Ruijters *et al.,* 1999).

The genome sequencing and subsequent annotation of the *A. niger* strain CBS 513.88 (Pel *et al.,* 2007) allowed for the identification of genes and transcription factors that may be the cause of the high CA production. CBS 513.88 is known for its high glucoamylase production and has undergone mutagenesis. Genes involved in the production of the citrate precursor, oxaloacetate, were found in number such as malate dehydrogenases, pyruvate decarboxylases and transporters. There were additionally, four citrate synthases. All of these would suggest an improved citrate reduction. Furthermore, several glucose oxidase genes, catalase and gluconate kinase were uncovered, revealing a method for production and metabolism of gluconic acid to 6-phosphate gluconate. It has been hypothesised elsewhere that this conversion of glucose to gluconic acid has developed evolutionarily to prevent utilisation by microbial competitors (Poulsen *et al.,* 2012; Andersen *et al.,* 2009). Further analysis and a comparison between the CBS 513.88 (glucoamylase strain) and the historic precursor to citric acid production strains ATCC 1015 provided a less clear distinction between the two which accounted for hyper CA production (Andersen *et al.,* 2011). Mutations were found in genes involved

in alternative oxidase pathways and transcriptomic studies showed that these resulted in up-regulation in ATCC 1015 (Andersen *et al.,* 2011).

Additionally, *A. niger* and the black aspergilli are used to produce hydrolytic enzymes such as amylases, pectinases, and xylanases (Andersen *et al*., 2011). Furthermore, *A. niger* is a widely used platform for the secretion of heterologous proteins due to its secretory pathway and versatile metabolism (Pel *et al*., 2007; Sun and Su, 2019; Conesa *et al*., 2001). Finally, *A. niger* is used in bioremediation, as it has potential in oxidation, hydroxylation and demethylation and tolerance of heavy metal stress (Pel *et al.,* 2007; Mukherjee *et al*., 2010; Srivastava and Thakur, 2006). Beyond these uses, members of *Aspergillus* section *nigri* are also used in the production of puerh tea (Wang *et al*., 2018; Ma *et al*., 2022), awamori, shochu and makgeolli distilled alcoholic beverages from Okinawa, Mainland Japan and Korea respectively (Ogawa *et al*., 1990; Choi *et al.,* 2014), nuruk; used for fermentations in Korea (Han *et al*., 1997), as well as harnessing the enzyme production of koji (Machida *et al.,* 2008; Hu *et al*., 2022; Mikami *et al*., 1987) for saccharification of raw materials such as barley and rice used in fermentation processes in these regions (Hong *et al*., 2013). Contrary to their usefulness in products, members of *Aspergillus* section nigri are also known as food spoilage organisms due to their production of mycotoxins (Nielsen *et al*., 2009; Perrone and Gallo., 2017; Taniwaki *et al*., 2018).

That there are genes shown to be responsible for differences in CA production, hydrolytic enzyme and protein secretion and fungal morphology is of interest to the application of the sexual cycle in *A. niger* for strain improvement purposes. Where high levels of recombination allow for large genetic differences between progeny that can be screened for traits of interest such as CA production, substrate utilisation, trace metal sensitivity and fungal morphology and rheology and how this relates to oxygen consumption and bioreactor use.

1.8 Aims of Present Study

Fungi are highly beneficial to human society, particularly as a source of a tremendous diversity of metabolites encountered in our every-day lives. However, fungi can also be highly detrimental, with long-established, and emerging, pathogens posing medical and agricultural problems. From processes as ancient as brewing and cheese-making, to the modern production of pharmacological chemicals such as antibiotics and statins, the production of enzymes for use as lab reagents, and proposed utilisation for biodegradation of plastics, we have long exploited the diverse properties of fungi (Raper *et al*., 1944; Manzoni *et al*., 1999; Daly *et al*., 2021). Regarding the exploitation of fungi in the biotechnology sectors, there is a long-standing demand for strain improvement for increased economic performance. Strain improvement strategies have classically utilised UV mutagenesis to alter the genetics of strains with the hope of increasing titres of desired products or reducing toxic by-products (Vu *et al*., 2009; Lotfy *et al*., 2007; Dashtban *et al*., 2009). However, recent advances in molecular genetics, genome sequencing and bioinformatics have provided opportunities for novel methods of strain improvement. For example, numerous 'silent' gene clusters that are not expressed under standard laboratory conditions have been discovered, whose activation (via techniques of gene manipulation) it is hoped will lead the way to a new wave of metabolite discovery (Brakhage and Schroeckh, 2011; Zhang and Elliot, 2019). In parallel, sets of genes responsible for sexual reproduction have been discovered in the genomes of a series of fungal species of economic and medical importance, which have traditionally been considered as obligate asexual species. The activation of a sexual cycle in such production organisms would provide a very valuable tool for strain improvement, with considerable benefits over UV mutagenesis and recombinant technologies (Pöggeler, 2001; Bohm *et al*., 2013; Seidl *et al*., 2009). For example, this would provide the ability to cross strains with desirable traits and/or the use of the sexual cycle to generate considerable genetic variation because of meiosis, thereby allowing for a great diversity of progeny to be screened for increased production of metabolites, or even novel metabolites. Finally, molecular genetic approaches combined with biochemical studies are also revealing aspects of the mechanisms of signalling that trigger and control fungal sexual development (Kues and Casselton, 1992; Dyer *et al*., 1992; Bennett, 2009; De Vries *et al.,* 2017).

The main aims of the current PhD thesis studies were therefore two-fold.

(1) Firstly, to build on recent genomic, experimental and bioinformatic studies to see if sexual cycles can be established and exploited in previously considered 'asexual' species of economic importance. A specific target species was *A. niger* given its economic importance in the biotechnology industry and recent discoveries of sexual cycles in other related species of black aspergilli. This was to be achieved by two-fold studies:

a) Investigations into an improved understanding of environmental and genetic factors regulating the development of sclerotia in *A. niger.* Given that the formation of sclerotia seems a pre-requisite for sexual development in the black aspergilli, then an enhanced ability to induce scleriotial development in *A. niger* is a key step in inducing a sexual cycle.

b) Secondly, to determine whether hormonal factors and other metabolites involved in fungal morphogenesis and sclerotia production, might have potential applications in agriculture for novel methods of fungal disease control and pest management. As explained above (**Section 1.5.5** and further in **Section 3.1.4**) there is accumulating evidence that endogenous signalling compounds produced by filamentous fungi might have the ability to regulate fungal growth, including the repression of asexual sporulation and growth, and thus the potential for commercial applications. However, this area has been little studied and warrants more attention.

2) Secondly, once conditions had been identified to reliably induce sclerotia in *A. niger*, then mating studies using complementary *MAT1-1* and *MAT1-2* isolates would be performed to determine if it were possible to induce sexual reproduction in *A. niger* and other control species from the aspergilli.

Chapter 2: General Materials and Methods

This chapter describes general materials and methods that are used in more than one of the experimental work chapters. By contrast, chapter-specific methods will be described in the respective experimental work chapters.

2.1 Materials

2.1.1 Media for culture growth

Unless specified the media for culture growth were sterilised before use by autoclaving at 121 °C for 15 mins.

Aspergillus Complete Medium (ACM)

Aspergillus complete medium (ACM) was prepared according to the recipe of Paoletti *et al.* (2005), and contained 10 g/L D-glucose powder (Sigma, U.K), 1 g/L yeast extract (Oxoid, U.K), 2 g/L peptone (Oxoid, U.K), 1 g/L casamino acids (Sigma, U.K), 0.075 g/L adenine (Sigma, U.K), 20ml/L of an *Aspergillus* salt solution, and 10 ml/L of an *Aspergillus* vitamin solution. Media was prepared and made up with distilled water and adjusted to pH 6.5.For solid media, 20 g/L agar (Oxoid, U.K) was added prior to autoclaving at 117 °C for 30 minutes.

The *Aspergillus* salt solution contained: 25 g/L potassium chloride (Fisher, U.K), 26 g/L magnesium sulfate (Fisher, U.K), 76 g/L Potassium dihydrogen phosphate (Fisher, U.K) and 100 ml/L *Aspergillus* trace elements solution. The solution was made up with distilled water.

The *Aspergillus* vitamin solution contained: 400 mg/L p-aminobenzoic acid (Sigma, U.K), 50 mg/L thiamine hydrochloride (Sigma, U.K), 2 mg/L biotin (Sigma, U.K), 100 mg/L nicotinic acid (Sigma, U.K), 250 mg/L pyridoxine hydrochloride (Sigma, U.K), 1.4 g/L choline chloride (Sigma, U.K) and 100 mg/L riboflavin (Sigma, U.K). The solution was made up with distilled water.

The *Aspergillus* trace elements solution contained: 40 mg/L sodium tetraborate decahydrate ($Na_2B_4O_7.10H_2O$) (VWR international, U.K), 800 mg/L copper sulfate pentahydrate ($CuSO_4.5H_2O$) (Fisher, U.K), 800 mg/L ferric orthophosphate monohydrate ($FePO_4.H_2O$) (Sigma, U.K), 800 mg/L manganese sulfate tetrahydrate ($MnSO_4.4H_2O$) (Fisher, U.K), 800 mg/L sodium molybdate dihydrate ($NaMoO_4.2H_2O$) (Fisher, U.K) and 8 g/L zinc sulfate ($ZnSO_4$) (Fisher, U.K). The solution was made up with distilled water.

Czapeks Yeast Autolysate Agar (CYA)

Czapeks yeast autolysate agar (CYA) was prepared containing 30 g/L sucrose (Sigma, U.K), 5 g/L yeast Extract (Oxoid, U.K), 1 g/L dibasic potassium hydrogen Phosphate (K_2HPO_4)(Sigma, U.K), 3 g/L sodium nitrate ($NaNO_3$)(Sigma, U.K), 0.5 g/L potassium chloride (KCl) (Fisher Scientific, U.K), 0.5 g/L magnesium sulfate heptahydrate ($MgSO_4.7H_20$) (Sigma, U.K), 0.01 g/L iron sulfate heptahydrate ($FeSO_4.7H_2O$) (Sigma, U.K) and 15 g/L Agar (Sigma, U.K). The medium was prepared in distilled water, with modifications (no trace additional trace metals used) from that described by Frisvad and Samson (2004). Autoclaved at 117 °C for 30 minutes.

Mixed Cereal Agar (MCA)

Mixed cereal agar (MCA) was prepared containing 50 g/L Gerbers mixed grain cereal (Gerber products, Gerber Products Co., Fremont, Michigan, U.S.A) and 20 g/L agar (Oxoid, U.K). The medium was prepared in distilled water as described by McAlpin and Wicklow (2005).

Malt Extract Agar (MEA)

Malt extract agar was prepared containing 20 g/L malt Extract (Sigma, U.K), 6 g/L Peptone (Oxoid, U.K) and 16 g/L agar (Oxoid, U.K). The medium was prepared in distilled water.

Oatmeal Agar (OA)

Oatmeal agar was prepared according to O'Gorman *et al.* (2009). The medium contained 40 g/L Oddlums pinhead oats and 20 g/L Agar (Sigma, U.K). The oats were simmered in tap water for 1 hour and then strained through muslin and made up to the correct volume with tap water (Nottingham, U.K) and agar added. Media was then autoclaved at 117°C for 30 minutes

Potato Dextrose Agar (PDA)

Potato dextrose agar (PDA) was supplied and made up according to manufacturer's instructions (Oxoid or BD/Difco). 39 g/L and 15 g/L agar (Sigma, U.K) made up in distilled water.

Yeast Extract Peptone Dextrose Broth/Agar (YEPD)

Yeast extract peptone dextrose broth or agar (YEPD) contained 20 g/L peptone (Oxoid, U.K), 10 g/L yeast extract (Oxoid, U.K). For solid media, 16 g/L agar was added (Sigma, U.K). The medium was made up with 900 mL distilled water. Separately, a 20 % glucose solution was made up by adding 200 g glucose (Fisher, U.K) in 1 L, which was then aliquoted into 100 mL aliquots. These aliquots were autoclave at 117 °C for 30 minutes. For the final YEPD, 100 mL aliquots of the 20% glucose solution were added to each 900 mL of broth or molten agar prior to pouring to result in a final 2 % total glucose.

Raisin media

In many instances, raisins were used as a modification to the media as described by Frisvad *et al.,* (2014). In these instances, the media will hereby be referred to by its standard abbreviation form with the addition of an 'R'. For example, CYA media modified with raisins will be called CYAR. Sun Maid Raisins (Kingsburg, California, U.S.A) were autoclaved at 121 °C for 30 minutes then dried for 24 hours at 50 °C in a drying cabinet. Raisins were then placed and left on the media for 24 hours before removal, prior to inoculation. Arrangements of raisins on the plate are discussed in the relevant sections.

4 % (w/v) Water Agar

A water agar was prepared by adding 40 g/L Bacto agar (Scientific Laboratory Supplies, U.K) to a final volume of 1L with distilled water.

2.1.2 General solutions and buffers

CTAB RNA Extraction Buffer

Stock solutions of 5 M sodium chloride, 1 M Tris-HCl pH 8 and 0.5 M EDTA pH 8 were prepared with DEPC treated water and autoclaved. RNA extraction buffer was prepared as follows: 1.4 M sodium chloride, 100 mM Tris-HCl pH 8, 25 mM EDTA pH 8, 2 % (w/v) polyvinylpyrrolidone (PVP) of molecular weight 40,000 (Sigma, U.K), hexadecyltrimethylammonium (CTAB) (Sigma, U.K), 0.5 g/L spermidine (Sigma, U.K) were made up with DEPC treated water and autoclaved. 2 % (v/v) β-mercaptoethanol was added to an appropriate volume of the buffer directly before use. The protocol for buffer preparation was taken from Jaacola (2001).

DEPC Treated Water

0.1 % diethyl-pyrocarbonate was made up dilution in an appropriate volume of distilled water. This was warmed overnight prior to autoclaving twice at 121 °C.

Nucleic Acid Loading Dye and Molecular Weight Markers

To make 6 mL of DNA gel loading buffer, 2.4 g sucrose, 15 mg xylene cyanol and 15 mg bromophenol blue were made up to a final volume of 6 ml with distilled water. Loading dye solution was not autoclaved. Both 100 bp and 1 kb weight markers were prepared according to the manufacturer's instructions (NEB, U.K).

Phosphate Buffered Saline (PBS)

Prepared by adding 1 preformed tablet (Oxoid) to 100 mL distilled water. Sterilised by autoclaving at 115 °C for 10 minutes.

Tween 80 Solution (0.01 % v/v)

0.01 % (v/v) Tween 80 (Sigma, U.K) was prepared in an appropriate volume of distilled water.

2.2 Methods

2.2.1 Culture Maintenance

Slopes of ACM agar were prepared by pouring 10 mL of molten agar into a 30 ml Universal container (Sterilin) and leaving to set. Agar plates were prepared by pouring 25 mL of Agar into 9 cm diameter (Sterilin) plates. For species that produce asexual spores (e.g., *Aspergillus spp.*), inoculation was via a sterile 10 µL loop from cultures maintained on ACM slopes. For non-asexual sporulating species (e.g., *Sclerotinia spp.*), a sterilised cork borer was used to cut a 5 mm section of a mycelial culture and plugs were placed face down on a fresh 9 cm PDA plate. After one week of growth at 28 °C under room lighting, the cultures were closed or sealed with a single layer of parafilm and refrigerated for storing.

There were certain instances where *A. niger* or *A. tubingensis* underwent a 'pre-freezing' step involving ACM slopes of the cultures being maintained at -20 °C for 2 weeks before defrosting and from which spore solutions prepared (see later **Section 3.3.1**).

For longer term storage of cultures, preparations of spores and mycelia were suspended in 10 or 20 % glycerol solution, of which 800 µL was added to a cryovial. The cryovials then underwent steps of decreasing temperature to gradually reduce temperature: 30 minutes at 4 C, followed by 30 minutes at -20 °C, before then being stored at -70 °C in liquid nitrogen.

Cultures from long term storage were revived by scraping material from the glycerol stocks using a 10 µL loop and using this as inoculant on fresh ACM slopes, which were then grown for one week at 28 °C.

Strain lists used for this thesis are given in the appendices. *Aspergillus nidulans* strains are listed in **Appendix 5**. *Aspergillus fumigatus* strains used are listed in **Appendix 6**. *Aspergillus tubingensis* strains are listed in **Appendix 7**. *Aspergillus sclerotiicarbonarius* strains used are listed in **Appendix 8**, and *Aspergillus niger* strains used are listed in **Appendix 9**. Other species are strains were used in this thesis but not broadly and where useful, they are described in the text.

2.2.2 Quantification of Spore Concentration

Spore suspensions were prepared by scraping the conidia from mycelia on prepared slopes using a sterile 10 µL loop and a suitable volume (e.g. 5 ml) of Tween 80. This suspension was then filtered through autoclaved Miracloth (Merck, U.K) to remove mycelial debris. Spore concentration was determined using an improved Neubauer haemocytometer. Serial dilutions of 1:10 were prepared so that a suspension yielding between 30-300 spores per count grid were present. Calculations and adjustments for spore suspensions were then made so that the spore suspensions could be diluted to a set concentration of 10^5 conidia/mL (or higher if required up to 10^8).

2.2.3 Setting up Cultures for Mating

For heterothallic species, plates were set up for mating between complementary opposite mating types. Petri plates of 9 cm diameter with 25 mL of media were poured and allowed to set. For CYAR plates, the organisation of raisins and points of inoculation were assayed for maximal sclerotial development (see later chapters for specific descriptions). After inoculation, plates were incubated upside down at a suitable temperature (given in the relevant sections) for 10 days before sealing with parafilm. Crosses were then examined after 3, 4, 5 and 6 months for signs of sexual development and possible production of sexual progeny.

2.2.4 DNA Extraction

Cultures were grown in ACM broth for 3 days before being filtered through sterile Miracloth (Merck, U.K) and flash frozen in liquid nitrogen. Frozen cultures were then ground to powder under liquid nitrogen using a sterile pestle and mortar. Powdered and frozen mycelia were then prepared for DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega, U.S.A) following manufacturer's instructions with minor modifications to 3.E (Isolating Genomic DNA from Plant Tissue): In step 2, 450 µL of Nuclei Lysis solution was used rather than 600 µL; In step 3, incubation was for 30 minutes rather than for 15 minutes; Step 4 was replaced with simply placing the material on ice for 5 minutes; In step 5, 225 µL of protein precipitation solution was used.

2.2.5 RNA Extraction and Amplification

2.2.5.1 RNA Extraction of Cultures Grown in Broth Media

Cultures were grown in ACM broth for 3 days under suitable inducing conditions before being filtered through sterile Miracloth (Merck, U.K) and flash frozen in liquid nitrogen. Frozen cultures were then ground to powder under liquid nitrogen using a sterile pestle and mortar.

Due to the low melanin content of the mycelium grown in broth, a standard version of the chloroform extraction protocol was suitable as described by Bok *et al*. (2006), the details of which were as follows.

Powdered mycelium was added to a 2 mL Eppendorf tube containing 1 mL of Tri reagent (Sigma, U.K) to make volume up to 1.5 mL and inverted several times to mix and left at room temperature for 10 minutes. 200 µl of chloroform (Fisher, U.K) was then added, the mixture was vortexed and left for 3 minutes. The mixture was vortexed again before centrifugation at 13,000 rpm for 10 minutes. 750 µL of the upper aqueous phase was removed and placed into a fresh 1.5 mL Eppendorf with 750 µL of 100% Isopropanol (Fisher, U.K) to precipitate RNA and DNA, tubes were inverted several times to mix and left at -20 °C for 20 minutes. The Eppendorf tubes were then centrifuged at 13,000 rpm for 10 minutes. The supernatant was then carefully poured off leaving a pellet of precipitated nucleic acids. The pellet was then washed with 700 µL of 75 % ethanol (Fisher, U.K, (v/v) made up with DEPC treated water) with agitation before centrifugation at 13,000 rpm for 10 minutes. The supernatant was again carefully poured off and the pellet was air dried in a laminar airflow cabinet (to not introduce RNase) for 15 minutes. The pellet was then be resuspended in 100 µL of DEPC treated water and left to solubilise for 30 minutes.

For DNA digest and RNA purification, the Machery-Nagel Nucleospin® RNA isolation kit (Fisher Scientific, U.K) was used following manufacturer's instructions [sections 5.5 (Clean-up of RNA from reaction mixtures) and 5.1 (RNA purification from cultured cells and tissue) from 5.1.5 onwards].

Successful DNase treatment was validated through PCR assays of the purified nucleic acid with β tubulin primers for 40 cycles (as described in **Section 2.2.6**). The absence of banding in the PCR product following TAE 1.5 % agarose gel electrophoresis was used to designate a successful DNase treatment, noting that gels were run with an accompanying positive control of β tubulin primers and genomic DNA.

RNA quality was assessed through visualisation of ribosomal RNA bands after TBE 1.5 % agarose gel electrophoresis. A clear double band pattern was interpreted as indicating low levels of degradation, whereas smears would indicate RNA degradation and low-quality nucleic acids.

2.2.5.2 RNA Extraction of Cultures Grown on Solid Media

Sexuality within the aspergilli is normally observed for cultures grown on solid media and therefore to assess RNA expression under these conditions, it was necessary to extract RNA from cultures grown on solid media. Where possible (due to number of plates) media in 5 cm diameter Petri dishes was covered with a sterile 5 cm diameter nylon filter disk (Merck Millipore Ltd., Co. Cork, Ireland) prior to inoculation, which was used to aid harvesting of mycelia later without contaminating agar and growth components from the media. In cases where these nylon disks were used, inoculation was made via spore suspension directly onto the surface of the nylon filter disk.Mycelia and sclerotia were harvested by scraping of the agar or nylon disk surface with a sterile number 10 scalpel blade (Swann-Morton Ltd., U.K). Mycelia was then transferred to a 2 mL Eppendorf tube before being snap frozen in liquid nitrogen. These samples could then be stored at -70 °C or immediately processed using a sterile pestle and mortar as previously described in **Section 2.2.4.1.** Mycelia harvested using this method yielded a much higher melanin content than that of mycelia grown in liquid culture due to the maturation of conidia on solid media. As such, the protocol used in **Section 2.2.4.1** rarely yielded viable RNA product when used with a variety of black aspergilli (data not shown).

A protocol that had previously been developed for the removal of polysaccharides by utilising CTAB as the detergent in the extraction buffer (Jaakola *et al*., 2001) was therefore adopted. This protocol was more successful in the retrieval of RNA product from cultures grown on solid media than that used in **Section 2.2.4.1,** with details of the extraction as follows.

100 mg of freshly harvested mycelia was obtained from scrapping growing mycelium from the surface of nylon disks placed on relevant media. This mycelium was flash frozen and processed by grinding using a sterile pestle and mortar and was immediately transferred to a 2 mL Eppendorf tube containing 750 µL of CTAB buffer [including 2 % (v/v) β-mercaptoethanol] (**Section 2.1.2**) preheated to 65 °C. Samples were vortexed and maintained at 65 °C for 15 minutes with frequent inversion. After this incubation period, an equal volume of chloroform (Fisher Scientific, U.K) was added with inversion and this was followed by centrifugation at 11,000 **g** at 4 °C for 10 minutes. After centrifugation, the upper aqueous phase is removed and added to a new 2 mL Eppendorf with 1/3 volume of 8 M Lithium chloride (LiCl) (Sigma, U.K) and precipitated overnight at 4 °C. Nucleic acids were then pelleted by centrifugation at 11,000 **g** at 4 °C for 60 minutes. After centrifugation, the supernatant was carefully removed and the pellet was washed with 200 µL 70 % ethanol [Fisher Scientific, U.K, (v/v) made up with DEPC treated water] followed by centrifugation at 11,000 **g** at 4 °C for 60 minutes. After this final centrifugation, the ethanol was removed, and the pellet allowed to air dry in a laminar airflow cabinet before solubilising in 65 °C 100 µL of RNase-free H_20.

For DNA digest and RNA purification, the Machery-Nagel Nucleospin® RNA isolation kit (Fisher Scientific, U.K) was used following manufacturer's instructions [sections 5.5 (Clean-up of RNA from reaction mixtures) and **5.1** (RNA purification from cultured cells and tissue) from 5.1.5 onwards].

Successful DNase treatment was validated through PCR of the product with β tubulin primers, and RNA quality was assessed through visualisation of ribosomal RNA bands as described in **Section 2.2.5.1.**

2.2.5.3 Complementary (cDNA) Synthesis (RNA Reverse Transcriptase)

Purified RNA was converted into cDNA using GoScript™ Reverse Transcription Mix (Promega, U.S.A) according to manufacturer's instructions. Incubations were performed on a Techne TC-512 FTC51F/H2D thermal cycler (Bibby Scientific Ltd, Stone, Staffordshire, U.K) using a cycling program recommended in the GoScript™ manufacturer's instructions.

2.2.6 Polymerase Chain Reaction (PCR)

All polymerase chain reactions (PCR's) were performed using Techne TC-512 FTC51F/H2D thermal cyclers (Bibby Scientific Ltd, Stone, Staffordshire, U.K).

The standard PCR protocol utilised the enzyme Phusion® High-Fidelity DNA polymerase (Fisher, U.K) for amplifications. Reaction volumes of 25µL included 5 µL of Phusion HF buffer, 0.5 µL of 10 mM dNTPs, 1.25 µL of 10 µM of each primer and 0.25 µL of Phusion DNA polymerase and a variable mass of template DNA or cDNA, made up with nuclease-free water (according to manufacturer's instructions). Standard cycle parameters (according to manufacturer's instructions) were initial denaturation at 98 °C for 30 seconds; followed by 25-35 cycles of denaturation at 98 °C for 10 seconds, annealing of between 45-72 °C for 30 seconds, elongation at 72 °C for 30 seconds (per kb); and then a final extension of 72 °C for 10 minutes. The template concentrations, cycling parameters and polymerase enzymes used (if different) will be described in relevant sections in the following chapters.

2.2.6.1 Primer Design

Genomic sequence data for species was available from NCBI (https://www.ncbi.nlm.nih.gov/) and AspGD (http://aspgd.org/). Primer3web (version 4.1.0; http://primer3.ut.ee/) was used for primer design.

2.2.6.2 Mating-Type Diagnostics

Mating-type diagnostics have previously been established and shown to be successful in determining mating type in the black aspergilli (Darbyshir, 2014). These *MAT* diagnostics were based around the presence of different genome sequence in isolates of different mating type, as a result of the different gene sets present at the idiomorphic *MAT* loci and were designed to amplify different products based on primer targets within one of the two available *MAT* loci. The diagnostic involved the use of two simultaneously run PCRs, one with a *MAT1-1* specific pair of primers, one with a *MAT1-2* specific pair of primers (**Table 2.1**). It would be expected that one PCR should give a product, indicating mating type, whilst the other was negative. However, this did require two PCR's to be run per isolate to reliably determine *MAT*-type. Two primer pairs were available for each *MAT*-type, and these are listed in **Table 2.1** and the nucleotide sequences given in **Appendix 2**.

Table 2.1. Primer pairs used for mating type specific mating type diagnostic.

MAT-type	Primer Pair 1	Primer Pair 2
*MAT*1-1	MAT1F and MAT1R	MAT1.1F and MAT1.1R
*MAT*1-2	MAT3-5 and MAT5-7	mat1.2for and mat1.2rev

20 µL reactions contained 2 µL Fast Start High Fidelity buffer with $MgCl_2$ to a final concentration of 1.8 mM; 200 µM dNTP's; 1.0 µL of each of the primers; between 10-50 ng of template DNA and 1 U of Fast Start High Fidelity enzyme blend (Roche, U.K) made up with distilled water.

The amplification protocol was performed using the following cycle parameters; initial denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at either 50 °C (MAT5-7 and MAT3-5), 54 °C (MAT1F and MAT1R), 55°C (MAT1.1F and MAT1.1R) or 60 °C (MAT1.2for and mat1.2rev) for 1 minute and extension at 72 °C for 30 seconds with a final extension step at 72 °C for 5 minutes. For primer nucleotide sequences see **Appendix 2.**

2.2.6.3 Multiplex Diagnostic PCR

An alternative multiplex PCR diagnostic, designed to determine mating-type identity based on the results of a single PCR has also previously been shown to be an effective method for *MAT* identification in certain species of the black aspergilli (Darbyshir, 2014). Three primers are used to give one of two possible bands and a positive result from a single PCR. Primer ATYP1 binds to sequence in the flanking region of the *MAT* loci (on the *SLA2* flank) which is highly conserved between the mating types. The other two primers ANMAT1F (*MAT*1-1-1 α-box domain) and MAT2amR (*MAT*-1-2-1 HMG domain) are *MAT*-type specific. The target sites of these primers within the MAT allele and flanking region are illustrated in **Figure 2.2**.

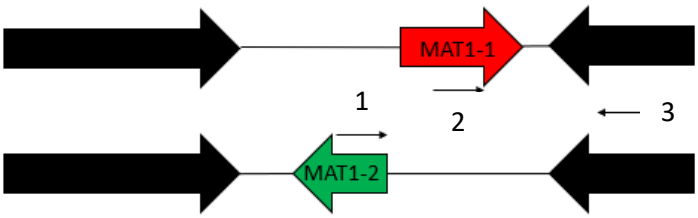

Figure 2.2. Diagram showing the binding positions of the primers used in the multiplex diagnostic PCR. Black sequences refer to flanking sequences/genes. Clear single lined sequence shows the *MAT* loci with its different idiomorphs and their respective directionality from 5'-3' as L-R. Primer labelled 1 refers to MAT2amR, primer 2 refers to ANMAT1F and primer 3 refers to ATHYP1.

20 µL reactions contained 2 µL Fast Start High Fidelity buffer with $MgCl_2$ to a final concentration of 1.8 mM; 200 µM dNTP's; 0.8 µL of each of the three primers; 50 ng of template DNA and 1 U of Fast Start High Fidelity enzyme blend (Roche, U.K) made up with distilled water.

The amplification protocol was performed using the following cycle parameters; initial denaturation at 95 ˚C for 2 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 60.5 °C for 30 seconds and extension at 68 °C for 4 minutes and 20 seconds increasing to 7 minutes over the 35 cycles. There was a final extension at 68°C for 7 minutes. For primer nucleotide sequences see **Appendix 2.**

2.2.7 Agarose Gel Analysis

Agarose gels were normally prepared to a final concentration of 1.5 % (w/v) by dissolving agarose in either 1 x TAE or 1 x TBE as specified in the relevant sections. Ethidium bromide was added to a final concentration of 100 ng/ml for visualisation of DNA (TAE) and RNA (TBE). Gel images were taken with a Bio-Rad ChemiDoc XRS+ using the Quantity One 4.6.9 program (BioRad, Hemel Hempstead, Hertfordshire, U.K).

2.2.8 PCR Product Purification, Gel Extraction and Sequencing

Bands for excision and purification from gels were visualised on an UVP Dual Intensity Transilluminator (Fisher Scientific, U.K). Desired bands were excised using a number 10 scalpel blade (Swann-Morton Ltd., U.K). DNA was then extracted from the excised band using the Machery-Nagel Nucleospin® Gel and PCR Clean-up kit (Fisher Scientific, U.K) according to manufacturer's instructions. Where required, PCR products were then for Sanger Sequencing by the DeepSeq unit (Medical School, University of Nottingham, U.K).

2.2.9 Nucleic Acid Quantification

Nucleic acids were routinely quantified using a Nanophotometer P330 (Implen GMBH, Munich, Germany). Where necessary, higher resolution quantification was completed with a Qubit v4 fluorometer from DeepSeq, Nottingham (ThermoFisher Scientific, U.K).

2.2.10 Preparation of Whole Genome DNA for Sequencing

Six *A. niger* strains were selected for whole genome sequencing. The strains were 8-160, 8-161, 8-162, 8-166, 8-169 and 8-175 as listed in **Appendix 9**. Strains were selected based on MAT-type and sclerotia production. Strains 8-161, 8-166 and 8-175 were identified as *MAT1-1* and 8-160, 8-162 and 8-169 were identified as *MAT1-2* using a multiplex PCR diagnostic (**Section 2.2.6.3** and **4.3.2**). Isolates 8-160, 8-161, 8-162 and 8-166 all reliably produced sclerotia on CYAR agar (**Section 2.1.1**) whilst 8-169 and 8-175 were never observed to produce sclerotia on the same media.

Strains were prepared for whole genome DNA extraction by growth in 25 mL ACM broth (**Section 2.1.1**) overnight at 28 ˚C. Mycelia were filtered through Miracloth and washed with sterile tap water (STW) before being pressed dry to removed water. The pellets were then flash frozen in liquid nitrogen and ground to powder using a sterile pre-chilled pestle and mortar. DNA was extracted from the powdered fungal material using a Qiagen DNeasy Plant Maxi kit according to manufacturer's instructions. DNA quality and RNA removal was confirmed by gel electrophoresis and DNA concentration determined by Qubit methodology (Deep-Seq University of Nottingham).

2.2.11 Sequencing of DNA

Purified DNA samples were then either sequenced using a NextSeq500 (Illumina) by the DeepSeq unit team (Medical School, University of Nottingham, U.K). Data was analysed using MacVector™.

Purified total genomic DNA for genomic sequencing was completed using 30 µL aliquots of 10 ng/µL (300 ng total) gDNA that were sent to Novogene (Cambridge, UK) for Next Generation Sequencing (NGS).

2.2.12 Analysis of Sequence Data

Sequence data was validated and trimmed for comparison with MacVector 17.0.3(34). Sequence alignments were against genomic or mRNA data of target gene sequences retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and AspGD (http://aspgd.org/) of CBS 513.88. Sequence alignments were performed with either ClustalW or Muscle.

2.2.13 Photography, Light Microscopy and Scanning Electron Microscopy

2.2.13.1 Photography

Images were collected with a Nikon D3200.

2.2.13.2 Light Microscopy

Sclerotia and cleistothecia used for light microscopy visualisation were prepared by rolling on 4% water agar to remove excess surface conidia and hyphae.

Images were collected with either a Leica S8APO Dissecting Microscope equipped with a QImaging Micropublisher 3.3RTV camera or a Motic BA310E Biological Microscope equipped with a Moticam 3.0MP. Images were processed using either QCapturex64 or Image-pro Insight respectively.

2.2.13.3 Scanning Electron Microscopy

Sclerotia of *Aspergillus spp.* were collected from crosses up to 1 year old, that had been grown on CYAR agar at 28 °C in the dark. Where other media and temperatures were used, these are discussed in their respective sections. These sclerotia were rolled on 4 % water agar to remove excess surface conidia and hyphae. Specimens were then either prepared as whole sclerotia, dissected sclerotia, or simply as the removed putative ascocarps.

2.2.13.4 Sample Fixation and Electron Microscopy

Prepared samples were fixed according to the protocol given by Horn, Ramirez-Prado and Carbone (2009) as follows. Samples were fixed for 24 h in 3 % glutaraldehyde/0.1 M sodium cacodylate buffer at 4 °C. All subsequent steps were at 10 x sample volume. Samples were then rinsed with agitation in three 1 h changes of cold 0.1 M sodium cacodylate buffer followed by 1 h changes of cold 30 % and 50 % ethanol. Samples were left in 70 % ethanol overnight at 4 °C, followed by an 8 h change of 4 °C 95 % ethanol with occasional agitation, before being moved to 4 °C 100 % ethanol and allowed to come to room temperature for 24 h. Two more changes of room temperature 100 % ethanol completed the dehydration. The samples were then ready for critical point drying (CPD)

Images for higher resolution SEM (imaging individual conidia or ascospores) required a separate approach for clear resolution. A protocol adapted from Joubert *et al.,* (2015) and used more recently by Ellena *et al.* (2021a) to image sclerotia, involved the use of a primary aldehyde – 2 % glutaraldehyde, to stabilise proteins, followed by the use of 2 % Osmium tetroxide solution (Sigma, U.K) to fix lipids. The fungal material was covered by 200 µL 2 % glutaraldehyde for an hour before drying and subsequent coverage with 200 µL Osmium tetroxide in a fume hood to allow evaporation.

2.2.13.5 Preparation of Stubs for Visualisation

Samples for visualisation were critical point dried in carbon dioxide and 100 % ethanol using a Leica EM CPD300. Program settings were 28 °C at 1 bar for 1 hour, with 14 exchange cycles. Following critical point drying, samples were placed on carbon stubs and were sputter coated in gold or platinum (~10 nm) using a polaron E5100 SEM coating unit. Coated stubs and samples were viewed and captured under a Jeol JSM-6060LV or Jeol 6490LV Scanning Electron Microscope, typically at 15 kV at The Nanoscale and Microscale Research Centre NMRC (University of Nottingham, U.K).

Chapter 3: Metabolomic, Genomic and Environmental Investigation of Sclerotia Formation

3.1 Introduction

3.1.1 Sclerotial Morphology

Sclerotia, here defined as hardened, pigmented hyphal aggregations containing metabolic reserves allowing for environmental survival which may also represent vestiges or precursors to fruiting bodies (Willetts, 1971; Calvo and Cary, 2015), have been observed in a wide diversity of fungal taxa (Smith *et al*., 2015). The development of sclerotia from a mass of undifferentiated mycelia has been divided into three stages termed 'initiation', 'development' and 'maturation'. In the 'initiation' phase, hyphae begin to aggregate in one of three ways 'loose', 'terminal' or 'lateral' (Willetts and Bullock, 1992). In the 'development' phase, the aggregation increases in size and accumulates metabolic reserves. In the final stage of 'maturation' differentiation of a hardened and melanised 'rind' occurs, alongside changes to the metabolic reserves (Willetts and Bullock, 1992). Any of these stages may be associated with exudation, although it is most noticeable in the latter stages and may evaporate.

Structurally, mature sclerotia consist of concentric zones of the 'rind' as previously mentioned, a 'cortex' and a 'medulla'. The 'rind' is variable and loosely defined amongst different sclerotia producing species but is generally thought of as a layer of hardened, melanised, pseudoparenchymatous hyphae which are devoid of cellular organelles and become decreasingly impermeable with maturation (Willetts and Bullock, 1992). Within the 'rind' is a continuous layer called the 'cortex' consisting of close-fitting hyphae which may be partially melanised, and this is the region where metabolic reserves accumulate (Willetts and Bullock, 1992). The majority of the sclerotium is made of the innermost 'medulla' consisting of prosenchymatous tissue and an extracellular matrix (Willetts and Bullock, 1992).

Sclerotia formation in *A. niger* is one of the main study areas of the present thesis work. Sclerotia were first observed to be produced in *A. niger* in the study of Frisvad *et al*. (2014) who reported the formation of sclerotia in certain isolates on agar-raisin media. This was followed by a study of *sclB* which identified formation of sclerotia in certain mutant isolates of *A. niger (*Thieme *et al.,* 2018*)* (also see **Section 3.1.2** and **3.1.3** below) and later in citric acid producing strains (Ellena *et al*., 2021a). More recently, a macro and microscopic analysis or the morphology of *A. niger* sclerotia was completed (Ellena *et al.,* 2021a). Sclerotia appeared as hard, elongated structures of between 1 - 5 mm in diameter. Sclerotia formed at the point of inoculation and colony periphery and around two months after inoculation detached from the mycelium (Ellena *et al.,* 2021a), suggesting 'maturation' of a true

sclerotium as defined by de Bary (Willetts and Bullock, 1992). Microscopically, the sclerotia were comparable to the descriptions of sclerotia in other fungi, and the authors used the same terms 'rind', 'cortex' and 'medulla'. The addition and visualisation of toluidine blue dye showed dense staining of the cortex, allowing visual differentiation of the three distinct tissues. The cells of the medulla, stained to a lesser extent than those of the cortex by toluidine blue, demonstrate large vacuoles and thick cell walls (Ellena *et al.,* 2021a). The three distinct tissues of *A. niger* sclerotia that can be visualised using both light microscopy and scanning electron microscopy (SEM) are illustrated in **Figure 3.1** (this study).

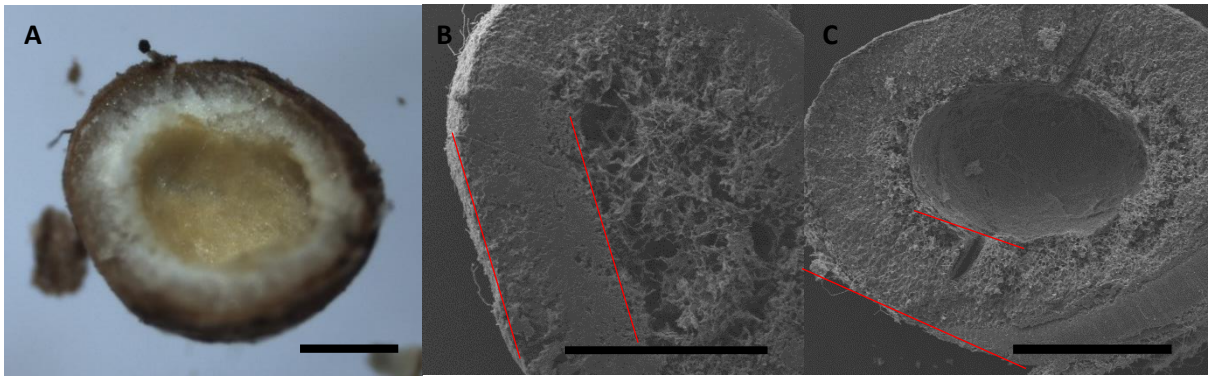

Figure 3.1. Light microscopy and scanning electron microscopy (SEM) of Sclerotia of *Aspergillus niger* demonstrating different structural zones of a sclerotium. A) Light microscopy of a sclerotium demonstrating melanised rind and differentiation of internal tissue and empty inner space. B) SE Micrograph of a sclerotium demonstrating rind and loosely defined cortex and medulla with no clearly defined internal space. C) SE Micrograph of a sclerotium demonstrating rind and differentiation of internal tissue into cortex and medulla and further differentiation of a clear internal empty space. Scale bar for each image is 500 µm. Red lines show approximate zones of tissue differentiation (all images from the present study).

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3.1.2 Genetic Control of Sclerotia Formation

Genes known to have a role in sclerotia formation in the aspergilli include the well described *laeA* and *veA* (velvet complex) genes as well as those involved in oxylipin biosynthesis (*ppo* genes) and others including various transcription factors. Genes linked with sclerotia development are reported as having impacts on conidiation, mycotoxin production as well as sexual development amongst other functions.

The products of *veA* and *laeA* are part of the velvet complex described in *A. nidulans* (Bayram *et al.,* 2008; Bayram and Braus, 2012). Homologs of *veA* are required for sclerotial production in both *A. parasiticus* (Calvo *et al.,* 2004) and *A. flavus* (Duran *et al.,* 2007). Additionally, Δ*laeA* deletion strains in *A. flavus* blocked the production of sclerotia, whilst overexpression increased sclerotia production (Kale *et al.,* 2008). The impact of *laeA* was determined to also govern differences in sclerotia production (Amaike and Keller, 2009) observed when different concentrations of spores were used as inoculum (Brown *et al*., 2008). Multi copy expression of *laeA* but not *veA* showed no difference in production of sclerotia at any spore concentration used, suggesting it is independent of the spore concentration dependent nature of sclerotia formation (Amaike and Keller, 2009). VeA in *Botrytis cinerea* is important for both pathogenicity and sclerotia formation (Schumacher *et al*., 2015).

Deletion of a lipoxygenase termed *lox* in *A. flavus* resulted in formation of increased numbers of sclerotia and is linked to the quorum sensing ability of fungi to generate sclerotia at different spore concentrations (as previously described) (Brown *et al*., 2008). In addition, four oxylipin generating dioxygenases were discovered in *A. flavus* which are homologs of the *A. nidulans ppoA*. These were termed *ppoA, ppoB, ppoC* and *ppoD* (Brown *et al*., 2009). Knockouts of these genes showed that *ppoA* had little effect on sclerotia number, *ppoC* and *lox* were repressors of sclerotia formation, and *ppoD* promotes sclerotia formation (note that the knockouts did not include *ppoB*). The opposition of roles of *ppoC* and *lox* to that of *ppoD* is similar to that seen in the opposing action of *ppoA* and *ppoB* in *A. nidulans* (Tsitsigiannis *et al*., 2004). The data showed that single deletion of *ppo* genes can be masked or compensated by the effect of others (Brown *et al*., 2009). These genes contribute to the governance of morphological transitions in *A. flavus* through the production of oxylipins.

The *pyrG* locus is commonly used as a selectable marker for genetic transformations. A recent study (Ellena *et al.,* 2021a) showed that deletion of *pyrG* in strain ATCC1015 of *A. niger* led to the ability to form sclerotia on media that did not normally support formation of such structures in this strain. Complementation reversed this phenotype. In *A. nidulans*, a *pyrG* mutant was shown to undergo enhanced sexual reproduction and reduced asexual conidiation (Sun *et al.,* 2013). That *pyrG* impairment causes the production of sclerotia and is likely to impact on the production of a raft of

metabolites associated with sclerotia, has important implications for the use of the gene in transformations of the aspergilli as there might be unexpected artefacts in the results. Indeed, *pyrG* deletion has been shown to increase citric acid production by 2-fold in *A. niger* (Zhang *et al.,* 2020).

A basic helix-loop-helix (bHLH) transcription factor called *sclR* has been identified in *A. oryzae* that acts as a positive regulator in the production of a sclerotium. A strain with *sclR* disrupted was found to produce abundant conidia and sparse sclerotia when compared to the parental strain (Jin *et al.,* 2009). Gene overexpression of *sclR* resulted in an increase in the number of hyphal aggregations and subsequent mature sclerotia (~5.5 fold), whilst at the same time delayed colony growth and decreased levels of conidiation (Jin *et al.,* 2011a). Additionally, the overexpression strain resulted in conidiophores that were unusually branched and intertwined. Another putative bHLH from *A. oryzae* called *ecdR* (Early Conidiophore Development Regulator) was shown to have a converse function to *sclR.* The deletion strain of *ecdR* produced very few conidia and abundant sclerotia whilst the overexpression strain produced a greater number of conidia, and at an earlier stage, with a reduction in sclerotia number. A yeast two hybrid assay showed interaction between the two bHLH transcription factors SclR and EcdR (Jin *et al.,* 2011b). SclR and EcdR were found to interact to form heterodimers that were competitive with their respective homodimers and regulated the balance between conidiation and production of sclerotia. Taken together, these results show that *sclR* and *ecdR* have functions in hyphal morphology, conidiophore formation and structure as well as development of sclerotia.

The discovery of an *A. niger* sclerotia-producing mutant termed *scl-2*, led to the elucidation of a $Zn(II)_2Cys_6$ finger binding domain-type transcription factor termed *sclB* following a bulk segregant style genome analysis (Jorgensen *et al*., 2020). Complementation of the mutant with the wild-type *sclB* led to reversal of the sclerotia producing phenotype. Further deletion of *sclB* in other strains of *A. niger* resulted in knock-out mutants which all produced sclerotia *in vitro*. The encoded protein SclB is therefore considered a repressor of sclerotia production (Jørgensen *et al.,* 2020). However, the production of sclerotia in these mutants was still dependent on other factors such as light, conidial density and media type.

Another $Zn(II)_2Cys_6$ finger binding domain type transcription factor from *A. flavus* termed *aswA* has also been shown to be necessary for full maturation of sclerotia. A knockout of this gene resulted in an *A. flavus* strain with an increase in number of sclerotia, but they were immature, irregularly shaped, and non-pigmented (Chang *et al*., 2017). The authors state that the change in sclerotia was due to the impact the absence of the transcription factor has on other unknown genes needed for proper development, as well as the production and storage of sclerotium-specific metabolites (Chang *et al.,*

2017). The presence of immature sclerotia would suggest that the role of *aswA* is downstream of other key development genes for the production of sclerotia.

A calcineurin-response gene *crzA* was shown to be involved in the production of sclerotia in *A. parasiticus*. Two deletion strains termed RHΔ*crzA* and BN9Δ*crzA* both showed decreased sclerotia production. The authors stated that the role of *crzA* is probably linked to unknown responses to physiological stress which has been shown to initiate sclerotial development (Chang*,* 2008).

Reactive Oxygen Species (ROS) have been implicated as being involved in the formation of sclerotia in a number of species (Wang *et al*., 2018; Liu *et al*., 2018; Grintzalis *et al*., 2014; Jorgensen *et al*., 2020). Studies have involved the addition of exogenous ROS inducers as well as the production of ROS at the site of sclerotia production (Wang *et al*., 2018, Georgiou *et al*., 2006). One study of Morels (*Morchella importuna*) showed that superoxide dismutase enzymes linked to ROS were highly expressed during sclerotia maturation, and that addition of hydrogen peroxide could induce sclerotia production (Liu *et al*., 2018). In the case of *A. flavus*, differentiation linked to sclerotia production was associated with a 3.2-fold increase in superoxide dismutase activity and differentiation was inhibited by exogenous antioxidants (Grintzalis *et al*., 2014). A key component in *S. sclerotiorum* are the NADPH oxidases (SsNox1 and SsNox2) which are involved in both the production of ROS and the 'Oxidative burst' associated with the infection process of this fungi on plants (Kim *et al*., 2011). Knockouts of the *noxA* (NADPH oxidase) and *noxR* (NADPH oxidase regulator) genes in *A. niger* abolished sclerotia production (Jorgensen *et al*., 2020).

The gene *fluG* in *A. nidulans* is best known for its role in the control of conidiation. Disruption of *fluG* leads to a fluffy phenotype and subsequent control of *brlA* which is integral to the conidiation central development pathway (Lee and Adams, 1996). In the sclerotia producing species *A. flavus*, deletion of *fluG* was found to lead to a ~30-fold increase in sclerotia production and delayed and decreased conidiation when compared to the parental strain (Chang *et al.,* 2012). By contrast to studies in *A. nidulans*, which lost the ability to produce sterigmatocystin, knockout strains of *fluG* in *A. flavus* retained their ability to produce aflatoxin (Chang *et al.,* 2012).

Deletion strains of the transcription factor *nrdA* (negative regulator of differentiation) in *A. nidulans* resulted in enhanced conidiation and increased production of cleistothecia (Jeon *et al.,* 2009). Conversely, in *A. flavus* and *A. parasiticus*, the deletion of the homolog *msnA* resulted in dense conidiation but a lack of sclerotial production (Chang *et al.,* 2010). The contrast between the production of cleistothecia and sclerotia and mycotoxin production confirm that the roles of both *fluG* and *nrdA* genes are different between species. This contrasting role of other genes may be the case in different members of the aspergilli too.

Finally, orthologs of the *A. nidulans* transcription factors *nsdC* and *nsdD* (Never in Sexual Development), which are necessary for sexual development were found in *A. flavus* and *A. parasiticus* (Cary *et al.,* 2012). Since there had been a long-understood relationship between the processes that govern sexual development and sclerotia development, these transcription factors made good targets for characterisation in *A. flavus*. *nsdC* is a C_2H_2 Zinc finger and *nsdD* is a GATA type zinc finger transcription factor. Deletion strains of these transcription factors showed the phenotype of reduced conidiation and absence of sclerotia when compared with parental strains (Cary *et al*., 2012).

3.1.3. Environmental Control of Sclerotia Formation

Varying external signals such as the pH or type of media, ratio and concentration of nitrogen, carbon and sulfur, presence of certain amino acids (McAlpin 2004; McAlpin and Wicklow 2005), media depth or water content (Nesci 2007) as well addition of linoleic acid (Calvo *et al*., 1999; Brown *et al.,* 2008) have all been shown to alter sclerotial production in a species-specific way in different *Aspergillus* species. Of relevance to the present study, many species of the black aspergilli, including *A. niger sensu stricto*, had not been previously reported to produce sclerotia, which would have been consistent with a purely asexual lifestyle (Frisvad *et al*., 2014). However, it has recently proven possible to induce the production of sclerotia by certain strains of *A. niger* on specific media such as Czapek's yeast autolysate supplemented with raisins. Thus, these studies provide some evidence that upon the addition of certain external signals, sclerotial production may be induced in other species where it has not yet been reported. Indeed, environmental sampling has previously discovered the presence of sclerotia in soils (with no fungi cultured from these), as well as fungi that produce sclerotia in the wild but have not yet been induced to produce cleistothecia in the laboratory (Smith *et al*., 2015). It is also noted that biotin has also been reported to be required for expression of the sexual cycle in *P. chrysogenum*, although this species does not produce sclerotia (Bohm *et al*., 2013).

3.1.4. Diffusible Metabolites and Sclerotia Formation

Diffusible hormones or 'morphogens' have been hypothesised to have an impact on fungal sexual development and have been characterised in a limited number of fungal species**.** Several different chemical sex factors have been identified correlated with fungal sexual morphogenesis, which can be divided into two classes; 'Sexual growth substances' and 'Sex hormones', defined as either those essential as nutrition or those involved in signalling and sexual morphogenesis, respectively (Siddiq *et al*., 1989; Dyer *et al*., 1992). Of particular interest to the present study are 'sex hormones' as these may effectively act as a switch from asexual to sexual reproduction. The connectivity between a sexual switch and sclerotia production within the sclerotia forming aspergilli may be linked or may constitute two separate pathways. The implications are that the sexual cycle or sclerotia production could either be induced, or if competitive analogues to these compounds were produced, could prevent the sexual cycle/sclerotia production. This is particularly true given that many of these compounds are thought to work on a concentration dependent basis.

Many of the identified fungal sex hormones are attractants or 'pheromones'. These have been discovered in water moulds such as Sirenin and Parisin from *Allomyces* (Machlis 1966) or the complementary hormones antheridiol and oogoniol from *Achyla spp* (Gooday 1974). Studies of *Zygomycotina* revealed that sexual development is here regulated by trisporic acids (Gooday 1974), with concentrations as little as 10^{-8} M resulting in a biological response, with each of these chemicals acting in tandem to induce a suitable sexual response.

As well as signalling pheromones there are factors that are responsible for the direct formation of fruiting bodies and sexual morphogenesis, which have been termed 'sex morphogens' (Dyer *et al*., 1992). In the model organism *A. nidulans*, one such factor was discovered which inhibited asexual reproduction and stimulated the formation of cleistothecia. This was termed a 'PSI' factor or 'Precocious Sexual Inducer' and was functional at masses as low as 50 ng (Champe *et al*., 1987). This 'PSI' factor was species specific as it had no effect on other related *Aspergillus* species. Meanwhile, another sex morphogen termed 'SF' has been discovered from the phytopathogen *Pyrenopeziza brassicae.* A lipid extract from mated cultures of *P. brassicae* was shown to induce the formation of immature apothecia (fruiting bodies) and repress asexual sporulation (Ilott *et al*., 1986; Siddiq *et al*., 1989; Tom Pearson, 2021). Of great interest was the discovery that 'SF' was also able to enhance sexual reproduction in six of ten ascomycete species tested (Siddiq *et al*., 1989) therefore demonstrating some inter-species efficacy amongst these factors. However, the bioactive chemical components of SF have yet to be identified.

The exogenous addition of linoleic acid can induce or increase sclerotial formation in some *A. flavus* strains as well as promoting conidiation and sexual spore formation *in A. nidulans* (Calvo *et al*., 1999). This was consistent with earlier studies which had identified that addition of linoleic acid increased perithecial formation in *N. crassa* (Nukina *et al*., 1981). Similar enhancement of production of perithecia and repression of asexual reproduction was observed in *Nectria haemotococca* (Dyer *et al*., 1993). As well as again identifying linoleic acid as a fungal sex factor (likely a sex growth substance), similar bioactivity was observed from lipid extracts from mated cultures in the latter study. When compared to unmated control cultures, lipid extracts from 10-day old, mated culture significantly enhanced the production number of perithecia in crosses but did not induce the production of immature perithecia in unmated cultures to which the extract was added. This would define the extract as a sex factor which it is essential for fruiting body formation but cannot act alone to induce sexuality. Interestingly, in the Dyer *et al*. (1993) study, the mass of linoleic acid needed to produce an effect was 100 µg. The mass of lipid extract from the mated culture, of which linoleic acid could reasonably only be expected to be a fraction, that had an effect was 167 µg. Therefore, it can be speculated that other factors than linoleic acid had an effect in repressing asexual development and promoting sexuality, so a 'sex morphogen' might be present at low levels.

Several endogenously produced and diffusible morphogens from sclerotia have been described. These studies have usually been conducted with the pathogenic *S. sclerotiorum* and *S. minor* which are of interest due to their impact on agriculture. Morphogens have been reported to have roles in pathogen plant interactions (Tanaka *et al*., 1974; Marukawa *et al*., 1975) as well as signalling and germination of mycoparasitic growth towards the sclerotium (Mischke *et al*., 1995). The presence of a darkness induced diffusible morphogen that triggers sclerotia production has been hypothesised previously (Chet and Henis, 1975; Hausner and Reid, 1999) but no conclusive evidence has been put forward. The most likely mechanism is the peroxidation of lipids via the ROS mechanisms discussed in **Section 3.1.2** and previously (Georgiou *et al*., 2000, Brown *et al.,* 2009).

To my knowledge, the addition of factors/extracts produced endogenously by *Aspergillus* species to gauge bioactivity or specifically sclerotia production has only been studied and recorded in *A. flavus* (Brown *et al*., 2008). Similar work has been conducted by Calvo *et al*. (1999) on *A. nidulans, A. flavus and A. parasiticus* but this was using exogenous components such as linoleic acid and as described above, these do not always correlate with whole extract effects. It is therefore of great interest to investigate whether the developmentally specific metabolites that form in sclerotia exhibit bioactivity when extracted and added to crosses or unmated cultures and whether any diffusible morphogens or hormones can drive sclerotia formation.

3.1.5. Aims of Chapter Work

Two overall aims of the present study were to study sexuality in the black aspergilli and investigate fungal sex hormones. Given that the formation of sclerotia is a pre-requisite for sexual development in this group (**Section 1.4** and **Section 1.6.4**), one general aim of this chapter was therefore to identify environmental (and genetic) factors that influence the production of sclerotia in members of the black aspergilli, with the aim of enhancing sclerotial production to optimise sexual reproduction for aspergilli of the petromyces sexusl morph. A second aim was to assess whether sex morphogens associated with sclerotia could be identified. Thus, the following specific aims.

1.) Determination of environmental factors that affect sclerotia production

As discussed in **Section 3.1.3** a range of environmental factors exist have been shown to influence the production of sclerotia in both the black aspergilli and other sclerotia-producing fungi such as *Sclerotinia sclerotiorum*. In this chapter, the impact of factors such as media composition, temperature, concentration of conidial inoculation, pH and a number of chemical additives will be explored.

2.) Characterisation of genes involved in sclerotia production

As discussed in **Section 3.1.2** there is some knowledge about the genetic control of the production of sclerotia although this subject has not been fully explored. In this chapter we will characterise the impact of transcription factor knockouts on sclerotia production, as well as asexual and growth characteristics of several sclerotia producing *A. niger* strains.

3.) Determination of the presence of a chemical sclerotial morphogen

As discussed in **Sections 1.5.4** and **3.1.4**, it has been hypothesised that biochemical sex morphogens may dictate the formation of sclerotia. In this chapter we will explore whether such morphogens can be extracted and used to influence the production of sclerotia in the black aspergilli.

3.2 Materials and Methods

3.2.1. Materials

3.2.1.1. Fungal Strains

Fungal stains of five species were used: *A. nidulans* (**Appendix 5**), *A. fumigatus* (**Appendix 6**), *A. tubingensis* (**Appendix 7**), *A. sclerotiicarbonarius* (**Appendix 8**) and *A. niger* (**Appendix 9**). Strains were maintained as described in **Section 2.2.1.**

3.2.1.2. Media

Many media used in the present chapter have already been described in the general media sections of **Chapter 2.** In addition, the following specialist media were used:

Aspergillus Minimal Media AMM (-N) Glc50 Gln10 (GG10) Agar

Chemical components were 9.9085 g/L D-glucose monohydrate (Fisher, U.K), 1.4614 g/L L-glutamine (Fisher, U.K), 1 mL 1000x Hutner's trace element stock, and 50 mL 20x salt stock, 20 g/L (2 %) Agar (Sigma, U.K).

Add salt stock, glutamine and make up to 0.5 L with distilled water, then adjust pH to 6.5 using NaOH (Fisher, U.K)/HCl (Fisher, U.K). Make up to 1 L with distilled water. Autoclave at 117 °C for 30 minutes.

For the Hutner's (1000x) trace element stock:

For 1 litre, for each trace element, the following chemicals were dissolved in the specified volume of water before mixing. 22 g/ 100 mL zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) (Sigma, U.K), 11.4 g/ 200 mL boric acid (H_3BO_3) (Sigma, U.K), 5.06 g / 50 mL manganese chloride tetrahydrate ($MnCl_2.4H_2O$) (Sigma, U.K), 1.61 g / 50 mL cobalt chloride hexahydrate ($CoCl_2.6H_2O$) (Fisher, U.K), 1.57 g / 50 mL copper sulfate pentahydrate ($CuSO_4.5H_2O$) (Sigma, U.K), 1.10 g / 50 mL ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_20$) (Sigma, U.K), 4.99 g / 50 mL iron sulfate heptahydrate ($FeSO_4.7H_2O$) (Fisher, U.K), 50 g / 250 mL EDTA disodium salt (Sigma, U.K).

All solutions other than EDTA were mixed and brought to the boil. EDTA was then added, and the pH adjusted with hot (~60 °C) potassium hydroxide (KOH) (Sigma, U.K) to bring to pH 6.5. The solution was brought to 1 Litre total volume. The container was stoppered with a cotton wool plug until the

solution turned a violet colour, it was then sterile filtered (to remove precipitate) and aliquoted into 10 aliquots of 100 mL. Store refrigerated within falcon tubes wrapped in foil to prevent UV degradation (Hutner *et al*., 1950).

For the Salt stock (20x): 10.4 g/L potassium chloride (Fisher Scientific, U.K), 10.4 g/L magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Sigma, U.K) and 30.4 g/L monobasic potassium hydrogen phosphate (KH_2PO_4) (Sigma, U.K). Use an initial 800 mL to allow for dissolution and make up to total 1 L after full dissolution. Be sure to add potassium hydrogen phosphate to water and not water to potassium hydrogen phosphate (as clumps may form that are very difficult to dissolve).

Pyrithiamine was used as a selectable marker in this media at a final concentration of 0.1 µg / mL. A 0.1 mg / mL stock (1000 x) was prepared by adding 1 mg pyrithiamine hydrobromide (Sigma, U.K) to 10 mL distilled water, agitating to dissolve, aliquoting and freezing at -20 °C to store prior to use. Stock was added to molten agar after allowing it to cool to 55 °C at 1 µL / 1 mL agar.

Hygromycin was used as a selectable marker in this media at a final concentration of 150 µg / mL. A 150 mg / mL stock (1000 x) solution was prepared by adding 1.5 g Hygromycin B (Fisher, U.K) to 10 mL distilled water, aliquoting and freezing at -20 °C to store prior to use. This stock was added to molten agar after allowing it to cool to 55°C at 1 µL / 1 mL agar.

Aspergillus Minimal Media AMM (-N) Glc50 Gln10 S1.2 (GG10 S1.2.) Agar

AMM was prepared as described above (This **Section 3.2.1.2**) but with addition of 218.6 g of sorbitol (Sigma, U.K) at the same time as the trace elements and glucose. Selectable markers were used in this media as described above for Aspergillus Minimal Media AMM (-N) Glc50 Gln10 (GG10) agar.

Luria Bertani (LB) Broth

Medium contained 10 g/L tryptone (Oxoid, U.K), 10 g/L sodium chloride (NaCl) (Fisher, U.K), 5 g/L yeast extract (Oxoid, U.K). Made up to 800 mL and adjusted to pH 7 with NaOH (Fisher, U.K), then adjusted to final volume of 1 Litre with distilled water. For Solid media (LB Agar) 15 g/L Agar (Oxoid, U.K) were added prior to autoclaving.

Super Optimal with Catabolite repression (S.O.C) Broth

Medium contained 20 g/L tryptone (Oxoid, U.K), 5 g/L yeast extract (Oxoid, U.K), 0.584 g/L sodium chloride (NaCl) (Fisher, U.K), 0.1863 g/L potassium chloride (KCl) (Fisher Scientific, U.K), 0.95 g/L magnesium chloride ($MgCl_2$) (Fisher, U.K), 1.2 g/L magnesium sulfate (MgSO4) (Fisher, U.K), 3.602 g/L D-glucose (Fisher, U.K).

3.2.1.3. Solutions and Buffers

Blocking Solution

Prepared fresh for each 40 mL use, combining 36 mL maleic acid buffer and 4 mL Southern blotting reagent as provided by the manufacturer (Roche, U.K).

Blocking Solution (+ antibody)

Prepared fresh for each 20 mL use, by adding 0.8 µL Anti-Digoxigenin AP Fab-Fragments as provided by the manufacturer (Roche, U.K) to 20 mL blocking solution as described above

Citrate-Phosphate Buffer

Buffer was prepared by combining 800 mL of 0.2 M sodium hydrogen phosphate (Na_2HPO_4) and up to 200 mL of 0.1 M citric acid solution (Record *et al*., 2002; Geib and Brock, 2017). The citric acid solution was slowly added to a stirring solution of the sodium hydrogen phosphate. A pH electrode was used to determine when the mixed solution was at pH 7.3 (this should use about 140 mL of the citric acid) and made up to a final volume of 1 L with distilled water and sterilised by filtration using a 0.22 µm sterile filter (Sartorius, U.K).

Colony PCR Buffer

The buffer was prepared by adding 0.585 g sodium chloride (10 mM NaCl) (Fisher, U.K), 10 mL 1 M pH 7.5 Tris/HCl buffer (to a final concentration of 10 mM) and 0.29g EDTA (Sigma, U.K) (1 mM final concentration) to 800 mL distilled water, dissolving and made up to a final volume of 1 L with distilled water.

Denaturation Solution

Per 1 Litre solution, 20 g of sodium hydroxide pellets (0.5 M NaOH) (Fisher, U.K) and 87.6 g sodium chloride (1.5 M NaCl) (Fisher, U.K) was added to 800 mL distilled water and allowed to dissolve before making up to a final volume of 1 Litre with distilled water.

Depurination Solution

Consisted of 0.25 M HCl solution. Prepared by necessary dilution with sterile distilled water of 13 M HCl solution (Fisher, U.K).

Detection Buffer

For 1 Litre total, 100 mL 1 M Tris/HCl buffer (to a final concentration of 0.1 M) and 5.8 g sodium chloride (to a final concentration of 0.1 M NaCl) were added to 800 mL distilled water. The pH was adjusted to 9.5 using NaOH and made up to 1 Litre total with distilled water.

Elution Buffer

To make up 100 mL elution buffer, 90 mL distilled water was added to 1 mL 1 M Tris/HCl buffer (to a final concentration of 10 mM) and adjusted to pH 7.5. Then adjusted to 100 mL using distilled water and filter sterilise using a 0.22 µM sterile filter (Sartorius, U.K).

Extraction Buffer

For 1 Litre total, 12.1g Tris [(hydroxymethyl) methylamine] (Fisher, U.K) (will be a final concentration of 100 mM), 29.23g sodium chloride (500 mM NaCl) (Fisher, U.K), 14.5 g EDTA (Sigma, U.K) (to a final concentration 50 mM) was added to 700 mL distilled water. The pH 8 was adjusted to pH 8 using NaOH/HCl and made up to a final volume of 1 Litre using distilled water. After autoclaving, β-mercaptoethanol (Sigma, U.K) was added to a final concentration of 10 mM (Gressler *et al*., 2015).

Directly prior to use, a master mix was prepared with the correct ratio of 500 µL extraction buffer, 60 µL 10 % SDS (sodium dodecyl sulfate) (Melford/Fisher, U.K) and 2 µL RNase (Promega, U.K) for 562 µL total volume / Eppendorf (Gressler *et al*., 2015).

For the 10 % SDS solution, 10 g SDS made up to 100 mL with distilled water (10 % w/v). The solution was stored at 28˚C to prevent precipitation.

Hybridisation Solution (Church and Gilberts buffer)

To make 1 Litre of buffer, start with 500 mL 1 M sodium phosphate buffer (final concentration 0.5 M), add 0.29 g EDTA (Sigma, U.K), 10 g bovine serum albumin (1 % w/v) (Sigma, U.K) and 20 g SDS (to a final concentration of 2 % w/v) (Melford, Fisher, U.K). The solution was then made up to 1 Litre with distilled water.

For the sodium phosphate buffer, 7.1 g/L dibasic sodium hydrogen phosphate (50 mM) (Na_2HPO_4) (Fisher, U.K) and 131.25 g/L (1 M) monobasic sodium hydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) (VWR, U.K) were added as described by Church and Gilbert (1984).

Lysing Enzyme Solution

1.3 g of Vinotaste Pro (Novozyme, U.K) and 0.1 g Lytic enzymes (from *Trichoderma harzianum*) (Sigma, U.K) were added to 10 mL osmotic solution (see below). The solution briefly underwent sonication (Soniprep 150, Sanyo, U.K) to aid solubility before being filter sterilised using a 0.22 µM sterile filter. These solutions were prepared fresh prior to use and stored short-term on ice (Lim *et al*., 2012; Daly *et al*., 2017).

Maleic acid Buffer

To make 1 Litre of Maleic acid buffer 11.6 g Maleic acid (to final concentration of 0.1 M) (Sigma, U.K) and 8.77 g Sodium chloride (to final concentration of 0.15 M NaCl) was added to 800 mL distilled water, allowed to dissolve and then the pH adjusted to pH 7.5 using NaOH/HCl before making up to a final volume of 1 Litre with distilled water.

Osmotic solution

To prepare the osmotic solution, 44.73 g potassium chloride (0.6 M KCl) (Fisher scientific, U.K) and 100 mL of 100 mM sodium phosphate buffer were added to 800 mL distilled water. The pH was adjusted to pH 5.7 using NaOH/HCl and the volume made up to 1 L with distilled water before filter sterilising using a 0.22 µM sterile filter (Sartorius, U.K).

For the sodium phosphate buffer, 710 mg/L dibasic sodium hydrogen phosphate (5mM) (Na_2HPO_4) (Fisher, U.K) and 13.125 g/L (100mM) monobasic sodium hydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) (Sigma, U.K) were combined.

PEG8000 solution

To make 100 mL of solution, 0.55 g calcium chloride (50 mM) ($CaCl_2$) and 1 mL 1 M Tris/HCl solution (10 mM final concentration) were added to 60 mL distilled water and allowed to dissolve. The volume as then adjusted to 100 mL using distilled water and 25 g (25 % w/v) PEG8000 (Fisher, U.K) was added. The resulting solution was warmed gently to dissolve, and filter sterilised using disposable 0.22 µM sterile filter (Sartorius, U.K).

Solution A (For Southern Blot)

Solution was made up as Maleic acid buffer (see above) but also adding 0.3 % Tween 80 (3 mL in 1 Litre = 0.3 % v/v) (Sigma, U.K).

Solution A (For Transformation)

To 800 mL distilled water, add 44.73 g potassium chloride (0.6 M KCl) (Fisher scientific, U.K), 5.5 g calcium chloride (50 mM $CaCl_2$) (VWR, U.K) and 10 mL of the 1 M Tris/HCl buffer (to a final concentration of 10 mM in 1 litre). The solution was adjusted to pH 7.5 using NaOH/HCl and made up to a final volume of 1 L with distilled water.

10x SSC Buffer

To make 1 Litre of buffer, 38.7 g anhydrous trisodium citrate (to final concentration of 0.15 M Na_3-citrate) (Sigma, U.K) and 87.7 g Sodium chloride (to final concentration of 1.5 M NaCl) (Fisher, U.K were added to 800 ml sterile distilled water and allowed to dissolve. Then made up to 1 Litre with distilled water.

TE Buffer

To make 100 mL of TE buffer, 2.5 mL 2 M Tris solution (50 mM) was added to 80 mL distilled water together with 0.29 g EDTA (Sigma, U.K) (1 mM), the pH was adjusted to pH 8 using NaOH/HCl and then distilled water added to final volume of 100 mL.

1 M Tris/HCl Buffer

To prepare 200 mL of buffer, 100 mL of 2 M Tris solution and used and added to 80.6 mL 2 M HCl (Fisher, U.K). Stirred thoroughly. Allowed to cool to room temperature before adjusting with further 2 M HCl to a final pH of 7.5. Adjusted to final volume of 200 mL using distilled water.

For the 2 M Tris solution, 48.54 g Tris (hydroxymethyl) methylamine (Fisher, U.K) was added to 150 mL and after dissolving, the volume adjusted to 200 mL using distilled water.

Wash Solution 1 (Southern Blot)

To prepare 1 Litre of wash solution 1, 700 mL distilled water, 200 mL 10x SSC Buffer (to final concentration 2x SSC) and 1 ml SDS (0.1 % v/v) were combined. Then the resulting solution was made up to 1 Litre with distilled water and stored at 28˚C to prevent precipitation.

Wash Solution 2 (Southern Blot)

To prepare 1 Litre of wash solution 2, 800 mL distilled water, 10 mL 10x SSC Buffer (to final concentration 0.1x SSC) and 1 mL SDS (0.1 % v/v) were combined. Then the resulting solution was made up to 1 Litre with distilled water and stored at 28˚C to prevent precipitation.

Wash Solution (For Transformation)

To prepare the wash solution, 44.73 g potassium chloride (0.6 M KCl) (Fisher Scientific, U.K) and 100 mL of a 1 M Tris/HCl buffer were added to 800 mL distilled water. The pH was adjusted to pH 7.0 using NaOH/HCl. The solution was then made up to a final volume of 1 L using distilled water and sterilised by filtration using a 0.22 μM sterile filter (Sartorius, U.K).

3.2.2 Methods

3.2.2.1 Counting Sclerotia

To determine the number of sclerotia produced by a given species, strain or growth condition, it was necessary to count the sclerotia produced. Mature sclerotia were defined as hardened pigmented structures typically around 1,000 µm in diameter. If plates produced ~300 or less sclerotia, whole plate counts were completed. If a greater number than this was produced, plates were divided into quarters and two were counted, averaged (mean of 2) and multiplied by four to give a mean of the whole plate. Plates were 'hoovered' to improve visibility of sclerotia using a vacuum pump set up as described by Ashton and Dyer (2019) (**Error! Reference source not found.** This helped to improve the clarity and reliability of counts by removing conidiophores and revealing sclerotia that may have been obscured. This method has been used extensively for cleistothecia and was modified for sclerotia (Swilaiman *et al*., 2020., Sugui *et al*., 2011., Ashton and Dyer, 2019).

In some instances, the width of sclerotia was determined by calibrating an eyepiece graticule to a stage micrometer under the respective magnification. Sclerotia were then examined under a dissecting microscope and the eye piece graticule was used to determine the width in 'graticule units' and then converted into µm.

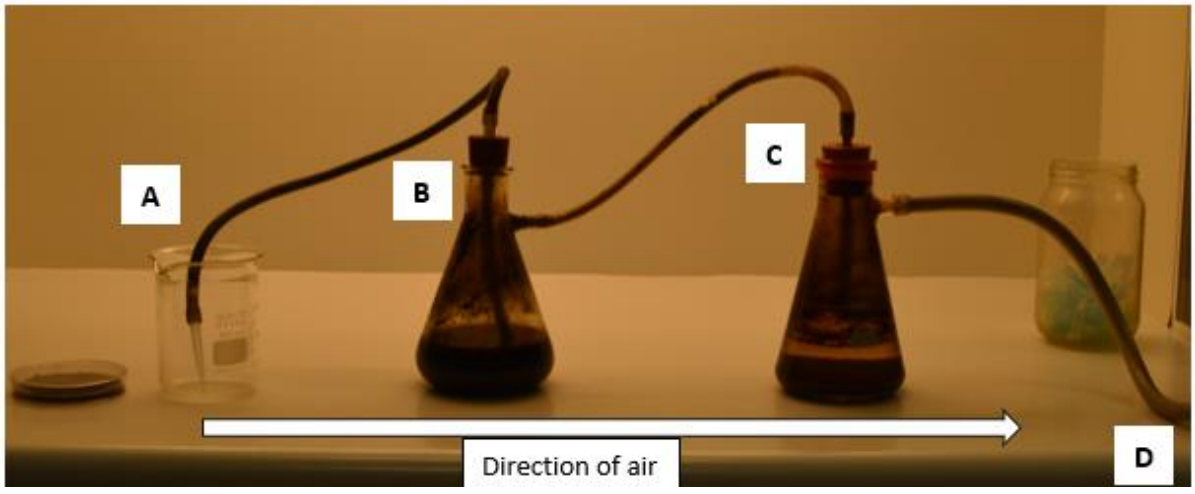


Figure 3.2. Apparatus used to 'hoover' conidia from *Aspergillus spp*. plates to improve ease of counting sclerotia. A) A 1000 µL pipette tip, used as point of contact with conidia. The suction could be precisely targeted, and the opening could be modified to increase diameter and increase suction efficiency, whilst simultaneously avoiding suction of target sclerotia. B) 2 % Trigene disinfectant; tubing moves air flow into solution for submersion and deactivation of conidia. (trigene routinely replaced). C) 100 % Isopropanol. Tubing entering flask is placed above surface of isopropanol to allow unobstructed airflow but deposition of remaining conidia into solvent. Air moving on to D is dry as bubbling is prevented. D) Tubing continues to Whatman 10 µM filter (not shown) to trap any remaining dry conidia, before moving on to vacuum pump. Direction of air flow is shown and moves from A to D.

3.2.2.2. Determining Number of Conidia

In some instances, it was necessary to determine the level of conidiation in a strain or transformation. Each strain or transformant was inoculated onto CYAR using a 5 µL point inoculation of a $1x10^6$ spores / mL suspension (i.e. 5,000 spores). CYAR was prepared with four equidistant raisins and inoculation was completed between the raisin positions. This yielded variation in conidiation between different points, therefore, determination of conidiation at each of these variable points was assessed to compensate for the natural variation in this method. Conidiation appeared dense where the raisin had been on the media, therefore the inoculation point yielded relatively high conidiation at this point and decreased towards the edge of the plate. To determine the conidia count, a sterile 1 mm cork borer was used to cut discs of agar containing the conidiophores (due to variation in conidiation, multiple bored disks were cut and this is described in **Section 3.4.2**) and the agar disc was suspended in 1 ml 0.01 and Tween 80 before agitation. Conidial suspensions were then further diluted (1 in 10 dilutions) before counting with a haemocytometer as described in **Section 2.2.2**.

3.2.2.3. Statistical Analysis of Sclerotia Counts

Sclerotia counts of the black aspergilli were converted into mean number of sclerotia per 9 cm or 5 cm diameter Petri dish. In cases where no sclerotia were produced for a particular isolate across all test conditions these data were excluded from analyses but they are reported.

In most cases, two-way or one-way ANOVA was the preferred statistical test. However, the use of either depends on assumptions being met; the absence of statistical outliers, a Gaussian (normal) distribution and homogeneity of variance. To test these assumptions, GraphPad Prism (version 9) was used. To test for the presence of outliers, the ROUT method was used due to the small sample sizes. To test indications of a normal distribution, the Shapiro Wilk test was performed (Hanusz *et al*., 2016; Motulsky and Brown, 2006).

If both assumptions of absence of outliers and normal distribution were met through the tests, then data was tested using ordinary two-way ANOVA (with Geisser-Greenhouse correction) with Tukeys multiple comparisons test or Welch's ANOVA with Dunnett's T3 multiple comparison test depending on how the data is divided (Herzog *et al*., 2019). Welch's ANOVA and the Geisser-Greenhouse correction allow for data to be used without assessing or assuming homogeneity of variance (Kozak and Piepho, 2018; Royston, 1995).

If the data failed to meet the Shapiro Wilk test for normality of data distribution, then data for a two-way ANOVA was subdivided by strain or single variable. The data was then re-assessed for suitability

for one-way ANOVA. If the data passed the Shapiro Wilk it was subjected to Welch's ANOVA as described above. If the data failed the Shapiro Wilk again then it was subjected to the non-parametric Kruskal-Wallis test with Dunn's multiple comparison test. In certain cases, other tests were used (such as the Mann-Whitney test) but if this was the case it is described in the relevant text.

3.2.2.4 In-Silico Development of Transformation Plasmids

Assemblies for transformation cassettes were generated using the NEBuilder® HiFi Assembly mix as discussed in **Section 3.2.2.5** Primers were designed using the NEBuilder® Assembly Tool (https://nebuilder.neb.com). A full list of primers used for the transformations can be found in **Appendix 3**. Primers were first designed using www.primer3.ut to find appropriate sites for annealing with a Tm ~ 60 ˚C and a GC content between 55-60 %. Further considerations were made so that the start and end of the primers had a GC clamp (GC) where possible and that no more than 3 of the bases at the end of the primer were GGG or CCC to avoid read non-specific annealing. NEBuilder was used to ensure that the end of fragments (such as those that would be generated by the primers just designed) contained the necessary overlaps to put the fragments together. Thus, the primers built by NEB contains two sequences connected by a synthetic 'bridge' sequence. NEBuilder utilises the ends of the fragments to anneal with the ends of separate fragments to ensure overlaps to bring them together. T5 exonuclease digests the 5' end of the dsDNA so that the primers can anneal with the 3' ssDNA overhangs. Hi Fidelity DNA polymerase fills the nucleotide sequences and DNA ligase closes the nick. The synthetic 'bridge' between the annealing ends of the NEB primers can be customised to contain sequences such as Restriction Enzyme sites to (re)generate RE sites in the finished constructs.

To avoid secondary structures, primers were analysed with the aid of the tool: https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/.

Regarding genetic markers used in the present study, *eGFP* and *tdTomato* are both commonly used genes that encode fluorescent proteins used in molecular biology research of the aspergilli (Kaur and Punekar, 2019, Geib and Brock, 2017). The hygromycin resistance cassette (*hph* cassette) and pyrithiamine resistance cassette (Geib *et al.*, 2019) were used as selectable markers. The marker *hph* was first used in *Aspergillus* species by Punt *et al*. (1987) and encodes for a phosphotransferase which inactivates the antibiotic hygromycin through phosphorylation. Pyrithiamine is an antagonist of thiamine and inhibits the production of thiamine pyrophosphokinase (TPK) and subsequently thiamine pyrophosphate (TPP). TPP is needed as a cofactor for many critical enzymes and its lack of production is fatal. The resistance gene *ptrA* was discovered in a mutant of *Aspergillus oryzae* (Kubodera *et al*., 2000) and its functionality ascertained as being due to a change in the promoter sequence whereby the wild type has the sequence **GAAAAGGATCATG** within this region whereas the mutant has **GAAAAGGGTCATG.** Kubodera *et al*. (2000) hypothesised that the resistance conferred may be due to an overproduction of thiamine and the removal of the antagonism.

The gene *akuB* is a *ku80* homolog and was found using the AspGD database (no longer available) and later NCBI database: https://www.ncbi.nlm.nih.gov/nuccore/AM270133.1 shows that An07g05980.

Upstream and downstream 1 kb sections were analysed for use as areas of homology for the construction of *akuB* knockout cassettes. However, due to the proximity of the upstream gene *vWFA* (An07g05970), the 'upstream' section encompasses several nucleotides of the 5' start of the gene itself. PSORT2 (https://psort.hgc.jp/) was used to locate the catalytic section of the gene and this was predicted favourably to fall within the 3' end of the *akuB* sequence (**Figure 3.3**).

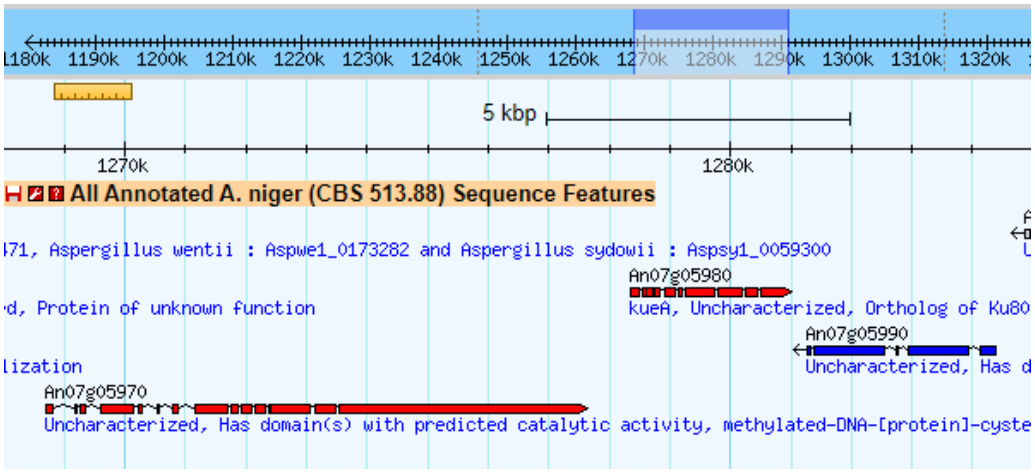


Figure 3.3. Diagram taken from NCBI showing the genomic location of akuB/kueA/An07g05980 within *A. niger* strain CBS 513.88 (Pel *et al.*, 2007).

Fragments were therefore designed using primers shown in **Appendix 3** to avoid fragment overlap with the neighbouring upstream gene. Therefore, the upstream region of *akuB* in this case includes the first two exons of *akuB*, as shown in **Appendix 19**. However, these first two exons of *akuB* are not responsible for functionality of the gene (as discussed above), therefore, their inclusion here will still result in a functional knockout without impacting on the upstream gene *vWFA*. This was achieved using the primers 'AKuB up F' 5'-gccagtgaattcgagctcggta**cccggg**TGGAGAAATGATCGGATCG-3' and 'AkuB up R' 5'-gtaa**gcggccgc**CTAGTCACTCACCATCTGTC-3' and primers 'AkuB down F' 5'-ggtgagtgactag**gcggccGC**TTACCGATATGGACGAG-3' and 'AkuB down R' 5'-tgcaggtcgactctagaggatc**ccc GGG**ATAAGCCTTGTTACTTAG-3' (**Appendix 3**) to generate the upstream and downstream fragments. The pUC19 backbone was designed to be cut at the *Sma*I site for annealing, and the primers regenerated *Sma*I sites (CCCGGG in bold) in the synthetic bridges between the backbone and each fragment respectively, whilst also generating a *Not*I site (GCGGCCGC in bold) between the up and downstream fragments. The resultant pUC19_AkuBup_AkuBdown plasmid is shown in **Figure 3.4.**

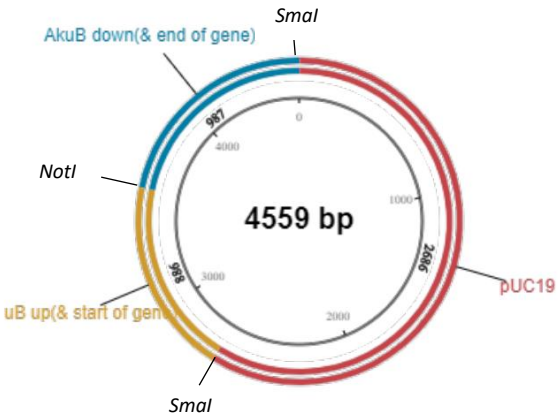


Figure 3.4. The pUC19_AkuBup_AkuBdown plasmid. The plasmid has *Sma*I sites regenerated between the pUC19 plasmid and both the AkuB up and downstream fragments, and a *Not*I site generated between the upstream and downstream fragments.

The resultant plasmid pUC19_AkuBup_AkuBdown could then be cut with *Not*I to generate a new 'backbone' and a further set of fragments could be assembled and integrated into the plasmid whilst keeping the total number of fragments for any one assembly to a minimum of 4. The fragments of the *A. nidulans gpdA* promoter and *trpC* terminator as well as the pyrithiamine resistance cassette *ptrA* used the following primers (also listed in **Appendix 3**):

'gpdA_fwd' 5'-caggacagatggtgagtgactag**gcggccgC**CTTATTCGTTGACCTAGCTG-3' and

'gpdA_rev' 5'-gtcggcatctact**ctcgag**CATTGTGATGTCTGCTCAAG-3', and

'trpC_fwd' 5'-acaatg**ctcgag**AGTAGATGCCGACCGGGATC-3' and

'trpC_rev' 5'-ccgtaatcaagcCAGTGTGATGGAATTCGCC-3' and additionally,

'ptrA_fwd- 5'-ttccatcacactgGCTTGATTACGGGATCCC-3' and

'ptrA_rev' 5'-cccgttctcgtccatatcggtaa**gcggccgc**CGTATTATACTGTCTTTCTTGTTACAC-3'.

The resulting construct is shown in **Figure 3.5**. The 'backbone' consisted of the pUC19 plasmid as well as the *akuB* upstream and downstream. The *akuB* upstream was then annealed with the *gpdA* fragment and regenerated the *Not*I site. The *gpdA* fragment annealed with the *trpC* and generated a novel *Xho*I site between the two fragments. The *trpC* fragment annealed with the *ptrA* cassette but no additional RE site was introduced. The *ptrA* cassette could therefore anneal with the *akuB* downstream fragment and regenerate another *Not*I site between the two fragments. The RE sites are shown in bold in the primers listed above. The generation of a *Xho*I site between the *gpdA* promoter and *trpC* terminator meant that a versatile chassis had been built for the separate introduction of either of the fluorescent protein genes – *tdTomato* and *eGFP*.

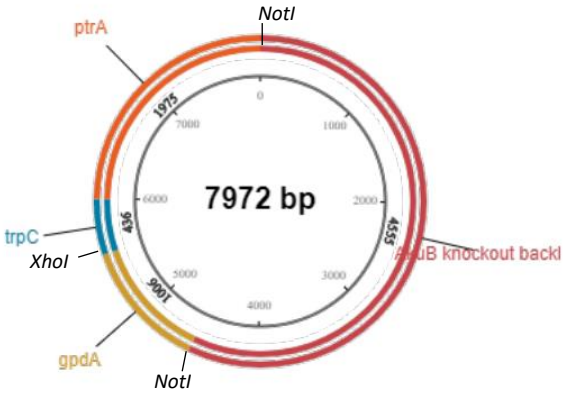


Figure 3.5. The pUC19_AkuBup_PgpdA_TtrpC_ptrA_AkuBdown plasmid. *Not*I sites were regenerated between the AkuB knockout backbone (consisting of *Not*I linearised pUC19_AkuBup_AkuBdown plasmid) and both the *gpdA* promoter and the *ptrA* cassette. A novel *Xho*I cut site was generated between the *gpdA* and *trpC* fragments. The *Xho*I site allowed for versatile and efficient addition of either of the *tdTomato* or *eGFP* fluorescent protein genes. The AkuB backbone contains the up and down regions with respective *SmaI* sites (from **Figure 3.4**) not shown.

Finally, the plasmid at this stage could be digested with *Xho*I at the introduced site and *tdTomato* or *eGFP* could be inserted via use of the HiFi assembly kit at this location using the following primers:

TdTomato_fwd: 5'- gacatcacaatg**ctcgag**ATGGTCTCCAAGGGTGAG-3'

TdTomato_rev: 5'- catctact**ctcgag**CTACTTGTAGAGCTCGTCC-3'

eGFP_fwd: 5'- gacatcacaatg**ctcgag**ATGGTGAGCAAGGGCGAG-3'

eGFP_rev: 5'- catctact**ctcgag**TTACTTGTACAGCTCGTCCATG-3'

The primers also allowed for the regeneration of the *Xho*I site on each site of either of the introduced fluorescent protein genes. The *Xho*I site '**CTCGAG**' for each of the primers is shown in bold above. The resulting plasmids for the introduction of either *tdTomato* or *eGFP* are shown in **Figure 3.6.**

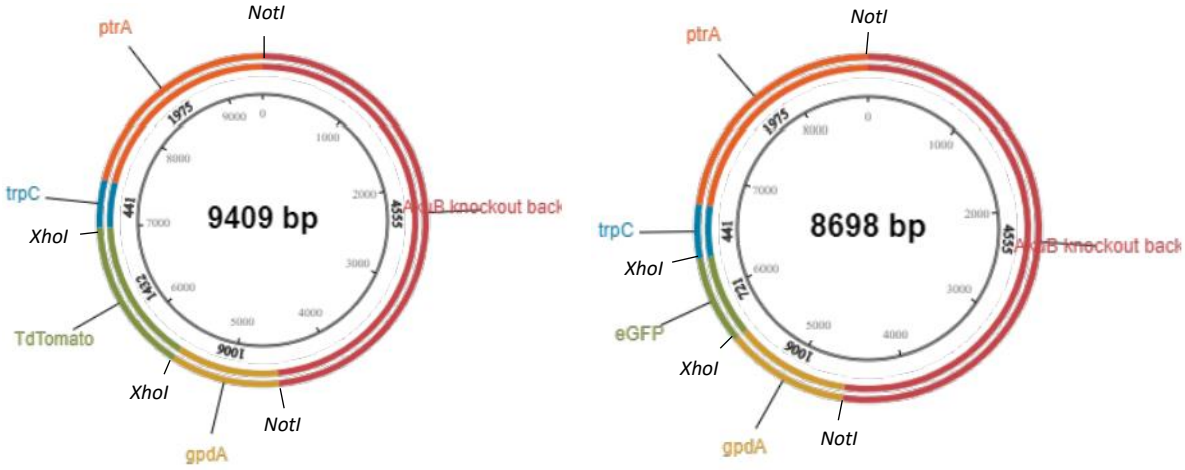


Figure 3.6. The pUC19_AkuBup_PgpdA_TdTomato_TtrpC_ptrA_AkuBdown (L) and the pUC19_AkuBup_PgpdA_eGFP_TtrpC_ptrA_AkuBdown (R) plasmids. *tdTomato* and *eGFP* (both shown in green) were introduced to the previous plasmid (linearised with *Xho*I) using HiFi assembly methodology. *Xho*I sites were regenerated either side of the introduced fluorescent genes. *The AkuB backbone contains the up and down regions with respective* SmaI *sites (from Figure 3.3) not shown.*

Following the generation of the Δ*akuB* plasmids, Δ*sclR* and Δ*sclB* knockout cassettes were designed containing *hph* as a selectable marker. The plasmids were pUC19_sclBup_hph_sclBdown and pUC19_sclRup_hph_sclRdown. Primers were designed to be used to generate the fragments as well as being used in the HiFi assembly of the plasmids. Primers used for the construction of the pUC19_sclBup_hph_sclBdown were as follows:

sclB up P3_fwd: 5'-gccagtgaattcgagctcggta**cccggg**TGGCCGTTGACAACTTGTAC-3'

sclB up P3_rev: 5'-aaga**gcggccgc**TTGAAACCTCACGCTTGC-3'

sclB down P3_fwd: 5'-cgtgaggtttcaa**gcggccgc**TCTTCGCATAGCACCTCC-3'

sclB down P3_rev: 5'-tgcaggtcgactctagaggatc**cccggg**TCTGGGGAAAGGAGATGG-3'

For the construction of pUC19_sclRup_hph_sclRdown primers were as follows:

sclR up_fwd: 5'-gccagtgaattcgagctcggta**ccc*ggg***CTGGGGTGATCTCTCGGTC-3'

sclR up_rev: 5'-gagggccatgaat***gcggccgc***ATGTGGATAGGCTCAAGGG-3'

sclR down Pair2_fwd: 5'-acat**gcggccgc**ATTCATGGCCCTCTCCTG-3'

sclR down Pair2_rev: 5'-tgcaggtcgactctagaggatc**cccggg**ACACTCTTCACAGCCCAC-3'

Both sets of primers allow for the regeneration of the *Sma*I site between each fragment and the plasmid backbone (**CCCGGG** in bold in the primers above) used to open the plasmid pUC19, as well as incorporating a *Not*I site (**GCGGCCGC** in bold in the primers above) between the two incorporated

fragments that could be used to further incorporate the *hph* cassette. The *hph* cassette was retrieved from a plasmid that required *Not*I digestion to release the cassette (Gressler *et al*., 2015). The *Not*I flanked *hph* cassette could then be ligated into the constructed plasmids after they had been digested by *Not*I (**Section 3.2.2.5.4**). The resultant plasmids are shown in **Figure 3.7.**

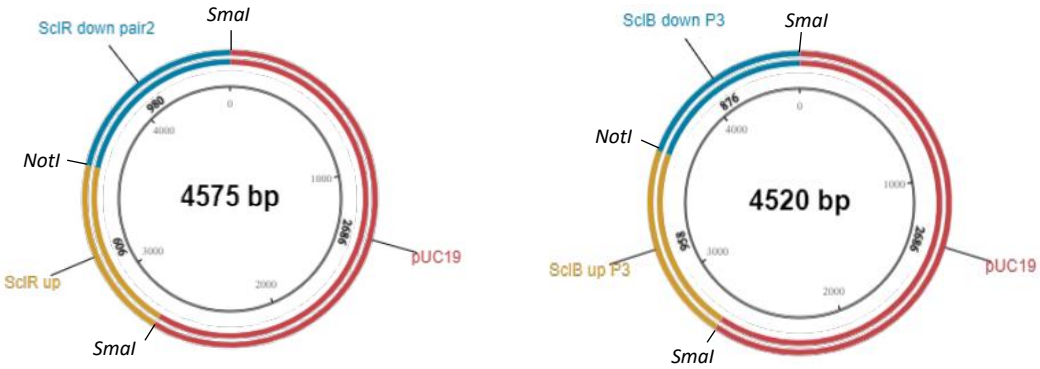


Figure 3.7. The pUC19_SclRup_SclRdown and the pUC19_SclBup_SclBdown plasmids. *Sma*I sites were regenerated between the inserted fragments and the pUC19 backbone. A *Not*I site was generated between the fragments to allow for further digestion, linearisation and later ligation of the *hph* cassette.

For all plasmids, no considerations of frame for constructs were needed. This was due to the inclusion of whole cassettes including separate promoter and terminator for the *ptrA* and *hph* and cassettes inclusive of the promoter and terminator sequences for both *tdTomato* and *eGFP*. There were no fusion proteins relevant to in-frame considerations, so here were not needed.

Serial cloner (https://serial-cloner.en.softonic.com/) was used to check nucleotide sequences of plasmids at all stages and look for restriction sites, and to ensure novel nature to prevent unintended restriction digest sites.

3.2.2.5 In-vitro Construction of Transformation Cassettes and Plasmids

Amplification of the ~1kb upstream and downstream fragments for *akuB*, *sclR* and *sclB* (of *A. niger* strains 160 and 161) and the *gpdA* and *trpC* fragments of *A. nidulans* strain 2.3 was completed using the Phusion® enzyme system according to manufacturer's instructions (NEB, U.K). For each fragment, 2 x 50 µL reactions consisted each of 4 µL 10 ng/µL of gDNA (obtained as described in **Section 2.2.4**), 10 µL 5x Phusion® HF Buffer, 2.5 µL of each of the primers named in **Section 3.2.2.4**, 1 µL 10 mM dNTP's, 29.5 µL nuclease-free water and finally 0.5 µL Phusion® DNA Polymerase. The reaction mixture was vortexed and micro-centrifuged to mix and settle before PCR under the following reaction cycle conditions: Initial denaturation at 98 ˚C for 30 seconds, 32 cycles of denaturation at 98 ˚C for 5 seconds, annealing at 67 ˚C for 30 seconds, extension at 72 ˚C for 30 seconds before a final extension at 72 ˚C for 10 minutes on a Techne TC-512 FTC51F/H2D thermocycler, all steps at maximum ramp rate. PCR products were then run on TAE agarose gel as described in **Section 2.2.7** and purified as described in **Section 2.2.8** and quantified as described in **Section 2.2.9**.

For the plasmid backbone, plasmid puc19 was digested with *Sma*I restriction enzyme (and subsequent reactions with *Not*I or *Xho*I) under the following conditions: 50 µL reactions consisting of ~1 µg (in ~20 µL) pUC19 plasmid, 5 µL (10x) CutSmart™ Buffer, 1 µL Enzyme (*Sma*I/*Not*I/*Xho*I) and made up to 50 µL with approximately 24 µL nuclease-free water. The reaction mixture was incubated at 28 ˚C (*Sma*I) or 37 ˚C (*Not*I/*Xho*I) for 1 hour. All enzymes were inactivated by incubation at 65 ˚C in a heat block for 10 minutes. *Sma*I produces blunt end fragments, so there was little need to prevent re-ligation. However, for *Not*I and *Xho*I, the use of Antarctic phosphatase (NEB, U.K) prior to inactivation was used to prevent self re-ligation. To the 50 µL reaction, 6 µL Antarctic phosphatase reaction buffer (10x), 2 µL Antarctic phosphatase and 2 µL nuclease-free water were added (to a volume of 60 µL) before additional incubation at 37 ˚C for 30 minutes and subsequent deactivation at 80 ˚C for 2 minutes.

The restriction enzyme products were then resolved by TAE agarose gel as described in **Section 2.2.7** and purified as described in **Section 2.2.8** and quantified as described in **Section 2.2.9**.

3.2.2.5.1 NEBuilder

To assemble the fragments into a required plasmid, the NEBuilder HiFi DNA assembly reaction protocol (NEB. U.K) was used. In short, a vector mass of between 50-100 ng was used with a 2:1 ratio of each Insert:Vector. NEBioCalculator was used to determine DNA masses needed: https://nebiocalculator.neb.com/#!/ligation. For example, ~70 ng of pUC19 and ~55 ng of each fragment were added to a suitable volume of water such that the total volume was 10 µL (usually ~7.5 µL nuclease-free water) and added to 10 µL NEBuilder HiFi DNA assembly master mix in a 0.2 mL PCR tube. The reaction mixtures were then incubated at 50 ˚C for 1 hour in a thermocycler for assembly.

3.2.2.5.2 Bacterial Transformation by Electroporation

Once the NEBuilder protocol had been completed, 50 µL aliquots of electrocompetent XL-1 blue (*E. coli*) (Agilent, U.K) were thawed on ice before addition of 2 µL of the resultant reaction mixture from the NEBuilder protocol. The cells and plasmids were then mixed before adding to a chilled sterile electroporation cuvette (Scientific Laboratory Supplies (SLS), U.K) and tapping to settle the mixture. The cuvettes were pulsed once in a MicroPulser (Bio-Rad, U.K) before immediate addition of 950 µL SOC Medium (pre warmed to 37 ˚C) (**Section 3.2.1.2**) and resuspension. The mixture was then transferred to a 2 mL Eppendorf for incubation at 37 ˚C with shaking at 150 RPM for 1 hour. Following this incubation, 5 µL, 20 µL and 50 µL aliquots were plated on to LB Agar plates (**Section 3.2.1.2**) containing ampicillin as the selectable marker compound (Fisher, U.K) to a concentration of 20 µg/mL and incubated at 37 ˚C overnight.

3.2.2.5.3 Restriction Enzyme Test Digest

After incubation overnight at 37 ˚C, ampicillin resistant colonies were numbered and selected using sterile toothpicks and streaked on to fresh LB agar plates containing 20 µg/mL ampicillin which were again incubated overnight at 37 ˚C before being stored as stocks. The toothpick used for streaking was also used to inoculate 10 mL of LB broth (**Section 3.2.1.2**) with a concentration of 20 µg/mL ampicillin. Cultures were incubated at 37 ˚C with shaking at 150 RPM in Falcon tubes with loosened lids overnight.

Once colonies were grown in broth overnight, the cells were collected by pelleting in 2 mL Eppendorf tubes by subsequent rounds of centrifugation at 13,000 RPM and subsequent disposal of supernatant. Once the pelleted cells were collected, the plasmids were extracted using the Nucleospin Plasmid DNA purification kit (Machery Nagel, U.K) according to manufacturer's instructions.

The extracted plasmids were then digested with an appropriate restriction enzyme as described in **Section 3.2.2.5** to conform correct integration, including control test digests. The size of the band produced by running on a TAE agarose gel as described in **Section 2.2.7** should conform to the size of the band described in **Section 3.2.2.4** for each stage of construction. Further fragment additions were then built into the plasmids as described in **Section 3.2.2.4** for ligation of the *hph* cassette.

3.2.2.5.4 Ligation

For the production of the Δ*sclR* and Δ*sclB* plasmids, the selectable marker for hygromycin resistance was introduced via introduction of the hygromycin B phosphotransferase gene (*hph*). The Δ*sclR* and Δ*sclB* plasmids were built as described in **Sections 3.2.2.4**, **3.2.2.5**, **3.2.2.5.1**, **3.2.2.5.2**, and **3.2.2.5.3**. However, to incorporate the *hph* cassette, ligation was necessary at an appropriate restriction enzyme site, therefore after *Not*I digestion of both the *hph* cassette (of flanking regions) and partially built plasmids (with reformed *NotI* sites between up and downstream fragments as described in **Section 3.2.2.4**) ligation was performed with T4 DNA ligase (NEB, U.K) as follows. A reaction mixture of 20 µL was made of 2 µL T4 DNA Ligase Buffer, 1 µL T4 DNA Ligase, and a 3:1 Insert:Vector ratio of DNA ends (this was calculated using NEBioCalculator) typically less than 1 µL each and made up to 20 µL with nuclease-free water. The T4 DNA Ligase was added last. The mixture was mixed and incubated at room temperature for 15 minutes (*Not*I produces 'sticky' ends). Following incubation, the mixture was inactivated at 65 ˚C for 10 minutes. Following ligation, 5 µL of the reaction mixture was transformed into electrocompetent cells as described in **Section 3.2.2.5.2**.

3.2.2.6 Protoplast Transformation of *Aspergillus niger*.

Protoplast transformation of *A.niger* was achieved largely according to the published methods of Daly *et al*. (2017). Pipette tips used during transformations were modified by cutting the ends prior to being sterilised by autoclaving, with the larger diameter of the tips thought to reduce shear forces on delicate protoplasts.

Conidia were prepared by growth of target strains on ACM (**Section 2.1.1**) and subsequent harvesting as discussed in **Section 2.2.2**. Harvested conidial suspensions were inoculated into both **ACM** broth and **YEPD** broth (**Section 2.1.1**) and cultivated overnight (~16 hrs) with shaking at 200 rpm. Mycelia were filtered through sterile Miracloth (Merck, U.K) and washed first with sterile tap water (STW) and then rinsed in citrate-phosphate buffer for osmotic balance. Mycelia was then transferred to 25 mL of citrate-phosphate buffer amended with dithiothreitol (DTT) (Fisher, U.K) (to 10 mM final concentration) in a conical flask for 1 hour at 28°C without agitation. Mycelia was then filtered again through Miracloth and washed again with STW and rinsed with osmotic solution (**Section 3.2.1.3**) for osmotic balance. Then approximately 4 g of wet mycelium was added to 10 mL osmotic solution in a fresh sterile 25 mL conical flask for 1 hour without agitation. To the mycelia and osmotic solution, freshly prepared lysing enzyme solution (**Section 3.2.13**) was added and agitated at 70 rpm at 28°C for between 1.5-3 hours (dependent upon strain). The progress of the cell wall lysing was monitored by periodically taking 10 µL aliquots from the lysing/osmotic solution and observing under a Motic BA310E until sufficient protoplasts (between 5 x 10^7 – 2 x 10^8 / mL) were observed (**Figure 3.8**).

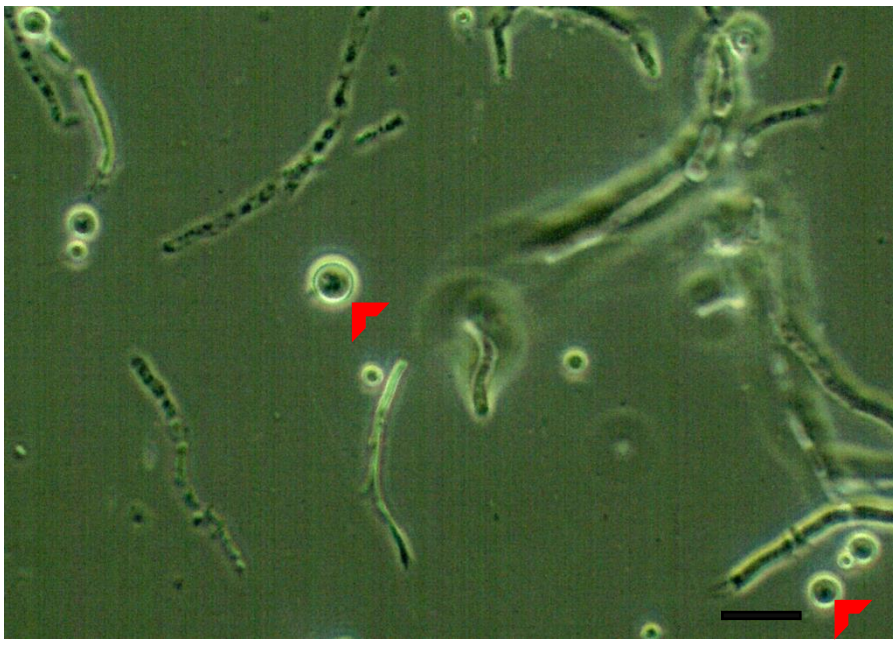


Figure 3.8. Light microscope (Motic BA310E with Moticam 3.0MP and QCapturex64) image showing the production of protoplasts from hyphae using lysing enzymes mix. Protoplasts are indicated by red arrow heads. Scale bar 10 µm.

Protoplasts were purified and stabilised by first filtering the lysing/osmotic solution through sterile Miracloth into an ice-cold Falcon tube before centrifugation at 4°C at 4,000 rpm for 8 minutes using an Eppendorf 5810R centrifuge. The lysing/osmotic solution was then immediately drained from the pelleted protoplasts before resuspension with 20 mL ice-cold wash solution (**Section 3.2.1.3**) and repeat centrifugation at 4°C at 4,000 rpm for 8 minutes. The wash solution was then drained from the pelleted protoplasts before resuspension again in 10 mL ice-cold Solution A (**Section 3.2.1.3**). 10 µL of this solution was used to determine the number of protoplasts as described in **Section 2.2.2** (modified to be counting protoplasts rather than conidia). The protoplasts in Solution A were then centrifuged a final time at 4°C at 4,000 rpm for 8 minutes and the Solution A drained from the pelleted protoplasts before a final resuspension in a volume of ice-cold Solution A that yielded 5 x 10^7 protoplasts / mL (pp / mL).

100 µL of the protoplast suspension (5 x 10^6 pp total) and 5 µL of 1µg / µL of the transformation construct (constructed as described in **Sections 3.2.2.4, 3.2.2.5** and **3.2.2.5.1**) DNA were mixed gently (with a control of 5 µL elution buffer; (**Section 3.2.1.3**). Subsequently 25 µL of ice cold PEG8000 solution (**Section 3.2.1.3**) was added, and the transformation mixture was then incubated for 25 minutes on ice. Further addition of 500 µL ice cold PEG8000 solution with careful mixing followed, with a further 5-minute incubation on ice. Finally, 1 mL Solution A as added and the solutions inverted twice to mix before aliquots of 600, 500 and 400 µL were each added to separate 12 mL molten (48°C) Aspergillus minimal media AMM (-N) Glc50 Gln10 S1.2 (GG10 S1.2.) agar aliquots (**Section 3.2.1.3**) before inversion and immediate pouring on to pre-poured and set 25 mL Aspergillus minimal media AMM (-N) Glc50 Gln10 S1.2 (GG10 S1.2.) agar plates (**Section 3.2.1.3**) with relevant selectable markers. Pyrithiamine and hygromycin resistance were used as selectable markers as described in **Section 3.2.2.7**. Controls (with no added DNA) were added to plates with an appropriate selectable marker (- control) and no selectable marker (+ control). Plates were incubated for up to 6 days at 30 °C (unsealed, incubated in the dark and inverted).

3.2.2.7 Screening of *Aspergillus niger* Transformants using Selectable Marker

Transformation plates (from **Section 3.2.1.2**) were used to select individual colonies. Sterilised toothpicks, wetted by immersion in Tween 80 solution (**Section 2.1.2**), were used to gently touch the asexual conidia of a colony and very gently inoculate these conidia on to a fresh Aspergillus minimal media AMM (-N) Glc50 Gln10 (GG10) agar plate with the appropriate selectable marker. These fresh plates were subsequently incubated at 30°C for at least 3 further days before observation.

3.2.2.8 Screening of *Aspergillus niger* Transformants using Fluorescent Microscopy

Colonies were selected for further screening based on their growth and conidiation, as this was a mark of uptake and incorporation of the *ptrA* resistance cassette to have potentially formed either Δa*ku80*::P*gpdA*$_{(Anid)}$*_TdTomato_*T*trpC*$_{(Anid)}$*_ptrA* or Δ*ku80*::P*gpdA*$_{(Anid)}$*_eGFP_*T*trpC*$_{(Anid)}$*_ptrA* as shown in **Figure 3.7.** These colonies were then selected for growth using a sterile toothpick wetted by immersion in Tween 80 solution (**Section 2.1.2**). The toothpick was gently inoculated with conidia from the centre of each colony and grown in 2 mL YEPD broth (**Section 2.1.1**) in plastic 30 mL universal bottles (STAR Labs, U.K) overnight at 28 °C. YEPD broth was found to give superior results due to its thinner consistency and lighter colour compared with ACM. Mycelium was then scrapped from the toothpick and prepared for observation under fluorescent microscopy by rinsing with distilled water, dab drying with paper roll and mounting. An Epi-fluorescent microscope (L3201LED4) using excitation wavelengths of 488 nm and between 544-581 nm were used to obtain images for eGFP and tdTomato expression respectively (Luis *et al*., 2020 and Schuster *et al*., 2015).

Colonies expressing eGFP or TdTomato as expected were selected for further screening by colony PCR.

3.2.2.9 Screening of *Aspergillus niger* Transformants using Colony PCR

To screen for correct integration of the Δ*ku80*::*ptrA* cassettes, colony PCR (Bergkessel and Guthrie, 2013) was completed on each of the strains that had completed previous screening rounds. To achieve colony PCR, mycelia was scraped from the edge of freshly grown colonies with care to avoid obtaining spores. The mycelium was added to 98 µl colony PCR buffer (**Section 3.2.1.3**) and 2 µL 1 M sodium hydroxide (final concentration of 20 mM) in 0.2 mL PCR tubes (STAR lab, U.K) and heated to 100 °C in a Grant dry block thermostat (Grant Instruments, U.K) for 10 minutes before being cooled on ice. 2 µL of this solution was then used as template for a PCR reaction with Phire Hot Start II DNA Polymerase (Fisher, U.K) under the following conditions: 2 µL DNA template solution, 10 µL Phire Buffer, 1 µL 10 mM dNTP's, 2.5 µL of 10 µM of each of three primers, made up to 49 µL reactions in PCR tubes before final addition of 1 µL Phire enzyme. Colony PCR conditions were: initial denaturation at 98 °C for 30 seconds, followed by 35 cycles of the following; denaturation 98 °C for 5 seconds, annealing 55 °C for 5 seconds, extension 72 °C for 15 seconds. Then a final extension of 72 °C for 1 minute on a Techne TC-512 FTC51F/H2D thermocycler, all steps at maximum ramp rate. An untransformed parental colony was used a negative (-) control.

PCR products were visualised after gel electrophoresis using TAE buffer as described in **Section 2.7**. Colonies showing an 847 bp product (+ transformant) and no 627 bp product (wildtype) were selected for Southern blot analysis. **Table 3.1** and **Figure 3.9** show the primers used for the colony PCR and their respective targets in both transformed and wild-type genomic DNA.

Table 3.1. The names and primers and respective sequences used for Colony PCR with the reverse primers showing the size of the product against the forward primer.

Primer name	Sequence	Product size
AkuB upstream F	atttcgccaacaaaggaccc	n/a
AkuB gene conf R	ttagcggtttctggacgttc	627bp
ΔAkuB gpdA conf R	cacaccagcctttccacttc	847bp

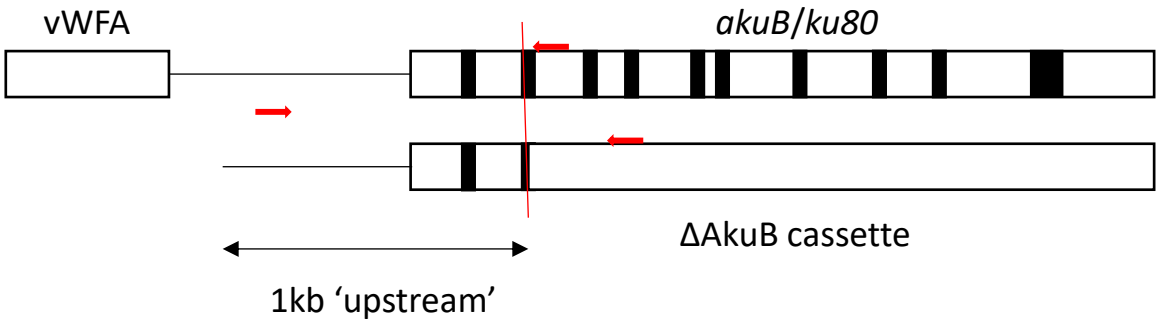


Figure 3.9. Diagram showing the targets and results of the Colony PCR screening for *Aspergillus niger* transformants. Red arrows show the primer sites. The forward primer is in the intergenic sequence of the 1kb 'upstream' sequence that both untransformed and transformed colonies share. The reverse primers are either for confirmation of *akuB* (AkuB gene conf R) yielding a product of 627 bp (binding to the third exon of the AkuB gene) or confirmation of integration of the ΔAkuB cassette (ΔAkuB gpdA conf R) yielding a product of 847 bp (binding to the *gpdA* promoter). *vWFA* is the upstream gene of *akuB* in *Aspergillus niger* CBS 513.88 (NCBI). Red line shows site of the end of the intended homologous recombination. Black bands indicate introns within the endogenous wildtype akuB sequence and the sequence upstream of the site of the intended homologous recombination.

3.2.2.10 Isolation of Genomic DNA of *Aspergillus spp*. for use in Southern Blot

DNA was prepared for Southern blot hybridisation using a method modified by that described by Dellaporta *et al*., (1983). Although RNAse was used in the extraction buffer, this method usually produced high levels of RNA, therefore the term 'nucleic acid' is used in place of 'DNA'. Strains of interest were grown in ACM broth overnight at 28 ˚C with 150 rpm agitation. Mycelia was then harvested from broth by filtration using Miracloth, briefly washed with sterile distilled water and pressed dry with paper roll. The mycelium was then transferred to a mortar and frozen with liquid nitrogen, before grinding to a fine powder with a pestle. Without allowing the powder to thaw, use it to fill a 2 mL Eppendorf to the 400 µL mark and add 562 µL of prepared extraction buffer (**Section 3.2.1.3**), followed by vortexing to wet and mix. The samples were then incubated for 15 minutes at 65 ˚C using a Grant dry block thermostat (Grant Instruments, U.K) with periodic vortexing. After incubation, the samples were placed on ice and 160 µL of 5 M Potassium acetate (Fisher, U.K) was added per sample. The samples were then vortexed and incubated on ice for a further 5 minutes before centrifugation at full speed for 10 minutes at 4 ˚C using a Thermo Heraeus Pico 17 centrifuge to separate cellular debris from dissolved nucleic acids. After centrifugation, the clear supernatant (containing nucleic acids) was transferred to a fresh Eppendorf containing 700 µL of isopropanol (Fisher, U.K) and then inverted slowly 30 times to thoroughly mix. This step precipitated DNA/RNA but to increase yield, the samples were then chilled at -20 ˚C for 20 minutes. After chilling, the samples were centrifuged for 10 minutes at 10,000 rpm to pellet the nucleic acids. After centrifugation, the supernatant was completely removed, and the nucleic acids were resuspended in 400 µL TE Buffer (**Section 3.2.1.3**) and incubated at 60 ˚C in a heat block until fully dissolved. To the dissolved samples were added 55 µL 3 M Sodium acetate (VWR, U.K) and subsequent centrifugation at full speed for 5 minutes to further purify nucleic acids from polysaccharides. The nucleic acids remain in the supernatant which is transferred to a fresh Eppendorf containing a further 500 µL isopropanol. The sample/s were then inverted gently 30 times and chilled again, this time at -20 ˚C for 10 minutes before centrifugation at 10,000 rpm for 10 minutes. Following the centrifugation, the supernatant was discarded, and the pellet was washed with 400 µL 70 % ethanol before a final centrifugation: 10,000 rpm for 10 minutes. After the final centrifugation, the supernatant was discarded, and the washed pellet of nucleic acids was air dried for 10 minutes. After this drying step, the pellet was redissolved in 60 µL elution buffer (**Section 3.2.1.3**). Samples were incubated at 60 ˚C for 30 minutes and lightly vortexed to aid dissolving of the nucleic acid pellet. Quality of nucleic acids could be checked by running 5 µL nucleic acid on a suitable TAE gel and subsequent visualisation as described in **Section 2.2.7**. Good quality was evaluated by lack of smear and tight banding of gDNA. DNA was quantified as described in **Section 2.2.9** and all samples standardised to an approximately equal concentration using nuclease free water prior to Southern blot analysis.

3.2.2.11 In Silico Determination of Suitable RE for Southern Blot Analysis

To generate fragments of DNA of differing sizes that would hybridize with the probe, a suitable restriction enzyme needed to be used. Analysis of the 5 kb regions upstream and downstream of the *akuB* upstream probe hybridization site allowed for analysis of predicted fragments containing the probe that were less than ~5 kb and was required to identify different restriction enzyme sites and which would need to generate differential sizes of fragment between successfully cloned and wild-type sites. **Appendix 20** shows the nucleotide sequences of the ~5 kb genetic sequence of *A. niger* CBS 513.88 comprising the upstream (2 kb), probe hybridisation (~1 kb) and downstream (2kb) regions. The sequence was obtained from the AspGD Database (www.aspgd.com; sadly, no longer available). The probe hybridisation site is underlined. *Bam*HI (sites highlighted in yellow) was selected as in-silico it was predicted to generate 1,812 bp fragments for the wild type (WT) and 3,891 bp and 3,180 bp fragments for the *tdTomato* and *eGFP* constructs respectively. *Bam*HI also had the benefits of a standard incubation temperature (37 ˚C) and generated fragments less than 5 kb that could resolve easily for improved resolution of expected band sizes in Southern blotting.

*Bam*HI digestion is at the location of the asterisk in the following sequence 5-G*GATTC-3 (**Appendix 20**). For all three sequences examined (WT, *tDTomato* and *eGFP*) the upstream site of the hybridisation site was 123 bp before the probe. This falls within an exon of the upstream gene *vWFA* and improves the probability of it being conserved amongst different wild-type *A. niger* strains and was predicted to be present in all WT strains and constructs. The *Bam*HI restriction site for the *tdTomato* and *eGFP* constructs is located 11 bp within the *ptrA* resistance cassette. Therefore, the only difference in size between the TdTomato and eGFP constructs was predicted to be in the difference in size of the respective fluorescence genes for each construct. *In-Silico* analysis of the WT show a *Bam*HI site 812 bp following the end of the probe hybridisation site. This is located within the 7th exon of the *akuB* gene and has an increased probability of conservation between WT strains whilst being expected to have been removed from successful constructs of either *tdTomato* or *eGFP* in all WT strains.

3.2.2.12 Southern Blot of *Aspergillus niger* Transformants

DNA was prepared for Southern blot analysis as described in **Section 3.2.2.10** (Dellaporta *et al*., 1983). Generation of the Southern blot probe used the primers for generation of *akuB* upstream ~1kb fragment and the probe was obtained by PCR using the following oligonucleotides:

AkuB upstream forward: 5- gccagtgaattcgagctcggtacccgggTGGAGAAATGATCGGATCG -3 and

AkuB upstream reverse: 5-gtaagcggccgcCTAGTCACTCACCATCTGTC -3.

Generation of the probe is based on non-radioactive digoxigen-11-dUTP in PCR incorporation as described by Green and Sambrook (2021). Benefits of this approach over the biotin labelled probes is that digoxigenin is not present in fungal cells and there is therefore no background compared to endogenous molecules such as biotin.

Thermopol Taq polymerase (NEB, U.K) was used for PCR amplification of the Southern Blot probes.

In advance, a 10 mM mixed dNTP (Invitrogen, Fisher, U.K) set with a ratio of A:C:G:T – 1:1:1:0.7 was prepared. Generation of the Southern Blot probe used volumes of 1 µL of 30 ng/µL (30 ng) of the respective linearised deletion cassettes (as described in **Section 3.2.2.5** and **Section 3.2.2.5.1**) as a template, 1 µL of each primer as seen in **Table 2.2**, 5 µl Thermopol Buffer and 1 µL of the prepared 10 mM dNTP set. 0.5 µL DIG-UTP should be added to probe reactions only and not negative controls. Reactions were made up to 49.5 µL /reaction and finally 0.5 µL Thermopol Taq Polymerase was added to each reaction.

Conditions for the PCR amplification and incorporation of DIG-UTP probe were as follows: initial denaturation of 95 ˚C for 30 seconds followed by 35 cycles of the following: denaturation at 95 ˚C for 30 seconds, annealing at 54 ˚C for 30 seconds, extension at 72 ˚C for 60 seconds, and a final extension of 72 ˚C for 5 minutes on a Techne TC-512 FTC51F/H2D thermocycler, all steps at maximum ramp rate.

After PCR, the probe was run on a TAE agarose gel as described in **Section 2.2.7**. Excision of the gel piece containing DIG-UTP probe and subsequent purification (described in **Section 2.2.8** with additional column incubation at 70 ˚C for 10 minutes) was completed. The probe DNA was quantified as described in **Section 2.2.9** and diluted where necessary such that its concentration was 50-200 ng / µL.

Table 2.2. Primers used for the genration of the ~1 kb (917 bp) upstream *akuB* probe for Southern Blot analysis.

Primer name	Sequence (annealing sequence in capitals)	Reverse complement
AkuB upstream F	gccagtgaattcgagctcggtacccgggTGGAGAAATGATCGGATCG	CGATCCGATCATTTCTCCAcccgggtaccgagctcgaattcactggc
AkuB downstream R	gtaagcggccgcCTAGTCACTCACCATCTGTC	GACAGATGGTGAGTGACTAGgcggccgcttac

DNA hybridization was carried out as described previously (Garcia *et al*., 2008).

DNA obtained from WT and transformants using the protocol from **Section 3.2.2.7** was digested with a suitable restriction enzyme e.g. *Bam*HI, (see **Section 3.2.2.11**). As a positive control, 1 ng of deletion cassette plasmid was also digested (made up to 20.5 µL volume with nuclease free water). Restriction enzyme digestions used 20.5 µL DNA of WT and transformants, with concentrations approximately standardised as described in **Section 3.2.2.10** and 2.5 µL of buffer and 2 µL restriction enzyme (to a total reaction volume of 25 µL). Reactions were incubated at 37 ˚C either overnight (WT and transformant gDNA) or for 1 hour (deletion cassette plasmid). Digested DNA was then resolved on a 0.8% agarose gel [volume of 130 ml containing 5.5 µL ethidium bromide (at a reduced final concentration of 0.042 µL / mL) in a 15 x 15 cm tray] for up to 3 hours at 100 V. Gels were visualised as described in **Section 2.2.7**, with the addition of a UV ruler as a marker for later comparisons.

Agarose gels containing digested DNA samples were then subjected to several rounds of washes with appropriate buffers. Buffers were poured from the gel and container after each wash and gels were briefly rinsed with distilled water and drained before next round. The buffers washes were as follows, with agitation: 2 x 7.5 minutes washes with depurination buffer (**Section 3.2.1.3**), 2 x 20 minutes washes with denaturation buffer (**Section 3.2.1.3**), and 2 x 15 minutes with neutralisation buffer (**Section 3.2.1.3**)**.** The gel was then transferred to a pre-prepared Hybond Nylon membrane (Cytiva) on a Southern blot transfer vacuum (Cytiva Amersham™ Vacugene™ XL). The transfer vacuum was prepared beforehand by wetting of membrane with distilled water and placing on top of the 'gel supporting screen' and underneath the blue plastic mask as shown in **Figure 3.10.** Care was taken to ensure correct orientation of lanes, and that the ladder was marked on the nylon membrane in pencil, so orientation could be determined later. The agarose gel containing the digested DNA was then placed on top of the nylon membrane as well as 1 cm of the periphery of the blue plastic sheet. Care was taken to make sure that the agarose gel wells were not directly over the nylon membrane, but were over the blue plastic mask, as this allowed for a more stable vacuum. Once the gel was in place, the vacuum was switched on and the gel was immediately covered with 10 x SSC Buffer (**Section 3.2.1.3**), and the vacuum was maintained at 5 Hg using the controls on the unit provided. Care was

taken to ensure that there were no air bubbles between the gel and the nylon membrane beneath it and that there was a good fit under vacuum pressure. The Vacugene™ XL was then left running for 1 hour with periodic checking to ensure the surface of the gel did not dry.

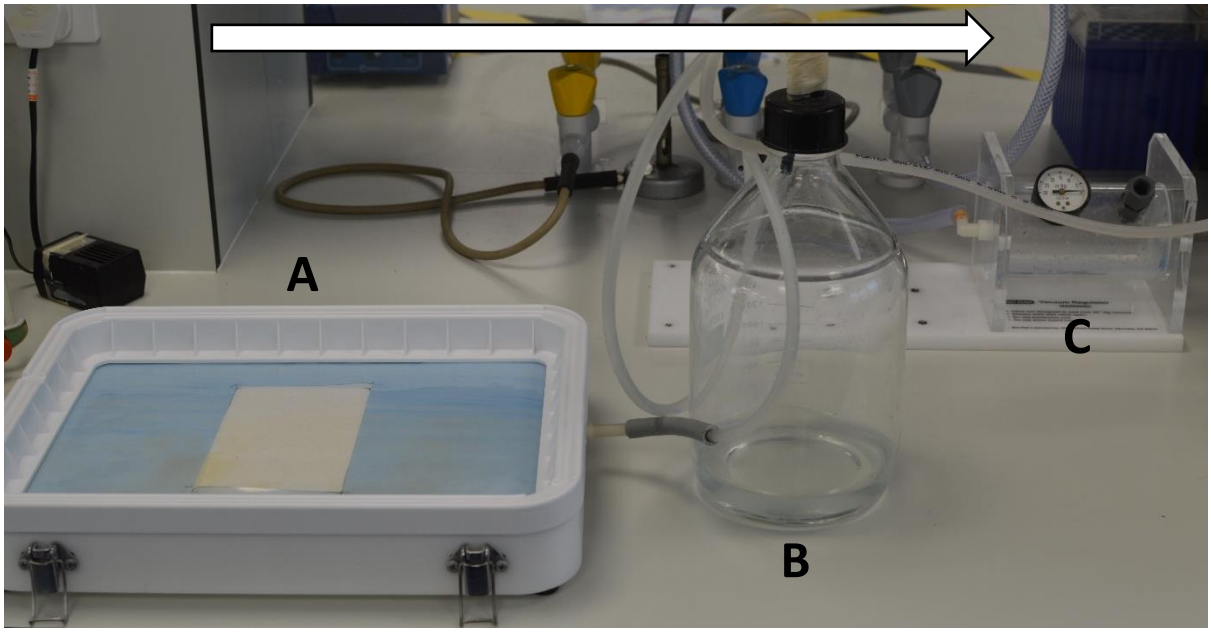


Figure 3.10. Image showing the Southern blot transfer vacuum set up. The nylon membrane should fit neatly in the 'window' of the blue plastic such that it will allow for a vacuum to form when the 'window' is covered with the agarose gel. This allows for the DNA from the agarose gel to be pulled down through the agarose gel and transfer on to the nylon membrane beneath it whilst maintaining a vacuum. The arrow shows the direction of airflow which creates a vacuum in the Vacugene™ transfer vacuum. A) Vacugene™ transfer vacuum base unit with blue plastic mask allowing for vacuum pressure to pull through the 'window' where the agarose gel sits. B) 940 mL liquid trap used to collect 10 x SSC buffer pulled through tubing by the vacuum. C) Vacuum control unit with Hg monitor (maintained at 5 Hg). Minor alterations in pressure can be made using the release valve in grey.

After the DNA had been transferred to the Nylon membrane, the gel was discarded, and the nylon membrane placed on filter paper and then DNA crosslinked onto the membrane using a GS Gene Linker® UV Chamber (Bio-Rad, U.K) on programme 3 (for wet Southern membranes). After crosslinking, the nylon membrane was trimmed to removed wasted space and placed into hybridization tubes (Fisher, U.K) with 20 mL hybridisation Solution (**Section 3.2.1.3**). Care was taken to ensure that there were no bubbles between the membrane and the tube before the tube was sealed and placed in a pre-warmed (65 ˚C) hybridisation oven SI30H (Stuart, U.K) with rotisserie for 1 hour. The DIG-UTP incorporated *akuB* upstream probe was then incubated for 95 ˚C in a heat block for 10 minutes, and then immediately transferred to an ice bath. Once the 1-hour hybridisation is complete, 10 µLof the Southern Blot probe was added (from 50-200 ng / µL to a final concentration of 25-100 ng / mL) and the mixture left to incubate overnight at 65 ˚C.

Following an overnight incubation with the probe, the membrane and hybridisation tube (herein referred to more simply as membrane) were drained of hybridisation solution (which could be reused). The solutions used in previous steps were removed before the addition of new solutions. The subsequent incubation steps were completed within the rotisserie. The following stringent washing steps were made at 65 ˚C: 2 x 20 mL wash solution 1 (**Section 3.2.1.3**), for 5 minute each and then 2 x 20 mL wash solution 2 (**Section 3.2.1.3**) for 5 minute each.

The following treatments were then all completed at room temperature, with timings and solution substitutions. The nylon membrane was incubated and keep moist with rotation on the rotisserie with 20 mL Solution A (**Section 3.2.1.3**) for 5 minutes, 20 mL maleic acid buffer (**Section 3.2.1.3**), for 2 minutes, 20 mL Blocking Solution (**Section 3.2.1.3**), for 30 minutes before replacement with 20 mL blocking solution (+ Antibody) (Anti-Digoxigenin AP Fab-fragments) (**Section 3.2.1.3**). After incubation with the antibody Fab-fragments, 2 x 20 mL Solution A for 10 minutes each, 20 mL maleic acid buffer for 5 mins and finally 20 mL detection buffer (**Section 3.2.1.3**) for 5 minutes.

Following the detection buffer step, a clear plastic wallet was cut out such that only one side remained sealed. The nylon membrane was carefully dried on paper roll and then the membrane slid into the plastic wallet and a few drops of CDP Star Reagent was added (Roche, U.K) to the membrane and evenly spread across the membrane, before dabbing with paper roll to remove excess before sealing the plastic wallet with tape.

Nucleic acids on the membrane were then visualised using the Bio-Rad ChemiDoc XRS+ together with the Quantity One 4.6.9 program (BioRad, Hemel Hempstead, Hertfordshire, U.K) with the filter switched to the **CHEMI** setting. The Iris was fully opened and the **CHEMI HI SENSITIVITY** option selected. This allows for detection of the chemiluminescent signal produced by CDP Star Reagent when it binds to the digoxigenin and anti-digoxigenin AP Fab fragment-labelled sites. The membranes

were orientated in the same way as the post restriction enzyme digest gel, this allowed for direct comparison of the two images. After selecting 'Live Acquire' the settings selected were; total exposure: 1800, starting exposure: 180 and number of exposures: 10. This allowed for a gradual increase in sensitivity of the image.

Post visualisation, the membrane could be manually developed using NBT/BCIP substrate solution (Fisher, U.K). The combination of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) yields an intense, insoluble black-purple precipitate when reacted with alkaline phosphatase (AP), supplied by the Anti-DIG AP Fab-fragments. A few drops of solution were added, spread evenly over the membrane, and left to incubate in the dark overnight. The membrane then showed the sites of probe hybridisation in visible light.

3.2.2.13 Metabolite Extraction and Enrichment

For metabolite extraction, whole plate cultures on agar media (grown on 9 cm diameter Petri dishes) were harvested and covered with a suitable volume of 100 % ethyl acetate (Fisher Scientific, U.K) or a 2:1 ratio mixture of chloroform:methanol (C:M; Sigma, U.K and Fisher, U.K respectively) (typically 200 mL for 10 x 9 cm Petri dishes). The cultures and solvents were then partially homogenised using an Ultra Turrax T25 (IKA Labortechnik) and left to allow for lipid metabolite extraction for 1 hour. The supernatant was then poured off and a second wash of the homogenate with 100 mL of 100 % ethyl acetate or 2:1 C:M was completed. For each solvent mixture, the two supernatants (first and second wash) were combined and filtered through Whatman no.1 filter papers. Sodium sulfate anhydrous (Fisher Scientific, U.K) was added and swirled to remove water content until clumping no longer occurred, indicating that total water/aqueous content had been removed. A second filtration step using Whatman no.1 filter papers (with a small amount of sodium sulfate) separated the supernatant from hydrated sodium sulfate and ensured another round of dehydration, and the supernatant was then collected in a 25 ml volume round bottomed glass flask. The supernatant was then concentrated using a rotary evaporator (Buchi RotaVapor R-124) set at 40 °C and 100 mBar until almost dry.

The concentrated supernatant and the dry contents of the round bottomed flask were then re-dissolved in either 10 mL of ethyl acetate or 10 mL of the 2:1 C:M mixture and aliquoted into 75 mm borosilicate glass test tubes (VWR, Germany). These aliquots were then concentrated to dryness in a Savant™ SPD131DDA SpeedVac™ Concentrator (Fisher Scientific, U.K). The dry mass was then measured and a final addition of ethyl acetate or 2:1 C:M of defined volume as made to bring the extraction to a set concentration (usually 10 mg/ml) before bottling in glass vials and treating the headspace with nitrogen gas to prevent oxidation, before storage at -20 °C.

3.3 Results

3.3.1 Identification of Environmental Factors that Impact Sclerotia Development in the Black Aspergilli

As discussed in **Section 3.1.3**, various environmental and chemical factors have been shown to have an impact on development of sclerotia. A number of these were investigated in relation to the black aspergilli to make attempts to maximise sclerotia production in advance of attempted sex in the black aspergilli (which will be discussed in **Chapter 4**). The protocols for counting sclerotia were described in **Section 3.2.2.1**. The improved clarity of these counts after hoovering is demonstrated in **Figure 3.11**.

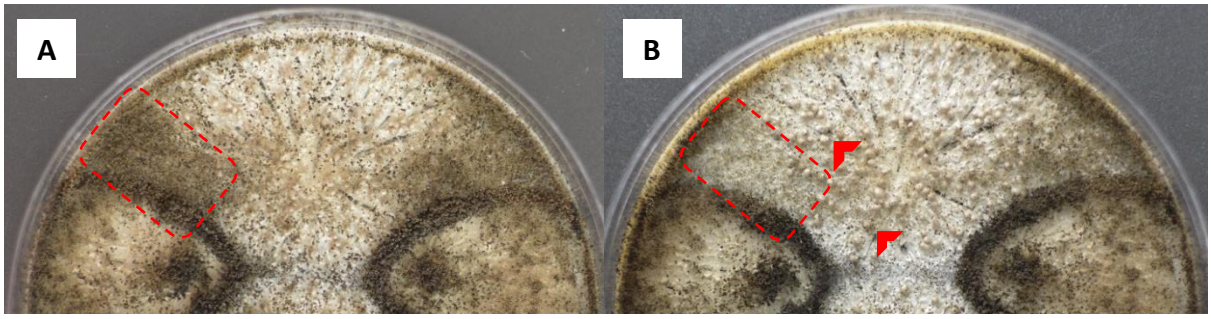


Figure 3.11. Light photograph showing typical *A. niger* Petri plate (9 cm diameter) with both dark brown/ black conidia containing conidiophores and pale white/grey sclerotia. Image A shows many conidiophores obscuring sclerotia and making counting difficult. Image B shows the same plate after 'hoovering' illustrating the function in making it easier to count sclerotia after hoovering to remove obscuring conidiophores. An area of abundant conidiophore growth which was cleared by the hoovering is indicated by the areas within the dashed red line boxes. Several sclerotia are indicated by the red arrowheads in image B.

For each of the following experiments, a base media of CYA with 3 Raisins (CYAR) was inoculated with a single drop of 5 µL of concentration 1 x 106 conidia / mL. This was then incubated for 24 hours at 28 °C for 24 hours before being wrapped with parafilm and returning to incubation at 28 °C for a further 13 days. Each of the variations below change at least one variable to analyse and improve upon sclerotia production.

Impact of media type on *A. tubingensis*

An assessment of which media would promote sclerotia formation in *A. tubingensis* was completed. It used media which had previously been shown to generate sclerotia in this species [CYAR, malt extract agar (MEA)] as well as widely used standard lab media to induce sexual reproduction in the aspergilli [Czapeks yeast autolysate (CYA), oatmeal agar (OMA), mixed cereal agar (MCA)]. The amendment of each with the addition of Sun Maid raisins was also trialled as described previously in

Section 3.2.2.1 and **3.2.2.2**. Four representative *A. tubingensis* strains were used for this study (76-9, 76-45, 76-46, 76-47). Sclerotia production was rarely seen at the site of the raisin location forming a non-sclerotia shadow. This is in keeping with results seen for *A. niger* and illustrated in **Figure 3.32**. When data was assessed, multiple outliers were found (ROUT). In addition, the data failed the Shapiro Wilk normality test. Therefore, the data was broken down into assessment within each strain. Welch's ANOVA test was used with Dunnett's T3 multiple comparisons. For *A. tubingensis* strain 76-9 Welch's ANOVA showed a highly significant difference [W (8, 7.3) = 102.7, p < 0.0001] between means of different media as shown in **Figure 3.11**. CYAR3 (as indicated by the presence of three raisins as opposed to a single raisin) produced the most sclerotia and this was statistically significant according to Dunnetts T3 test compared with all other media used, with the lowest significance being compared with MEAR3 (p < 0.05), the p < 0.001 for the remainder of comparisons.

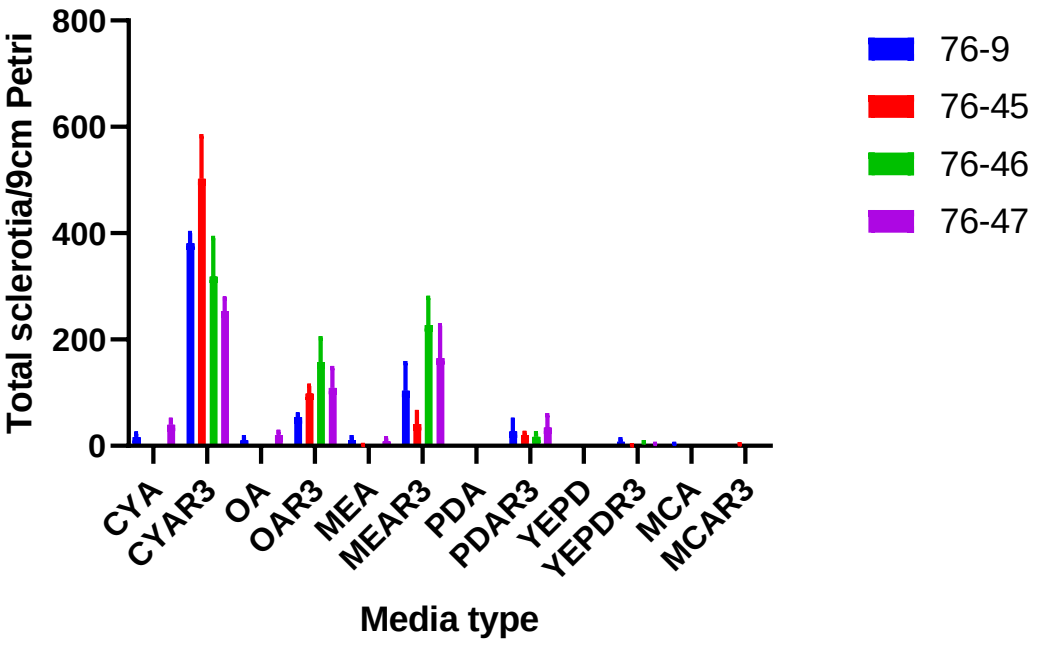


Figure 3.11. Graph showing the number of sclerotia produced on various media with and without the addition of raisins (latter indicated by suffix R3). Four strains of *Aspergillus tubingensis* were assessed (9, 45, 46 and 47) which had already shown high sclerotia production on CYAR (data not shown).

For strain 45 Welch's ANOVA showed a highly significant difference [W (6, 5.7) = 31.4, p = 0.0004] between means of sclerotia produced on different media. CYAR3 produced more sclerotia than all other media although Dunnett's T3 multiple comparison revealed no statistical difference between CYAR3 and OAR3 or MEAR3 (p = 0.0657 and p = 0.0531 respectively). For strain 76-46, data failed the Shapiro Wilk test and therefore the non-parametric Kruskal-Wallis test was used. This showed a highly significant difference between means, with CYAR3 producing more sclerotia than any other media [H

(5) = 13.1, p < 0.0001]. For strain 47, Shapiro Wilk normality test was failed and Kruskal-Wallis test was used and showed a significant difference [H (8) = 21.93, p = 0.0026] between means. Again, CYAR3 was the media that produced the highest number of sclerotia. To compare within CYAR3 as media, a Kruskal-Wallis test was used and showed that there was a statistical difference between strains [H (4) = 8.44, p = 0.0108]. Dunnetts T3 test showed that strains 76-45 and 76-47 have a statistically significant difference (p = 0.0279) but there was no significance when comparing any other strains. CYAR evidently produced more sclerotia than any other media tested here for *A. tubingensis*.

Impact of inoculum spore concentration (quantity of spores) on *A. tubingensis*

The effect of spore concentration on development of sclerotia has been described previously (Brown *et al*., 2008, Affeldt, Brodhagen and Keller, 2012) as part of a quorum sensing mechanism in *A. flavus*. In these studies, it was found that a lower density spore concentration (100-10,000 conidia / plate) led to increased sclerotia production which was abolished at high spore concentrations (1,000,000 conidia / plate). In the present study the effect of varying the spore concentration and method of spore load were examined on CYAR media using *A. tubingensis* isolate 76.27 as a candidate due to its high production of sclerotia on CYAR (pilot data not shown). Five solutions of varying spore concentrations were prepared (as described in **Section 2.2.2**) and assessed using a 5 µL inoculation point containing; 1,000, 5,000, 10,000, 50,000 and 100,000 conidia. For contrast, spread plates were also prepared using the same number of spores, but in which the point of inoculum was spread over the whole surface of the plate using a sterile plate spreader. Plates were incubated and scored as described previously (**Section 3.2.2.1**).

The results of the assay are shown in **Figure 3.13**. Data was processed and ROUT revealed no outliers. Data also passed the Shapiro Wilk normality test so passed the requirements for two-way ANOVA. The two-way ANOVA revealed statistically significant differences between means for both concentrations [F (4, 20) = 6.49, p = 0.0016] and between point and spread inoculation [F (1, 20) = 24.82, p < 0.0001). The results of Tukey's multiple comparison test are shown in **Figure 3.13.** The use of 5,000 spores as inoculum gave the highest number of mature sclerotia compared to all other concentrations. However, Tukey's MCT showed no significance between 5,000 and 1,000 conidia inoculated point wise (p = 0.0649). All other comparisons with 5,000 spores were statistically significant, with p < 0.001 compared to 10,000 and 50,000, p < 0.0001 compared to 100,000. Tukey's MCT revealed there were no statistical differences in sclerotial production between the different inoculum spore numbers using the spread plate method. Between the two methods there was a statistically significant increase in sclerotia production using the point inoculation method (p<0.001), and this was most evident at lower concentrations, which saw a 6-fold increase at 5,000 spores when using the point rather than spread

inoculum method ($p<0.0001$ for both 1,000 spores and 5,000 spores). These findings suggest that to optimise sclerotia production within *A. tubingensis*, the point inoculation method and 5,000 spores in a 5 µL inoculum should be used in future assays and any proposed crosses.

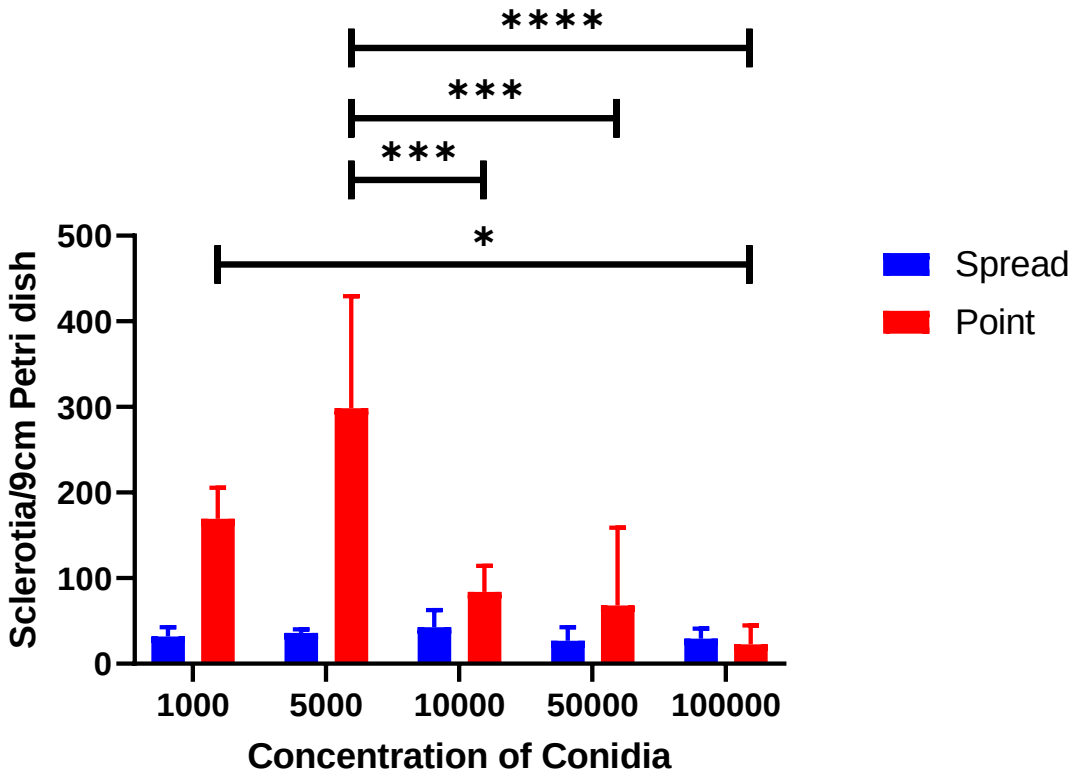


Figure 3.13. A graph showing the number of sclerotia produced by *A. tubingensis* strain 76.27 at different concentrations of conidial load using two different methods of spore load (spread and point inoculation). Within methods, there was no statistical difference between spore concentrations for the spread plate method and there was a statistically significant increase in sclerotia production for 5000 spores/ 5 µL compared to all other concentrations other than 1000 spores/ 5 µL (*** $p<0.001$, **** $p<0.0001$). Between methods there was a statistically significant increase in sclerotia production due to the point inoculation method compared to the spread inoculation method for 1000 spores/ 5 µL and 5000 spores/ 5 µL ($p<0.0001$, two-way ANOVA).

Impact of inoculum spore concentration (quantity of spores) on size of sclerotia on *A. tubingensis*

The same assay was used as an opportunity to explore how changes made would impact on the width of sclerotia as a proxy for size. Measurements of sclerotial width are discussed in **Section 3.2.2.1**. A Kruskal-Wallis test comparing means of sclerotial width showed that there was no statistical difference in sclerotia sizes between concentrations for either of the methods used (**Figure 3.14** and **Figure 3.15**). However, analysis of the size of sclerotia between the conditions (using an inoculum volume of 5 µL of containing 5,000 conidia), revealed a statistically significant difference in the variation of sclerotia size between the two methods [$H(5) = 11.64$, $p = 0.0202$] (**Figure 3.16**).

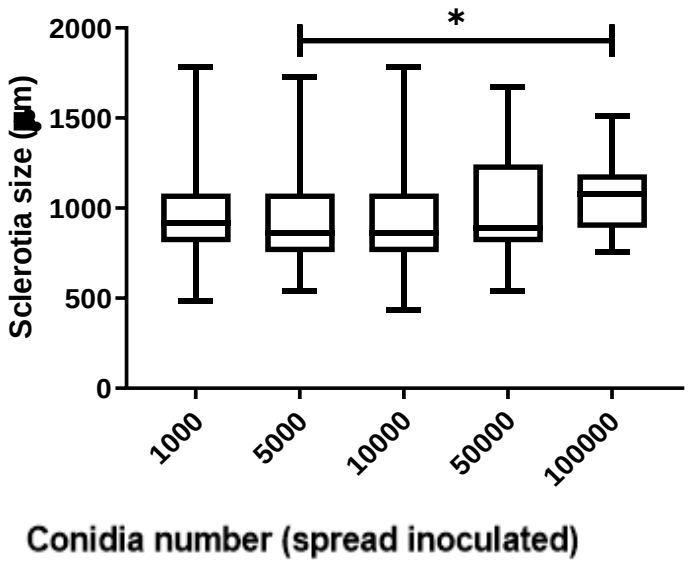


Figure 3.14. Box and whisker plot showing the variation in sclerotia size (diameter) of *Aspergillus tubingensis* isolate 76.27 between spore concentrations that were spread inoculated. There was no statistically significant difference in sclerotia size shown between concentrations.

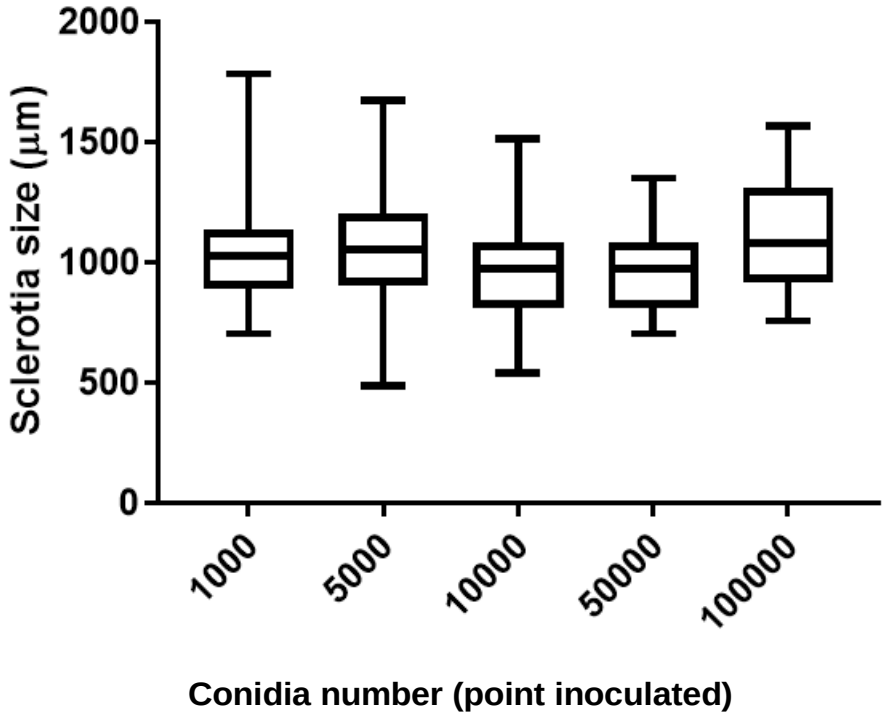


Figure 3.15. Box and whisker plot showing the variation in sclerotia size (diameter) of *Aspergillus tubingensis* isolate 76.27 between spore concentrations that were point inoculated. There was no statistically significant difference in sclerotia size shown between concentrations

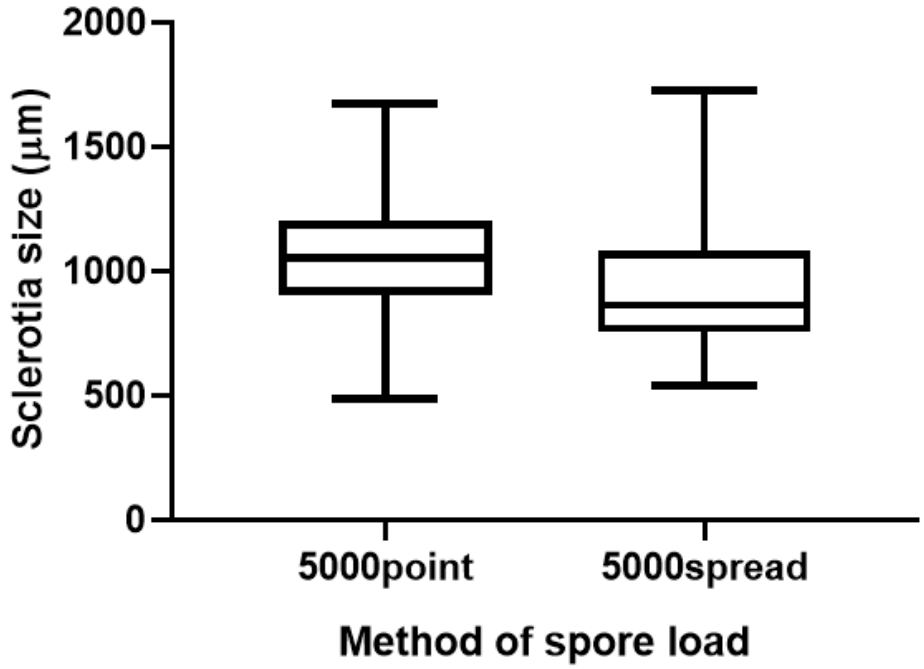


Figure 3.16. Box and whisker plot showing the variation in sclerotia size (diameter) between spore inoculation methods used for *A. tubingensis* strain 76.27. There was a statistically significant difference in sclerotia size between the two samples (Kruskal Wallis $p < 0.05$).

Impact of pre-freezing step on *A. niger*

Research in *A. niger* has shown that a 'pre-freezing' step of -18°C for three weeks enhanced sclerotium production (Frisvad *et al*., 2014). A similar step is required for sexual carpogenic growth of apothecia from sclerotia of *S. sclerotiorum* (Hegedus and Rimmer, 2005; Bardin and Huang, 2001), it has been suggested this mimics winter given that seasonality is an important part of the infection cycle of *S. sclerotiorum* (Clarkson *et al*., 2003).

To assess whether a 'pre-freezing' step would enhance sclerotium production in *A. tubingensis*, two slopes of a series of representative isolates were prepared and left to grow and mature for 10 days as described in **Section 2.2.1**. Slopes were then either frozen for three weeks at -20°C, or spores were harvested after three weeks for the 'non frozen' control. Spore solutions for each were then prepared as described in **Section 2.2.2**. These spore suspensions were then used to inoculate 5 cm diameter CYAR plates and left to incubate as described in **Section 3.1.1.3**, before scoring plates both for sclerotium density and sclerotium size.

Data analysed by ROUT found several outliers, and data additionally failed the Shapiro Wilk test for normality. Therefore, a Wilcoxon matched-pairs signed rank test was completed between unfrozen and frozen samples. The analysis showed that there was no statistically significant difference in

number of sclerotia between the two treatments ($p < 0.2817$). However there was a statistically significant difference in the Kruskall-Wallis analysis of size of sclerotia between conditions [$H(23) = 129.2$, $p<0.0001$] because of this 'pre-freezing' step with larger sclerotia found in the unfrozen samples (**Figure 3.17** and **Figure 3.18**). Differences in sizes between sclerotia have previously been discussed in relation to importance of mode of germination and form of lifecycle (Willetts and Bullock, 1992).

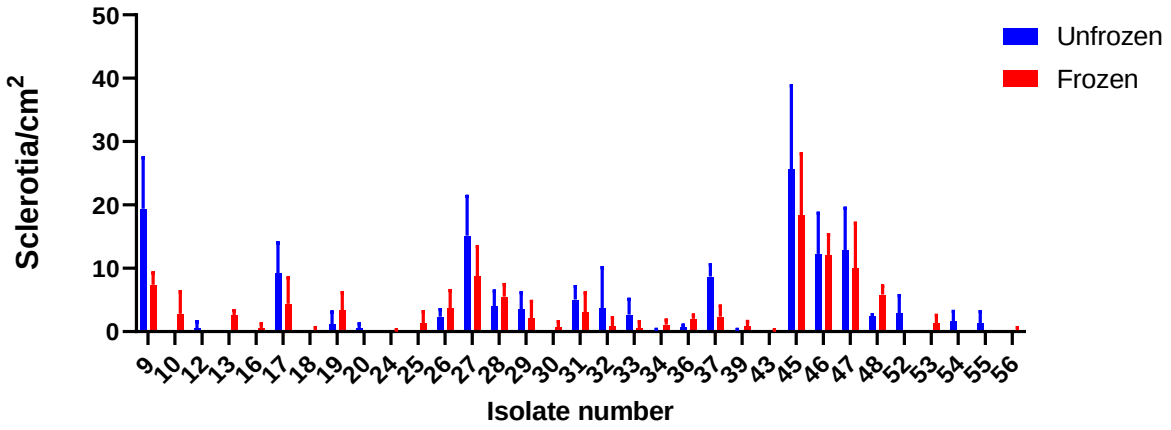


Figure 3.17. Graph showing the sclerotia production of *A. tubingensis* isolates, both with a 'pre-freezing' step and without. Many isolates produced no sclerotia under either condition, those isolates are not shown. Analysis showed no statistical difference between the two groups ($p<0.2817$). *A. tubingensis* strains have the prefix 76 but this has been omitted from the X axis for clarity.

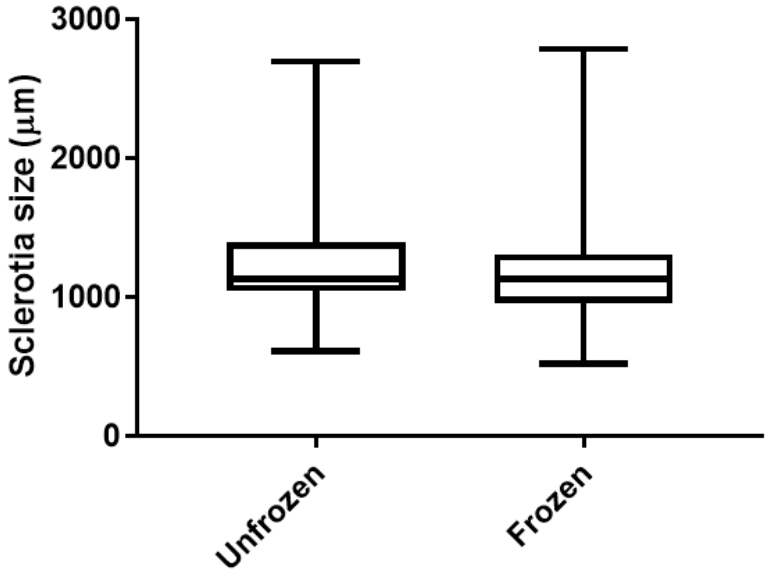


Figure 3.18*.* Box and whisker plot showing the variation in sclerotia size between samples (all *A. tubingensis* sclerotia producing isolates) grown from frozen and unfrozen conditions. There is a statistically significant difference between the unfrozen and frozen samples ($p<0.0001$).

Impact of temperature on *A. niger*

To determine the best temperature for sclerotia production in *A. niger*, three representative strains were assessed for sclerotia production at a range of temperatures [isolates chosen as there was previously possible evidence of sexual development in these isolates (Ashton 2018)]. The temperature range was intervals from 15 °C to 37 °C. Lower and higher temperatures (15 °C, 18 °C, 20 °C and 37 °C) were used but data was trimmed as some temperatures prevented sclerotia production or growth in the strain used. The 9 cm diameter CYA plates had raisins added for a day before removal. Plates were inoculated with three 5 µL points of inoculation (each with 100,000 conidia / mL i.e. 500 spores). Plates were grown in triplicate and incubated unsealed at the particular temperature for 14 days. Plates were removed and sclerotia counted as described in **Section 3.2.2.1** and **Section 3.2.2.2**.

Analysis by ROUT showed no outliers and the data passed the Shapiro Wilk test for normality. Therefore, two way ANOVA was selected for data analysis. Results showed statistically significant differences between strains [$F(2, 30) = 142.5$, $p < 0.0001$] and more importantly temperatures [$F(4, 20) = 32.66$, $p < 0.0001$]. Tukey's multiple comparison test was used to further analyse differences between temperatures and revealed statistically significant results for each comparison other than between 23 °C – 25 ˚C and 28 ˚C – 30 °C. Multiple comparisons that revealed statistically significant results of $p < 0.0001$ are indicated in **Figure 3.19.** Maximum numbers of sclerotia were produced at both 28 ˚C and 30 °C for all three strains.

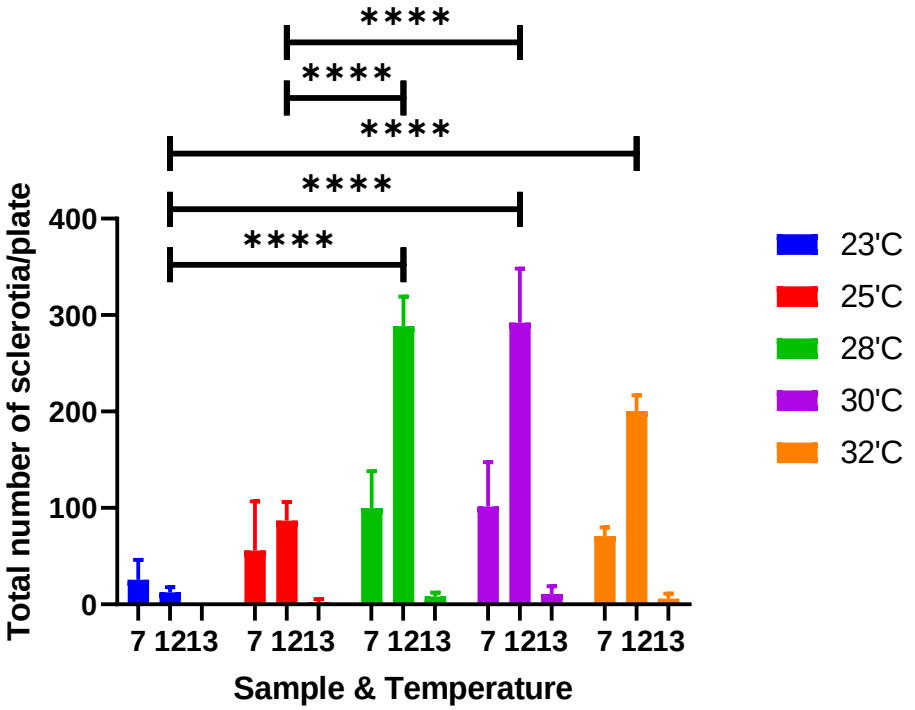


Figure 3.19. Mean number of sclerotia produced (per 9 cm diameter Petri plate) on CYAR (three raisins per plate) at different temperatures. Three strains of *A. niger* were used (shown as 7, 12 and 13 which are 8-160, 8-166 and 8-161 respectively).

The experiment was repeated but with the use of additional raisins (six instead of three, arranged as described in **Section 4.2.2.4**). Analysis by ROUT again showed no outliers and the data passed the Shapiro Wilk test for normality. Therefore, two way ANOVA was selected for analysis. The data showed statistically significant differences between strains [$F(2, 30) = 112.1$, $p < 0.0001$] and between the temperatures used [$F(4, 30) = 29.14$, $p < 0.0001$] resulting in differences in sclerotia production number, these data can be seen in **Figure 3.20.** Tukey's multiple comparison test was used to analyse differences between single variables and revealed significant differences within the temperatures. A grouping appeared with differences in sclerotia production between the temperatures 23 ˚C and 25 ˚C not statistically different from one another, but highly statistically significant from each of the other temperatures; 28 ˚C, 30 ˚C and 32 °C (significances are shown where $p < 0.0001$ for all but between 25 °C and 32 °C $p = 0.0002$). The grouping of 28 °C, 30 °C and 32 °C had no significant differences between them.

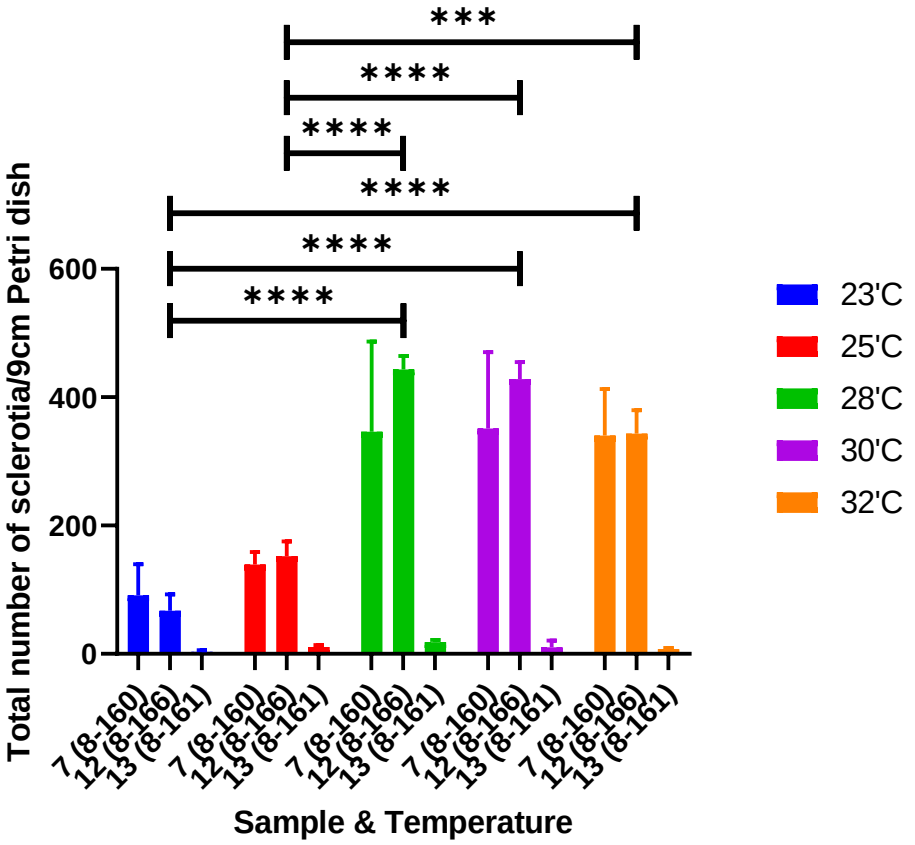


Figure 3.20. Mean number of sclerotia produced (per 9 cm diameter Petri plate) on CYAR (six raisins) at different temperatures. Three strains of *A. niger* were used (shown as 7, 12 and 13 which are 8-160, 8-166 and 8-161 respectively).

Impact of media type on *A. niger*

A similar approach to that of the *A. tubingensis* experiment analysing sclerotia production on different media (**Figure 3.11**) was repeated using *A. niger* strains as the organism for analysis. A broad range of media were used but sclerotial production was dominated by only two media types - CYA and MEA both with 3 raisins added (as previously described **Section 3.2.2.1 and Section 3.2.2.2**). Therefore, data was trimmed to show only strains that produced sclerotia (as opposed to *all* non-GM strains – see **Appendix 9**) and only on CYAR and MEAR. CYA and MEA were both used, but neither produced sclerotia reliably or in any number, many strains produced no sclerotia on these media types. As observed for A. tubingensis, sclerotia were not observed in the shadow of the where the raisins had been laid. Data analysed by ROUT showed outliers, and after removal of outliers, the data was subjected to a Shapiro-Wilk normality test but failed, therefore, a Wilcoxon matched pairs rank test was used to compare between CYAR and MEAR. Results showed no statistical difference between media types ($p < 0.3223$). Sidak's multiple comparison test showed significance between means of different media within specific strains. **Figure 3.21** shows the variation between media types within strains, and for clarity, any strains with no statistical difference between media types are not shown. Strains 8-152 and 8-162 were most interesting as they produced sclerotia on both CYAR and MEAR media but had statistically significant differences between sclerotia number on the different media. Strain 8-152 produced significantly more sclerotia on MEAR ($p < 0.0001$), whilst 8-162 produced significantly more sclerotia on CYAR ($p < 0.0001$), demonstrating a lack of species-wide media specificity between these two media. This was even more in contrast to the species-wide sclerotia production on these media whilst failing species wide to reliably produce sclerotia on any other media.

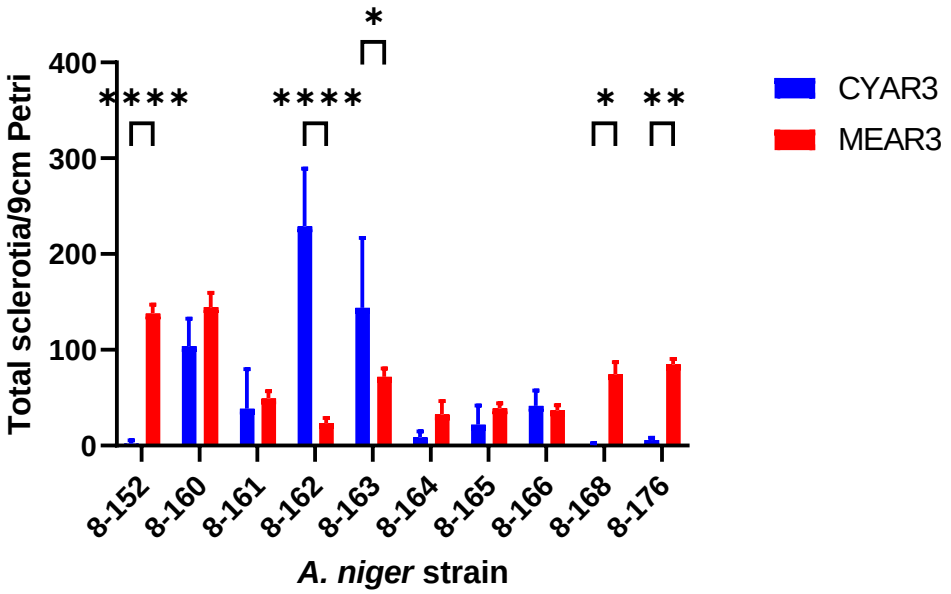


Figure 3.21. Graph showing the number of sclerotia produced by sclerotia-forming *Aspergillus niger* strains on both CYAR and MEAR media. Data was trimmed to remove data for other media that irregularly produce few sclerotia as well as other strains that failed to produce sclerotia. No statistically significant differences were evident between CYAR and MEAR but multiple comparisons showed statistically significant differences between media types for individual strains (these are shown) and notable in both strain 8-152 and 8-162.

Impact of metal cations on *A. niger*

Several microbiological growth media have a well defined metal cation content. In the case of Hutner's media, the added trace element solution can provide several transition metal cations for use as cofactors in normal metabolism. CYAR media lacks any specifically added trace metal solutions but does contain yeast extract, which would be expected to contain trace elements. Therefore, the addition of Hutners trace element solution was analysed for its possible impact on sclerotia production in three strains of *A. niger*; 8-160, 8-161 and 8-166 (**Appendix 9**). Hutner's trace element was added to the CYAR media as described in **Section 3.2.1.2**.

Data was analysed for outliers by ROUT and none were found. Additionally, the data passed the Shaprio Wilk test for normality. Therefore, a two-way ANOVA was performed which found statistically significant differences between strains [$F(2, 14) = 7.039$, $p = 0.0077$] and more importantly between the +Hutner's and -Hutner's conditions [$F(1, 14) = 37.54$, $p < 0.0001$]. The addition of Hutner's trace elements almost completely abolished the production of sclerotia in the three *A. niger* strains tested on CYAR. Additionally, the media enriched with Hutner's trace element solution produced conidia with stark black conidia, whilst on CYAR conidia were black/brown as has been reported previously (Samson *et al*., 2007b). Data is shown in **Figure 3.22**.

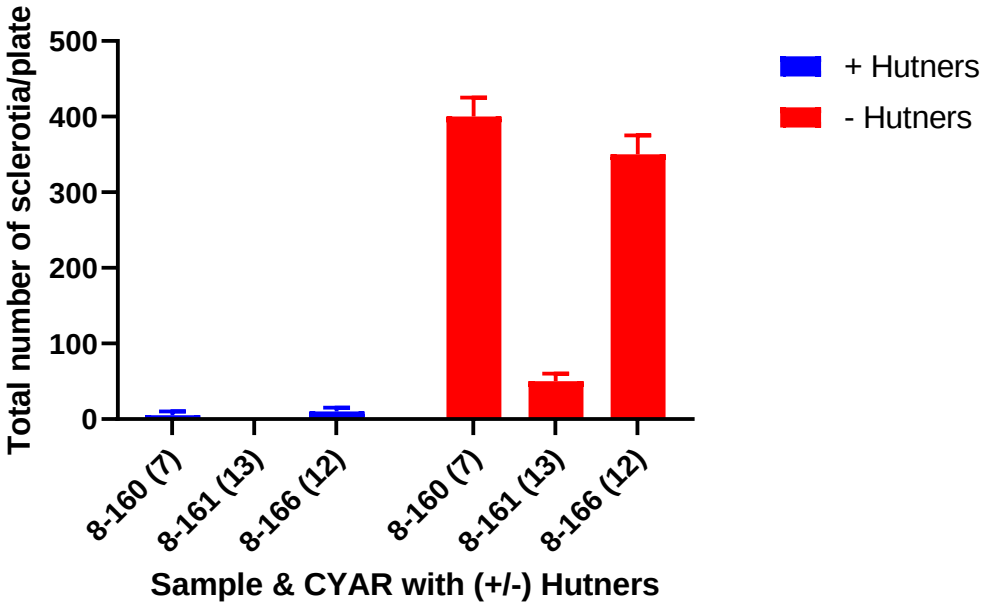


Figure 3.22. Graph showing the number of sclerotia produced from three *A. niger* strains on CYAR either without Hutner's trace element solution and with Hutner's trace element solution added.

Impact of farnesol on *A. niger*

Farnesol is a widely reported quorum sensing molecule in fungi, particularly yeast (Nickerson *et al*., 2006; Machida *et al*., 1998). It has been reported as having a role in the cAMP pathway and ROS generation (Machida *et al*., 1998; Lindsay *et al*., 2012). Addition of farnesol has been reported to reduce conidiation in *A. niger* (Lorek *et al*., 2008) under specific conditions. Given the hypothesis that conditions drive fungi into either sexual or asexual pathways in aspergilli (Ojeda-Lopez *et al*., 2018; Dyer and O'Gorman, 2012; Adams *et al*., 1998; Jorgensen *et al*., 2020), it was hypothesised that the addition of farnesol to media that induces sclerotia, may inhibit conidiation and promote further sclerotia production.

The research examining farnesol impact on *A. niger* conidiation used several conditions which were not adopted as they were not inducive to sclerotia production such as the use of Wort Agar in place of CYAR. The use of 25 °C would also likely produce fewer sclerotia than 28 °C, 30 °C or 32 °C as previously demonstrated (**Figure 3.19** and **Figure 3.20**)**.** Therefore, a narrower set of CYAR and 28 °C were selected for experimental use. An additional condition of the previous study was the sealing of plates immediately after inoculation. Immediate sealing of plates after inoculation was found to alter normal colony formation for strains of *A. niger* in this study with abnormal floccose growth and irregular conidiation (data not shown). Triplicate plates were prepared for each farnesol concentration as well as for conditions of sealing plates immediately, 24 hours post inoculation and leaving the plates

unsealed. Strain 8-160 was selected as it produced sclerotia and had less variation in sclerotia number than other sclerotia-producing strains.

Data was analysed by ROUT and no outliers were found and additionally data passed the Shapiro Wilk test for normality. Data revealed no statistically significant difference between the different concentrations of farnesol added [$F(7, 96) = 1.616$, $p = 0.1400$] but did reveal a statistically significant difference between sealing conditions [$F(2, 96) = 11.26$, $p < 0.0001$]. Tukey's multiple comparison revealed highly statistically significant differences between the unsealed plates than either of the conditions of sealing plates immediately (0) and after 1 day of unsealed growth (1) (**Figure 3.23**). Despite the lack of statistical significance supporting the impact of farnesol, there is an approximate correlation from the data showing a reduction in sclerotia production with increasing farnesol concentration.

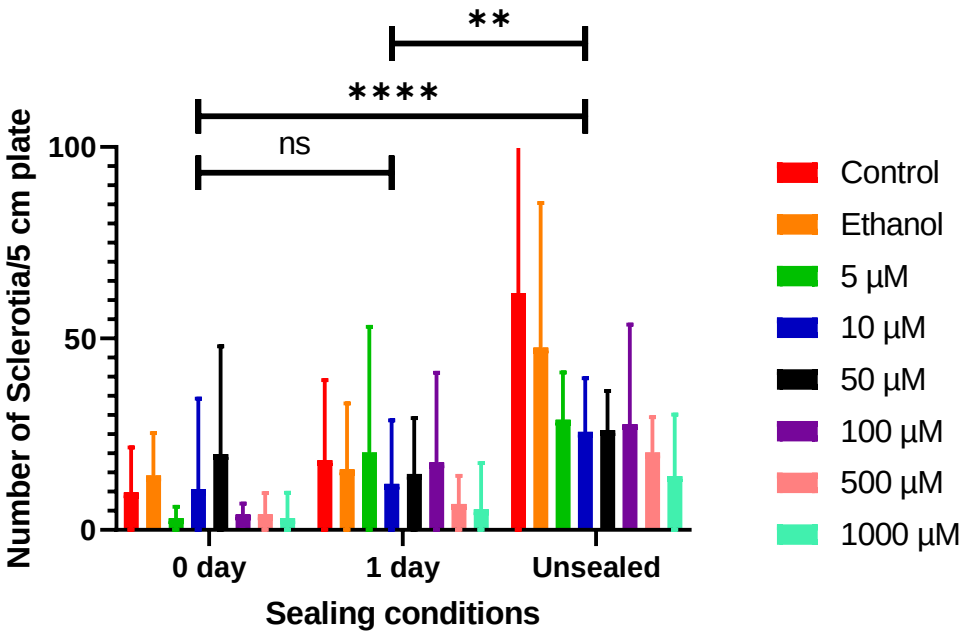


Figure 3.23. Graph showing the number of sclerotia produced by *Aspergillus niger* strain 8-160 on CYAR with varying concentrations of added farnesol and different plate sealing methods. Analysis reveals no statistical difference in sclerotia production between farnesol concentrations despite a broad pattern of decrease in unsealed plates. There was a statistically significant difference between sclerotia number produced by different sealing time (shown) with unsealed plates producing greater number of sclerotia.

3.3.2 Identification and Expression Analysis of Sclerotia Development Regulatory Genes *sclB* and *sclR (Sequencing Data and Up/Downstream)*

Six *A. niger* strains were sequenced using the CBS 513.88 strain as a reference sequence as described in **Section 2.2.10** and **2.2.11**. Four of the strains were selected as they reliably produced sclerotia (8-160, 8-161, 8-162 and 8-166). The other two strains were never seen to produce sclerotia (8-169 and 8-175). The sequence data was then used for generating consensus sequences amongst the strains as well as against the CBS 513.88 (which has not been reported to produce sclerotia). This enabled a search for potential SNPs within *sclB* and *sclR* that have a consensus amongst all the sclerotia producing strains but not amongst the sclerotia non-production strains (or vice versa) as well as looking into individual SNP's and their potential impact on the functionality of either *sclB* or *sclR*.

The gene *sclB*

Aspergillus niger sclB is a 1,782 bp gene with a single predicted intron at 646-780 (135 bp inclusive). It could be accessed via the NCBI database as C6 transcription factor (*A. niger* CBS 513.88 and the accession number XP_001392888.1). The 1,647 bp transcript translates into a 548- amino acid sequence. PSortII was used to analyse the protein product. Subcellular localization was predicted as nuclear in nature, and a $ZnII_2Cys_6$ binuclear cluster domain was predicted. The predicted domain is underlined in the full amino acid sequence in **Figure 3.24**.

MQSLVLPPSSFIATEFGHPRFDPGPERLSLNLPRSTNARRYPPRDLPLPRSMSGSVPADDPLDTSGPVRRPGHPELP
QAATTVTAATSVSAGLSGPVLPPGSAGAVTTHESVTQRVAPASADEVLRQPFPVGDAFASSRLPPSLVGQGISQAT
ATAYAQPSFGTSPPGTTARALPQKPTRRTKAHVASACVNCKKKHLGCDPARPCRRCVLSGKEATCVDVTHKKRGR
PPLKAEEASLRTYAAHMDNRATQGEQHGPQSRRTLHRATSSREIRPMTDLQMPGAQTGAMAMRASAGHPQR
WAAPVYSQAIDPSIMQRSVGHRRFSSSGSAQSITAASPPGYVPMPVGYNPALGGQRMPMGMGRPLSSYTHQG
MNPTTTPPQYQQSFVPISPYPESARMSNRMPMGESPMSRDPREGYLESPVRLPPIYPPTMGTPASTSQGHRLSDP
YPGAWSPRTREEFLQQEHRQQMPSHGFIDPLSPSSQMRHAASDMGYGEPVPRQLGPGSTAERHAMHMSLVPA
PDEPPTTEADTEGSRPAKRRKMALDDMVND-

Figure 3.24. The amino acid sequence of *A. niger sclB*. The $ZnII_2CYS_6$ binuclear cluster domain is underlined. The non-synonymous amino acid changes as results of SNPs are highlighted in red.

Across the 6 six strains that were sequenced as well as using the CBS 513.88, a multiple sequence comparison yielded several single nucleotide polymorphisms (SNP's) between the strains. The complete multiple sequence alignments for both *sclB* nucleotide and amino acid sequences can be seen in **Appendix 11**. SNPs that are within the intron and those that result in a synonymous amino acid sequence are shown in **3.3** below but are not discussed.

The SNP at nucleotide 448 results in a change of amino acid from serine to proline. This is a non-synonymous change resulting in either a possible turn or helix break. The amino acid change is 29 amino acid residues before the $ZnII_2Cys_6$ binuclear cluster domain and might predictably have a major effect. The SNP is conserved amongst the 6 strains sequenced in this study and suggests that the SNP is within the genome of CBS 513.88. It should be noted that sclerotia production in strain CBS 513.88 has not been reported on any media tested (this study) and had previously been described to fail to produce sclerotia on CYAR (Frisvad *et al*., 2014).

The SNP at nucleotide 124 (CCC>TCC) results in a non-synonymous change in amino acid residue from proline to serine. This is again a turn or helix break and occurs before the $ZnII_2Cys_6$ binuclear cluster domain. This SNP only occurs in strain 8-169 which was one of two strains selected for sequencing due to the absence of sclerotia production on all media tested (in this study).

The remaining two SNP's that result in non-synonymous amino acid substitutions occur in Strain 8-166. This strain was selected for sequencing as it produced sclerotia reliably, although its sclerotia were often aberrant in shape and were observed to have another unusual phenotype – when being washed they were often observed to float where sclerotia of all other strains fully submerged. Many of the crosses arranged with this strain led to interesting phenotypes (as will be discussed in **Chapter 4**).

Table 3.3. The SNPs recorded from multiple sequence alignments of *sclB* across six strains used in this study and CBS 513.88. Synonymous amino acid changes are highlighted in red. Strains reliably forming sclerotia have prefix (S), those lacking sclerotia are shown as (NS).

Strain	nt 124 aa 42	nt448 aa 150	nt 511 aa 171	nt 673	nt 679	nt 724
8-160 (S)		TCG>CCGSer>Pro			T>C Intron	
8-161 (S)		TCG>CCGSer>Pro				
8-162 (S)		TCG>CCGSer>Pro				
8-166 (S)		TCG>CCGSer>Pro	GCG>ACG Ala>Thr	C>T Intron		A>G Intron
8-169 (NS)	CCC>TCC Pro>Ser	TCG>CCGSer>Pro				
8-175 (NS)		TCG>CCGSer>Pro				

Strain	nt 1027	nt 1120	nt 1342	nt 1594	nt1636 aa500
8-160 (S)	CCA>CCG Pro>Pro	TCT>TCA Ser>Ser			
8-161 (S)	CCA>CCG Pro>Pro	TCT>TCA Ser>Ser			
8-162 (S)	CCA>CCG Pro>Pro	TCT>TCA Ser>Ser	CCA>CCT **Pro>Pro**		
8-166 (S)				GCC>GCT Ala>Ala	TTA>TTT Leu>Phe
8-169 (NS)	CCA>CCG Pro>Pro	TCT>TCA Ser>Ser			
8-175 (NS)	CCA>CCG Pro>Pro	TCT>TCA Ser>Ser			

The gene *sclR*

Aspergillus niger sclR was identified as an ortholog of the described *sclR* in *A. oryzae* (Jin *et al*., 2011). It could be accessed via the NCBI database as An14g02540 (and the accession number XM_001400843.2). It reads 3'-5' and *sclR* is a 1,010 bp nucleotide sequence with a single predicted intron of 60 bp length (331-390 inclusive). The translated transcript yields an amino acid sequence of 316 residues which is shown in **Figure 3.25** for *A. niger* and for comparison, the 302 bp *A. oryzae* in **Figure 3.26**. PSortII predicted a 36-residue coil region towards the C' end of the sequences (underlined) which does not contain any glycine (G), proline (P), tryptophan (W) or tyrosine (Y) and is rich in both alanine (A) and leucine (L) (A and L make up 7 of 36 residues). This distribution of amino acids is a common motif amongst helices, which are disrupted by the imino acid nature of proline as well as by the R-chain size of both tryptophan and tyrosine whilst alanine and leucine are helix stabilising (Padmanabhan *et al*., 1990., Williamson, 1994). The *sclR* genes of both species also contains a conserved basic motif consisting of arginine (R) and histidine (H) residues (combined 5 of 17 residues) and this region is highlighted in green. The conserved basic region and the conserved helix region constitute an 81 amino acid sequence with high conservation between the *A. niger sclR* and the *A. oryzae sclR* with only a short sequence of 4 residues containing 3 amino acid substitutions between them (highlighted in purple). Taken together these data describe the basic helix-loop-helix nature of these genes and demonstrate high conservation within the predicted basic helix-loop-helix domain previously described in *A. oryzae sclR* (Jin *et al*., 2009).

MAYPRPDSFSLDDERMYSMSHPSPLTRPNDTFAKGPDPLSANWSYDNAIDLFSLNTMMPETFPLEMSNEMMNL
DPKDFPADFFAPPPDISAFTISNHSGEDAASCGSLSSDLDSDDQSWSPTCRVSPLEPIHMELPKPAARTSRTSTRRKT
ASQPKPREVTATRWSSSPEITPQDYPATSVSPPPAPSSPAANNTARKTTRSLSSDSNASTGQAQTTTGRNAAKRAA
HNIIEKRYRTNMNAKFVALEKAMCGGVQKPTKGGSASLKKSEILTNAITFMQELQEENKVLQKELAMLKQSMVPN
GMWRHSKGVRRFTL

Figure 3.25. The amino acid sequence of *A. niger sclR* (316 aa) strain CBS 513.88. A presumed basic region containing a high proportion of arginine (R) and histidine (H) residues is highlighted in green. A predicted coil region (PSortII) containing high levels of alanine (A) and leucine (L) and absent glycine (G), proline (P), tryptophan (W) and tyrosine (Y) is underlined. Between the two is a short sequence of 4 residues with low conservation to *A. oryzae sclR*. This *A. niger sclR* has a 14 aa inclusion upstream of both described regions compared with the *A. oryzae sclR*. The three amino acids highlighted in red are the positions of three non-synonymous SNP substitutions amongst the sequenced strains from this study.

MAYTRTDPSFTLSDDERMYMSHHSPMHRPYDTFAAPKGPDPLSANWNYDSAIDLFSLNTMMPENFALDVPNEP
MGVDPKDFPADFFAPPPDISGFTISNHSGEDAGSITSDLESDDQSWSPTYAAPAEMLPAPGRQSTRRKTTPAVKRE
TTWSSSPELAPQEYPAHTSPQTTPTSPPVNRKMTRTTSVDSNASTGQTTTATTTSGRNAAKRAAHNIIEKRYRTNM
NAKFVALEKAMCGGVQKSNKSGSASLKKSEILTNAIAYMQELQEENKALQKELALFKQNMVPSGMWRHTKGAET
FRA

Figure 3.26. The amino acid sequence of *A. oryzae sclR* (302 aa). A presumed basic region containing a high proportion of arginine (R) and histidine (H) residues is highlighted in green. A predicted coil region (PSortII) containing high levels of alanine (A) and leucine (L) and absent glycine (G), proline (P), tryptophan (W) and tyrosine (Y) is underlined. Between the two is a short sequence of 4 residues with low conservation to *A. niger sclR*.

For *sclR*, again the six strains that were sequenced were aligned with CBS 513.88, and a multiple sequence comparison yielded several single nucleotide polymorphisms (SNPs) between the strains. The complete multiple sequence alignments for both *sclR* nucleotide and amino acid sequences can be seen in **Appendix 12**. In these multiple sequence alignments, there were a greater number of SNPs but most of them were within introns or resulted in synonymous amino acid substitutions. The full list of SNPs (n=11) is shown in **Appendix 12,** but the shorter list of non-synonymous amino acid substitutions is shown in **Table 3.4** (n=3). These will now be discussed.

In a similar way to the *sclB* gene, all the newly sequenced strains differed from the CBS 513.88 strain in that a SNP was seen at nucleotide 491 of *sclR*. This was an A**G**A > A**A**A nucleotide substitution, which would result in an arginine > lysine amino acid substitution at amino acid 164 when compared with the CBS 513.88 strain. However, due to the consensus of this change amongst the sequenced strains of this study, it would suggest that the SNP is within the CBS 513.88 genome in this case. The shared basic nature of these residues and the upstream (of the conserved domain) location of the change is unlikely to have an impact on the functionality of SclR. This SNP is one of three that exist which all sequenced strains in this study have in consensus but is different in CBS 513.88, the other two are synonymous (**Table 3.4, Appendix 12**).

Of the total number of 11 non-consensus SNPs within the sequenced strains when compared to CBS 513.88, 10 of them were seen within strain 8-161. Of these 10, four were novel to this strain and one of these is the change at nucleotide 641, which results in a non-synonymous substitution of amino acid 194 (after intron removal). The nucleotide change is A**A**C > A**G**C and this results in an asparagine > serine substitution. This change results in an amidic to hydroxylic change in residues but is upstream of the basic and DNA binding domains and within a region that has low consensus between the *A. niger* and *A. oryzae sclR* sequences (shown in **Figure 3.25** and **Figure 3.26**).

The final SNP is one that evenly divided between the sequenced strains and CBS 513.88 and is located at nucleotide 238. CBS 513.88 and strains 8-161 and 8-166 from this study maintain **G**CC and have an

alanine residue (amino acid residue 80). Strains 8-160, 8-162, 8-169 and 8-175 have an **A**CC threonine residue. Interestingly, this division almost matches the division of MAT-types, with the exception being 8-175 which is MAT1-1 but is grouped into the 80Threonine with the three MAT1-2 strains. Threonine is hydroxylic in that is has an additional OH-group, and this substitution is within a region of the sequence with low conservation between the *A. niger* and *A. oryzae sclR*. Taken together, this substitution is not predicted to have a strong impact on protein functionality.

Table 3.4. The SNPs recorded from multiple sequence alignments of *sclR* across six strains used in this study and *A. niger* reference strain CBS 513.88. Synonymous amino acid changes are highlighted in red and listed, for the complete list of SNPs, refer to **Appendix 12**. (S) denotes a strain that produces sclerotia. (NS) denotes strains that have not produced sclerotia in this study.

Strain	238	491	641
8-160 (S)	GCC>ACC Ala>Thr	AGA>AAA Arg>Lys	AAC>AGC Asn>Ser
8-161 (S)		AGA>AAA Arg>Lys	
8-162 (S)	GCC>ACC Ala>Thr	AGA>AAA Arg>Lys	
8-166 (S)		AGA>AAA Arg>Lys	
8-169 (NS)	GCC>ACC Ala>Thr	AGA>AAA Arg>Lys	
8-175 (NS)	GCC>ACC Ala>Thr	AGA>AAA Arg>Lys	

Screening of *akuB*, *sclR* and *sclB* transformants

Due to the number of transformants arising from the transformation protocol, several screening methodologies were employed to reduce the downstream workload. In the first instance, it would be simple to screen for good growth on the selective media (hygromycin and pyrithiamine resistance). Secondly, screening could be completed based on TdTomato and eGFP expression. This would indicate which strains had been successful in taking up the construct DNA but not whether this was in the correct location, and strictly in that location. For the detection of correct location and strictly that location, Southern blotting was employed.

As discussed in **Section 3.2.2.7**, potential transformants were screened for the presence of either eGFP or TdTomato. Transformants containing the *tdTomato* gene appeared variable shades of pink under ambient lighting as shown in **Figure 3.27**.

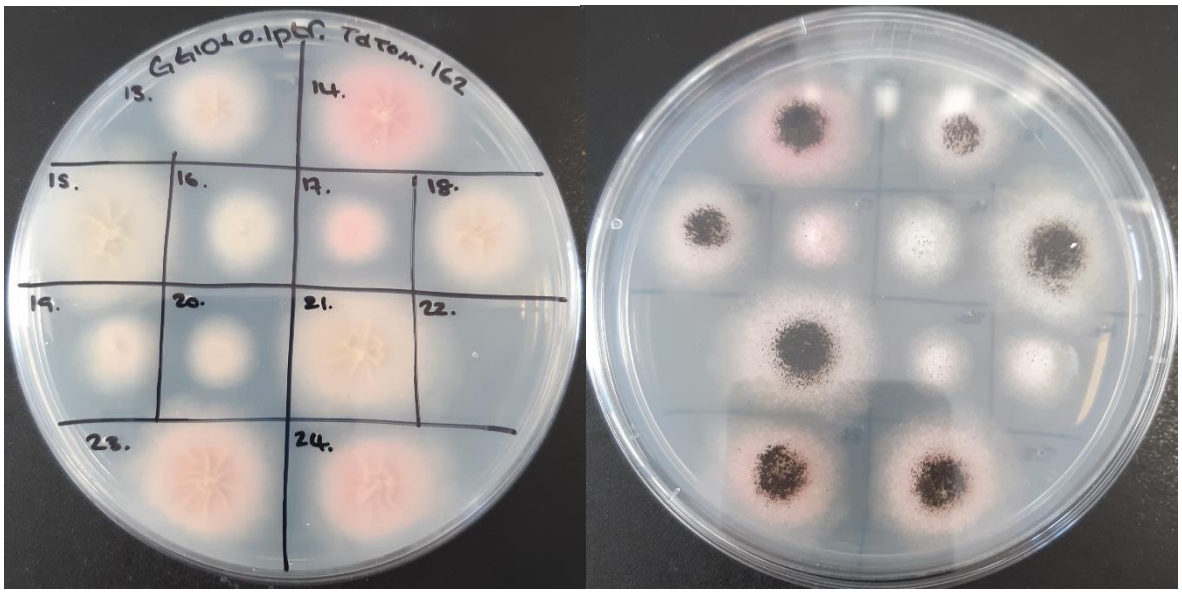
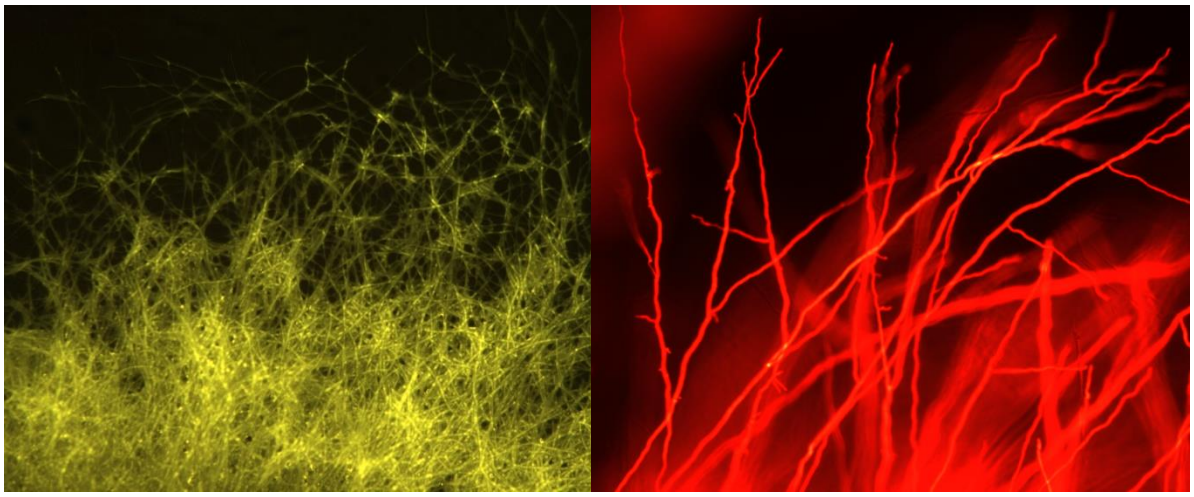


Figure 3.27. *Aspergillus niger* transformation plates showing transformants of Strain 162 growing on *Aspergillus* minimal media AMM (-N) Glc50 Gln10 (GG10) agar with 0.1 µg / mL pyrithiamine as a selectable marker after 5 days of growth at 30°C. Transformants have been transformed with the Δ*ku80*:: P*gpdA*$_{(Anid)}$_*tdTomato*_T*trpC*$_{(Anid)}$, *ptrA* puc19 plasmid. A) Reverse view numbered 13-24 for independently selected colonies, note the absence of growth for colony 22, indicating that the previous growth had been a false positive. B) Front view of plates. Note that not all colonies show conidiation and some show retarded growth. Colonies were selected from those that have growth and conidiation for further screening.

Screening was completed using fluorescence microscopy and typical positive results for each of eGFP and TdTomato can be seen of positive expression of both proteins is shown in **Figure 3.28**.



F**igure 3.28.** Fluorescence Micrographs showing the expression of eGFP (Left) and TdTomato (Right). These allowed for easy screening of cultures for insertion of these genes.

Probe preparation for Southern blotting was described in **Section 3.2.2.12. Figure 3.29** demonstrates the shift in size between the DIG-UTP incorporated probe and the negative control.

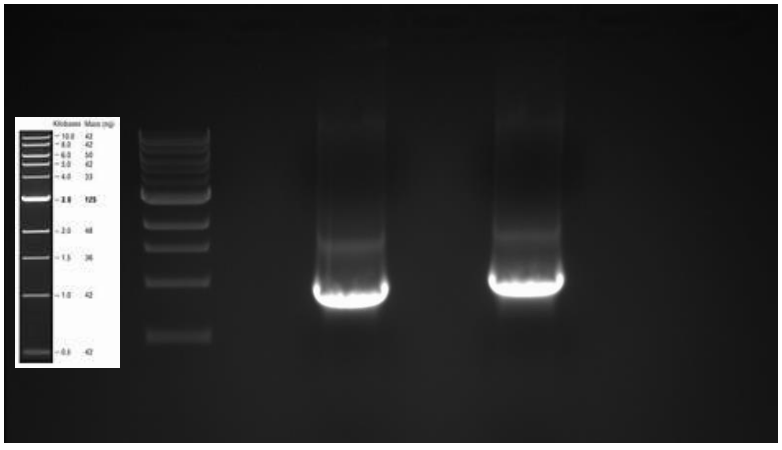


Figure 3.29. Gel electrophoresis image of the digoxigenin-11-dUTP probe generation. 1kb ladder. Lane A is the band for the probe without the addition of DIG-UTP as a negative control. Lane B shows the probe with the incorporation of DIG-UTP. Probe size for the akuB upstream target sequence was 917 bp. Note the size shift upwards for the DIG-UTP probe (Lane B).

Knockout of *akuB* (*ku80*) gene

The colony PCR results allowed for quick observation of strains that had a correct integration at the intended site using the positional PCR approach (**Appendix 17**). However, this approach does not determine whether there had been ectopic integrations and as such a Southern blot was also developed to check for single integration at the intended site.

Confirmation of correct construction of Δ*akuB* (also known as *ku80*) knockouts were completed using Southern blots. A total of 10 µg DNA was digested overnight with *Bam*HI. As a positive control, 5 ng of plasmid containing the Δ*akuB* construct was also digested (with *Xho*I for 1 hour). Prior to Southern blotting, the digested gDNA was run via gel electrophoresis to show the efficacy of the digestion. An example of these agarose gels can be seen in **Figure 3.30**. A UV ruler was used to measure the migration of the ladder bands. Therefore, the Southern blot probe bands could have their size determined by comparing the Southern blot membrane with the gel image and determining sizes using the ladder calibrated to migration length.

Transformations yielded numerous clones that passed the early screens, but these screens detected the genomic presence of both the selectable marker (pyrithiamine resistance) and fluorescence. Neither of these screens determine the location of the cassette insertion, and therefore cannot determine successful knockout of the target gene, as they may be ectopic insertions.

Gels were visualised as described in **Section 2.2.7**, with the addition of a UV ruler as a marker for later comparisons.

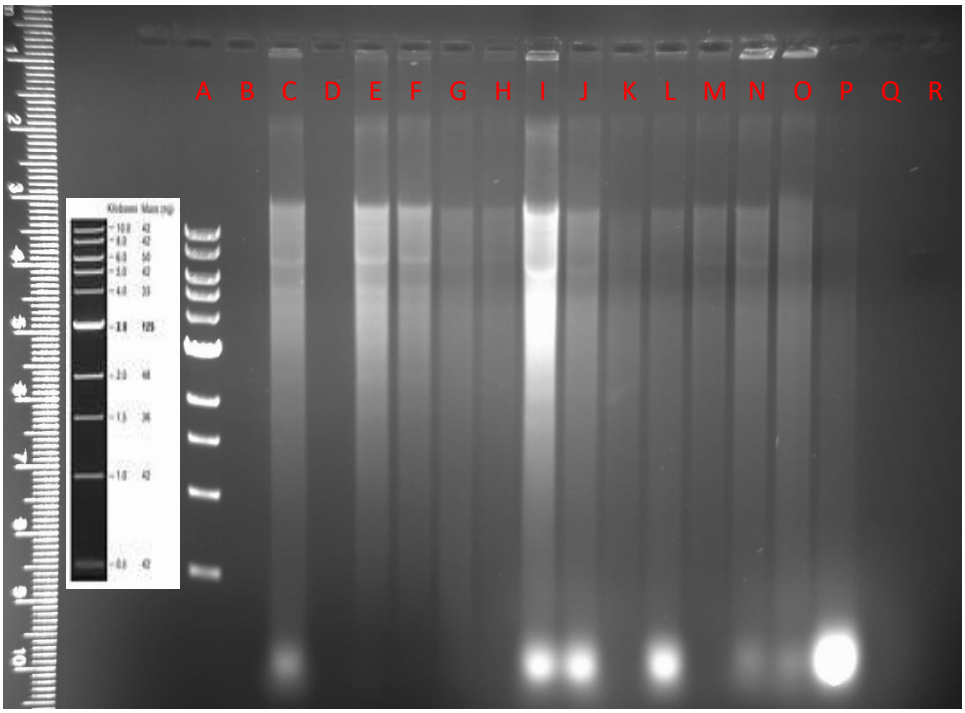


Figure 3.30. Gel electrophoresis image showing the configuration of DNA samples of *A. niger* 166 post digestion with *Bam*HI. ready for Southern blot analysis. Lane A) 10 µL 1 kb ladder (NEB, U.K). Lanes B, D and Q are all blank. Lane C 28 µL WT 166 RE digested gDNA (prepared with loading dye). Lanes E-P are each 28 µL digested transformant gDNA. Lane R is the deletion cassette plasmid (not apparent at low concentration). UV ruler is used to relate actual distance to distance supplied by ladder, allowing for cross reference of expected band sizes between this image and that of the Southern blot. Note the presence of RNA contamination as illustrated by very small band smears towards the bottom of the image. This was despite the use of RNAse A in the gDNA preparation.

Ten different *A. niger* strains were selected for transformation hosts to yield Δ*akuB* and downstream Δ*sclB* and Δ*sclR* knockouts. The 10 strains selected were 8-152, 8-160, 8-161, 8-162, 8-163, 8-164, 8-166, 8-169, 8-175 and 8-178, chosen to reflect a range of sclerotial production (**Figure 3.21**). Strains 8-169, 8-175 and 8-178 were selected as they did not produce sclerotia on any media selected. Southern blot revealed a lack of successful transformations in three of the strains (8-152, 8-164 and 8-175) despite repeated attempts. 8-152 had many clones exhibiting pyrithiamine resistance but all were shown to be ectopic by Southern blot. 8-164 and 8-175 had very poor transformation efficiencies and rarely yielded clones on selective media containing pyrithiamine in the first instance. Strains 8-160 and 8-162 yielded typically >50 clones per transformation (across three selection plates), all other strains yielded less than 20 clones (across three plates) per transformation. In many cases, this meant that multiple transformation attempts were needed to yield enough clones that could be used to give a positively targeted insert. Strain 8-160 had a successfully targeted insert efficiency of 13.5 % (17/52) and strain 8-162 had a successfully targeted insert efficiency of 7.4 % (4/54), All other strains that had successfully targeted insert had an efficiency less than 2 %. This is comparable to previously reported transformation efficiencies in *A. niger* of 1.78 % (Zhang *et al*., 2011) utilising a similar protocol of transformation by polyethylene glycol and calcium ions. These results also provide evidence that both 8-160 and 8-162 are both particularly amenable to transformation. It was anecdotally noticed that the cassettes including eGFP had greater transformation success than the TdTomato cassettes, although it could only be conceived that this was due to the decrease in size between the two (3,180 bp to 3,891 bp).

In silico determination of a suitable restriction enzyme for southern blotting is described in **Section 3.2.2.11**. This revealed that *Bam*HI would be a suitable restriction enzyme for this purpose. In all three comparable genomes (wildtype gDNA and those of Δ*akuB_tdtomato* and Δ*akuB_eGFP*) the products of digestion containing the 1 kb probe were less than 8 kb and could be distinguished clearly. The absolute sizes of the three products were determined in silico to be 1,812 bp for the wild type, 3,180 bp for the knockout including eGFP and 3,891 bp for the knockout including the Tdtomato. All three contain the same *Bam*HI site 121 bp upstream of the start of the probe annealing sequence (termed *akuB* upstream 1kb). The wild type contains a *Bam*HI restriction site within the *akuB* gene, whilst the other two transformant classes were predicted to contain the same *Bam*HI site 14 bp into the pyrithiamine resistance cassette. The reason for the difference in size between the eGFP and Tdtomato constructs is the difference in size between the eGFP gene cassette (721 bp) and the Tdtomato gene cassette (1,432 bp), both of which are upstream of the *Bam*HI site in the pyrithiamine resistance cassette.

Southern blots were used to determine a shift in size from the wild-type sequence containing the probe to the larger fragments containing the fluorescent *akuB* knockout as well as the probe. **Figure**

3.31 demonstrates a typical Southern blot membrane after both NBT/BCIP and gel doc CHEMI exposure. The gel doc CHEMI exposures demonstrating more noise and were more difficult to analyse than the NBT/BCIP images. In the left-hand result in **Figure 3.31** the wild type (wt) band is clearly demonstrated in its control lane and is additionally seen in Lanes 1-3 and Lane 5. Each of these transformants demonstrate multiple ectopic copies of the probe sequence and are negative. Lane 4 shows a clear band at the expected 3,891 bp for the inclusion of the Δ*akuB_Tdtomato* and an absence of other bands showing no other insertions of the probe targeting sequence. This was taken to be a positive result. The right-hand image shows positive results for the targeted insertion of the Δ*akuB_eGFP* cassette. The plasmid (P) was used as a positive control and shows a dominant product band despite the greatly reduced concentration of plasmid DNA compared to gDNA because of its greater target proportionality.

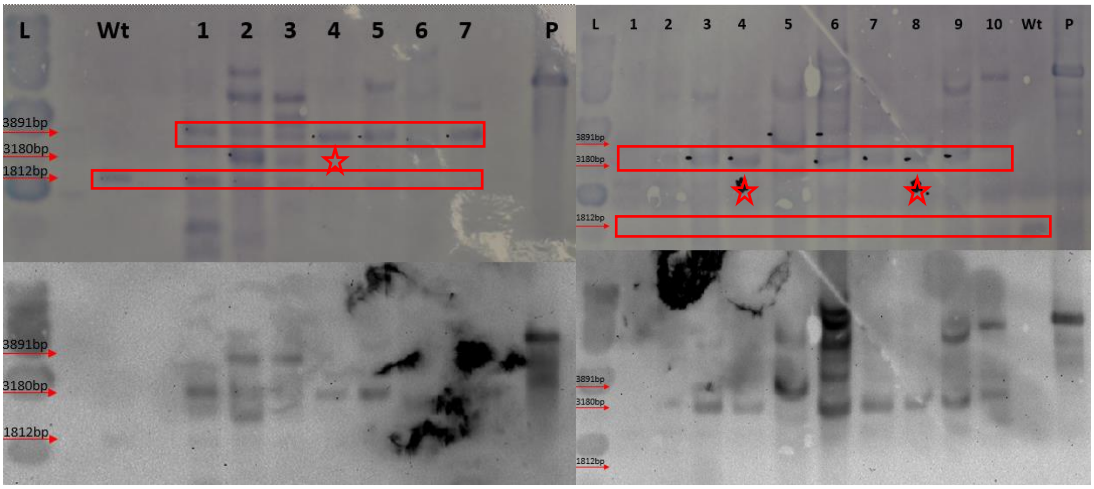


Figure 3.31. Southern blot results after digestion with *Bam*HI. Top images are both photos taken after exposure of membrane with NBT/BCIP substrate solution. Bottom images are Gel-doc images produced after CHEMI exposure. Left hand images are the same membrane as one another as well as the right-hand images being the same membrane as one another. Expected restriction digest product sizes are given in red arrows (see **Appendix 20** for full details but simply wt is 1812bp, TdT insert 3891bp and eGFP insert 3180bp). Lanes 1-7 are transformations that have passed all screening to this point. Left hand lane 4 is 169_t3 is marked with a red star (confirmation of Δ*akuB_tdtomato* as has confirmation band at 3891bp with no other probe binding sites – so no ectopic insertions). Right hand image Lane 4 is 8-162_e25 and Lane 8 is 8-162_e15, both are marked with red stars (both are confirmation of Δ*akuB_eGFP* as have confirmation band at 3180bp with no other probe binding sites, so no other ectopic insertions). L – Ladder, Wt – Wildtype, P – Plasmid. Plasmid digested as positive control (with *Xho*I) was the linearised *pUC19_AkuBup_PgpdA_TtrpC_ptrA_AkuBdown plasmid* (as shown in **Figure 3.5**) yielding a product of 7973bp.

<u>Knockout of *sclB* and *sclR* genes</u>

Successful transformants of the Δ*akuB* genotype were generated and then subsequently transformed with the Δ*sclB_hph* and Δ*sclR_hph* cassettes. This was completed for strains 8-160 (twice), 8-161, 8-162 (twice), 8-163, 8-166, 8-169 and 8-178. Successful transformations of these were confirmed with growth on hygromycin as well as colony PCR (described in **Section 3.2.2.9**). An additional Δ*akuB*

transformant from 8-166 was retrieved but subsequent transformations with Δ*sclB* and Δ*sclR* yielded phenotypes that were not statistically significant from the controls. Given that this was so unusual against the findings of all the other transformed strains, it was ascertained that this transformant had not integrated the Δ*sclB* and Δ*sclR* DNA successfully and the data collected from it has been excluded from the later analyses.

The Δ*sclB_hph* and Δ*sclR_hph* transformants were used to characterise the role of the respective transcription factor genes. **Figure 3.32** shows images of representative Δ*sclR* and Δ*sclB* knockouts of strain 8-160 (e2) with 8-160 Δ*akuB* (progenitor) as a control. There were stark differences in the number of sclerotia produced as well as the conidiation patterns, similar results were seen with the other strains. It had previously been described that sclerotia were not produced where the raisins had been laid on the CYAR agar (Ellena *et al*., 2021a), and this was particularly apparent in the Δ*sclB* transformants for each strain.

Controlled experiments were then completed to determine differences in radial growth of colonies, conidiation, sclerotia number and sclerotia size amongst these transformants.

Figure 3.32. Light photographs of strain 8-160_*akuB*:: PgpdA$_{(Anid)}$_*eGFP*_TtrpC$_{(Anid)}$ (left) 8-160_Δ*akuB*::*PgpdA*$_{(Anid)}$_*eGFP2_TtrpC*$_{(Anid)}$_Δ*sclR*_hph (centre) and 8-160_Δ*akuB*::*PgpdA*$_{(Anid)}$_*eGFP_TtrpC*$_{(Anid)}$_Δ*sclB*_hph (right). Sclerotia are small spherical white structures (shown with red arrowhead on in central image). Note that even when the culture is producing hundreds of sclerotia, it rarely does so where the raisins were laid on the media, but conidiates heavily instead, shown with the red circles.

A comparison of the radial growth of colonies could be made between Δ*sclB* and Δ*sclR* against a control strain. The control was the Δ*akuB* knockout that was used to generate the Δ*sclB* and Δ*sclR* knockouts and was itself subject to the transformation protocol but as the positive control. This ensured that the control had been through as similar a set of experiments so that any differences could be attributed to the nature of the knockout and not the stress of the transformation itself. Radial growth of colonies was measured after 3 days of growth on CYAR, as this gave a large enough diameter

to measure differences accurately without causing overlap or intergrowth of colonies. Inoculation of CYAR was completed as described in **Section 4.2.2.5**. **Figure 3.33** shows the results of the experiment.

The effect of deleting *sclB* or *sclR* on radial growth was evaluated on all strains that had been successfully transformed. A similar study had been completed on the Δ*sclR* in *A. oryzae* (Jin *et al.,* 2011). The data had no outliers (ROUT) and passed the assumptions of normality with Shapiro Wilk test. Two-Way ANOVA showed that there were highly statistically significant differences in radial growth (mm) both between strains [$F(8, 243) = 102.9$, $p < 0.0001$] and between gene knockouts and control within strains [$F(2, 243) = 270.5$, $p < 0.0001$]. There was also interaction between the two [$F(16, 243) = 12.33$, $p < 0.0001$]. Tukey's multiple comparison test revealed that there was no statistical difference between the control and Δ*sclB* for any strain. For all but two strains, there was a highly statistically significant difference between the control and Δ*sclR* as well as the Δ*sclB* and Δ*sclR*. The two instances where there was no statistical difference between any of the control, Δ*sclB* and Δ*sclR*, both instances from the same source strain – 8-160 (e15 and e25). These data suggest that Δ*sclB* has no impact on radial growth but Δ*sclR* reduces radial growth. By contrast, Δ*sclR* in *A. oryzae* was described as having an increase in radial growth (Jin *et al.*, 2011).

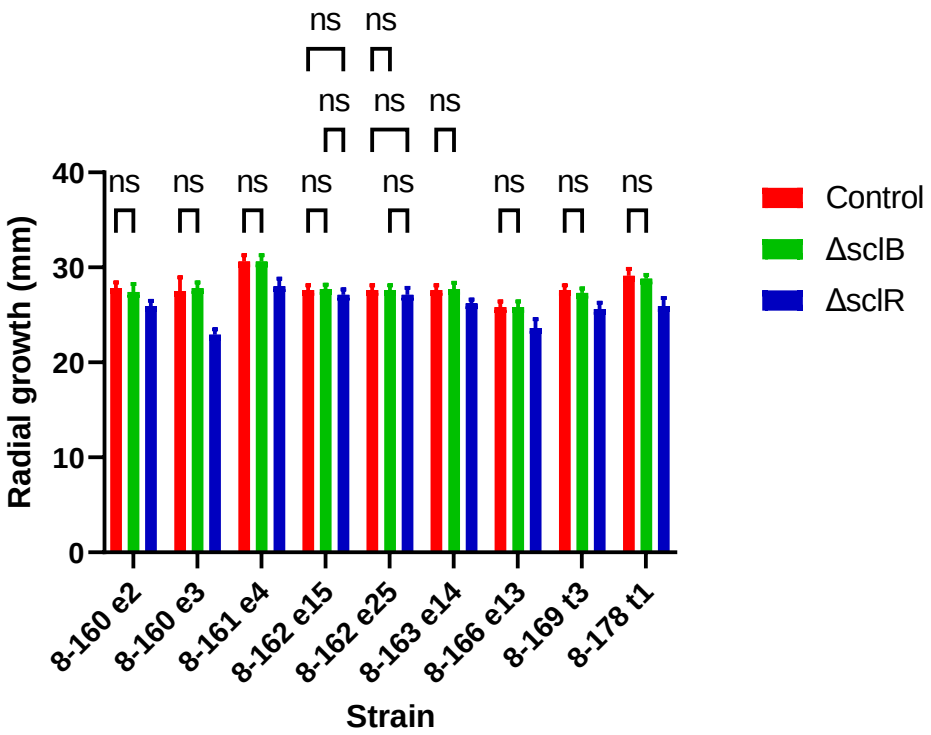


Figure 3.33. Graph showing radial growth (mm) of different *A. niger* strains and gene knockouts after 3 days growth on CYAR. Only non-significant differences are shown (ns), for all other comparisons $p < 0.0001$ (not shown).

A comparison of conidiation of one strain (8-160) was completed. Data was collected from conidiation in the control, as well as two successful Δ*sclB* and two Δ*sclR* transformants of the strain (i.e. five isolates in total including control). Conidiation varied across the plates when strains were grown on CYAR, with dense conidiation in the area where the raisin is placed on the plate (See **Figure 3.32**) and anecdotally appeared to decrease from that point towards the edge of the plate. Cultures were grown for 10 days to maturity at 28 °C. Conidia were counted as described in **Section 3.2.2.2**.

Data collected had no outliers according to the ROUT test and passed the Shapiro Wilk test for normality. Two-way ANOVA showed a statistically significant difference between both plate areas (here defined as area where either raisin had been laid, not laid or periphery of culture) [$F(2, 117) = 44.73$, $p < 0.0001$] and between control and transformants [$F(4, 117) = 122.3$, $p < 0.0001$]. Additionally, there was interaction between plate areas and isolates [$F(8, 117) = 4.903$, $p < 0.0001$]. Tukey's multiple comparison test described a pattern whereby the Δ*sclB* strains consistently produced fewer conidia than either the Δ*sclR* and the control with a minimum of $p < 0.01$ for each comparison between either Δ*sclB* transformant and the Δ*sclR* and control at any plate location. Data is shown in **Figure 3.34** and multiple comparisons are shown between control, Δ*sclB* and Δ*sclR*, for simplicity, all comparisons that are $p < 0.0001$ are removed from the figure. Results for the two Δ*sclB* strains were consistent and there was no statistical difference between them at any point. Between the control and Δ*sclR* transformants, there was less consistency. Within the raisin location there was statistical difference between the control and each of the Δ*sclR* transformants ($p=0.0322$ and $p<0.0001$ respectively) and equally between the two Δ*sclR* transformants themselves. At the inoculation points, there was no statistical difference between the number of conidia produced by the control and either of the Δ*sclR* mutants, nor between the Δ*sclR* mutants themselves.

Conidiation at the edge of the colonies was more variable with no statistical difference between the control and Δ*sclR*1, but a small difference between the control and Δ*sclR*12 ($p = 0.0101$). However, there was a surprisingly large difference between the two Δ*sclR* isolates ($p < 0.0001$). This is likely because of the increased variation of conidiation towards the edge of the plates, particularly in the Δ*sclR* strains that produce consistently fewer sclerotia.

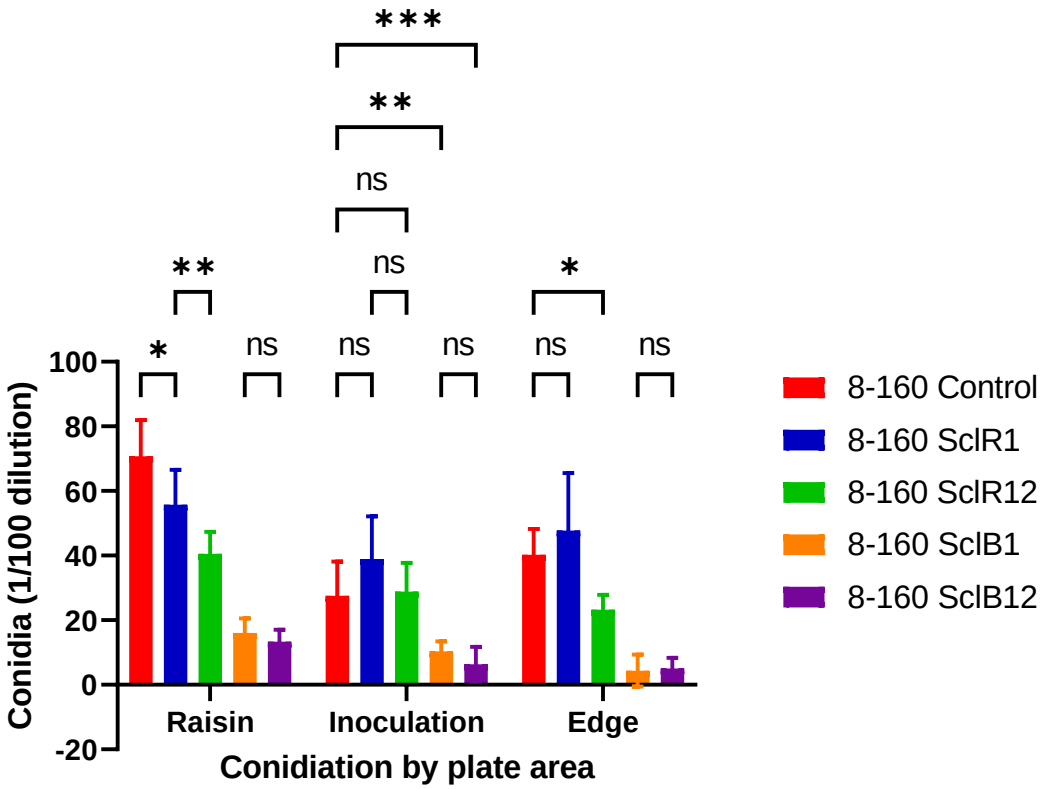


Figure 3.34. Graph showing the number of conidia between different transformants and host control strain of *A. niger* 8-160. Conidiation varied across a plate, therefore subgroups of raisin, inoculation (inoculation point) and edge (of plate) were used to demonstrate these differences. Two-way ANOVA confirmed statistically significant differences between conidiation areas (Raisin, inoculation point and plate edge) as well as between control and transformations of 8-160 (both $p<0.0001$). Tukey's multiple comparison test results are shown other than all $p < 0.0001$ comparisons (*** $p<0.001$, ** $p<0.01$, *$p<0.1$).

Sclerotia production was assessed for the impact of gene knock out in the Δ*sclB* and Δ*sclR* mutants. Sclerotia were counted as described in **Section 3.2.2.1**. Sclerotia were not produced by all strains and these strains were removed from analysis, but it is worth stating that strain 8-169 and 8-178 had not produced sclerotia previously under any conditions, and deletion of *sclR* and *sclB* did not change this phenotype.

Data was analysed for outliers using ROUT and none were found, and data then passed the Shapiro Wilk test for normality. Two-Way ANOVA revealed highly statistically significant difference between strains [$F (6, 84) = 52.11$, $p < 0.0001$]and within each transformation condition within a strain [$F (2, 84) = 775.4$, $p < 0.0001$], between the knockouts and controls. Additionally, there was interaction between the strains and transformation conditions [$F (12, 84) = 18.70$, $p < 0.0001$]. **Figure 3.35** shows the results and includes the multiple comparisons, specific comparisons that had a $p < 0.0001$ made the majority of comparisons and were excluded from the figure. Importantly, in every remaining strain, the Δ*sclB* transformant produced more sclerotia than the control and Δ*sclR*, and this was highly statistically significant ($p < 0.0001$) for all strains except 8-163 which had a highly statistically

significant increase compared to the Δ*sclR* but only a weakly significant difference between the control and the Δ*sclB*. There was no statistically significant difference between the control and the Δ*sclR*. However, the Δ*sclR* knock out of 8-163 produced no sclerotia across any of the three plates observed. This was the one instance of the complete abolition of sclerotia production in a strain that previously produced sclerotia. These data suggest that Δ*sclB* confers an overproduction of sclerotia phenotype compared to the control wild type and Δ*sclR* results in an under production of sclerotia compared with the control wild type, and in one instance abolishes sclerotia production completely (8-163 Δ*sclR*). However, in the strains in which the controls did not produce sclerotia, Δ*sclB* did not enable the production of sclerotia as had been previously described (Jorgensen *et al.,* 2020).

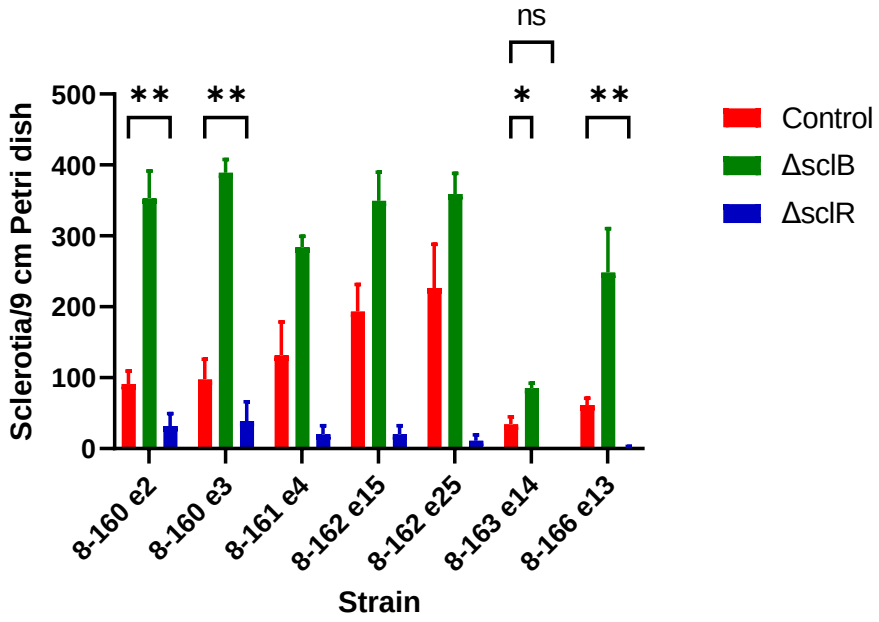


Figure 3.35. Graph showing the number of sclerotia produced per 9 cm diameter diameter Petri dish (CYAR) of different *A. niger* strains and transformations. Graph shows relationship between strains and within each strain, between transformants of the control, Δ*sclB* and Δ*sclR*. Results of Tukeys multiple comparisons test were all p<0.0001 other than those displayed (** p<0.01 and * p<0.1). Strain 8-163_e14_Δ*sclR* failed to produce sclerotia on any culture (n=5). Strain 8-166_e13_Δ*sclR* failed to produce sclerotia on 3 of 5 cultures. Strains 8-169_t3 and 8-178_t1 are absent but produced no sclerotia under any conditions. The control in each case is the strain transformed to Δ*akuB* and produced as the positive control of the respective transformations that yielded Δ*sclB* and Δ*sclR* mutants of that strain.

To determine any further differences to the sclerotia produced by the Δ*sclB* and Δ*sclR*, sclerotia were harvested from the same plates that were used to count sclerotia. Sclerotia widths were measured as described in **Section 3.2.2.1**. Again, all transformation forms and controls of 8-169 and 8-175 were removed from analysis as they all failed to produce sclerotia.

Data were shown to be absent outliers using ROUT and data passed both the Shapiro Wilk normality test. Two-way ANOVA revealed no statistical difference within strains (between control, Δ*sclB* and

Δ*sclR*) [F (2, 342) = 1.322, p < 0.2680]. However, it did reveal a difference in widths between strains [F (5, 342) = 16.90, p < 0.0001] (**Figure 3.36**).

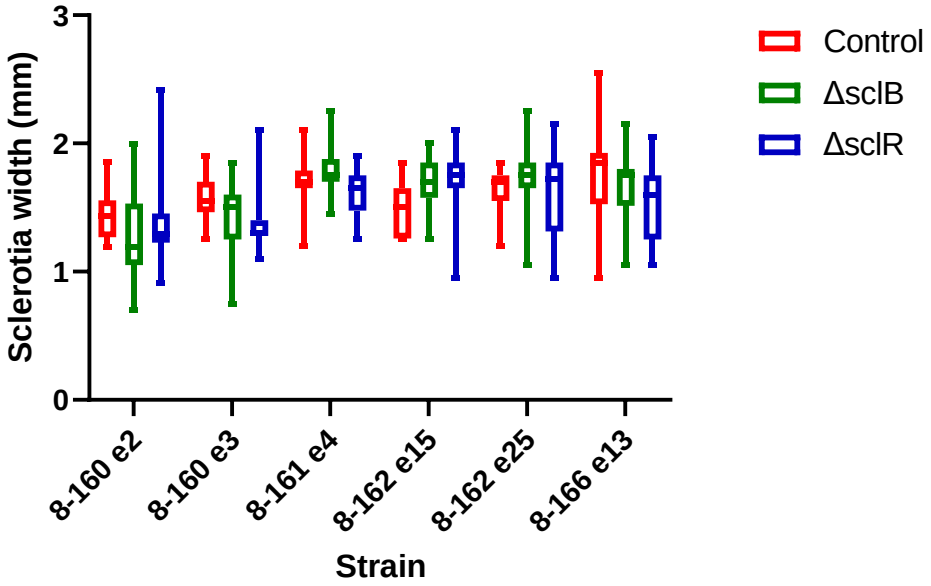


Figure 3.36. Box and violin graph showing the widths of sclerotia. Each strain used here produced sclerotia under each condition (Strain 8-163_e14 is absent due to the lack of sclerotia under Δ*sclR*, 8-169_t3 and 8-178_t1 are absent as they did not produce sclerotia under any condition). Two-way ANOVA found no statistical difference between the controls or transformants of any one strain but confirmed a statistical difference between strains (p<0.0001).

3.3.3 Investigation of Hormones from Sclerotia as a Potential Source of Novel Applied Metabolites

Potential hormones from 'crosses' of *A. niger* were collected as described in **Section 3.2.2.13**. Once optimal conditions for sclerotia production in two isolates of opposite mating type were determined, hormone extractions were completed. Firstly, extracts from a 4-day cultures that had been inoculated with isolate 8-166 (MAT1-1) were collected**.** The step of concentrating the extract occasionally yielded a black tar-like substance that was only soluble after repeated pipetting and as such two collections were made where one was the whole extract (black), and the other was only the readily soluble extract (green). These extracts are shown in **Figure 3.37.** Additionally, an extract was made from sterile CYAR plates as a negative control. The black from the extract is likely to have contained components of fungal melanin from *A. niger* conidia (Gomez and Nosanchuk, 2003) as this was not present in the non-inoculated plates.

Figure 3.37. Light photograph of two extracts, one whole extract with a characteristic black colour, one with only the readily soluble components of the extract which has a characteristic green colour.

To determine whether the extracts had any bioactivity, 1:10 serial dilutions were made up with ethyl acetate or chloroform: methanol depending on their extraction method. For each extract, 100 µL of each of these serial dilutions (concentrations) were pipetted on to the centre of 5 cm CYAR plates in triplicate and allowed to air dry in a laminar flow hood. After drying, a noticeable deposit was left on the surface of the plate where the extracts had been added. Additional controls of pure ethyl acetate and no solvent/extract plates were also prepared. These plates were then inoculated with 5000

conidia in 5 µL inoculation points and incubated at 28 °C for 10 days in the dark to promote sclerotia formation. After 10 days the plates were sealed and after 14 days were scored for sclerotia production by counting total number of sclerotia produced per plate (as described in **Section 3.2.2.1**). Results of this initial sclerotia scoring assay can be seen in **Figure 3.38**. ROUT found no outliers and the data passed the Shapiro Wilk test for normality. There was no statistically significant effect between any of the extracts but a weak statistically significant difference due to changes in concentration (p=0.0379). However, the data was consistently highly variable and there was no systematic increase or decrease in sclerotia production correlated with addition of increased extract.

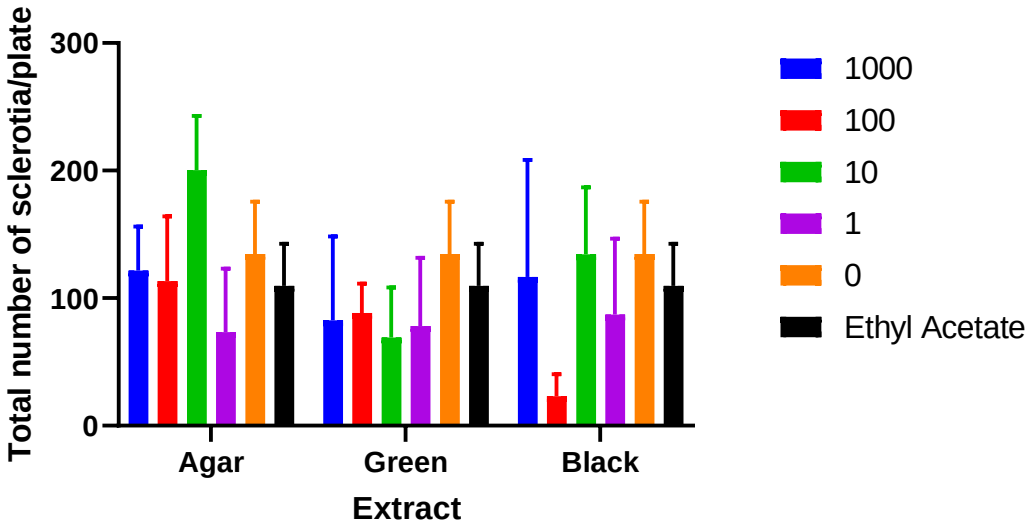


Figure 3.38. Graph showing the number of sclerotia produced on CYAR after addition of varying concentrations of the different extracts. CYAR agar was used as an extract control. Extracts were retrieved after 4 days from an axenic culture of isolate 8-166. There was no statistically significant difference between extracts or concentrations when compared to controls.

Additionally, the same protocol was used to determine whether any of the extracts could induce sclerotia formation on CYA rather than CYAR media. The addition of raisins has been determined to be necessary for sclerotia formation, and if the extracts from CYAR could induce sclerotia formation on CYA, it would be evidence that whatever the nutritional cue on the raisins which induced sclerotia formation would be soluble in ethyl acetate.

The results (**Figure 3.39**) show clearly that raisins on the CYA media are necessary for sclerotia development and that the nutritional cue is not soluble in ethyl acetate (the same result was seen with chloroform: methanol). CYAR plates produced approximately 200 sclerotia per plate whilst the

CYA plates produced none ($p<0.0001$). The effect was the same for each extract, with a small non statistically significant reduction in sclerotia production in the plates prepared with black extract.

Similar experiments were completed at varying time points of 3, 5, 6, 7, 8, 9, 10, 30 and 60 days and no statistically significant increase or decrease in sclerotia production was ever found (extensive dataset not shown). Experiments using extracts of one mating type and inoculated with conidia of the opposite mating type also had no statistically significant results (not shown). Finally, modifying the extraction protocol using chloroform: methanol yielded no statistically significant results (not shown).

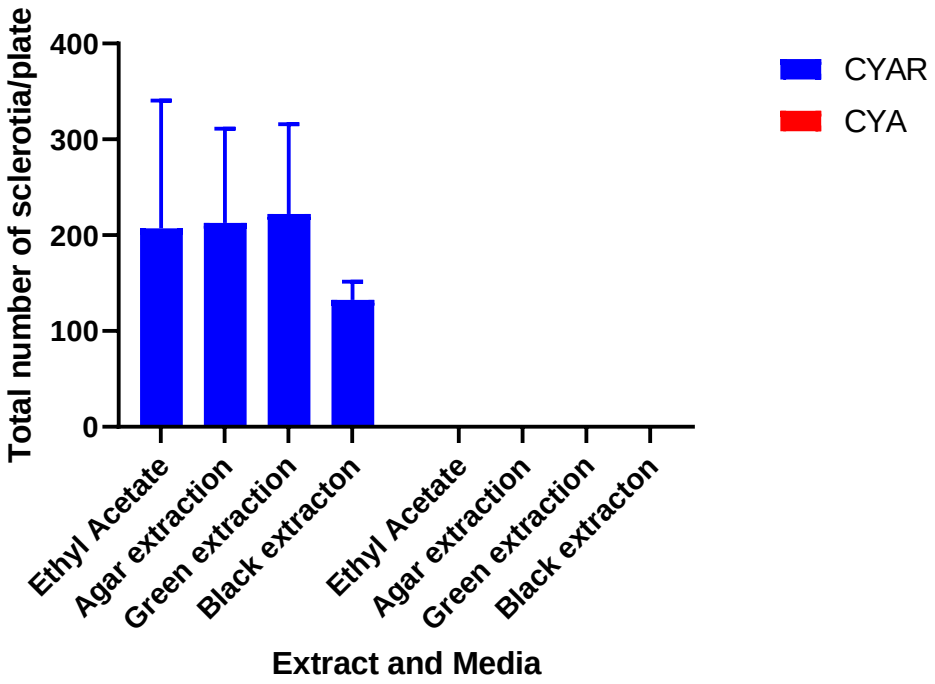


Figure 3.39. A graph showing the number of sclerotia produced by different extracts on different prepared media (CYAR and CYA). There was no statistical difference in sclerotia produced between the different extracts but sclerotia were only produced on CYAR at a mean of ~200 sclerotia per plate, and CYA produced no sclerotia on CYA with any added extract ($p<0.0001$). This provides evidence that the nutritional factor in CYAR that induces sclerotia production is not soluble in ethyl acetate. Similar results were seen with chloroform:methanol.

3.4 Discussion

As discussed previously, by the onset of this PhD, a sexual cycle had been demonstrated in a very limited number of species of the black aspergilli (Rajendran and Muthappa, 1980; Horn *et al*., 2013; Darbyshir *et al*., 2014). The sexual cycles of the respective species (*A. japonicus Saito, A. sclerotiicarbonarius* and *A. tubingensis*) had been elucidated only under very specific nutritional and environmental conditions. Additionally, the necessity for sclerotia production was found as a prerequisite for these sexual cycles, suggests that conditions allowing for production of sclerotia in *A. niger* will be an essential component of elucidating sex in *A. niger*. Therefore, work was undertaken to analyse a number of nutritional or environmental conditions from the scientific literature that suggest they may have an important role in sclerotia production in members of the black aspergilli, most notably *A. niger*. Additionally, a characterisation of two transcription factors, *sclR* and *sclB*, was completed across several strains of *A. niger*. Finally, experiments were completed to identify any endogenously produced hormones or morphogens that may impact sclerotia production.

3.4.1 Environmental and Nutritional Factors and Their Role in Sclerotia Production

Recent findings of a sexual cycle in members of *Aspergillus* section *Nigri* (Rajendran and Muthappa, 1980; Darbyshir, 2014; Olarte *et al.,* 2015) and section *Flavi* (Horn *et al*., 2009a; Horn *et al*., 2009b) have revealed that the formation of sclerotia is a prerequisite for sexual reproduction. The sexual morph of *Aspergillus* species that produce ascospores within the section *Nigri* have been defined as saitoa and petromyces and those of the sclerotia of section *Flavi* have been defined as petromyces (Rajendran and Muthappa, 1980; Dyer and O'Gorman, 2012). Therefore, conditions that either promote or increase sclerotia production in the black aspergilli, and notably *A. niger* were analysed. It was anticipated that arising results would aid efforts in inducing sexual reproduction in *A. niger*.

One of the more notable conditions that have been implicated in success in inducing sexual cycles amongst section *Nigri* and section *Flavi* is the growth media used. The recent discoveries of sex in *A. flavus*, *A. parasiticus* and *A. tubingensis* were all completed on a relatively undefined mixed cereal agar (MCA) media (Horn *et al*., 2009a; Horn *et al*., 2009b; Olarte *et al*., 2015; McAlpin and Wicklow, 2005). Sclerotia production in *A. niger* has been reported historically, however, it has been argued that this likely involved mis-identification and was due to other members of section *Nigri* rather than *A. niger sensu stricto* (Samson *et al*., 2004; Frisvad *et al*., 2014). Sclerotia production was first conclusively identified in several strains of *A. niger* (identified using a polyphasic approach) when grown on Czapeks yeast autolysate (CYA) enriched by addition of biotin and certain types of raisins (Frisvad *et al*., 2014). Additionally, sclerotia production in *A. niger* and other members of the section *nigri* has been reported on malt extract agar (MEA) (Samson *et al*., 2004; Ellena *et al*., 2021). Meanwhile, previous work had shown that the sexual cycle of *A. fumigatus* (neosartorya sexualmorph) could be induced on a relatively undefined oatmeal agar (OA) (O'Gorman *et al*., 2009).

In the present study, representative isolates of two species, namely *A. niger* and *A. tubingensis* were analysed for their sclerotia production on varying media. *A. tubingensis* had been previously described to produce sclerotia on various media (Samson *et al*., 2007b; TePaske *et al*., 1989). Results of this study confirmed that *A. tubingensis* strains can produce sclerotia on various growth media. However, not all strain were able to produce sclerotia, with several strains not producing sclerotia on any media tested (33/55 strains tested produced sclerotia – 60%). Interestingly, the use of CYAR, OAR and MEAR produced a statistically significant increase in sclerotia when compared with basal media without raisins and other media trialled. Furthermore, *A. tubingensis* strains rarely produced sclerotia on MCA or MCAR, and those that did, produced very few sclerotia in number (**Figure 3.11**), highlighted in particular was the use of the four highest scoring strains on CYAR (9, 45, 46 and 47), only two of which produced any sclerotia on ME (9 and 47) and even then less than 10 / cm^2. This contrasts with the reports of Horn *et al*. (2013) in which strains regularly produced sclerotia on MCA and on which sex

was elucidated. Explanations for this discrepancy may be changes from the protocol used, in which slopes were bagged by Horn *et al*. (2013) to limit air exchange whereas the present study used Petri dishes that were sealed with Parafilm, or changes to the formulation of Gerber's MCA used between the studies (the original mixed cereal baby food formulation was unfortunately no longer available). Meanwhile, results from this study were in agreement with earlier studies showing *A. niger* sclerotia production on CYAR (Frisvad *et al*., 2014) (reliable production was only 7 out of 28 – 25%) as well as demonstrating regular production of sclerotia on MEA amended with raisins (MEAR) (reliably this was 10 out of 28 – 36%). It is noted that these growth tests used a specific type of raisin, namely 'Sun Maid' raisins, as Frisvad *et al.* (2014) and Ashton (2018) had previously found the type of raisin used was important for induction of sclerotia. The data showed no statistically significant differences between the two media (**Figure 3.21**). Strains that produced sclerotia on one media type invariably did so on the other media type. However, many other strains failed to produce sclerotia on any media tested. Interestingly, of the ten strains that did produce sclerotia on these media, six were obtained environmentally from raisins (**Appendix 9**), which is indicative of a possible evolutionary adaptation to raisins as a nutritional cue.

Previous studies of the aspergilli in general have failed to find a single set of conditions that can be used to induce sclerotial formation in phylogenetic groupings of *Aspergillus* taxa that show sclerotia production. Conditions as varied as pH, carbon source, nitrogen source, C:N ratio, light and oxygen levels have been evaluated for impact on sclerotial production in such *Aspergillus* species (McAlpin and Wicklow, 2005; Agnihotri, 1967). These studies were completed before the discovery of sclerotia production in *A. niger sensu stricto*. Furthermore, additional species, such as the aptly named *A. sclerotiicarbonarius* have previously been reported. *A. sclerotiicarbonarius* is very broad in the media range in which it produces sclerotia (this study, Petersen *et al.,* 2015; Noonim *et al*., 2008). However, as discussed earlier, sclerotia production in *A. niger* and *A. tubingensis* is more fastidious, with *A. niger* sclerotia production only occurring on MEA and CYA amended with raisins of various media so far tested (Frisvad *et al.,* 2014). Both MEA and CYA contain or are composed entirely of undefined components. MEA is composed of malt extract which likely contains proteins, vitamins and salts. CYA has many defined components, but the use of yeast extract introduces undefined levels of additional amino acids, proteins, vitamins, salts and trace elements (Grant and Pramer, 1962). The addition of exogenous trace metal solutions such as Hutner's trace element solution has been widely used in defined minimal media for *Aspergillus* growth (Alves de Castro *et al*., 2021). The usage of such trace elements in media have been reported with CYA prepared with zinc and copper added (Silva *et al*., 2011), and without (Frisvad *et al*., 2014). Indeed, the use of zinc has been implicated in gliotoxin production in *A. fumigatus* (Seo *et al*., 2019) and manganese (absence) plays an important role in citric acid overproduction in *A. niger* (Karaffa and Kubicek, 2003). In the present study it was found that the

addition of Hutner's trace metal solution to CYAR abolished the production of sclerotia in three strains of *A. niger* which produce sclerotia on CYAR without Hutner's added (**Figure 3.22**). Additionally, the addition of Hutner's produced stark, black conidia. This indicates a link between the development of pigments in the asexual cycle linked to trace elements. Indeed, work has been completed demonstrating the need for copper in normal pigmentation (Agnihotri, 1967). Several trace elements and vitamins such as biotin, copper and manganese have been implicated in enhancing sexual reproduction in ascomycete fungi (Kent *et al*., 2008; Bohm *et al*., 2013; Frisvad *et al*., 2014). Therefore, developing an understanding of the balance of trace elements needed for sclerotia production, without compromising sexual activity would be insightful.

It has previously been shown that the density (amount) of spores used as an inoculum can have a major impact on subsequent fungal growth. For example, the impact of spore density on development has been studied in the switch between yeast and hyphal growth habits in several yeasts such as *Candida albicans* and *Saccharomyces cerevisiae* (Sudbury *et al*., 2004; Jagtap *et al*., 2020). This suggests a mechanism of quorum sensing within these fungi; indeed, farnesol has been demonstrated as a quorum sensing molecule in *C. albicans* (Davis-Hanna *et al*., 2008) as well as having function in conidial development in *A. niger* (Lorek *et al*., 2008). Inoculum conidia density has been shown to impact sclerotia formation in *A. flavus* (Brown *et al*., 2008) where it was found that lower spore density resulted in higher sclerotia production. Additionally, extracts prepared from the spent media of low spore density grown cultures, was shown to reduce conidiation and stimulate sclerotia formation (Brown *et al*., 2008) suggesting a diffusible chemical morphogen responsible for development pathway based on spore density. Spore densities of 3500/7 µL and 1,000 per point (volume not disclosed) have been used in *A. niger* and *A. tubingensis* for sclerotia production and sex, respectively (Horn *et al*., 2009a; Ellena *et al*., 2021) but there have been no specific studies in *A. niger* and *A. tubingensis* emulating those completed for *A. flavus* density dependent development. Therefore, we assessed the impact of conidia concentration on sclerotia production within the black aspergilli. Studies had previously been completed in the Nottingham University research group suggesting an optimal concentration of 5,000 spores in a 5 µL volume for *A. niger* sclerotia production (Ashton, 2018) so this study focussed on *A. tubingensis* to examine differences if they existed between the closely related species. It was found that the same inoculum levels (i.e. 5 µL of a 1,000 spores/µL suspension – so 5,000 conidia) was also near optimal for sclerotial production in *A. tubingensis*. These results are in consistent with other studies previously discussed. However, in contrast to findings for *A. flavus*, lower concentrations created through the spreading of equal numbers of conidia (as opposed to point inoculation) led to drastically reduced sclerotia production. This may be due to an increased distance between conidia reducing the concentration of any quorum sensing molecules for germinating conidia, directing a separate developmental pathway leading to reduced sclerotia. At high

concentrations, this signal may be too 'noisy' for a distinct pathway to be selected by germinating conidia. A similar effect has been described in *N. crassa* which have different hyphae called conidial anastomosis tubes (CATs) produced in response to a high concentration of neighbouring germinating conidia (Roca *et al*., 2005).

Farnesol has been demonstrated to have an impact on conidiation in *A. niger* (Lorek *et al*., 2008). The possible impact of farnesol on sclerotia production was therefore an interesting topic of study, as a reduction in conidiation may result in an increase in sclerotia formation. This hypothesis was gleaned from studies that have found increasing conidiation with decreased sclerotia production, a phenomenon which has been reported both between cultures and within single cultures (Brown *et al*., 2008; Ellena *et al*., 2021). Indeed, similar hypotheses which describe a morphological pathway switch towards either sexual or asexual reproduction have been proposed (Dyer *et al.* 1993; Dyer and O'Gorman, 2012; Frawley *et al.,* 2020). The sexual development pathway may consist of two separate linked or unlinked pathways, with one necessary to produce accessory tissues such as sclerotia and Hülle cells, and the other – the formation of cleistothecia and ascospores (Dyer and O'Gorman, 2012). These pathways also result in differences in secondary metabolism and mycotoxin production (Calvo and Cary, 2015). However, it was found that addition of varying levels of farnesol had no statistically significant impact on sclerotia production (**Figure 3.23**), although there did appear to be a trend of decreasing sclerotia number with increasing farnesol concentration. That farnesol has an impact in both limiting or abolishing conidiation and lowering sclerotia production may suggest a link to the tetrameric pheromone module SteC-MkkB-MpkB-SteD (Frawley *et al*., 2020), MpkB being homologous to the Fus3 pheromone module in yeast (Maeder *et al*., 2007). The module has been shown to regulate both asexual and sclerotia production in *A. flavus* by directing environmental signals into transcriptional responses and therefore development (Frawley *et al*., 2020). Indeed, knockouts of any component of the module resulted in abolishment of both asexual sporulation sclerotia production.

Experiments where the use of a freezing step was employed (prior to conidial harvest for plate inoculation) was assayed to assess whether it had an impact on sclerotia production within the black aspergilli. A freezing step had previously been employed in other studies of sclerotia production in *A. niger* (Frisvad *et al*., 2014) and cold conditioning is also discussed as a trigger for the carpogenic or myceliogenic germination of sclerotia of other species such as *S. sclerotiorum* (Huang, 1991; Lane *et al*., 2019; Clarkson *et al*., 2004). In the present study, the use of a freezing step was found to result in no significant difference in sclerotia number when compared to cultures prepared without freezing in *A. tubingensis*. However, the use of a freezing step did produce a slight, but statistically significant difference in size (diameter) of sclerotia, with the addition of a pre-freezing of cultures before conidial harvest resulting in slightly smaller sclerotia. Sclerotia size has been discussed in relation to the mode of germination. Larger sclerotia have been reported to result in greater levels of carpogenic

germination in *S. minor* (Wu and Subbarao, 2008) and *S. sclerotiorum* (Taylor *et al*., 2018). The increased carpogenic germination of larger sclerotia is likely due to the accumulation of greater nutrient reserves which are utilised during germination and production of stipe, apothecia and ascospores (Willetts and Bullock, 1992).

A re-assessment of the optimal growth temperature for sclerotia production in *A. niger* was completed. Three strains (8-160, 8-161 and 8-166; **Appendix 9**) were selected which reliably formed reproducible numbers of sclerotia (these strains were also used in later studies). Temperatures for sclerotia production have been reported between 25 °C (Frisvad *et al*., 2014; Ashton, 2018) to 30 °C (Ellena *et al*., 2021; Seekles *et al*., 2022). *A. niger* grows optimally between 24 °C – 36 °C as previously reported on MEA (Samson *et al*., 2007b). Lower and higher temperatures of this range limited growth radius or conidiation. However, to the best of my knowledge, no direct reporting of an assay assessing sclerotia production of *A. niger* at different temperatures has been completed. Our findings corroborated those of optimal growth range on MEA when grown on CYAR and additionally provided evidence of optimal temperature for sclerotia production. For all three strains used in the assay, there was an increase in sclerotia production as temperatures increased to the optimal of 28 °C and 30 °C before declining in a non-statistically significant way at 32 °C and being completely abolished by 37 °C with accompanied limitation to growth. This was the case whether using the three raisin or six raisin inoculum approaches. A greater number of data temperatures between these points would indicate the statistical drop off temperature more clearly. The results of 30 °C being an optimal temperature for production of sclerotia corroborates the temperature used in more recent studies (Ellena *et al*., 2021; Seekles *et al*., 2022) and appears to offer an improvement for the purposes of increasing sclerotial production over the methods of Frisvad *et al*. (2014) where 25 ˚C was used. This is particularly evident as the present study used one strain in common (8-161) with the study of Frisvad *et al*. (2014) (therein referred to as IBT29019). Optimising temperature for sclerotia production may be beneficial but caution should be applied for utility in examining the sexual potential of sclerotia produced in crosses at these temperatures. Temperature has been recorded as one of the fastidious elements of closely related species in induction of a sexual cycle and what may be an optimal temperature for sclerotia production may not be for sexual development. Greater production of sclerotia at higher temperatures may be a survival mechanism in response to adverse environmental stimuli (Chang *et al*., 2020) rather than stimuli inducive to sexual recombination. As mentioned before, there may be two distinct, linked or unlinked pathways, one for development of accessory tissues (in this case sclerotia) and the other for development of cleistothecia (Dyer and O'Gorman, 2012). Thus, experimental manipulations such as a drop in temperature once sclerotia have been formed might be beneficial to inducing a sexual cycle.

3.4.2 Transcription Factors *sclR* and *sclB* and Their Role in Sclerotia Production

Several transcription factor genes have been identified in different *Aspergillus* species with roles in sclerotia development as discussed in **Section 3.1.2**. Two were selected for further study in this work, *sclR* and *sclB*. The aim of this work was to characterise both the impact of the transcription factors on sclerotia production across several strains of *A. niger*, as well as to look for any SNPs that may account for differences in sclerotia production. To that aim, six strains were selected for pilot sequencing of the coding region of the gene, two of which do not produce sclerotia under any tested conditions whilst the other four produced sclerotia reliably. Additionally, knockouts mutants of both transcription factors were completed for a number of strains, the wild type of which give a spectrum of sclerotia production, allowing for comparison between phenotypes.

Analysis of sequencing data of the coding region of these transcription factors revealed various findings. A key outcome was that, importantly, all of the strains appeared to encode functional SclB and SclR proteins, with no evidence of mutation (e.g. indels or frameshifts) within the coding regions. Thus, the lack of sclerotial production in strains 8-169 and 8-175 was apparently not due to mutational change within the coding region. Mutations within coding regions have been used to identify these targets previously, such as the *sclB* mutant reported by Jorgensen *et al*. (2020). A number of SNPs were found for each TF between the various strains, but again there was no consistent link between a given SNP and presence or absence of sclerotial production, and no single SNP was identified that could explain the differences in levels of sclerotia production between strains. There was a single residue in both TFs that was conserved amongst the six strains of this study, suggesting that the SNP causing this SNP was from the scaffold strain CBS 513.88. In *sclB*, the conserved SNP resulted in a residue change at amino acid 150 (**Table 3.3**). In *sclR*, the SNP caused a change in amino acid residue 491 (**Table 3.4**). Alternatively, the suggestion may be that the six strains belong to the same evolutionary clade and diverged from the CBS 513.88 strain. For either TF, strain 8-169 had a unique residue change at amino acid 42 resulting in a Pro>Ser, which may have a functional effect and explain the strains inability to form sclerotia, although this is unlikely given that *sclB* is a repressor and loss of function would most likely result in increases in sclerotia production. Indeed, the knockout of *sclB* from 8-169 resulted in no change to sclerotia production (see below). Additionally, across the two TF's, strain 8-175 and 8-162 shared an identical SNP profile compared to CBS 513.88. This is in stark contrast to the differences in sclerotia production between these two strains, with 8-162 being the foremost producer of sclerotia of all strains assessed (**Figure 3.21**), and 8-175 never having produced sclerotia under any condition. Therefore, any explanation for the differences in sclerotia production between these strains are likely found elsewhere than these TF's in the strains assessed. However, it is cautioned that this sequencing work was only a pilot study, as sequence analysis of the upstream

promoter region was not made, and mutation in this region can impact greatly on gene expression (Chettri *et al*., 2018; Albarrag *et al*., 2011; Amore *et al*., 2013). Also, if sufficient time had been available the expression levels of *sclB* and *sclR* would have been evaluated (e.g. by qRT-PCR) to determine any link between gene expression and production of sclerotia.

Knockouts of these TF's were then completed for several strains. As part of this work it was first necessary to create Δ*akuB (*Δ*aku80*) strains of the various wild-type strains to allow efficient gene targeting for knock-out construction (Zhang *et al.,* 2011). The gene *sclR* was previously characterised in *A. oryzae* (Jin *et al*., 2009; 2011), where both a knockout and overexpression strain were constructed. The knockout strain resulted in greater colony diameter, abolishment of sclerotia production and an increase in conidiation (~1.5 fold). The overexpression strain had reduced colony diameter, 4-fold increase in sclerotia and a drastic reduction in conidiation. Transcripts analysis of the overexpression strain revealed reduced transcript levels of *abaA*, *veA*, *brlA* and *wetA*, all of which are implicated in conidiation or control of sexual/asexual switching. Additionally, the transcript levels of a homolog of *ssp1* were increased. Ssp1 has previously been shown to be a major protein of sclerotia in *S. sclerotiorum* (Amselem, 2011; Li and Rollins, 2009). These results show that *sclR* is a TF upstream of the conidiation pathway, and key in mediating early switching to a sclerotia production phenotype. The results of Δ*sclR* knockouts from several strains of *A. niger* in the present study revealed some similarities but also unexpected changes to those described in *A. oryzae*. Unexpectedly, Δ*sclR* resulted in a decreased radial growth, in contrast to that seen in *A. oryzae*. Conidiation in the *A. niger* cultures was heterogeneous in nature due to the way the CYAR was prepared. Patterns of conidiation altered depending on the location of the plate. Results for strain 8-160 (**Figure 3.34**) show Δ*sclR* causing a reduction in conidiation where the raisins had been placed, and variable but not statistically significant differences at both the inoculation sites and cultures edges. Conclusions from the conidiation data are hard to draw, particularly due to the nature of variability in conidiation in these strains on CYAR media. The knockouts showed a stark reduction in sclerotia production in all strains where sclerotia were present. However, sclerotia production was surprisingly only totally abolished in one strain, 8-163, in contrast to findings for *A. oryzae*. This may be due to intracellular protein or transcript levels remaining above a critical threshold level that allowed for sclerotia production at a limited level, implies a second pathway that regulates sclerotia production, or may mean the presence of some functionally redundant regulatory genes. It is also noted that the study in *A. oryzae* was completed only on a single strain, and the results of total abolishment may be specific to that strain (as seen in this study with 8-163), as opposed to a species-wide effect, thus masking a broader species wide conclusion that *sclR* is not essential for sclerotia production but is required for high levels of sclerotial production. *A. oryzae sclR* forms a heterodimer with *ecdR*, with competing homodimers and heterodimers resulting in differences in conidial or sclerotial development (Jin *et al*., 2011b). It may be that a similar competitive

heterodimer system operates within *A. niger*, and the knockouts resulting in loss of *sclR* in our strains results in an increase in levels of the heterodimer partner (a homolog of *ecdR* or similar, a BLASTn search was inconclusive). It may be that under specific conditions, the heterodimer partner do not form the necessary homodimer to abolish sclerotia production.

The gene *sclB* was first identified in a sclerotium-producing mutant strain of *A. niger* (*scl-2*) (Jorgensen *et al*., 2020) and identified as a repressor of sclerotium formation. Results from the present work are in agreement with those of the earlier study. Δ*sclB* mutants had no differences in radial growth compared to the wild-type progenitors (**Figure 3.33**). There was a reduction in conidiation (at all plates points tested) (**Figure 3.34**) and an increase in sclerotia production (**Figure 3.35**) compared to the wild-type progenitors. This concurs with the hypothesis of the earlier study, that *sclB* is a repressor of sclerotia whose removal increases sclerotia production. Additionally, the reduction in conidiation is because of the new developmental direction, where a strain either develops asexually or sexually under the correct conditions. This switch is from asexual development and growth to sexual or dormant growth, resulting in reduced conidiation and increased sclerotia production. By contrast to the previous study of Jorgensen *et al*. (2020), where a mutation in *sclB* 'awoke' sclerotia production, and was confirmed by the Δ*sclB* knockout, Δ*sclB* knockouts of strains used in this study that produced no sclerotia (8-169 and 8-175), continued to fail to do so. This suggests that the reason for an absence of sclerotia production in these strains is distinct from *sclB*. Additionally, this is in agreement with the conclusions drawn from the *sclB* SNP analysis, which revealed no changes to *sclB* that may direct the difference in sclerotia production amongst the strains assessed. It was anticipated that deletion of *sclB* might enhance production of sclerotia in *A. niger* strains that already exhibited sclerotia production, noting that the study of Jorgensen *et al*. (2020) had only shown induction in a previously non-sclerotial strain. In fact, it was found that strains with no previous sclerotia production did not awaken sclerotia production potential upon deletion of *sclB*. For those strains that did already produce sclerotia, there was an increase in sclerotia production up to an approximate maximal production of between 300-400 sclerotia / 9 cm Petri plate. This increase did not seem to be a fold increase upon the already existing sclerotia production. Indeed, strain 8-160 was transformed from a relatively low sclerotia producer (when compared to untransformed 8-162 which had an approximately 2.5-fold increase in sclerotia to 8-160) to form two Δ*sclB* strains that outproduced all other Δ*sclB* transformed *A. niger* strains in this study. It appears there is hidden genetic intricacies that require further analysis.

3.4.3 Hormone/Morphogens

Diffusible chemicals such as hormones or morphogens have a specific role in fungal development but can exhibit a broader spectrum of activity in fungi (Dyer *et al.,* 1992). They have been implicated in sexual development, most notably the pheromones of *S. cerevisiae* (Bobrowicz *et al*., 2002) and *N. crassa* (Kim and Borkovich., 2006), as well as in conidial attraction and homing (Roca *et al*., 2005), and also appear to have a role in the attraction and germination of mycoparasites towards a sclerotium (Mischke *et al*., 1995). Within the aspergilli, studies of *A. flavus* demonstrated a potential diffusible morphogen that impacted both conidiation and sclerotia formation (Brown *et al*., 2008). Lipoidal extracts of growth media from cultures derived from low conidia concentrations formed sclerotia more readily than extracts from cultures derived from high conidia concentrations. When extracts from the cultures derived from low conidia concentrations were added to fresh cultures, conidiation was reduced and sclerotia number increased. This diffusible morphogen was hypothesised to be an oxylipin derived from the action of oxygenase family genes (*ppoA*-*ppoD* and *lox*) (Brown *et al*., 2009). Similar control of sexual development by Psi factor has been described as a result of the action of these enzymes in *A. nidulans* (Tsitsigiannis and Keller, 2006). Oxylipins are a broad class of oxygenated (poly)unsaturated fatty acids (Brown *et al*., 2009; Brodhun and Feussner, 2011).

Results of the present study failed to detect any bioactive extract from sclerotia-producing cultures that had an additional impact on cultures that already produced sclerotia, nor cultures that had yet to produce sclerotia (**Figure 3.38**). This was despite extensive experimental efforts in which extracts were made over a 3-60 day time period, given that production of other fungal sex morphogens has been shown to be dependent on maturity of cultures (Siddiq *et al*., 1989; Pearson, 2020; L Humbert unpublished data) (full dataset not shown). Similarly, there was no effect observed when lipoidal extracts from a control culture of one MAT-type were added to growing cultures of the opposite *MAT-type*. These results were repeated for two different lipid solvent extraction protocols. An observation could be made that these cultures produced sclerotia only when raisins were added. If the raisins themselves contain certain fatty acids, these could be modified into oxylipins for development of sclerotia by the same suite of oxygenase enzymes as recorded in *A. flavus* (Brown *et al*., 2009; Amaike and Keller, 2009). Indeed, homologs of *ppoA, ppoB* and *ppoD* have been found in *A. niger* (Wadman *et al*., 2009). However, when extracts where derived from CYAR media (without cultures) and were used as an addition to cultures grown on CYA, no sclerotia were produced as would be expected of growth on CYA alone (**Figure 3.39**). Therefore, any fatty acids (or other bioactive lipids) soluble in either of the solvents used in this study, are not alone responsible for sclerotia production. It is also noted that sclerotia production within *A. niger* on CYAR plates never occurs in the location at which the raisins had been placed on the plate (Ellena *et al*., 2021; **Figure 3.32**). Indeed, the use of

homogenous macerated raisins to CYA has been found to abolish sclerotia production (Frisvad *et al*., 2014; Ellena *et al.,* 2021). Taken together, these observations suggest a gradient effect, whereby fatty acids (or other potential morphogen) only induce sclerotia because of translocation of these morphogens across a concentration gradient. Indeed, concentration gradients of differing nutrient cues have been hypothesised as a key factor involved in sclerotia formation in other well researched models (Georgiou *et al*., 2006; Willetts, 1972). Alternatively, it could be that some other lipid factors extracted with the morphogen(s) interfered with morphogen activity, noting the colouration of the lipid extracts (**Figure 3.37**). Finally, it is possible that any morphogen(s) inducing sclerotial formation in *A niger* might have a different chemical nature to the previously reported lipid factors (e.g. might be hydrophilic in nature), so will not have been recovered using the methods applied here.

3.5 Conclusions

Findings from these studies reveal that the black aspergilli appear to be fastidious in sclerotia production. Closely related species of *A. niger* and *A. tubingensis* have different sclerotia production profiles on the media on which they do so. The ability of *A. tubingensis* to complete a sexual cycle on MCA (although not replicated in this study) may be linked to the appearance of sclerotia of this species on OA (in this study). *A. niger* wild-type strains were found to produce sclerotia only on CYAR and MEAR other than in exceptionally rare instances. It is fair to state that no set of conditions exist that promote sclerotia production across all species.

Bioinformatic analysis of the gene sequences of the transcription factors *sclB* and *sclR* revealed no mutation(s) or SNPs that could account for the differences in sclerotia production amongst the strains assessed. Knockouts of these TF's revealed patterns similar to those described previously in relation to sclerotia production in other aspergilli, but differences in conidiation and radial growth. The *sclR* knockouts failed to totally abolish sclerotia production in all but one strain, this is likely a result of different interactions between competing hetero and homodimers that influence sexual or asexual development. By contrast to previous studies, knockouts of *sclB* failed to awaken sclerotia production in strains whose wild type did not produce sclerotia. This suggests a block in sclerotia production elsewhere in the genome in these strains.

No morphogens were identified that impacted on sclerotia production. It is likely that amongst many other confounding variables, the necessity for a concentration gradient of redox, oxylipins, fatty acids or sugars is necessary for sclerotia production. Any potential morphogens soluble in solvents used in this study could not reproduce the use of raisins on CYA(R) to promote sclerotia production in *A. niger*, even when taken directly from prepared CYAR.

Chapter 4: Sexual Reproduction in the Black Aspergilli

4.1 Introduction

Heterothallic mating in fungi requires two mating partners of different (complementary) mating type and is obligately out-breeding. Within the last fifteen years, there has been a fungal 'sexual revolution' amongst the aspergilli, with the discovery of a heterothallic arrangement of MAT loci together with functional sexual cycles in *Aspergillus* species that were previously considered asexual organisms (Dyer and O'Gorman, 2011). Amongst the economically and medically important species that have had their sexual cycles described are *A. fumigatus*, *A. parasiticus*, *A. flavus*, *A. nomius*, *A. lentulus*, *A. terreus*, *A. tubingensis*, and *A. clavatus* (O'Gorman *et al*., 2009, Horn *et al*., 2009a, Horn *et al*., 2009b, Horn *et al*., 2011, Arabatzis and Velegraki, 2013, Swilaiman *et al*., 2013, Horn *et al*., 2013, Swilaiman *et al*., 2017). These heterothallic species have a remarkably fastidious set of requirements for sex as compared to homothallic species such as *A. nidulans*. The fastidiousness of these heterothallic species relates to factors such as media used, gas exchange and even intraspecies differences in mating ability (fertility). The intraspecies differences in mating has been particularly highlighted with the description of 'supermater' strains of *A. fumigatus* which exhibit far higher levels of sexual fertility (as judged by numbers of cleistothecia formed in crosses) than the bulk of wild strains (Sugui *et al.,* 2011, Swilaiman *et al.,* 2020). This difference in sexuality between strains of the same species may be due to factors such as the accumulation of mutations, epigenetic control of sex, vegetative compatibility group, *het* genes and possibly the role of anisogamy (Dyer *et al.,* 1992; Dyer and Paoletti., 2005; Gell *et al*., 2019; Mehl and Cotty, 2010,; Coenen *et al*., 1994; Van Diepeningen *et al*., 2009; Bruggeman *et al*., 2003; Kwon-Chung and Sugui., 2009; Scazzocchio, 2006).

The media used in crosses of these heterothallic species seems to be a particularly critical factor in determining success (or not) of crossing efforts, with sexual reproduction of a given heterothallic species only induced on a single or very limited number of media. For example, crosses have been reported on oatmeal agar (O'Gorman *et al*., 2009), mixed cereal agar (Horn *et al.,* 2014) and even corn (Horn *et al*., 2013). These media all contain an organic element, and in the case of corn being used for *A. flavus*, is a substrate known to exist as a saprophytic host for wild isolates of the species.

The report that *Penicillium ochrosalmoneum (given as Eupenicillium ochrosalmoneum)* can inhabit the sclerotia of *A. flavus* and produce ascospore bearing ascocarps within the stromal tissue, suggest something of an interplay between species, involving changes to the rind permeability and hyphal ingrowth leading to fertilisation (Horn *et al.,* 2014). A similar phenomenon has been reported in the homothallic *S. sclerotiorum*, in which mycelia of a compatible mating partner can penetrate openings or channels in sclerotia (Colotelo, 1974). The presence of such channels has been hypothesised in

sclerotia of *A. flavus*. A study utilising fluorescent strains of the species found evidence of the presence of mycelia of one mating type partner amongst the sclerotial tissue of the opposing mating type (Luis *et al.,* 2020). The study utilised a method of 'spermatisation' of a sclerotium produced axenically and grown alongside conidia of the opposite mating type. This provided evidence that sclerotia can be infiltrated and fertilised and suggests the presence of channels within sclerotia or that changes to the rind allow permeability. In these instances, the sclerotium acts as a female organ and suggests an element of anisogamy.

Prior to the onset of this PhD, a full sexual cycle and evidence of recombination had only been found in *Aspergillus japonica, Aspergillus sclerotiicarbonarius,* and *Aspergillus tubingensis* of the section *nigri* (all Saitoa sexual morphs). This opens the possibility that a fully functioning sexual cycle might be present in the closely related and economically important species *Aspergillus niger* and indeed some preliminary evidence had already been gained in studies at Nottingham (see below; Darbyshir 2016, Ashton 2018). The overall aim of the work in this chapter was therefore to attempt to induce the sexual cycle in *A. niger*. An initial part of this work involved demonstrating sex in related species as a means to confirm that the methodologies used were appropriate and could be used to identify if ascospores were functional. The putative heterothallic nature of sex in *A. niger* (Pel *et al.,* 2007; Darbyshir 2016, Ashton 2018; Ellena *et al.,* 2021) meant that it was pertinent to identify the mating types of strains used prior to attempts to cross strains. MAT1-1 and MAT1-2 mating types that could then be paired appropriately to increase the likelihood of successful crossing. Media for increased production of sclerotia has already been described in **Chapter 3**. Crosses were therefore attempted on appropriate media, as well as allowing for production of sclerotia prior to mating on alternative media. Methods used for crossing were used which have been previously been described as proving successful for a range of fungi (Houbraken and Dyer, 2015).

The species used to exemplify sex in the aspergilli and more importantly, sex in *Aspergillus* species in section *nigri* will now be described below.

4.1.1. *Aspergillus nidulans*

The homothallic species *Aspergillus nidulans* (emericella sexual morph) was selected as a model organism for this experiment due to its established and easy to replicate conditions for sex (Paoletti *et al*., 2007). The sexual cycle and asexual cycle are conditional based on environmental factors. Factors that determine which cycle is entered are light, temperature, gas exchange and temperature (Han *et al*., 2003). Whilst homothallic, *A. nidulans* can outcross with compatible strains, and it has been reported that this is in fact favoured in a process termed 'relative heterothallism' (Pontecorvo, 1953, Hoffman *et al.,* 2001). Therefore, a distinction between 'selfed' and 'outcrossed' sexuality is made (Paoletti *et al*., 2007). Outcrossing results in the generation of novel genetic diversity due to meiotic recombination.

Nuclear fusion and reproduction occur within cleistothecia which are surrounded by Hülle cells as they develop. Cleistothecia have been described as being between 125-200 µm (Adams, Wieser and Yu, 1998) and 120-170 µm in diameter (Sohn and Yoon, 2002). Cleistothecia yield 4 µm wide, lenticular, smooth walled ascospores bearing two crosslinked crests about 0.5-1 µm in diameter (Thom and Raper, 1939) and have been described as having the appearance of a bivalve mollusc (Champe and Simon, 1992). Conidia are smaller at around 2 µm and are described as globose and verruculose (Adam, Wieser and Yu, 1998).

4.1.2. *Aspergillus fumigatus*

Aspergillus fumigatus is a ubiquitous fungus first described by Fresenius (Samson *et al.,* 2007). Mating-type like genes and pheromone precursors and receptors were identified during genomic studies (Poggeler, 2002; Dyer *et al.,* 2003). Evidence of a MAT1-1 α box mating-type gene at the MAT locus provided further evidence of a heterothallic life cycle (Paoletti *et al*., 2005). A global survey of 290 isolates was found to have no statistical divergence from a 1:1 distribution of MAT1-1 and MAT1-2 isolates. This was consistent with a sexually reproducing population (Paoletti *et al*., 2005). A survey of the distribution of mating types of 91 isolates from a population from Dublin, Ireland also showed no statistically significant divergence from a 1:1 ratio of both mating types. A functional sexual cycle was then induced by crossing isolates of opposite mating types from this Irish population, utilising a barrage-type crossing method (O'Gorman *et al*., 2009). The study reporting the discovery of the sexual cycle described a particularly fastidious set of requirements for sex, with a temperature of 30 ˚C and specific media being required (oatmeal agar), as well as control of gas exchange and subsequent 6-month incubation. Light yellow cleistothecia between 150 – 500 µm in diameter were produced singly or in small clusters along the barrage zone of mycelial contact (O'Gorman *et al.,* 2009). Yellowish to greenish lenticular ascospores with two equatorial crests were observed using SEM. This is not unlike the equatorial furrow described in ascospores of *A. nidulans*, but *A. fumigatus* ascospores have additional ornamental crests on the faces away from the equatorial furrow (Lim and Park, 2009). The sexual morph name given to this state was neosartorya.

It has since been shown that in addition to the barrage methods, that sex can also be induced utilising a vegetative mass mating (VeM) method (Lim and Park, 2019). The VeM method involves overnight growth of conidia in liquid culture (ACM) to from pellets, and then inoculation of pellets of opposite mating type mixed together to induce sex. This method still requires the use of oatmeal agar media and control of gas exchange as per the method of O'Gorman *et al.* (2009), but cleistothecia were reported to be produced much more abundantly as there were multiple opportunities for mycelial contact between the two mating types, rather than just at the barrage zone. This study also detected cleistothecial primordia as early as 7 days post inoculation, and ascospores were reported as early as 13 days post inoculation. However, no evidence of recombination amongst these ascospores was provided and this method had only proved successful for a limited number of isolates (Alghamdi, Du, *et al.* unpublished results).

4.1.3. *Aspergillus sclerotiicarbonarius*

Aspergillus sclerotiicarbonarius was first identified as a novel member of section *Nigri* in 2008 (Noonim *et al.*, 2008). A polyphasic approach was used to distinguish it from other members of section *Nigri* and it was noted that it produced abundant sclerotia. The sexual cycle in *A. sclerotiicarbonarius* was first described by Darbyshir (2014). Two strains from the CBS collection (CBS 121056 and CBS 121853) were crossed to produce cleistothecia, which were shown to contain ascospores (visible under SEM) that germinated to produce viable colonies. Genome sequencing of progeny later showed evidence of recombination (Ashton, 2018). Due to the production of asci within a stroma held within the sclerotia, its teleomorph was described as a petromyces state by Darybshir (2014) similar to other species with a similar sclerotial/stroma held asci such as *A. alliaceus, A. flavus* and *A. tubingensis* (Dyer and O'Gorman, 2011 Horn *et al*., 2013., Olarte *et al.*, 2015). The study that isolated *A. sclerotiicarbonarius* also encountered other black aspergilli such as *A. niger* and *A. carbonarius* in the same environment (Thai coffee beans), providing evidence that this is a particularly strong niche habitat for members of the section *Nigri* (Akbar and Magan., 2014). The sclerotia of *A. sclerotiicarbonarius* were shown to produce sclerotial-specific metabolites with antiinsectan activity when grown on CYAR agar (Petersen *et al*., 2015). This illustrates that the induction of sclerotia can express otherwise silent secondary metabolite pathways. Sclerotia of *A. sclerotiicarbonarius* were described as yellow to red-brown and as having a diameter of 600-1600 µm (Frisvad *et al.,* 2014, Noonim *et al.,* 2008).

The species was described as heterothallic by Darbyshir (2014), who had already characterised the mating type of all strains of *A. sclerotiicarbonarius* available in the University of Nottingham BDUN culture collection (**Appendix 7**). Despite having four MAT1-2 strains available and two MAT1-1 strains available, sex was only recorded in a single cross between CBS 121056 (MAT1-1) and CBS 121853 (MAT1-2). A total of 11 progeny were collected from this cross which were subsequently genotyped for MAT, revealing an additional 7 MAT1-1 and 4 MAT1-2 strains in the offspring.

4.1.4. *Aspergillus tubingensis*

A. tubingensis is another described heterothallic species from the section *Nigri* which was first formally described as a separate species to *A. niger* based on differences in restriction fragment length polymorphisms (Kusters-van Someren *et al.,* 1991). The same study also noted that once separated into two species, there were no heterokaryon formation between the species. Species of section *Nigri* are used extensively within the biotechnology industry due to their high levels of production of various organic acids (Ruijter *et al*., 2002), enzymes (van Dijck *et al*., 2003) and for the bioremediation of soils (Srivastava and Thakur., 2006). A study exploring the range of mycotoxins formed within different members of the black aspergilli, found that most the common two – ochratoxin A and fuminosin B_2 were both absent in all strains of *A. tubingensis* tested (Horn *et al.,* 2013). This would suggest that *A. tubingensis* is not only safe but possibly favourable to *A. niger* to produce food-based biotechnological products.

A. tubingensis CBS strain 121047 was identified from coffee beans, again showing that this is a niche habitat for members of the section *Nigri*, which also include soil and grapes/raisins as niches for the black aspergilli (Samson *et al.,* 2007). The same study also illustrated that *A. tubingensis*, like *A. niger*, is able to grow at temperatures as high as 40 ˚C.

Prior to the onset of this PhD thesis, sex in *A. tubingensis* was first recorded by Horn *et al.* (2013) and later, evidence of recombination provided (Olarte *et al*., 2015). The authors first genotyped strains collected from soil sampled at a North Carolina Research station for mating type. Interestingly, 94 % of all strains MAT-typed were MAT1-1, even though these strains were then shown to be sexually functional. The conditions used to induce sex involved using preparations of mixed spores as an inoculum, and addition to slants of mixed cereal agar as a growth media (Horn *et al*., 2013). The mixed spore suspension consisted of 5 x 10^5 conidia/mL of which 7 µL was used to inoculate 7 mL slopes of MCA. These were incubated at 30 ˚C in the dark for 14 days and then sealed in plastic bags to prevent desiccation and incubated for a further 5-6 months.

4.1.5. *Aspergillus niger*

Aspergillus niger is the species type for *Aspergillus* section *Nigri*. However, the taxonomy of section *Nigri* is complex as the differences in morphology between species is increasingly subtle (Silva *et al*., 2011). *Aspergillus niger sensu stricto* is widely used in biotechnology, particularly to produce citric acid and hydrolytic enzymes (Karaffa and Kubicek., 2003, Pel *et al*., 2007). *A. niger* has GRAS (Generally Regarded As Safe) status, but recent studies have shown that some strains can produce Ochratoxin A (Perrone *et al.,* 2007, Silva *et al.,* 2011). *A. foetidus*, *A. niger* and *A. tubingensis* are difficult to distinguish based on morphology, as they are biseriate, and their conidia have the same diameter (3-5 µm) and shape and ornamentation (globose to subglobose and verruculose to smooth) (Silva *et al*., 2011). It should be noted that *Aspergillus foetidus* is no longer accepted as a distinct species to *A. niger* (Bian *et al*., 2022). Many members of *Aspergillus* section *Nigri* had been reported to produce sclerotia on standard media such as CZA, but *Aspergillus niger sensu stricto* had not been reported to do so until 2014 (Frisvad *et al*., 2014). Here it was shown that cultivation on CZA with raisins (now referred to as CYAR agar) reliably induced sclerotium formation. The description of sexual reproduction within the sclerotia/stroma of the closely related *A. tubingensis* infers that sclerotial production is a prerequisite for sex in *A. niger* (Horn *et al*., 2013).

Whole genome sequencing of *A. niger* (Pel *et al*., 2007, Andersen *et al*., 2011) had revealed the presence of a series of sex-related genes including the identification of a MAT locus containing a MAT1-1 gene, indicating a likely heterothallic breeding system. The complementary MAT1-2 gene was later reported by Ellena *et al*. (2021b) who described the development of primers for mating-type assays (Ellena *et al*., 2020).

Meanwhile so far unpublished studies by Darbyshir (2014) and Ashton (2018) at the University of Nottingham had also provided major insights into the possibility of sexual reproduction in *A. niger.* A survey for mating type of over 110 isolates of *Aspergillus niger sensu stricto* [provided by collaborators at the CBS (Netherlands) and J. Perrone and A. Susca (Italy)] found a very strong bias towards the MAT1-1 genotype (approx. 95% MAT1-1 and a few, rare MAT1-2 isolates). Attempts were made to cross representative MAT1-1 and MAT1-2 isolates under a range of conditions in pilot experiments in which sclerotia were known to be produced. Later inspection of sclerotia from such 'crosses' revealed that almost all sclerotia showed no signs of sexual development. However, in certain attempted crosses in which raisins were left on media for 24 hours but then removed, red coloured structures were occasionally seen within the sclerotia - and of great promise was the rare discovery of apparent cleistothecium containing putative ascospores in one cross (8-161 x 8-160; **Figure 4.1**). However, it was never possible to germinate the putative ascospores from this cross, and it proved very hard to reproduce this crossing result (Ashton 2018).

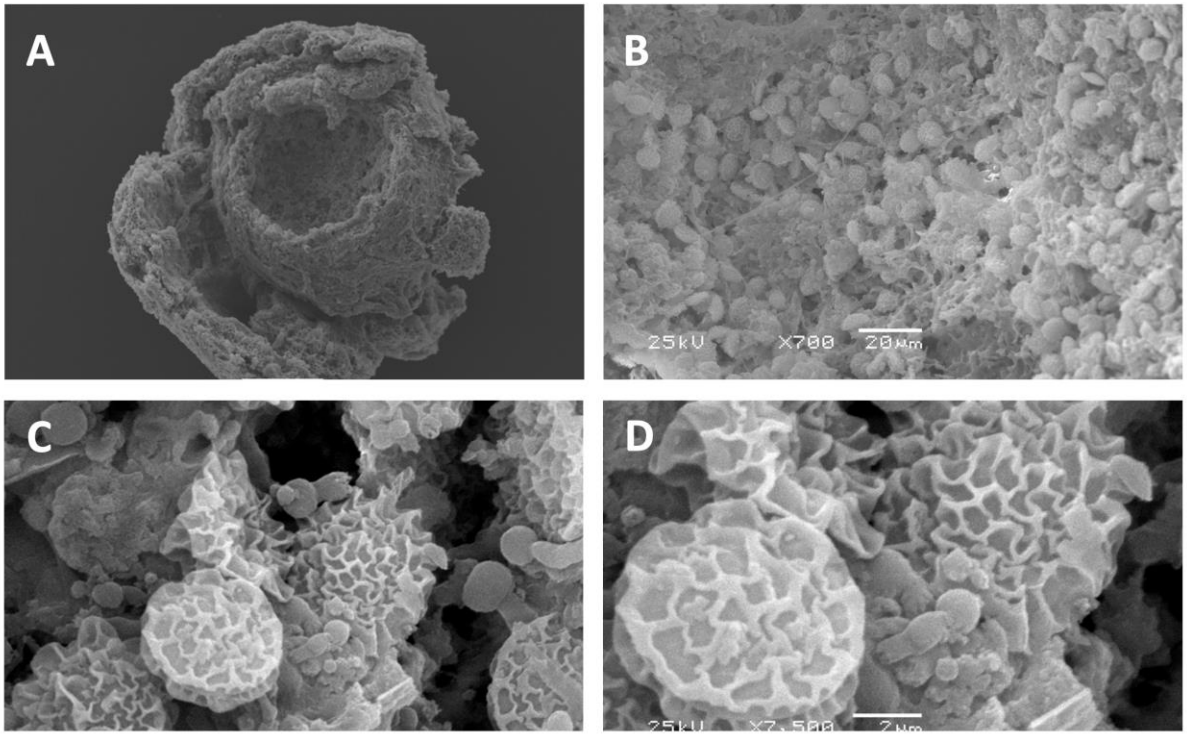


Figure 4.1. SEM imaging of a sclerotia cross-section from attempted sexual cross of *A. niger* isolates 8-161 x 8-160. A) X90 magnification displaying the whole sclerotia cross section, showing the outer sclerotia section and an inner structure; B) X700 magnification of the inner structure observed in A), revealing smaller spherical structures; C) X4,300 magnification showing 'frilled' structures embedded into the wall of the inner structure; D) X7500 magnification showing 'frilled' structures at a greater level of detail (Ashton 2018).

4.1.6. Aims of Chapter Work

(1) **Determination of the mating type of strains of *Aspergillus tubingensis* and *Aspergillus niger.*** This was undertaken to enable crossing between isolates of opposite mating types.

(2) **Determination of whether *Aspergillus niger* can reproduce sexually**. Directed crosses were set up between isolates of opposite mating types. This was done under conditions suitable to produce sclerotia as described in **Chapter 3**. A range of crossing methods, utilising new methodologies employed in fungal sexual research, were used including the setting up of sexual crosses of other *Aspergillus* species (including members of the black aspergilli) as control crosses.

(3) **Identification of putative progeny and/or changes within sclerotia/stroma**. Light and scanning electron microscopy (SEM) observational approaches were used to characterise any morphological changes within scleriotia. Using sex in *Aspergillus nidulans* and *Aspergillus fumigatus* as a model for sex, methods were developed to further investigate if any progeny and structures found were functional/viable.

(4) **Development of a method utilising flow cytometry and cell sorting to separate putative ascospores from conidia.** In the eventuality that sex was induced in *Aspergillus niger*, this method would allow separation of ascospores and conidia and allow downstream investigation of recombination.

4.2 Materials and Methods

4.2.1 Materials

***Aspergillus* Minimal Media (10x)**

To 50 mL of distilled water were added: 6 g sodium nitrate ($NaNO_3$) (Sigma, U.K), 0.5 g potassium chloride (KCl) (Fisher Scientific, U.K), 0.52 g magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Sigma, U.K), 1.52 g monobasic potassium phosphate (KH_2PO_4) (Sigma, U.K), 0.008 mg sodium tetraborate decahydrate ($Na_2B_4O_7.10H_2O$) (VWR International, U.K), 0.16 mg copper sulfate ($CuSO_4.5H_2O$) (Fisher, U.K), 0.16 mg iron phosphate monohydrate ($FePO_4.H_2O$) (Sigma, U.K), 0.16 mg manganese sulfate tetrahydrate ($MnSO_4.4H_2O$) (Sigma, U.K), 0.16 mg sodium molybdate dihydrate ($NaMoO_4.2H_2O$) (Fisher, U.K), 1.6 mg zinc sulfate ($ZnSO_4$) (Fisher, U.K). A carbon source was then added (see below) and the solution made up to 100 mL. For solid media 15 g agar / Litre was added. This is similar to the *Aspergillus* trace elements solution used in **Section 3.2.1.2.**

Wheat Straw on Agar

Wheat straw (gift from Rothamsted Research, UK) was collected and cut into 3 cm long sections and autoclaved. 4 % water agar was poured, and the autoclaved 3 cm long wheat straw sections were overlaid as it set. This provides a wheat surface fixed into media onto which inoculation could occur. This medium was adapted from both Quiroz-Castaneda *et al.* (2009) for growth of basidiomycete fungi and Benslimane *et al.* (2004) for growth of the ascomycete fungus *Pyrenophora tritici-repentis* in particular to induce the sexual cycle or morph.

Wheat Steep Liquor Agar (WSLA)

Sections of wheat (gift from Rothamsted Research, UK) 3 cm long were cut and boiled for 2 hours. After boiling, the solution was recovered by filtering through Miracloth. This wheat steep liquor was then bottled with 15 g/L agar and autoclaved at 117 ˚C.

Wheat Steep Liquor Agar + (WSLA + AMM)

As above, but 100 mL AMM (10x) was added to 900 mL before 15 g/L agar and autoclaved at 117 ˚C.

Cut Willow Agar

Willow (*Salix* sp.) variety Tora was cut with a Fritsch Pulverisette 19 knife-mill (Fritsch, Germany). The product was then screened through first a 2 mm screen and then a 0.5 mm screen. This provided small size material that could be used in media. 50 g of this material was added per litre (5% w/v) of distilled water, with 15 g/L agar per litre and autoclaved at 117 ˚C. Adapted from Pullan *et al.* (2014).

Cut Willow Agar + AMM

As above, but 100 mL AMM (10x) was added to 900 mL before 15 g/L agar and autoclaved at 117 ˚C.

Adapted from Pullan *et al.* (2014).

Ground Willow Agar

5 g of the cut willow was ball-milled in a 80 mL stainless steel grinding bowls with 25 10-mm-diameter steel balls in a Planetary Mill (Pulverisette 5 classic line, Fritsch, Germany), at 400 rpm for 20 min. This resulted in a particle size of ~75 µm. This was repeated to produce enough material. 50 g of this material was added per litre (5 % w/v) of distilled water, with 15 g/L agar per litre and autoclaved at 117 ˚C. Adapted from Pullan *et al.,* (2014).

Ground Willow Agar + AMM

As above, but 100 mL AMM (10x) was added to 900 mL before 15 g/L agar and autoclaved at 117 ˚C.

Adapted from Pullan *et al.,* (2014).

Birch Tree Water Agar

Birch wood was collected and ground under Pestle and mortar and screened first with a 2 mm and then a 0.5 mm screen. This small sized material was then boiled with 800 mL distilled water for 2 hours, filtered with Miracloth, made up to 1 Litre and 15 g/L agar added. Autoclaved at 117 ˚C.

4.2.2 Methods

4.2.2.1 Sex in *Aspergillus nidulans.*

Two isolates (2-3 and 2-258) from the University of Nottingham BDUN collection were used for experimentation. For both, sexual reproduction was induced by preparing 5,000 spores in 50 µL volume solution, which was then spread over the surface of a fresh ACM agar plate (**Section 2.1.1**) using a sterile spreader. Strain 2-258 is a ΔpyrG knockout and the ACM used for this strain was supplemented with 5 mM uracil and 5 mM uridine (Weidner *et al.,* 1998). Plates were left unsealed at 28 °C in the dark for 24 hours before being sealed with parafilm to induce sex, given that reduced gas exchange favours sexual reproduction (Paoletti *et al*., 2007). Incubation was then for a minimum of an additional four days but normally at least 14 days to allow maturation of cleistothecia and ascospores.

Cleistothecia were removed from the plates using sterile forceps and cleaned as described in **Section 4.2.2.7** and previously described by Todd *et al.* (2007). Ascospores were prepared by rupturing cleistothecia in PBS and centrifuging to concentrate. Ascospores and cleaned or uncleaned cleistothecia were imaged using SEM as described in **Section 2.2.13.3**.

4.2.2.2 Sex in *Aspergillus fumigatus.*

The heterothallic species *A. fumigatus* was selected as a model organism for further experiments due to its fastidiousness nature for sex (Kwon-Chung and Sugui, 2009). Being able to replicate the sexual cycle and then apply SEM amongst other tools, would be useful to demonstrate that the methodologies applied in the current work in application were suitable for investigations of the sexual potential for other species with no known sexual cycle.

4.2.2.2.1 Mating using Barrage Method.

Due to its heterothallic nature*, A. fumigatus* requires two strains of opposite mating type to induce sexual reproduction. Mating-type determination of strains used in this study had previously been conducted by O'Gorman *et al*. (2009). Two strains (47-59/AFRB2: MAT1-1 and 47-52/AFIR964: MAT1-2) were selected due to their previously reported high level of cleistothecia production (Swilaiman *et al*., 2020., Sugui *et al*., 2011). Conidial suspensions of each strain were prepared by growing each strain on ACM slopes for 7 days before using the method described in **Section 2.2.1**. Barrage crosses, with 2

drops of spore suspension of each mating type diagonally placed in square formation to create extended mycelial contact or barrage zones was used (**Figure 4.2**). For each drop of the spore suspensions, 1 µL of spore suspensions of concentration 5 x 10^5 spores/ mL was used. Spore concentrations were determined using the method described in **Section 2.2.2**. Oatmeal agar was used as the growth media. After inoculation, drops on plates were allowed to dry, the plates immediately sealed with parafilm and incubated inverted at 30 ˚C in the dark for 6 months.

Figure 4.2. *An oatmeal agar plate marked with the inoculation points for the 'Barrage' crosses (reverse of plate shown). Blue dots represent the position of the MAT1-1 and red dots represent the position of the MAT1-2.*

Cleistothecia were removed from the plates using sterile forceps and cleaned as described in **Section 4.2.2.7** and previously described by Todd *et al.* (2007). Ascospores were prepared by rupturing cleistothecia in PBS and centrifuging to concentrate. Ascospores and cleaned or uncleaned cleistothecia were imaged using SEM largely as described in **Sections 2.2.13.3**. Specifically, ascospores of *A. fumigatus* were prepared for SEM by both dissection of cleistothecia and spreading of a pellet of centrifuged ascospores directly on to stubs. Cleistothecia were dissected using a number 10 scalpel blade (Swann-Morton Ltd., U.K) under a dissecting microscope. Preparation of ascospores pellets for SEM was completed by rupturing cleistothecia in PBS and centrifuging at 13,000 until a pellet was formed. The supernatant was then removed carefully to leave a solid mass of ascospores (and debris). Subsequent preparation of the pellet for SEM was with osmium tetroxide to reduce the number of wash steps (**Section 2.2.13.3**). The ascospores were then prepared on the carbon stub by smearing a spatula of the pellet across the surface of the stub.

4.2.2.3. Attempted Induction of Sex in *Aspergillus sclerotiicarbonarius*.

Sexual reproduction was previously identified in *A. sclerotiicarbonarius* under particular conditions by Darbyshir (2014). A similar methodology was applied in this study. Cultures were grown on ACM slopes for 5 days at 28 ˚C. Then 5 mL Tween 80 solution was used with a sterile inoculating loop to harvest conidia and homogenised by vortexing. The spore suspension was then filtered through Miracloth (rather than nylon wool) to remove remaining hyphal debris. Spore density was estimated using a haemocytometer as described in **Section 2.2.2**. Spore suspensions were then diluted to a concentration of $1x10^5$ conidia/mL. Spore suspensions were then used immediately. Crosses were set up on CYAR or oatmeal agar (Oddlums pinhead organic oats). Crosses were set up between each of the available MAT1-1 and MAT1-2 strains (listed in **Appendix 8**). Strains of opposite mating types were crossed on 9 cm Petri plates containing 25 mL of growth media. Two aliquots of 5 µL of each parental strain were inoculated as in **Figure 4.2** from the spore suspension of 1×10^5 conidia/ mL (500 spores). Plates were either sealed immediately or after 24 hours with a single layer of Parafilm. Plates were incubated at 28 ˚C in the dark. All crosses were set up in triplicate other than the 80.5x80.3 (CBS121056 x CBS121853), which was set up ten times, as this was previously described as successfully undergoing sexual recombination. Plates containing sclerotia were examined for internal structures and ascospores after 6 months and 8 months. Examination for light microscopy and SEM were completed as described in **Section 2.2.13.2**, **2.2.13.3**.

4.2.2.4 Attempted Induction of Sex in *Aspergillus tubingensis.*

4.2.2.4.1 Mating-type Assays

Multiplex Diagnostic PCR for *MAT*-typing of *A. tubingensis*

A multiplex *MAT*-type diagnostic had been previously developed by Darbyshir (2014) which allows for confirmation of the mating type of an isolate from a single PCR reaction (**Section 2.2.6.3**). 56 isolates of *A. tubingensis* from the University of Nottingham BDUN collection and a single isolate from DSM foods (designated the industrial strain) were subjected to this multiplex diagnostic to determine *MAT*-type identity. Isolates were predicted to yield either a 3.3kbp (*MAT1-2*) or 1.9kbp (*MAT1-1*) product. For each diagnostic, control isolates of either *MAT*-type were run as positive controls and water was run as a negative control.

Individual *MAT*-type Diagnostics for *MAT*-typing of *A. tubingensis*

Where the multiplex diagnostic of Darbyshir (2014) was not able to generate a clear outcome, an alternative degenerate primer-based PCR diagnostic (**Section 2.2.6.2**) was used to determine (and also confirm) the mating type of isolates of *A. tubingensis.* This diagnostic has a greater success rate but is more time consuming, requiring 2 reactions per isolate. Again, each reaction product was run alongside those of known MAT1-1 or MAT1-2 as positive controls and water as a negative control.

4.2.2.4.2 Mating using Barrage Method

CYAR agar was used the primary crossing medium due to the high production of sclerotia of *A. tubingensis* on this medium, and the lack of availability of the MCA media previously used to induce sex in *A. tubingensis* (Horn *et al.,* 2013). Spore suspensions for each strain of *A. tubingensis* were prepared by growing them on ACM for 7 days and then following the protocols described in **Section 2.2.1** and **2.2.2** to prepare spore concentrations of 1 x 10^6 spores / mL. CYAR agar was then inoculated with 5 µL of each spore suspension as shown in **Figure 4.3** (noting that raisins were left on media for 24 hours, and then removed). These methods allowed for formation of a 'barrage zone' of mycelial interaction with has been shown to induce sexual reproduction for other heterothallic *Aspergillus sp.* such as *A. fumigatus* (O'Gorman *et al*., 2007). These 'crosses' could then be assessed for sexual reproduction by sampling sclerotia and looking for signs of sexual reproduction using either light or SEM microscopy.

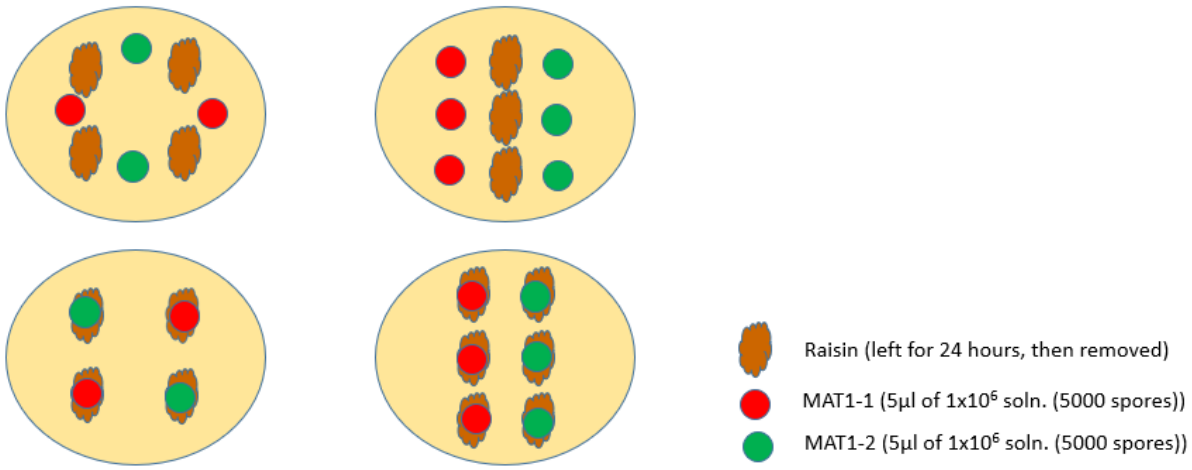


Figure 4.3. Diagram showing the arrangements of both raisins and point inoculation targets on CYAR media of *Aspergillus tubingensis* (and later *Aspergillus niger*).

4.2.2.4.3 Mating using Mixed Spore Suspensions

Attempted crossing using a mixed spore suspension involved the same steps as for the barrage crosses. Instead of inoculating opposite mating types (of sclerotia producing strains) individually and allowing 'barrage zones' to emerge, spore suspensions of $1x10^6$ spores/ mL were added to one another to mix, vortexed and added as 10 µL, 20 µL and 50 µL of the mixed spore suspension as shown in **Figure 4.4**. The spore suspensions were spread using a sterile spreader before incubating at 30 ˚C in the dark. After 48 hours, cultures were wrapped with Parafilm. Incubation continued for up to 12 months and were sampled at multiple time points to look for signs of sexual reproduction using both light and SEM microscopy.

Figure 4.4. Diagram showing the methods of spread plate crosses of *Aspergillus tubingensis*.

4.2.2.5 Attempted Induction of Sex in Aspergillus niger

4.2.2.5.1 Mating type Assays

The mating type of all *A. niger* strains (University of Nottingham BDUN culture collection) were determined as necessary using the multiplex diagnostic PCR described in **Section 2.2.6.3** and **4.2.2.4.1**. Strains were grown in ACM broth and gDNA was prepared for the mating-type diagnostic using the protocols in **Section 2.2.1** and **Section 2.2.4**.

4.2.2.5.2 Mating using 'Barrage'

This was as described in **Section 4.2.2.4.2** for *A. tubingensis*.

4.2.2.5.3 Mating via Vegetative Mycelial Balls

A distinct method of inducing sex in *A. fumigatus* to that described in **Section 4.2.2.2** was recently described by Lim and Park (2019). In this study, they inoculated liquid complete medium with conidia and used the resulting balls of mycelium produced after overnight growth as an inoculum to initiate sexual reproduction.

To ascertain whether this 'vegetative mass mating' method could be used in *A. niger*, conidia of strains of opposite mating type were prepared as described in **Section 2.2.1** and spore suspensions were made as described in **Section 2.2.2**. 500 µL of 5 x 10^5 spores / mL was inoculated into 25 mL ACM and 25 mL CYA broth for 1 and 2 days to produce mycelial balls of each strain. Liquid cultures were shaken at 250 rpm in the light. Balls of mycelia were then filtered through from the media through sterile Miracloth and washed in distilled water. Cleaned mycelial balls were then paired with strains of opposite mating types on media inducive to sclerotia production (CYAR agar). These cultures were incubated at 30 ˚C, inverted and in the dark. Parafilm wrapping of these cultures was completed immediately, after 24 hours and after 48 hours. This was to determine whether sexual reproduction could be induced using this technique.

4.2.2.5.4 Mating using Mixed Spore Suspensions

This was as described in **Section 4.2.2.4.3** for *A. tubingensis*.

4.2.2.5.5 Mating using Spermatisation of Sclerotia

The process of spermatization of a female organ to induce sexual reproduction has been described for various fungal species (Houbraken and Dyer, 2015). Fertilisation of a sclerotium of one strain by hyphae grown from the conidia of a strain of the opposite mating type has been demonstrated in *A. flavus* by Luis *et al*. (2020).

To investigate whether a similar approach might induce sexual reproduction in *A. niger*, strains of *A. niger* were grown ACM agar slopes before subsequent production of spore suspensions as previously described (**Sections 2.2.1** and **2.2.2**). These spore suspensions were used at a concentration of 5 x 10^5 spores / mL and 5 µL was used to axenically inoculate CYAR agar**.** These cultures were inoculated at 30 ˚C in the dark without parafilming as method of producing both conidia and sclerotia. For strains that did not produce sclerotia, they were still grown on CYAR agar, but used only as a source of spermatising conidia rather than sclerotia.

To harvest conidia from plates rather than slopes, 1 mL of PBS (**Section 2.1.2**) was first pipetted on to the centre of plates and agitated with a sterile cotton tipped swab (SLS, U.K). Spore suspension on the plate was then removed by pipette and filtered through sterile Miracloth to remove mycelial debris before adjusting to 5 x 10^5 spores/ mL with PBS as described in **Section 2.2.2**.

To harvest sclerotia, two methods were used. In the first method, sclerotia produced after 14 days growth on CYAR media were removed from the agar and washed sequentially in a 50 mL Falcon tubes containing 2 x changes of 50 mL Tween and 3 x changes of 50 mL PBS to solution to remove contaminating conidia of the same mating type. Sclerotia could then be considered cleaned and plated with conidia of the opposite mating type. The second method for removing and cleaning sclerotia is described in **Section 4.2.2.6**.

To set-up 'spermatising crosses' the harvested conidia were used to inoculate plates with 50 µL of the 5 x 10^5 spores/mL solutions and spread using an L-shaped spreader. A range of different media were used and these are listed in **Sections 4.2.1** and **2.1.1**. Sclerotia that had been cleaned were then plated on this 'bed' of conidia, usually in a 5 x 5 grid of 25 sclerotia and incubated at 30 ˚C in the dark in both atmospheric gas and supplemented with 5 % carbon dioxide.

An exception to the above-described methods is the inoculation of **Wheat Straw Agar** which dues to its uneven surface was difficult to inoculate as a flat 'bed' of conidia. Therefore, sclerotia were saturated with the spore suspension prior to plating and placed on the wheat straw directly.

4.2.2.5.6 Confocal Microscopy of Fluorescent Strains of Opposite Mating type

Strains of complementary mating type that had been transformed to contain fluorescent TdTomato or eGFP (described in **Chapter 3**, summarised in **Section 3.2.2.5**) were used as mating pairs in a range of the crossing methods described above. To visualise the fluorescence in situ (within sclerotia) of fixed cells, a Zeiss LSM880 confocal laser scanning microscope was used. This microscope utilised both 488 nm and 561 nm excitation wavelengths for eGFP and TdTomato respectively. Images were obtained and analysed using Fiji software.

4.2.2.6 Ethanol Washing of Sclerotia

Sclerotia of strain 8-162 were produced by growth on CYAR agar. Sclerotia were then removed from the plates after 14 days growth at 28 °C using sterile forceps and washed in 500 µL 70 % ethanol for 1 min with vortexing. Following the 1 minute in ethanol, the sclerotia were removed and then washed in 500 µL Tween 80 solution twice. The ethanol and each of the subsequent Tween 80 wash step solutions were kept. Additionally, a control experiment using water instead of 70 % ethanol was completed. And the solutions and sclerotia kept. The sclerotia and 10 µL of each solution were placed or spotted three times on each plate of ACM, CYA and PDA for observation of germination of conidia or outgrowth of sclerotia. A similar protocol has been used recently by Ellena *et al*. (2021a).

4.2.2.7 Separation of Conidia and Ascospores by Flow Cytometry

Cell suspensions containing both *A. nidulans* (strain 2.3) conidia and ascospores, and a separate mixture of both *A. fumigatus* (a cross of strains 47.52 x 47.59) and *A. niger* (strain 8-160) conidia were prepared for flow cytometry.

Conidia were collected from asexual plates as described in **Section 2.2.2**. Ascospores were prepared from plates of *A. nidulans* and *A. fumigatus* that had undergone sex and produced cleistothecia as follows. Cleistothecia were prepared by removing them from the plate that had undergone sex and cleaned on a fresh 4 % water agar plate. By adding 5 µL Tween 80 and rolling the cleistotheica under a dissecting microscope with a dissecting needle, the majority of conidia could be removed. Afterwards the cleaned cleistothecia were moved singly to fresh 1.5 ml eppendorf tubes, containing 100 µL PBS and crushed with a sterile dissecting needle. This released red ascospores in the case of *A. nidulans*. The ascsospore solution could then be made up to 500 µL using PBS. This protocol was taken and modified from Todd *et al.* (2007). Combinations of solutions were then prepared. Axenic conidia or ascospores were set aside as well as a combination of both types of spore. It should be noted that despite cleaning the cleistothecia, some contaminating conidia may have survived but this would not present a problem as discussed below.

In order to ascertain whether the flow cytometer could be cleaned of conidia by 70% ethanol, an assay was prepared by preparing five aliquots of 5 µL of each of the conidial and ascospore solutions. One 5 µL aliquot of each solution acted as a positive control. The other 5 µL solutions were then subjected to the addition of 50 µL of 75 % ethanol (to a final concentration of ~70 % ethanol) for either 5, 10, 15, 20 or 25 minutes. To terminate the ethanol treatment, the suspensions were centrifuged for 1 min at full speed, before total removal of the ethanol and resuspension of the pellet in 50 µL PBS. Three aliquots of 10 µL of each solution were then plated on fresh ACM and incubated for 1 day at 28 ˚C in the light. Colonies were then counted for each plate and a mean was taken of the technical replicates. A volume of 100 µL of each spore suspension was then analysed using a Beckman Coulter Astrios EQ flow cytometer (University of Nottingham flow cytometry unit) as follows.

Data Processing:

Analysis of data was completed using Kaluza analysis software. Parameters were set as forward scatter (FSC) for size and side scatter (SSC) for granularity. Cellular debris was identifed and ignored and general populations of either ascospores or conidia were identifed and gated. Mixed population samples could then be separated according to the characterisitcs of either population.

4.2.2.6.1 Microbiological validation of spore type separation by Cell Sorting.

To validate whether the spore suspensions had been separated by flow cytometry and cell sorting, microbiological validation of the resultant spore suspensions was completed. Each spore suspension was representative of either *A. niger* conidia, *A. fumigatus* ascospores (although this may still have contained some contaminating *A. fumigatus* conidia), a deliberate mixture of the two spore types, or the *A. niger* conidia and *A. fumigatus* ascospores as separated by flow cytometry according to their distinct characteristics. 10 µL of each solution was then used to inoculate fresh ACM agar plates and spread using Lazy-L spreaders (Sigma, U.K). Alongside this, the same source spore suspensions were subjected to heat shock of 30 minutes at 80 ˚C in a heat block before subsequent inoculation of ACM plates using 10 µL of each solution per plate and spreading. Plates were left to grow for 5 days at 28 ˚C, inverted and in ambient light before analysis.

4.3. Results

4.3.1 Sex in Various *Aspergillus* species

4.3.1.1 Sexual Development in *A. nidulans*

Sexual development of the homothallic *Aspergillus nidulans* (sexual morph emericella) was induced as described in **Section 4.2.2.1** on ACM Agar and was completed over a relatively short time frame of around 14 days. Two sets of selfed cultures were set up using strains 2-3 and 2-258. Strain 2-258 (Δ*pyrG*) was found to excelled at undergoing selfing, showing abundant production of cleistothecia under the conditions described, whereas strain 2-3 tended to favour asexuality even under sex inducing conditions.

Sexuality was easily identifiable as a decreased level of conidiation and associated increase in cleistothecia, observable as small (> 50µm diameter) black to dark red globose structures. Sexual development was particularly favoured towards the centre of plates, with an increase in asexual conidiation towards the exterior edge. This was likely due to the slightly elevated levels of gas exchange near the edge as opposed to the centre (Kim *et al*., 2002, Troppens *et al*., 2020). Mature cleistothecia were easily observed under light microscopy and dissecting light microscopy even when covered in hyaline mycelium due to the stark contrast of their pigmentation as can be seen in **Figure 4.5**. Cleistothecia were easily removed from the mycelium using a sterile dissecting needle and cleaned where necessary as described in **Section 4.2.2.1**.

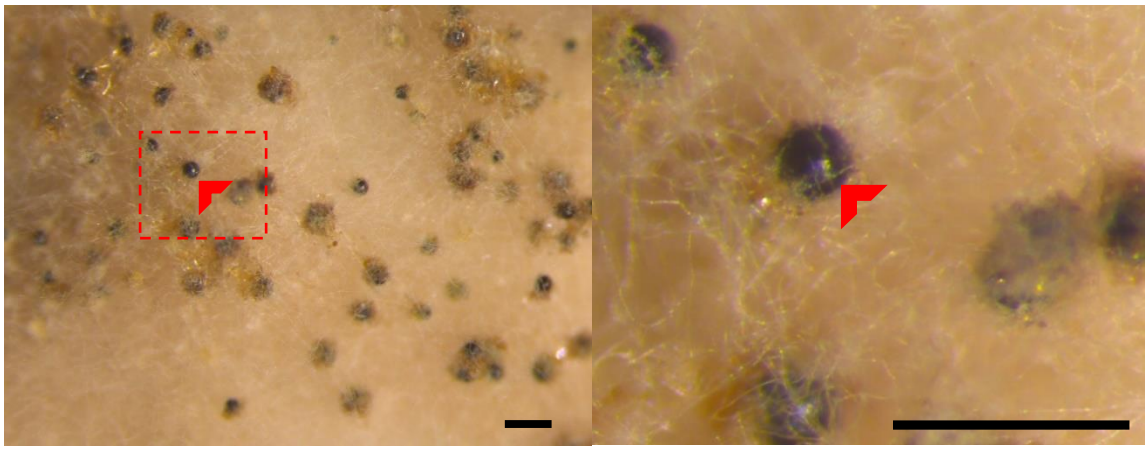
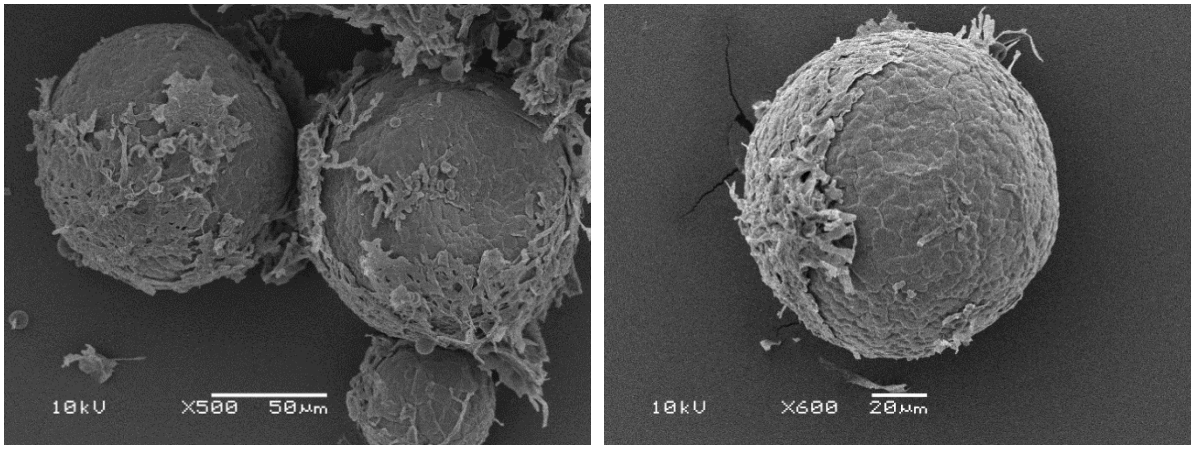


Figure 4.5. Light microscopy image of developing cleistothecia of *Aspergillus nidulans* (2-258) A cleistothecium is indicated with a red arrowhead. Image magnification indicated with dashed box. Grown on ACM supplemented with 5 mM uracil and 5 mM uridine and conditions stated in **Section 4.2.2.1.** Images were collected using a Leica S8APO dissecting microscope equipped with a QImaging Micropublisher 3.3 RTV camera. Scale bar on each image 250 µm.

Cleistothecia were prepared for visualisation using scanning electron micrography as described in **Sections 2.2.13.3**. Sizes were typically within the range of 50 µm to 150 µm, with the majority around 80 µm. This is smaller than the 120 - 170 µm previously reported (Sohn and Yoon, 2002). Size difference may be due to differences in strain or the reduction in number of observed Hülle cells (Troppens *et al*., 2020). Differences in number of Hülle cells, and particularly genetically modified strains have been discussed (Carvalho, Baracho and Baracho, 2002). Intact and cleaned cleistothecia can be seen in **Figure 4.6.** Hülle cells were observed on all uncleaned cleistothecia as part of the attached physcial milieu of *A. nidulans* cleistothecia (**Figure 4.7**).



F**igure 4.6**. Scanning electron micrographs of intact *Aspergillus nidulans* cleistothecia. Separate scale bars for each image shown indicate approximate width of cleistothecia as ~80 µm..

Hülle cells were observed on all uncleaned cleistothecia as part of the attached physcial milieu of *Aspergillus nidulans* cleistothecia. Hülle cells have a recognised role in the rearing of cleistothecia, with links between their number and cleistothecia size and ascosporogenesis made (Ellis, Reynolds and Alexopoulous, 1973., Hermann, Kurtz and Champe, 1983). Hülle cells begin as unordered hyphae that pack closely together and then differentiate into multinucleate, thick walled and globose cells (Geiser 2009., Poggeler, Nowrousian and Kuck, 2006). Although the size of Hülle cells is not often explicitly stated, studies on the subject provide evidence that they are often around ~10 µm in width (Kim *et al*., 2002., Paoletti *et al.,* 2007). This matches the size of the Hülle cells seen in this study where they were often between 6-10 µm as can be seen using the scale bars on the images in **Figure 4.7.**

The observation of these structures was no more or less evident between the two methods of preparing samples for SEM, namely between the use of cacodylate buffer and Osmium tetroxide (as described in **Section 2.2.13.3**). The use of Osmium tetroxide was deemed likely to be of superior application in these contexts, due to the vast reduction in number of washing steps, and therefore reduced loss of attached or internal structures. However, for *Aspergillus nidulans* cleistothecia this

consideration was unnecessary, as attached structures remained and comparison provided little insight. Cleistothecia were covered in flattened, smooth cells consistent with the descriptions given for the sexual morph emericella (Dyer and O'Gorman., 2011).

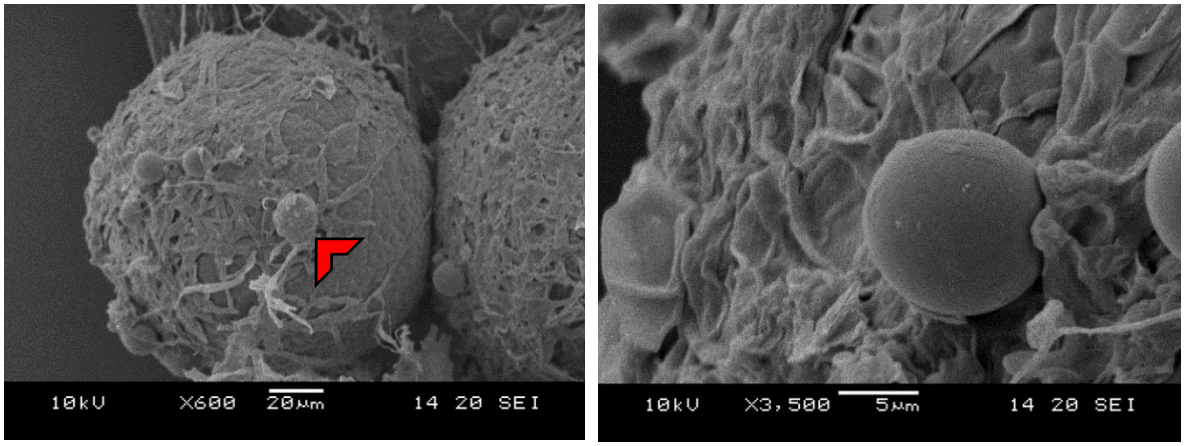


Figure 4.7. Scanning electron micrograph of Aspergillus nidulans cleistothecia with Hülle cells. On the left-hand image is an attached Hülle cell indicated by the red arrow. Separate scale bars for each image indicate the approximate width of Hülle cells to be between 8-10 µm.

Ascospores were visible by light microscopy as dark red pigmented masses as previously described (Brown and Salvo, 1994). Preparation of ascospores for SEM involved opening of cleistothecia either prior to fixing and mounting, or post fixing and mounting. Opening prior to fixing and mounting had the risk that wash steps and vacuum use during platinum or gold coating would disrupt and disperse ascospores. Opening of cleistothecia post fixing and mounting would ensure good maintenance of the internal ascospores but poor conductivity for the imaging and commonly resulted in loading or drifting during SEM imaging. Therefore, attempts were made to conserve internal ascospores by using preparation techniques that required the fewest wash steps. This was achieved by using osmium tetroxide as the fixative.

Ascospores were found to be around 4-5 µm in diameter, slightly smaller than the 6-7 µm previously recorded (Chrenkova *et al.,* 2017). Ascospores were lenticular to globose and ornamented with two equatorial crests that were crosslinked at the furrow. This description matches those previously of both *A. nidulans sensu stricto* and other members of section *Nidulantes* (Chrenkova *et al*., 2017; Sklenar *et al*., 2019). Of particular importance was the stark difference between the ascospores and conidia in both size and ornamentation. High magnifications of up to x21,000 allowed resolution of the ascospores and conidia and could be used to easily distinguish between spore types as shown in **Figure 4.8.**

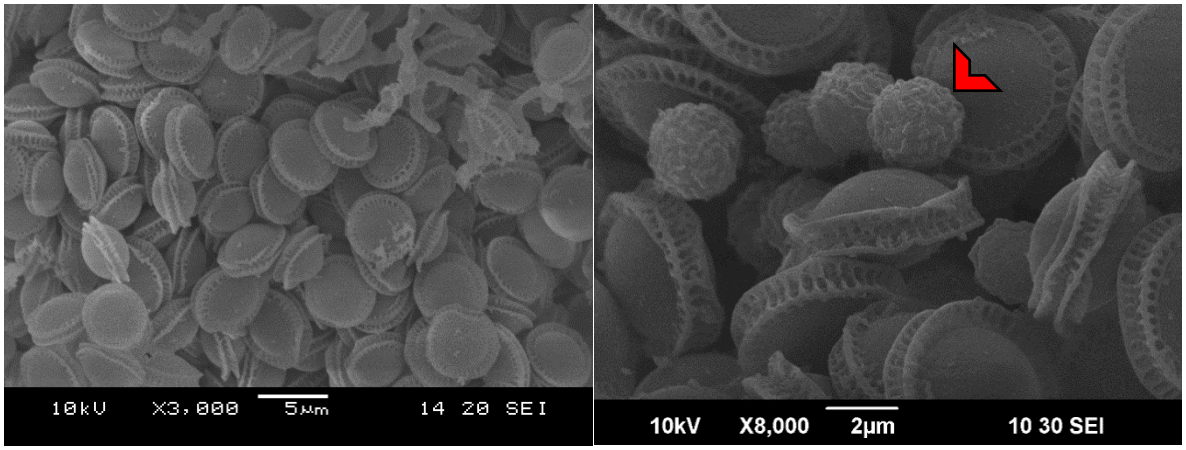


Figure 4.8. Scanning electron micrograph of *Aspergillus nidulans* ascospores. On the right-hand image is a conidium, indicated by the red arrow. This contrasts with the larger and more ornamented ascospores. Separate scale bars on each image indicate the approximate width of ascospores as ~4-5 µm and conidia as ~2.5-3 µm.

4.3.1.2. Sexual development in Aspergillus fumigatus

Cleistothecia were formed reliably from the cross between isolates 47-59 (AFRB2: MAT1-1) x 47-52 (AFIR964; MAT1-2) on oatmeal agar plates which had been left for a period of 6 months under the conditions previously described in **Section 4.2.2.2**. Cleistothecia were seen at the 'barrage zone' of mycelial contact between two colonies of opposite mating types as previously recorded (O'Gorman *et al*., 2009). Between 15-50 cleistothecia were clearly visible per 9 cm plate (n=10). This was lower than previously reported (Sugui *et al.,* 2011) but still sufficient for imaging purposes. The cleistothecia were hyaline to white against the blue-green of *A. fumigatus* conidia. Cleistothecia were removed by sterile dissecting needle and cleaned in Tween and PBS (**Section 4.2.2.2**). The cleistothecia were either then prepared for imaging by SEM or ruptured within 1.5 ml Eppendorf tubes containing 100 µl PBS with a dissecting needle to yield suspensions of ascospores for SEM (**Section 2.2.13.3).**

Cleistothecia were prepared both cleaned and uncleaned for SEM to show that attached structures could survive the SEM preparation steps. Cleistothecia were determined to be between 250-400 µm in diameter which is in line with previous reports (O'Gorman *et al*., 2009., Dyer and O'Gorman., 2011 Alves de Castro *et al*., 2021). The cleistothecial wall consisted of irregularly flattened hyphae concomitant with the teleomorphic genus neosartorya (Dyer and O'Gorman., 2011) (**Figure 4.9**).

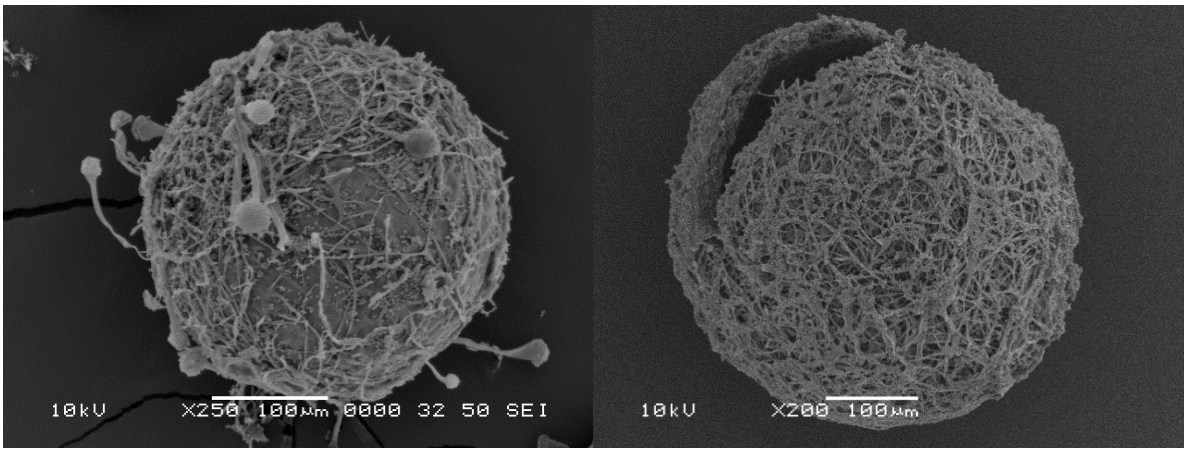


Figure 4.9. Scanning Electron Micrographs of *Aspergillus fumigatus* cleistothecia. The image on the left was uncleaned prior to preparation and has visible attached conidiophores. The scale bars from the separate images suggest widths of between 250-400 µm. Image on the left published in Swilaiman *et al*., 2020).

The ascospores mass of *A. fumigatus* was found to be hyaline to white and lacked the pigmentation seen with *A. nidulans*. Ascospores were measured to have a range between 3-4 µm, clustered around the mean of 3.5 µm (n = 20), in agreement with previous studies (O'Gorman *et al*., 2009). Ascospores showed two primary equatorial ridges and a number of smaller accessory ridges as part of their ornamentation (**Figure 4.10**) and were distinct from those of *A. nidulans* (sexual morph emericella*)*, in that the equatorial ridges lacked linkages between them (**Figure 4.8**). Conidia for *A. fumigatus* were smaller at around ~2 µm in diameter (smaller than average than the *A. nidulans* conidia of ~2.5-3.0 µm). The surface of the conidia for *A. fumigatus* were generally flattened with some exhibited raised bumps on the surface, in direct contrast to the complex system of small verrucose ridges apparent in all *A. nidulans* conidia observed. Either of these structures may be the rodlets previously described (Paris *et al*., 2002). As seen in *A. nidulans*, the size and stark difference in ornamentation between the conidia and ascospores allows for easy differentiation.

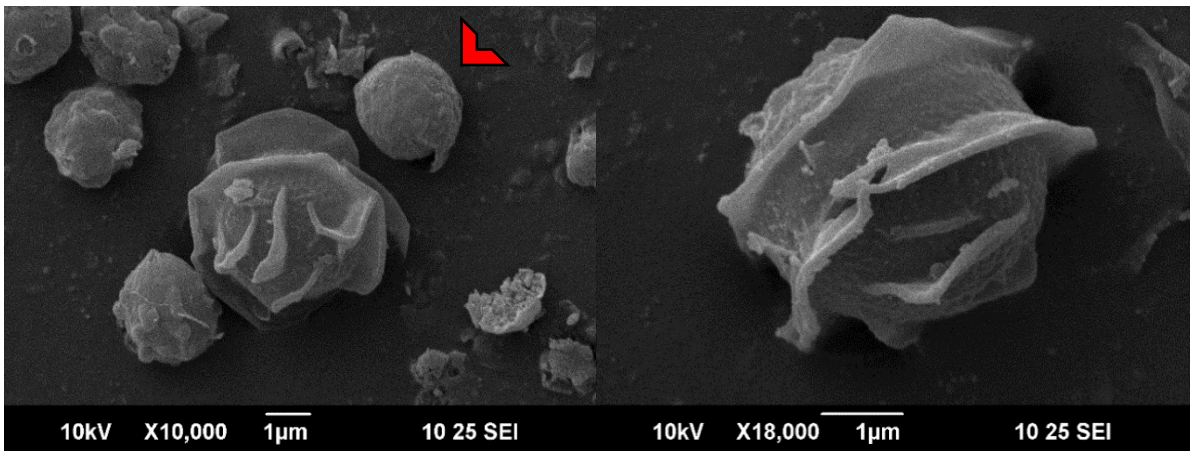


Figure 4.10. Scanning electron micrographs of *Aspergillus fumigatus* ascospores. The image on the left shows a conidium for direct comparison as indicated by the red arrow. Note the size difference and difference in ornamentation between the conidium and the ascospore. Additionally, note the two equatorial crests on the ascospores. Scale bars indicate that conidia are ~2 µm in width and ascospores are ~3.5 µm in width.

4.3.1.3. Sexual development in *A. sclerotiicarbonarius*.

Attempts were made to induce sexual reproduction in *A. sclerotiicarbonarius* as well as to discern ascospores from conidia. Crosses were attempted with all MAT1-1 to MAT1-2 isolates available in the BDUN collection as well as negative controls of axenic cultures or two strains of the same MAT-type. Particular emphasis was placed on the cross for which Darbyshir (2014) had previously reported success. As well as the barrage method, spermatization of sclerotia by placing them on a bed of conidia of the opposite mating type was trialled based on the results of Luis *et al.,* (2020). Sclerotia were prepared for this 'spermatising' as described in **Section 4.2.2.2.1**.

For the barrage crosses, fewer sclerotia were formed at the mycelial boundary than had previously been described by Darbyshir (2014). Variable numbers of sclerotia were formed in crosses and despite dissecting over 500 sclerotia, visualising the contents yielded little evidence of structures such as cleistothecia that are concomitant with sex in this species. In summary, it was not possible to replicate the results of Darbyshir (2014) using the barrage method. This was true even after colonies had been left to incubate for periods of up to a year. Sclerotia that were removed from these crosses tended to have an outermost layer of plush mycelium and hardened pigmented, packed centre. Sclerotia were up to 1 mm in diameter but typically around 800 µm which is smaller than the 1,000-1,400 µm previously reported by Darbyshir (2014). The mycelium surrounding these sclerotia was typically yellow in contrast to the background white mycelium where they were not produced.

In tandem, sclerotia were produced by inoculating CYAR agar as described in **Section 2.2.3** and harvesting the sclerotia. In contrast to other species, *A. sclerotiicarbonarius* rarely exhibited asexual and sexual differentiation in close proximity. This resulted in reduced numbers of contaminating conidia but the sclerotia were usually covered in a plush layer of attached mycelium with a hardened centre. Sclerotia with both intact attached mycelium and those cleaned of mycelium were then inoculated on to oatmeal agar plates (**Section 4.2.2.5.5**). Plates were then incubated as described in **Section 4.2.2.3** with a variable of time of wrapping with Parafilm of either 0 hours or after 24 hours. Notably for the crosses that had not been subject to 24 hours of gaseous exchange, they had reduced conidiation and increased volume of mycelium. This was most noticeable around the sclerotia themselves and seemed to encase the structures as can be seen in **Figure 4.11.** These crosses were then incubated as previously described for up to one year.

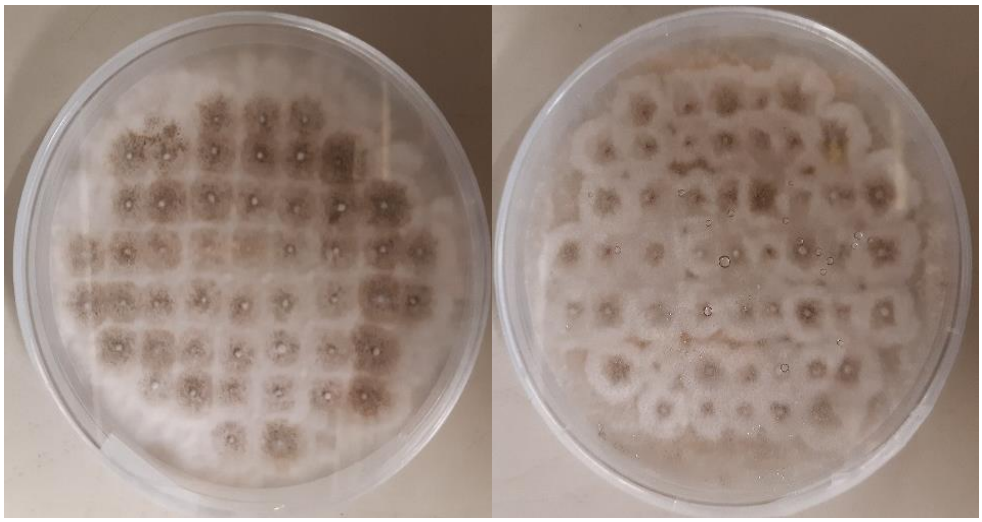


Figure 4.11. Photographs of the 'spermatising' method of crossing. A) Sclerotia of *A. sclerotiicarbonarius* CBS 121056 (MAT1-1) placed on a lawn of CBS 121853 (MAT1-2) conidia. B) Sclerotia of CBS 121853 (MAT1-2) placed on a lawn of CBS 121056 (MAT1-1) conidia. This image is taken after approximately 14 days and shows an interesting reduction in overall conidiation and regrowth of white mycelia over the previously washed sclerotia.

Harvesting and sectioning of the sclerotia produced by this 'spermatising' method, which had had restricted gas exchange by sealing with parafilm at 0 hours, yielded structures of interest. When dissected, as many as 80 % of sclerotia produced this way had a clear and obvious cream coloured structure in the interior of the sclerotia, visible against the otherwise contrasting red to purple pigmentation of the sclerotium (**Figure 4.12**). This contrasts with a lower figure of ~10 % which had this morphology in those cultures which had been exposed to gaseous exchange for 24 hours prior to sealing with parafilm. Importantly, these structures were conspicuous by their total absence in negative controls and crosses of the 'barrage' type in all sclerotia examined.

Amongst all of the cream structures inspected, there were an equal number of dried and hollow structures as seen in **image A** of **Figure 4.12** and smaller, globose moist structures as seen **image B** of **Figure 4.12**. Further sectioning of the dry structures revealed no further interest. Sectioning of the smaller, moist structures by removal with a clean dissecting needle and mounting on a coverslip revealed irregularly large spores, with occasional pigmentation, when viewed by light microscopy as can be seen **Image C** of **Figure 4.12**. The centre of sclerotia described here for this species is very different to the cross sections of sclerotia described previously as consisting of just a rind and pale pseudoparenchymatous medulla (Abu El-Souod *et al.,* 2017). It should be noted that in that study, Czapek's yeast autolysate agar without raisins was used to induce sclerotium formation.

The spores seen in **image C** of **Figure 4.12** were approximately 4-5 µm which is larger than the conidia of both *A. nidulans* (~2.5-3 µm) and *A. fumigatus* (~2 µm) but in keeping with the range of 5-6 µm for *A. sclerotiicarbonarius* previously reported (Noonim *et al.,* 2008).

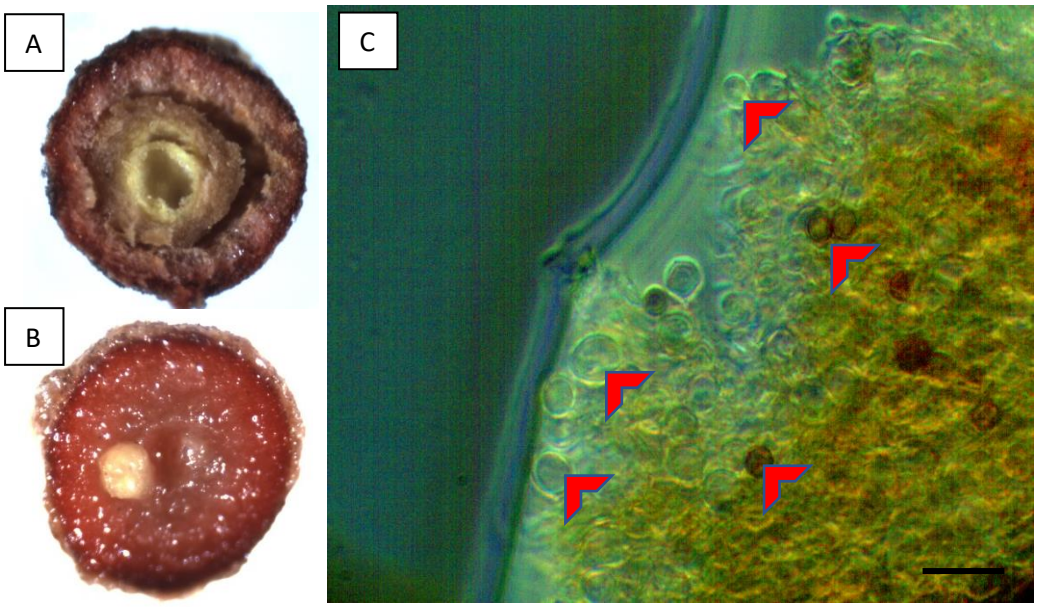


Figure 4.12. Light microscopy images of *Aspergillus sclerotiicarbonarius*. A and B) Light microscopy images of cleaned, dissected *A. sclerotiicarbonarius* sclerotia. Note the pale hollow cavity in the centre of A and the intact pale yellow structure in the centre of B. C) A light microscopy image of the sectioned central structure from B, showing putative ascospores (red arrowheads).

Subsequent preparation of the cream coloured structures for SEM was completed as described in **Sections 2.2.13.3** using osmium tetroxide as the fixative to reduce the number of wash steps. SEM revealed a stark contrast in the cellular organisation of the cream structures against the sclerotium. Whereas the sclerotium was made of broad vascular hyphae - pseudoparenchymatous and typical of sclerotial medullae (Willetts and Bullock, 1992), the internal structure consisted of finer hyphae making up several structures of flattened cells, each with a hollow cavity at the centre. This cellular arrangement was suggestive of the outer surface of cleistothecia – the peridium (Dyer and O'Gorman, 2011), but in this case without any obvious ascospores. Analogous to other members described as petromyces/saitoa such as *A. tubingensis (*teleomorph/sexual morph previously named petromyces*)*, where central flattened cells were described as the ascocarp peridium (full of ascospores) and showing a clear delineation with the stromal matrix of pseudoparenchymatous cells (Horn *et al*., 2013) and again illustrated with *Aspergillus parasiticus* (previously teleomorph petromyces) (Horn *et al*., 2009) and *A. flavus* (previously teleomorph petromyces).

Previously described petromyces sexual morphs named above show a clear ascocarp matrix full of ascospores except for *A. flavus* (sexual morph petromyces). Within this species, the ascospores are described as being held within asci-forming hyphae (**h** in **Image A** of **Figure 4.13**) rather than being many discontinuous asci and ascospores populating the space. During the observation of *A. sclerotiicarbonarius* structures, a delineation between two cell types (ascocarp peridium and outer

matrix) became obvious (**Image B** of **Figure 4.13**), although to a lesser extent than (**w**) in **Image A**. Additionally, magnification of the structure's internal cavity (from *A. sclerotiicarbonarius* cross 80.5 x 80.3) appeared to show both spores 'budding' from the internal cavity and spores with ornamentation which is synonymous with ascospores (**Image C,** indicated by the red arrows). These two features are similar indeed to the asci-forming hyphae described for *A. parasiticus* (Horn *et al.,* 2009).

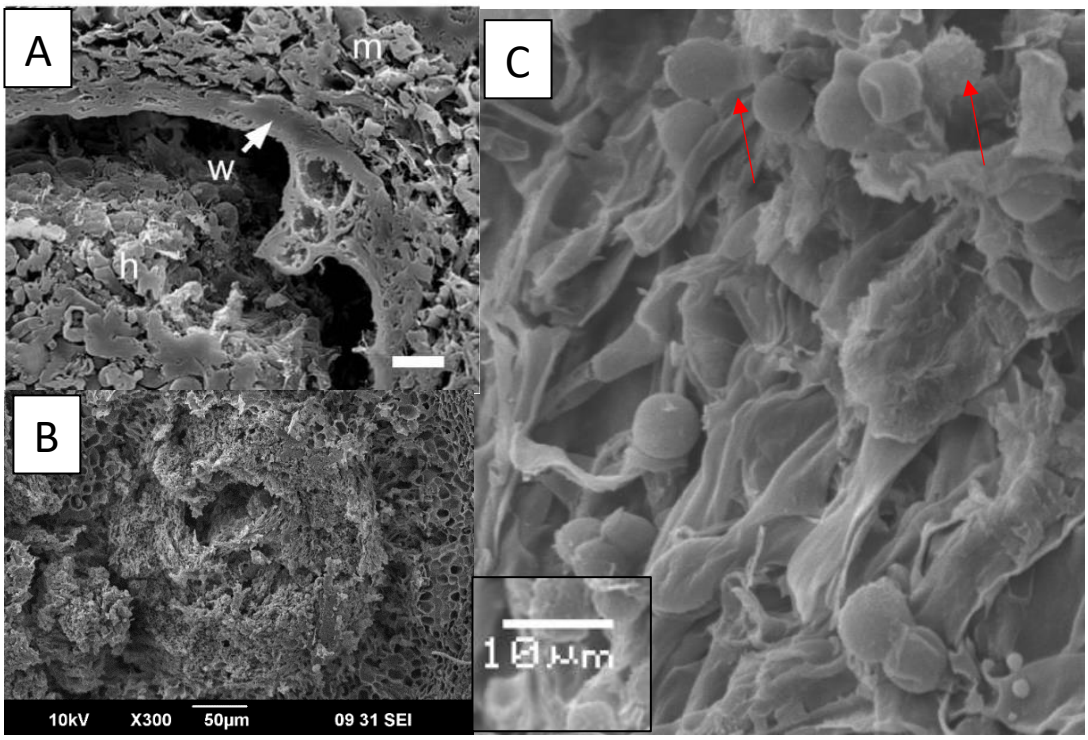


Figure 4.13. Scanning electron micrographs of: A) *Aspergillus parasiticus (*sexualmorph petromyces) stroma featuring an outer matrix (m), wall of the ascocarp or ascocarp peridium (w) and ascus forming hyphae (h). B) *A. sclerotiicarbonarius* internal structure featuring delineation between pseudoparenchymatous hyphae and flattened cell in layers like peridium. At the centre of the image is a hollow cavity. C) Magnification of the hollow cavity from B. Spores appear to be 'budding' from the walls of the structure and some have ornamentation. Red arrows indicate 'budding' or 'ornamented' cells. Image A is taken from Horn *et al*., 2009. Image C is from the *A. sclerotiicarbonarius* cross 80.5 x 80.3.

Spores collected that may have been ascospores were around 4-6 µm in diameter, smaller than the 4.8-9.5 µm diameter described for ascospores elsewhere in the aspergilli (Samson *et al.,* 2007). This means that any size difference between conidia and ascospores in this species is less discernible than for the other species so far described e.g. *A. nidulans* (sexual morph emericella) and *A. fumigatus* (sexual morph neosartorya). Another feature observed is that the conidia were often seen depressed or deflated which would have altered the measurable diameter of the spores as well as masking any ornamentation, further hindering discernability between the spore types. This is likely an artefact of the preparation for SEM, termed 'collapse' (Khalil and Hashem, 2018), although critical point drying (CPD) was undergone to try and avoid this problem. Spores can be seen in the internal structures of

sclerotia in **Figure 4.14** as well as the collapse of likely conidia. The absence of definitive evidence of the production of ascospores (and downstream evidence of recombination) illustrated the need to design a protocol able to differentiate spore types as will be described in **Sections 4.2.2.7** and **4.3.4**.

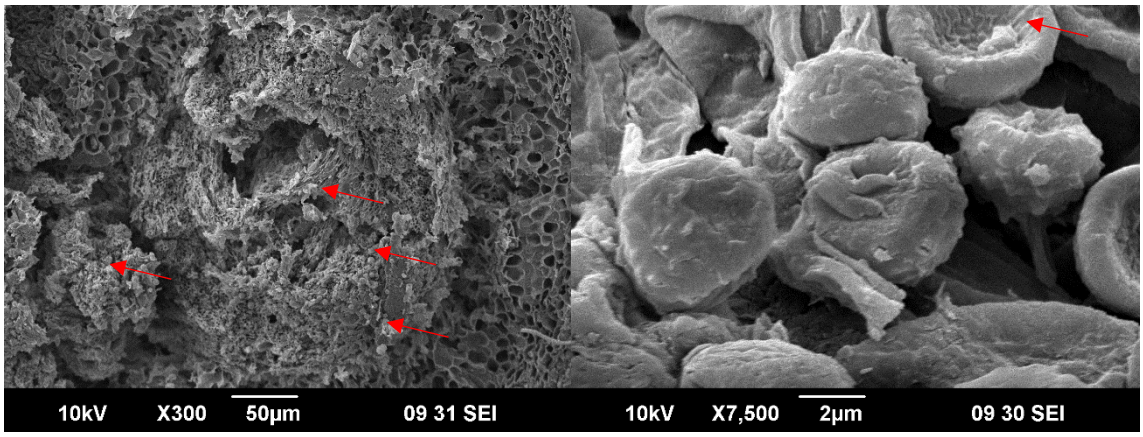


Figure 4.14. Scanning electron micrographs of *Aspergillus sclerotiicarbonarius* structures. A) The centre of an *A. sclerotiicarbonarius* sclerotia, showing the in-situ central structure observed under many of the 'spermatising' crosses. Spores are indicated by red arrows. B) Probable *A. sclerotiicarbonarius* conidia. The scale bars on B indicate an average diameter of ~5 µm. This is in keeping with the sizes of conidia in this species. Note the flattened, almost deflated conidia on in the top right of the image indicated by the red arrow.

4.3.1.4 Attempted Induction of Sex in *A. tubingensis*

At the onset of this PhD, a sexual cycle in *A. tubingensis* had been described (Horn *et al*., 2013) and evidence of recombination then shown (Olarte *et al*., 2015). The sexual cycle was fastidious in nature, but this species was the most closely related to *Aspergillus niger* within section *nigri* to have a sexual cycle described. The species is heterothallic and therefore required prior determination of the mating types of the strains available in the University of Nottingham BDUN culture collection such that opposite mating types could be paired for crossing. Two different mating-type PCR diagnostics were used, either a multiplex or degenerate-PCR based method (**Section 2.2.6.3**).

Application of the multiplex PCR MAT-type assay to the 44 strains tested, yielded positive results for all but four of the *A. tubingensis* strains (one additional strain was missing from stocks) (**Appendix 7).** This is a similar success rate for this type of diagnostic with the black aspergilli as recorded in other studies (Darbyshir, 2014). Isolates that yielded a result gave either a characteristic 3.3kbp (*MAT1-2*) or 1.9kbp (*MAT1-1*) product. The results of a typical multiplex diagnostic can be seen in **Figure 4.15.** The results of this assay were recorded for each strain (**Appendix 7**). For example, results confirmed that the DSM Foods industrial citric acid production strain, in this thesis referred to as 76-57, was indeed of the MAT1-2 genotype.

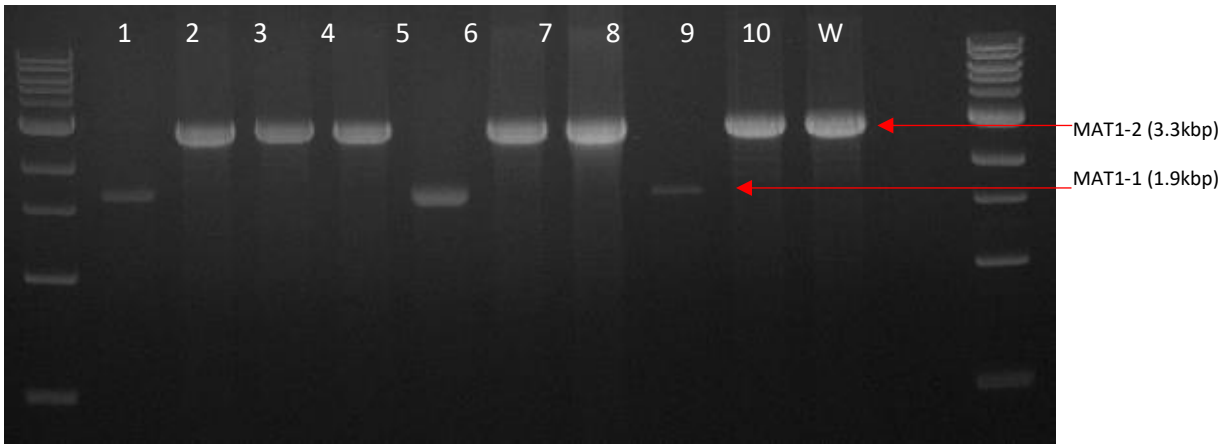


Figure 4.15. Agarose gel electrophoresis image of the products of a multiplex diagnostic PCR using the three primers ATYP1, ANMAT1 and MAT2amR on strains of *A. tubingensis*. 1 kb ladder is used. Lane 1 is a known MAT1-1 control (1.9 kbp product). Lane 2 is a known MAT1-2 control (3.3 kbp product). Lanes 3-10 are test products (Nottingham strains 76; 15, 16, 19, 21, 22, 25, 32 and 34 respectively) here all identified as either MAT1-1 or MAT1-2 by matching their sizes to the control products. W denotes the water control.

Degenerate primers were then used to determine the mating type of the remaining isolates (**Section2.2.6.2**). This diagnostic had greater success but was more time consuming, requiring 2 reactions per isolate. It was found that MAT1-1 primers MAT1.1F and MAT1.1R were more consistent than MAT1F and MAT1R and these were used for the diagnostic. Similarly, MAT1-2 primers MAT3-5 and MAT5-7 were consistent and used for the diagnostic, whereas mat1.2for and mat1.2rev failed to yield product. These primers gave characteristic banding of ~270bp for MAT1-2 and ~360bp for MAT1-

1 (**Figure 4.16** and **Appendix 2**). When overlaid the lanes show a product in one lane for one assay and an absence in the corresponding assay. Any lanes showing neither band, or a band in each lane could be repeated (See Lane 7 in **Figure 4.16**) or could be matched with the multiplex diagnostic. This assay yielded the mating type identity of the remaining isolates except 76.39 and 76.41. Additionally, 76.35 and 76.38 were absent from stocks which were then excluded from future work. The mating types of all isolates of *A. tubingensis* are summarised in **Appendix 7**.

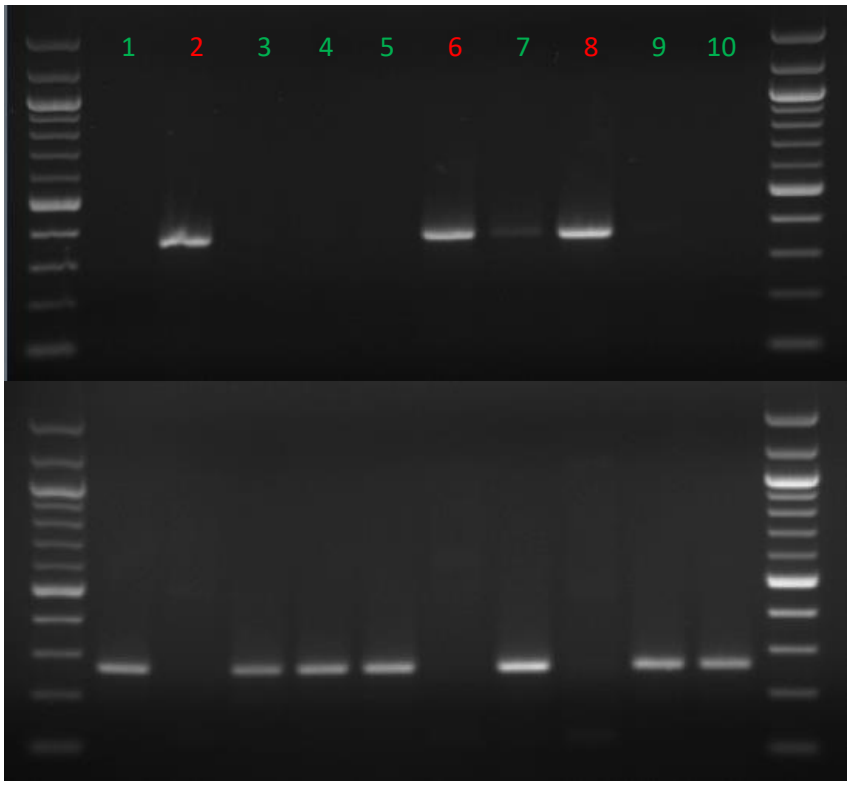


Figure 4.16. Agarose gel electrophoresis images of the products of single MAT-type diagnostic PCR. Top image) Products of primers MAT1.1F and MAT1.1R (*MAT1-1*), Lane 2 is a *MAT1-1* control. Products are ~360 bp. Bottom image) Products of primers MAT3-5 and MAT5-7 (*MAT1-2*) with Lane 1 as a *MAT1-2* control. Products are ~270 bp. Lane numbers are shown at the top of the image and are red for *MAT1-1* and green for *MAT1-2*. Generally, the products for MAT1-2 were more intensely amplified. Lane 7 showed a faint band for *MAT1-1* and confirmation of *MAT1-2* when repeated.

The results overall showed that the BDUN collection of fifty isolates consisted of 22 MAT1-1 and 28 MAT1-2, which slightly favoured MAT1-2. Chi squared analysis revealed that this is in-line with the expected 1:1 ratio of mating type of a sexually active species with no statistical difference evident (n=50, 22:28, p = 0.5478). These results were combined with parallel findings about which isolates of *A. tubingensis* scored most highly with respect to sclerotium production (**Section 3.2.2.2** and **Table 4.1)**, together with findings about which media had provided greatest sclerotia production and the most productive methods for inoculation (**Figure 3.11** and **Figure 3.13**).

Crosses were set up in all iterations between eight sclerotia producers of *A. tubingensis* of each mating type (including the MAT1-2 industrial strain 76-57, which did not produce sclerotia; **Table 4.1**). Additional control 'crosses' between isolates of the same MAT-type were also set up.

Table 4.1. Table showing lists of all *A. tubingensis* isolates that produced sclerotia separated into *MAT*-type columns (numbers in brackets show average sclerotia formed per cm^2 on plate). The DSM industrial strain 76-57 is included despite its ability to form sclerotia, as it was valued for its potential source of variation in case of any sexual recombination.

Highest scoring MAT1.1 isolates	Highest scoring MAT1.2 isolates
76-17 (9.2)	76-9 (19.4)
76-26 (2.4)	76-27 (15.2)
76-29 (3.6)	76-28 (4.0)
76-33 (2.7)	76-31 (5.1)
76-46 (12.2)	76-37 (8.6)
76-47 (12.9)	76-45 (25.6)
76-53 (2.9)	76-48 (2.4)
76-56 (1.3)	76-57 Industrial Strain (0.0)

The spread plate groups described in **Section 4.2.2.4** produced no sclerotia in any of the test isolates and were therefore discounted from further analysis. Amongst the other arrangements of raisins and inoculation points (as shown in **Figure 4.3**) most isolates produced abundant sclerotia, although notably none on any of the portions of plates inoculated with the DSM industrial strain, nor at the margin of mycelial contact. Sclerotia were dissected and sectioned, and examination of a number of these dissected sclerotia yielded a red pigmented structure within the central sclerotial cavity (**Figure 4.17**), whereas the internal space otherwise showed an unpigmented buff colouration throughout. SEM of the sectioned and intact structures are shown in **Figure 4.19**. It then became prudent to score the plates and crosses depending on the number of these red pigmented structures were observed. The results of this red pigmented structure assay are shown in **Figure 4.18**. The results were consistent amongst the four methods of inoculation (A-D in **Figure 4.3**) in terms of strains and crosses. Most red structures were produced in crosses involving MAT1-1 strains 76-17, 76-33 and 76-46, with 76-17 particularly highly scoring in production across all strains it was crossed with. This indicates a pattern whereby the production of these structures is strain specific regardless of mating partner and seems to be concentrated on three strains, all of which are MAT1-1.

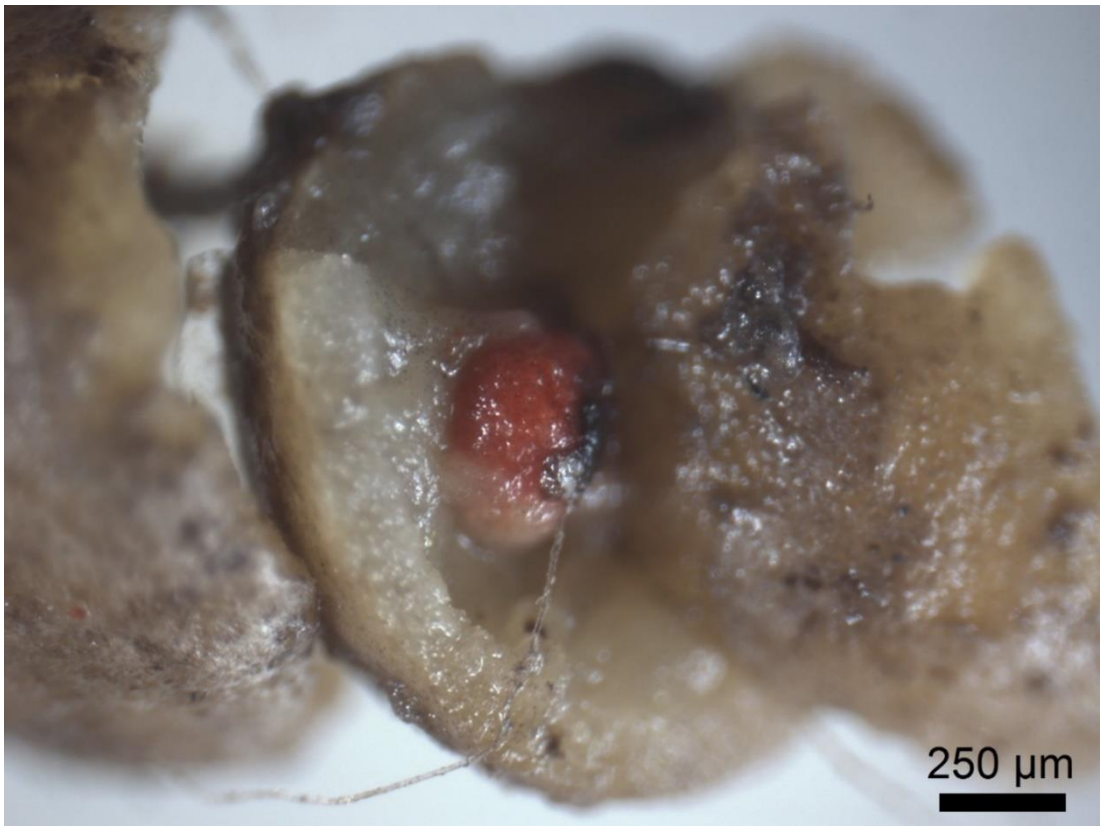


Figure 4.17. Light microscopy image showing a red pigmented structure that was observed in some of the dissected sclerotia from attempted crosses with *A. tubingensis*. It was possible to differentiate the rind, cortex and medulla. Image taken from the cross 76.17 x 76.48.

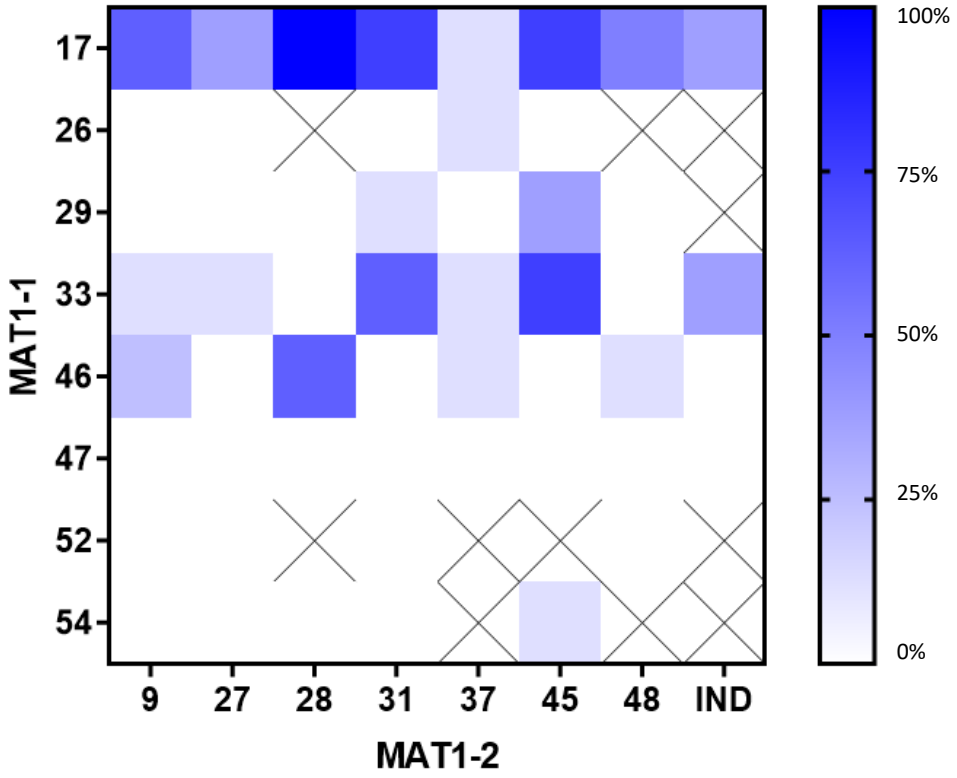


Figure 4.18. Heat map showing % examined sclerotia that yielded red pigmented structures from dissected sclerotia. X represent mating crosses that yielded no sclerotia to examine.

The red internal structures seen were either excised from sclerotia or left in situ and prepared for SEM for further visualisation. The steps involved in preparation for SEM resulted in total reduction of pigmentation. Images presented in **Figure 4.19** show two distinct tissue types of the structures. A hardened centre with a discrete shape made of flattened cells was contrasted with a mass of attached flocculose hyphae. Spores were seen on the surface but these matched conidia in size (3-4 µm) and both shape and lack of ornamentation seen in ascospores in other *Aspergillus* species.

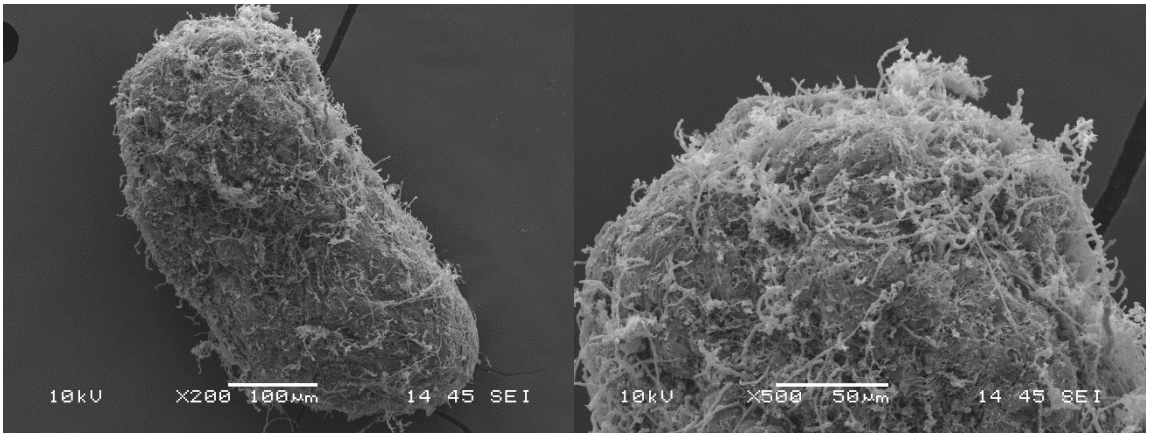


Figure 4.19. Scanning electron micrographs of the red pigmented structure in the central cavity of certain *A. tubingensis* strains.

Preparation of whole sclerotia for imaging wholly reduced the pigment seen. This result, taken with the similar result seen with red pigmentation lost when red pigmented structures are prepared directly, indicates that the pigment is highly soluble in ethanol and that the soluble pigment can be washed from the centre of sclerotia during critical point drying. A similar result was seen with *Sclerotinia sclerotiorum* (results not shown), whose sclerotia beyond the rind have a pinkish hue that is lost during CPD. Similarly, Asperthecin from *Aspergillus nidulans (*sexual morph emericella*)* is seen to be highly soluble in methanol and is used to concentrate the pigment for analysis (Szewczyk *et al*., 2008). This made it harder to observe any red pigmented structures from sclerotia that had been prepared as whole sclerotia and later dissected.

Figure 4.20 illustrates what may be an example of one of these structures from a whole prepared sclerotium therefore illustrating its location in-situ. The figure also illustrates the stark difference in tissues between the pseudoparenchymatous hyphae and irregularly flattened cells that constitute the internal cavity wall. This difference in tissues was also seen previously in *A. sclerotiicarbonarius* (Darbyshir, 2014) Higher magnification of this structure (**Figure 4.21**) shows a different composition than the whole excised structure in **Figure 4.19**. This may be due to the structures having multiple forms, damage to the in-situ structure caused by sectioning, or this structure may be a relic of the cortex or medulla as opposed to the red pigmented structures seen under light microscopy (**Figure 4.17**). Again, this highlights the difficulty of imaging these structures in-situ, where the key identifier of their presence (red pigmentation) is lost during preparation.

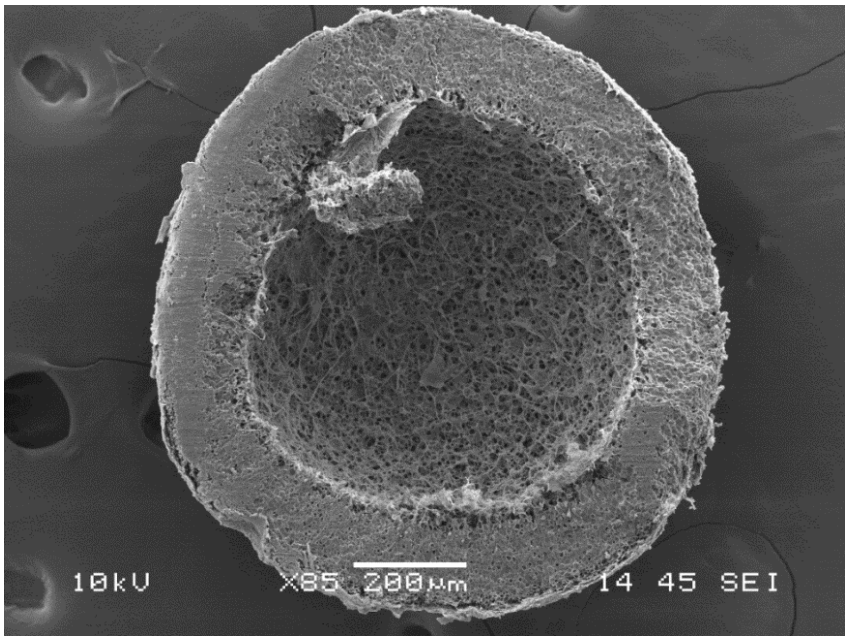


Figure 4.20. Scanning electron micrograph across a cut sclerotia of *A. tubingensis*. Two notable features are the roughly and irregularly flattened cells lining the central cavity and an example of the inner red pigmented structure commonly observed, here apparent in the top left of the cavity space.

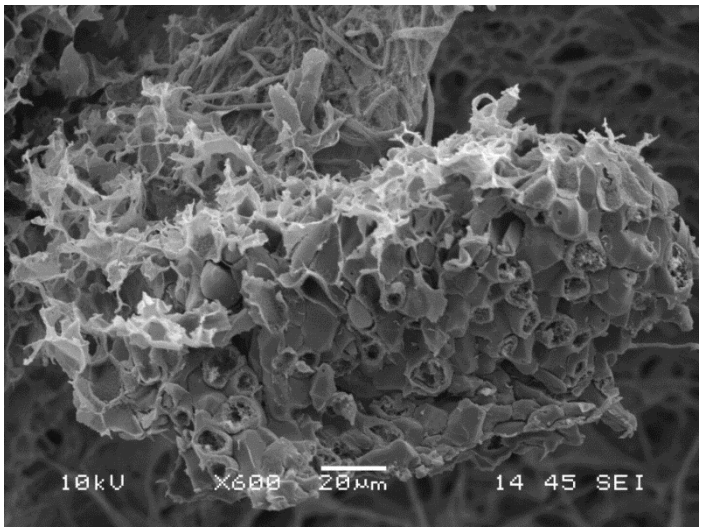


Figure 4.21. A Scanning electron micrograph at high magnification of the red pigmented structure. Clearly pseudoparenchymatous in this example and lacking any conidia or ascospores.

The irregularly flattened cells that make up the wall of the internal cavity of sclerotia is evident in **Figure 4.22.** This is comparable to the outer wall or rind of the sclerotium as seen in **Figure 4.23**. In both images, conidia can be seen and these are measured at a diameter of 3-4 µm, which is similar to previously described (Horn *et al*., 2013, Samson *et al.,* 2007b). Conidia in this species are described as aculeate, and this agrees with images seen here, with some conidia having a single, very large spike. However**,** in none of the attempted crosses that were investigated, even after 12 months of incubation, were the discrete and discontinuous ascospores observed by Horn *et al*. (2013) observed**.**

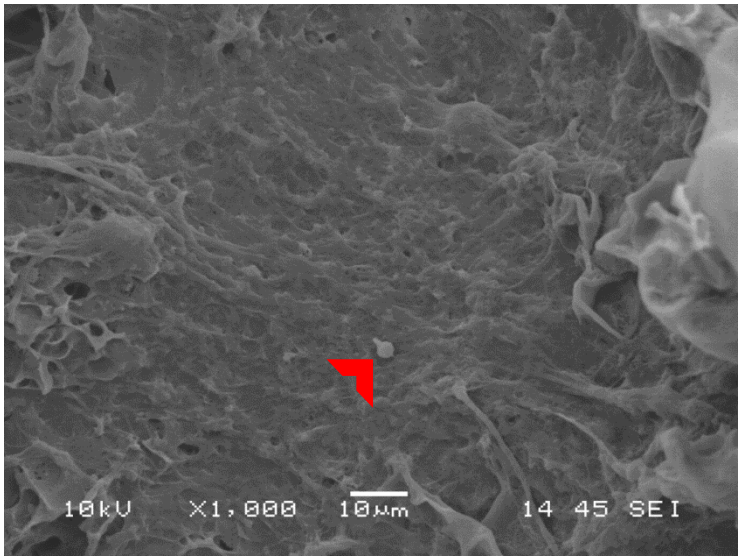


Figure 4.22. A Scanning electron micrograph of the interior cavity of a sclerotium of *A. tubingensis*. A single conidium is shown (marked with red arrowhead) against irregularly flattened cells forming the internal wall of the cavity.

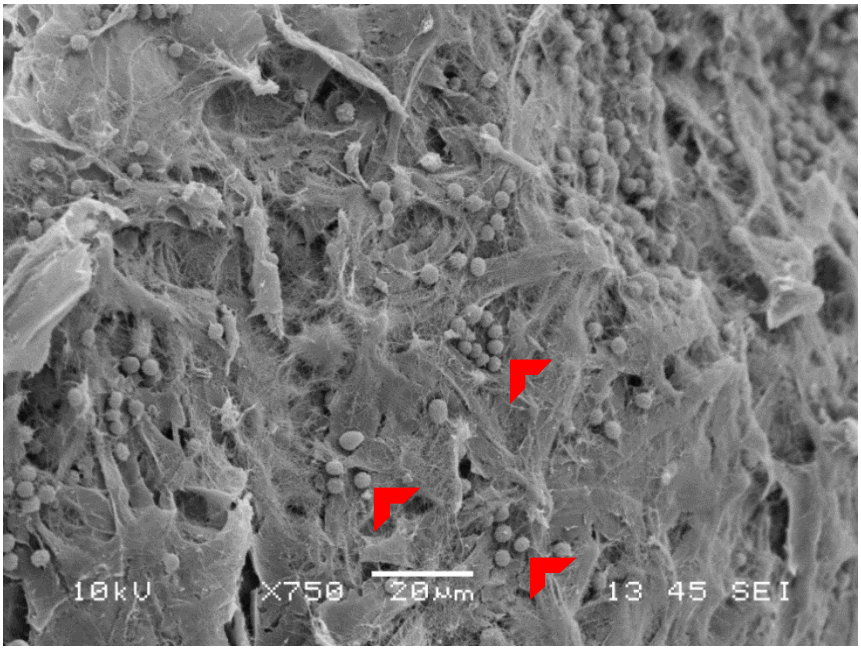


Figure 4.23. A Scanning electron micrograph of the outside surface of a sclerotium showing abundant conidia (several marked with red arrow heads) of approximately 3-4 µm diameter.

4.3.2 Attempted Induction of Sexual Reproduction in *A. niger*

4.3.2.1 Mating-type Assays of A. niger

At the onset of these PhD studies no sexual cycle had yet been described in *A. niger* and it was therefore a valuable target for research. Evidence of a heterothallic lifestyle and the known presence of mating-type genes meant that designation of the MAT-type of each strain of the University of Nottingham culture BDUN collection was imperative. The multiplex diagnostic described in **Section 2.2.6.3** proved successful to determine the mating-type identity of strains of *A. niger* and results are shown in **Appendix 9**. Twenty-eight strains were MAT-typed, revealing a distribution of 18:10 MAT1-1 to MAT1-2 with MAT1-1 yielding a 1.5 kbp product and MAT1-2 yielding a 2.9 kbp product (strains 8-178 and 8-179 were used as MAT1-1 and MAT1-2 controls respectively; **Figure 4.2.4**). A chi-squared test showed there was no statistically significant difference between these values (n=28, p=0.28) and therefore it is not deviant to the expected 1:1 ratio. A tendency towards MAT1-1 had also previously been seen in other studies with reports of 75-95 % MAT1-1 in sample cohorts (Darbyshir 2014; Ashton 2018; Mageswari *et al.,* 2016). The isolate collection in the present study did not come from a single source and is therefore not a predictor of local population biology but can be used to infer balance between the MAT-types.

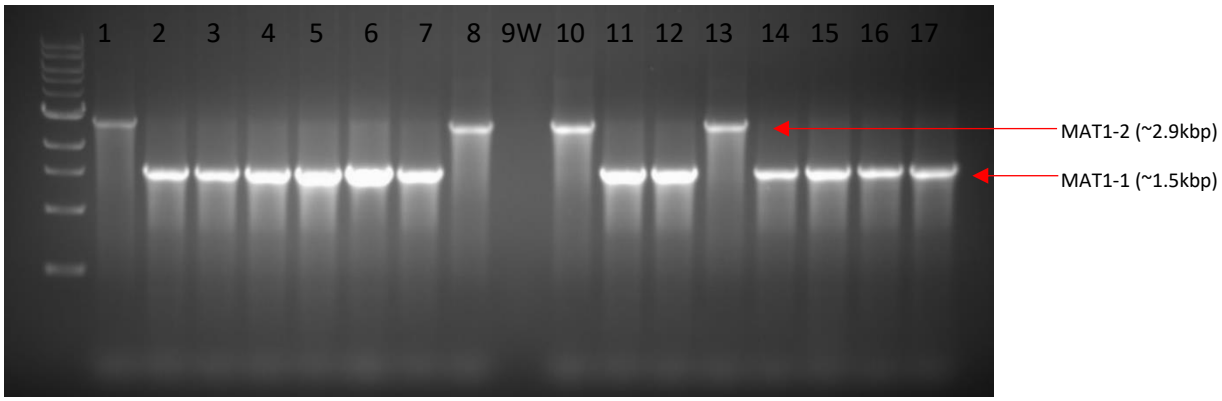


Figure 4.24. Gel electrophoresis image of the results of a multiplex diagnostic of *Aspergillus niger* strains from the University of Nottingham BDUN culture collection. Lanes 1-7 are experimental, and Lane 8 is the 8-179 MAT1-2 control. W-Lane 9 is water control. Lanes 10-16 are experimental. Lane 17 is the MAT1-1 control. MAT1-1 products are ~1.5 kbp and MAT1-2 products are ~2.9 kbp.

4.3.2.2 Attempted Sexual Crossing of A. niger

Crosses were set up between *A. niger* isolates of complementary mating types using the conditions and media identified in **Section 4.2.2.5**. These crosses were based upon successful crosses in *A. fumigatus* (O'Gorman *et al*., 2009), *A. flavus* (Horn *et al*., 2009a), *A. parasiticus* (Horn *et al.*, 2009b) and *A. tubingensis* (Horn *et al*., 2013) and the pilot *A. niger* crosses of Ashton (2018). An important consideration was the identification of sclerotia-producing strains. This was explored in and had revealed a set of sclerotia producing strains of both mating types. Crosses were primarily incubated at 28 ˚C and 30 ˚C, given that these temperatures yielded the highest sclerotial production. Finally, strains of opposite mating types which had been transformed to contain eGFP and Tdtomato (discussed in **Chapter 3**) were used to inform whether heterokaryon formation occurred within the sclerotia.

Sclerotial initials were typically observed within 3-4 days of colony incubation and mature sclerotia around 10 days. Mating via the barrage method led to the greatest number of sclerotia produced of any of the crossing methods. CYAR was used a reliable means to induce sclerotium production, but noticeably, sclerotia were very rarely produced where the raisin has been resting on the agar. This has been previously reported (Ellena *et al.,* 2021a). The inoculation point for the barrage crosses tended to be the position where groups of sclerotia subsequently formed for most isolates (i.e. rather than at the margin interaction barrage zones), with sclerotia formed elsewhere in the plates only for those strains that produced many sclerotia. Thus, strains that produced few sclerotia only did so at the point of inoculation. CYAR reliably induced the production of sclerotia but all other media produced very few or none. In particular it was observed that incubation in 5 % atmospheric carbon dioxide at 30 ˚C often yielded sclerotia that had a yellow mycelial covering, which was never observed in crossing on CYAR with normal atmospheric gas exchange (**Figure 4.25**).

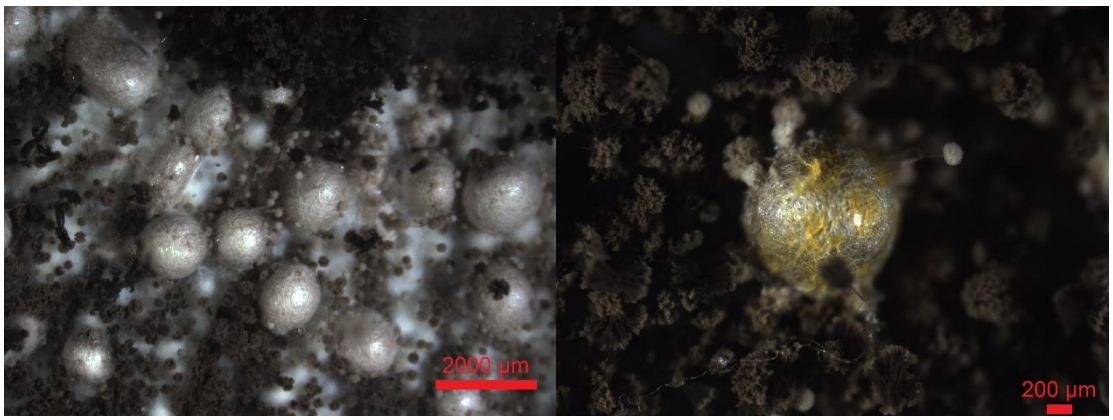


Figure 4.25. Light microscopy images of *A. niger* sclerotia produced on **CYAR**. On the left are sclerotia exhibiting the previously described white/gunmetal grey colouration, grown at 30 ˚C in the dark with standard sealing of cultures with parafilm (strain 8-160 axenic). On the right is a sclerotium with yellow/golden mycelium on the exterior, grown at 30 ˚C in the dark supplemented with 5 % atmospheric carbon dioxide. The size of sclerotia are no different between the conditions, with typical mean diameters ~1000 µm (cross of strains 8-161 x 8-160).

The use of vegetative mass mating (VeM, also referred to herein as the vegetative mycelial ball method) technique led to a more rapid production sclerotia than other crossing methods (mature sclerotia visible after around 5-6 days) but a drastic reduction in the number of sclerotia produced. Examination/dissection of these sclerotia yielded little of interest at all time points up to 1 year.

The mixed spore suspension inoculation method yielded very few sclerotia. Examination of these sclerotia yielded little of interest over all time points up to 1 year. This is likely due to the considerable reduction in concentration of spores across the surface of the plate rather than a high concentration of spores at the inoculation point used during barrage method, as discussed previously (Brown *et al.,* 2008).

The method of spermatising sclerotia, based on a recent study investigating sex in *A. flavus* (Luis *et al*., 2020) allowed for crosses to be set up between strains that produced sclerotia and strains of the opposite mating type, regardless of whether the latter strains actually produced sclerotia themselves as the latter strains were only needed as a source of spermatising conidia. Sclerotia were therefore produced on CYAR, cleaned as described in **Section 4.2.2.5.5** and plated on new media amongst a bed of conidia of the opposite mating type before incubation as described in **Section 4.2.2.5.5**. Cultures were incubated both with normal atmospheric gas exchange (plates sealed with parafilm) and also in the presence of atmospheric 5 % CO_2. Media used for these spermatising crosses included standard media such as oatmeal agar, mixed cereal agar, malt extract agar and a number of alternative media utilising forms of organic matter (**Section 4.2.1**). Examination of such spermatised sclerotia at times points up to a year often yielded the commonly described sclerotia structural components of a rind, cortex and medulla. The rind was often pigmented lightly grey, but rarely white as widely described (Ellena *et al*., 2021a; Frisvad *et al*., 2014). Interestingly, sclerotia of one strain, namely 8-162, displayed a reddish-brown inner hue turning white and tapering where the sclerotia detached from the mycelium. In terms of both size and colouration, this was highly reminiscent of radish seeds. The cortex was consistent amongst all sclerotia examined and consisted of pseudoparenchymatous hyphae. The medulla was highly variable, with many consisting of loose hyphae as widely described in sclerotia (Willetts and Bullock, 1992, Horn *et al*., 2013) but differing greatly in diameter. Light microscopy and SEM of standard sclerotia consisting of rind, cortex and medulla can be seen in **Figure 4.26.** However, such a layered morphology was absent from many sclerotia, and instead there was an empty central cavity, lined with irregularly flattened cells, not unlike descriptions of cleistothecial peridium (Dyer and O'Gorman, 2012) and very similar to the flattened cells seen on the surface of *A. nidulans* cleistothecia (**Section 4.3.1**). Light microscopy and SEM of this peridium-like lining of the cavity are shown in **Figure 4.28**. A smaller number of sclerotia examined yielded a globose red pigmented structure held within the medulla and surrounded by loose hyphae. These were relatively common amongst 'crosses' of either 8-160 (MAT1-2), 8-162 (MAT1-2) and 8-162 (MAT1-1) and never

observed in sclerotia of axenic cultures or 'crosses' of the same mating type. Light microscopy of the red pigmented structure can be seen in **Figure 4.27**. A similar structure has been described in *A. tubingensis* as a sterile ascocarps (Horn *et al*., 2013).

Crosses using the spermatising sclerotia method that were then grown on wheat straw agar at 30 ˚C in 5 % carbon dioxide were very distinct. Upon examination after 6 months, the sclerotia and straw were both indistinguishable and had formed a dense mat of fungal mycelium. The sclerotia from these crosses and those grown on oatmeal Agar had both degraded the rind of their sclerotia to allow prominent mycelial outgrowth. Examination of these sclerotia revealed some sclerotia that had prominent channels from the central cavity. Scanning electron micrographs of these channels can be seen in **Figure 4.29**.

Figure 4.26. Light microscopy and scanning electron micrograph of intact sterile sclerotia of *A. niger* with a classic rind of hardened pigmented cells, cortex of pseudoparenchymatous hyphae and a medulla of loose hyphae.

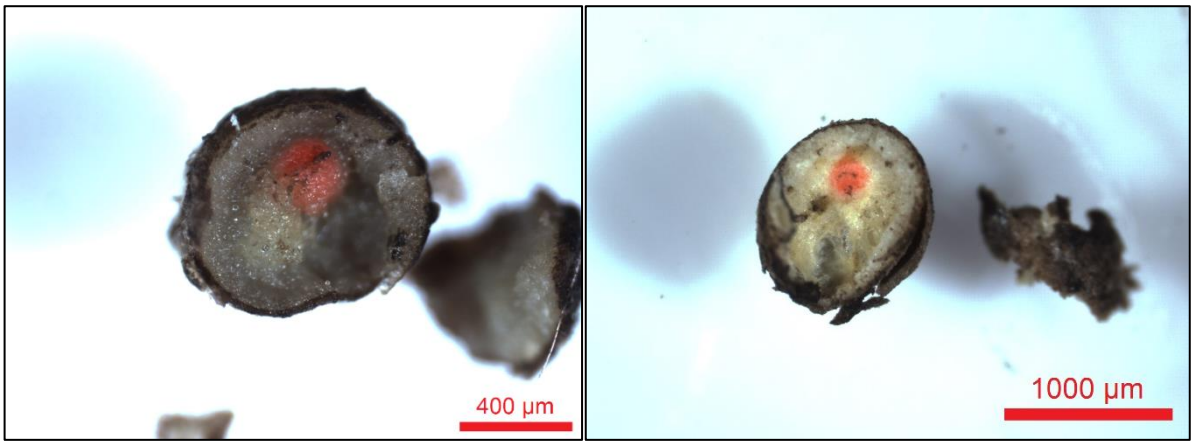


Figure 4.27. Light microscopy images of sectioned sclerotia of *A. niger* bearing a red pigmented structure. This structure was never seen in axenic cultures or 'crosses' between cultures of the same mating type. Sclerotia are typically 800 µm in diameter and the red structures are 150-250 µm in diameter. Similar structures were seen in this study in *A. tubingensis* and have previously been described as sterile ascocarps (Horn *et al.*, 2013). Sclerotia from cross between 8-161 x 8-160.

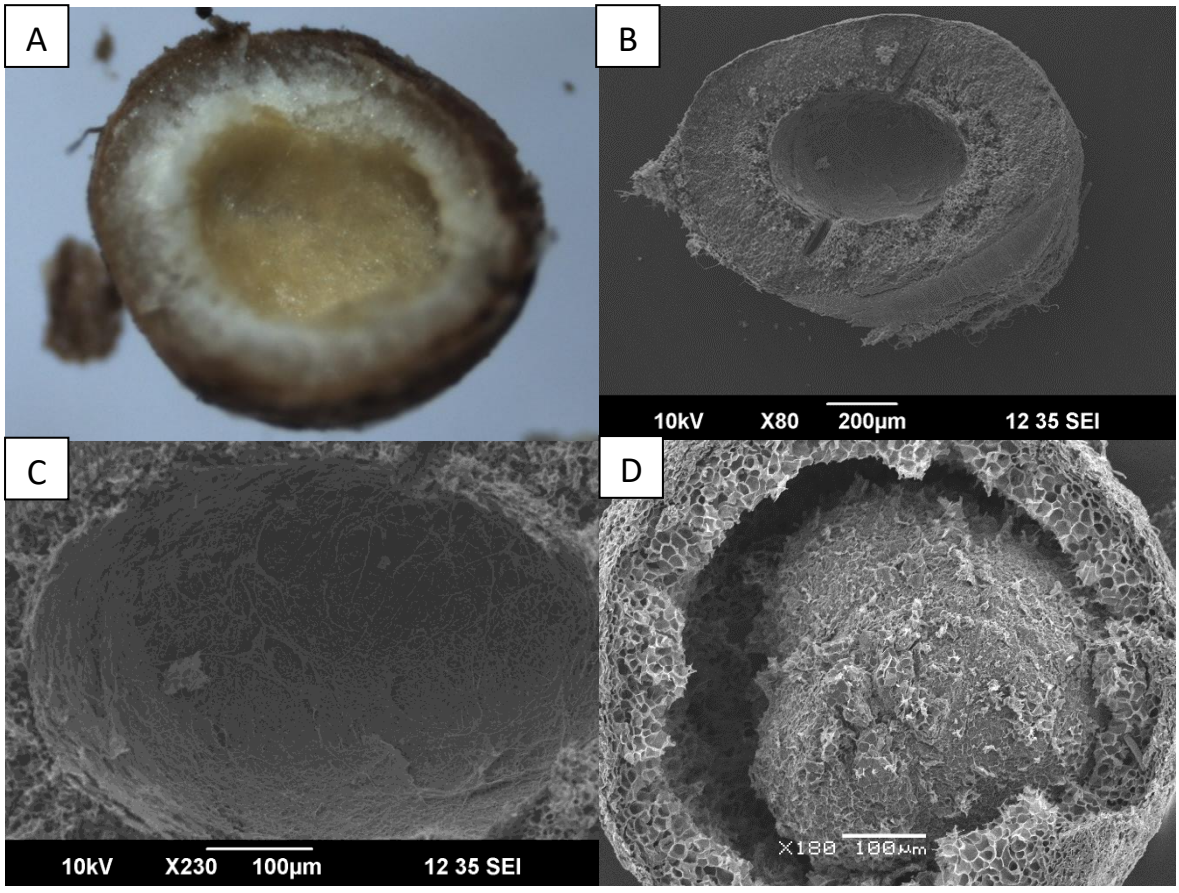


Figure 4.28. Light microscopy and scanning electron micrographs of sclerotia of *A. niger* with a central cavity in the place of the medulla of disorganised hyphae. These images all show the cavity formed from flattened cells similar to a peridium but without the spores present. Image D shows how this central cavity can become detached as described previously for *A. parasiticus* (Horn *et al.*, 2009a). Image A is an axenic sclerotium of strain 8-160. Image B and C are a sclerotium of strain 8-162. Image D is from a cross between strains 8-161 x 8-160.

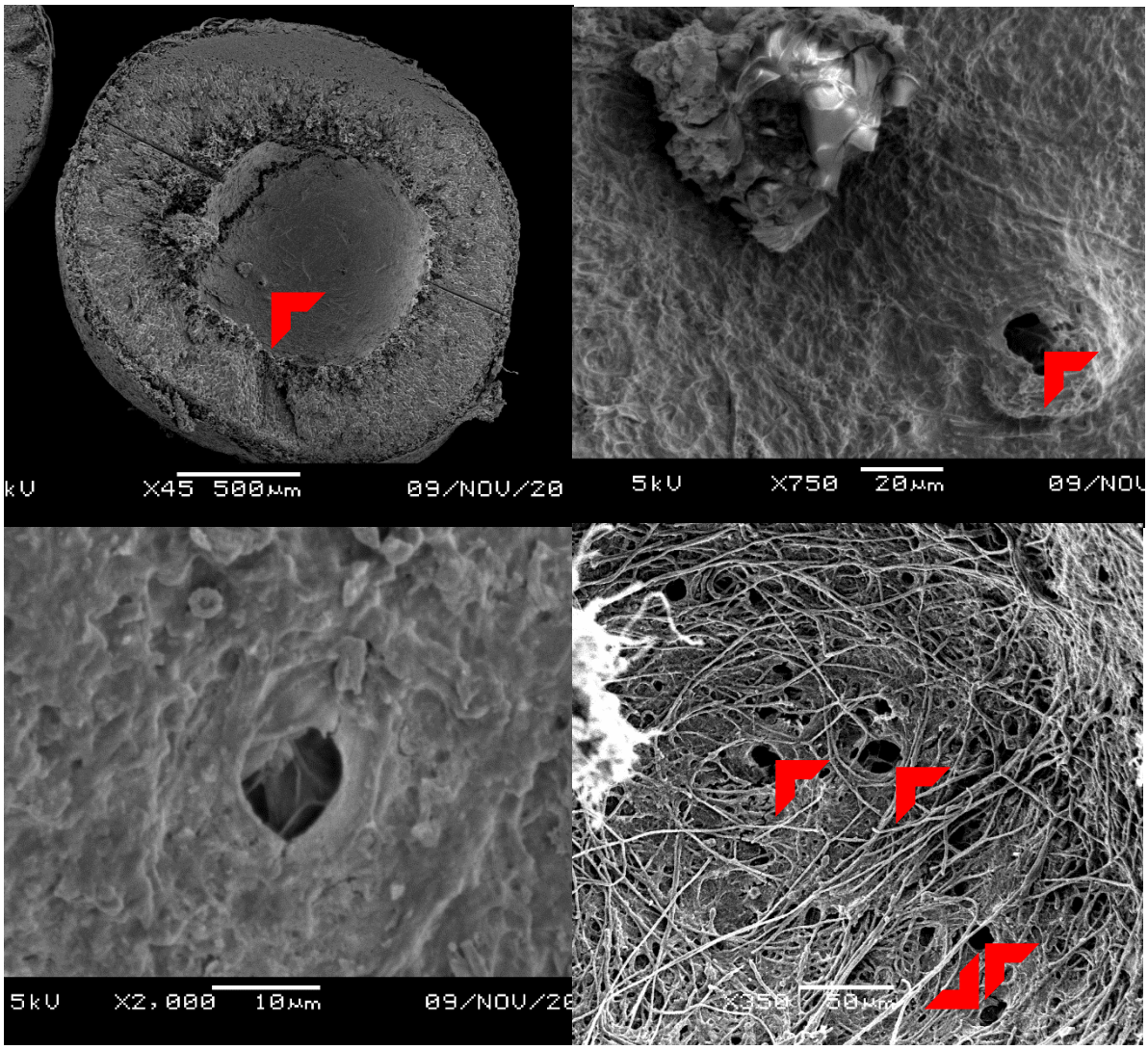


Figure 4.29. Scanning electron micrographs showing channels through the peridium-like cavity wall lining of *A.niger* sclerotia. Red arrows indicate the channels. Sclerotia from multiple crosses.

Despite intense efforts, involving examination of well over several thousand sclerotia, none of the sclerotia showed any direct signs of ascospore production under any of the conditions tested either through light microscopy or SEM. However, one cross from the spermatising sclerotia method grown on wheat straw agar and one on malt extract agar, both grown at 30 ˚C in 5 % carbon dioxide for 8 months yielded additional novel structures of interest within the sclerotia. Within a cavity of the medulla of loose hyphae, was a suspended structure made of distinct fine tissue. These structures were in stark contrast to the broad hyphae surrounding them and may be some form of early or aborted sexual structure or receptor or sexual tissue type. SEM of the internal structure viewed from the cross on wheat straw agar are shown in **Figure 4.32**. It was also apparent that the mycelium from this cross had degraded the wheat straw significantly. The cross on malt extract agar, used a sclerotium harvested from strain 8-160 grown on CYAR and inoculated on a bed of conidia. The conidial suspension was from all utilised strains of the opposite mating type. The internal structure of the cross on malt extract agar can be seen in **Figure 4.31**.

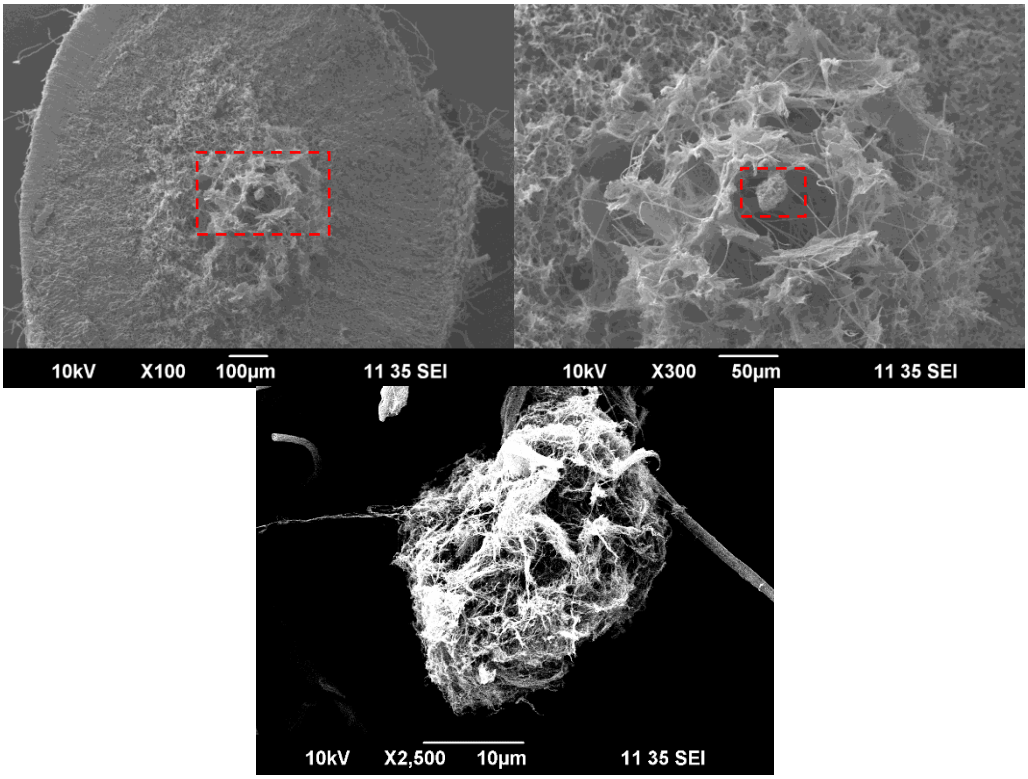


Figure 4.30. Scanning Electron Micrograph of an internal structure within the medulla of an *A. niger* sclerotium. Each image is at increased magnification. The structures fine tissue is in stark contrast to the broad hyphal tissue surrounding it and it is housed within a small central cavity within the medulla of loose hyphae. This sclerotium was of strain 8-162 grown on conidia of strain 8-161 on wheat straw on agar at 30 ˚C with 5 % carbon dioxide. Red dashed boxes indicate area of magnification for the following image (In order: Top left, top right, bottom).

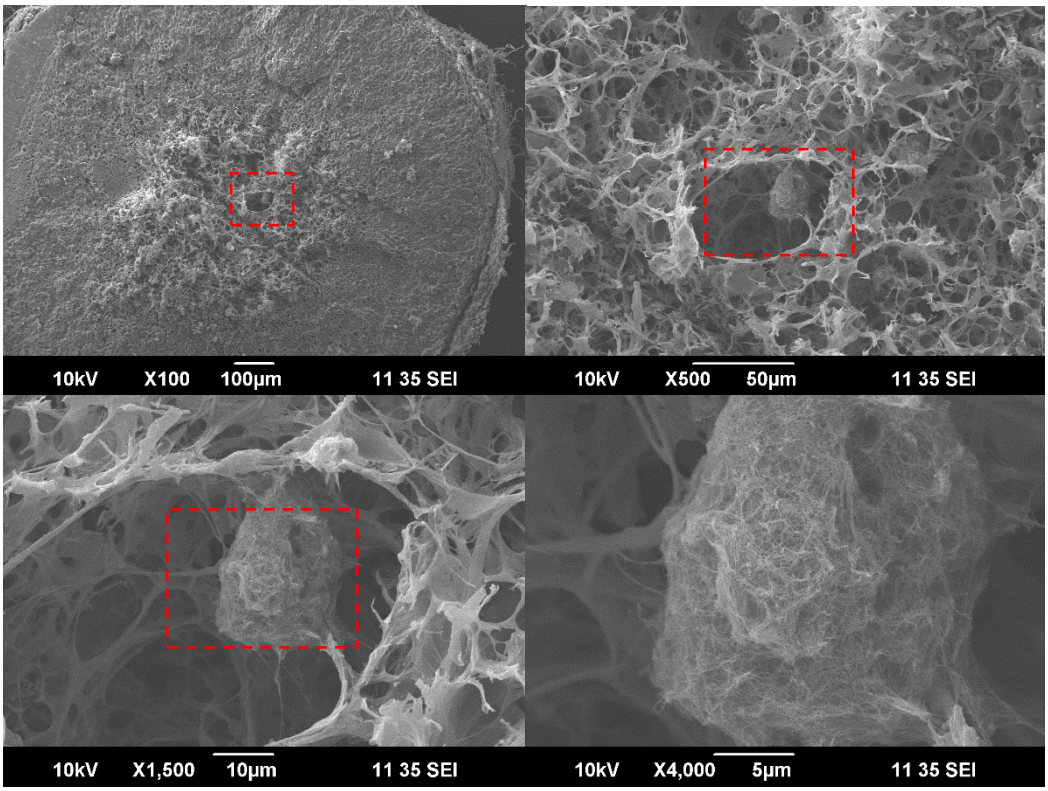


Figure 4.31. Scanning Electron Micrographs of a second structure within the medulla of an *A. niger* sclerotium. The structures fine tissue is in stark contrast to the broadened loose hyphae surrounding it. The structure is located within a small central cavity within the medulla of loose hyphae. Sclerotium of 8-160 crossed on a bed of mixed spores of the opposite mating type. Grown on MEA at 30 ˚C with 5 % carbon dioxide. Red dashed boxes indicate the area of magnification for the following image (In order: Top left, top right, bottom left, bottom right).

4.3.2.3 Fluorescence Studies of Hyphal-Sclerotial Interactions

The strains that produced the majority of the red pigmented structures were identified as targets for the use of fluorescent mutants to investigate potential hyphal-sclerotial interactions between different mating partners. Strains 8-160 (MAT1-2), 8-161 (MAT1-1), 8-162 (MAT1-2) and 8-166 (MAT1-1) had been observed to be involved in the majority of interactions yielding sclerotia with internal structures of interest. Therefore, transformants of these strains with the fluorescent protein genes eGFP and Tdtomato were selected to examine potential hyphal-sclerotial interactions using confocal microscopy. The transformation of these strains was described in **Chapter 3**. A limited set of crosses using the strains above were set up using both the barrage and spermatising sclerotia methods. The media used were malt extract agar, oatmeal agar, mixed cereal agar and CYAR. These crosses could only be examined up to 2 months post inoculation due to time constraints.

Interestingly, of the sclerotia observed from these crosses, those using sclerotia produced from the barrage method only exhibited Tdtomato or eGFP fluorescence and can therefore be described as single culture structures (at least for the ca. 100 sclerotia examined). By contrast, crosses set up via the spermatization method, involving the production of sclerotia of a single strain on CYAR, before cleaning and inoculating on the conidia of the opposite mating type (**Section 4.2.2.5.5**), produced several sclerotia exhibiting fluorescence from both mating type partners. **Figure 4.32** shows confocal microscopy images of sclerotial transects exhibiting both fluorescent markers. The cross of 8-166Δ*AkuB_eGFP* sclerotia placed onto a bed of 8-160 Δ*AkuB_Tdtomato* conidia and inoculated on MEA at 30 ˚C with 5 % CO_2 showed clear ingrowth of Tdtomato exhibiting 8-160 hyphae into the sclerotium of 8-166 (**Figure 4.32 A**). This demonstrates the ability for conidia to germinate and interact within the sclerotia of a mating-type compatible strain. Additionally, it was observed that there were regions of hyphae of 8-166 exhibiting brighter eGFP fluorescence at the interaction zone. The promoter for the eGFP gene is gpdA used in sugar metabolism, and these results suggested that there is an increase in metabolism in these regions where Tdtomato hyphae interact with pre-existing and aged eGFP expressing cells.

Another cross of interest consisted of a sclerotium of 8-161 Δ*akuB_eGFP* which was incubated on a bed of conidia of 8.162 *ΔAkuB_Tdtomato* conidia on oatmeal agar at 30 ˚C with 5% CO_2. This cross yielded a sclerotium whose transect was dominated by expression of both Tdtomato and eGFP fluorescence as well as highlighting the presence of a central cavity by the absence of fluorescence in the same plane (**Figure 4.32 B**). Hyphal growth of these two strains produced what appeared to be a network of interlocking hyphal strands and demonstrates that there is the potential for interaction between isolates of opposite mating type within the sclerotial matrix.

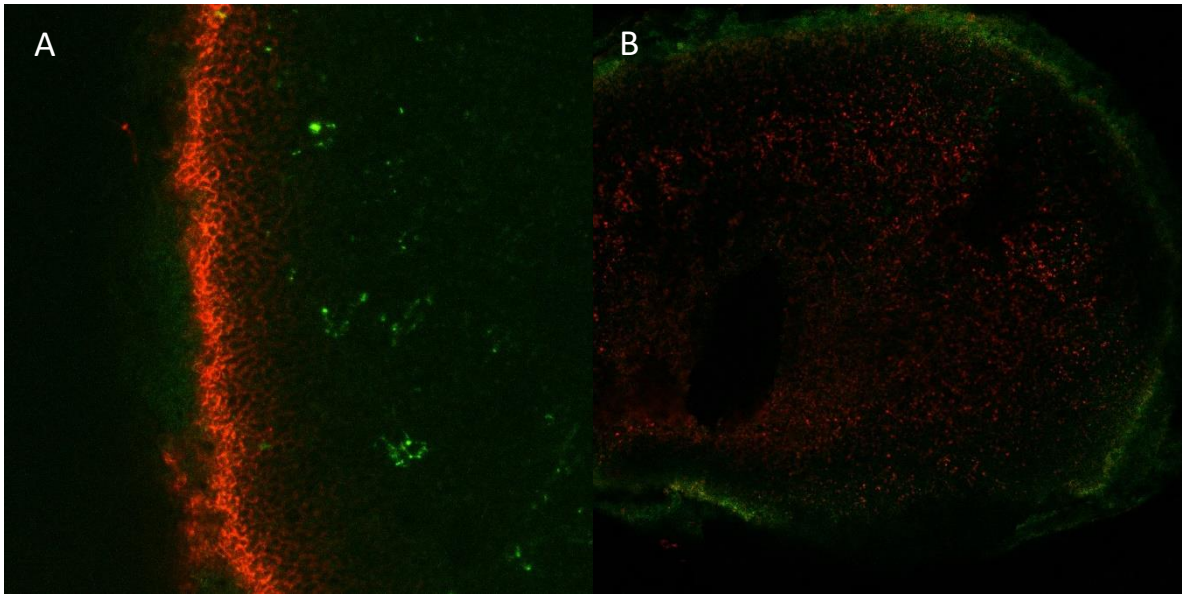


Figure 4.32. Confocal microscopy images of sclerotial transects. The two mating types used in the production of these sclerotia are the *eGFP* and *TdTomato* strains generated in **Chapter 3**.
(A) Cross of *8-160ΔAkuB_Tdtomato* x *8-166ΔAkuB_eGFP*. The *8-166ΔAkuB_eGFP* sclerotium was inoculated on to conidia of *8-160ΔAkuB_Tdtomato* on MEA at 30 ˚C with 5 % CO_2. (B) Sclerotium of *8-161ΔAkuB_eGFP x 8.162ΔAkuB_Tdtomato* conidia on oatmeal agar at 30 ˚C with 5 % CO_2.

4.3.3 Ethanol Washing of Sclerotia

Sclerotia were used for various crossing protocols of *A. niger* as described above. It was therefore of interest to confirm that ethanol washing of sclerotia was able to inactivate any attached conidia on the surface of sclerotia, as well as confirm that sclerotia remained viable after such wash treatmeant. Various different approaches to this end were trialled. Jorgensen *et al*. (2020) recorded success using 0.9 M sodium chloride and Tween 80 solution to surface sterilise sclerotia. Although they concluded that this allowed for sclerotial germination, they did not ensure that contaminating conidia were indeed removed or deactivated using this approach. Meanwhile, the use of 70 % ethanol to deactivate conidia of *Aspergillus* species has long been described [as reviewed by Visconti *et al*. (2021)] and has been applied to *A. niger* (Bundegaard-Nielsen and Nielsen., 1996; Gabler *et al*., 2004)

Sclerotia were therefore washed with either 70 % ethanol or a PBS-Tween 80 solution as described in **Section 4.2.2.6**. **Figure 4.33** shows the results of this experiment when sclerotia were transferred onto ACM. Sclerotia washed with PBS+Tween 80 (**Figure 4.33 B**) showed clear germination after 24 hours, whereas sclerotia washed with ethanol (A) only showed clear germination after 48 hours inoculation (**Figure 4.33 A**). This indicates that despite ethanol washing allowing for sclerotial myceliogenic germination, there is a delay of ~24 hours when compared with PBS-Tween 80. The ethanol wash solution containing conidia (C) did not show germination for any of the replicates when transferred on to ACM Agar, or indeed on any replicate of other media used (PDA and CYA Agar). By contrast, the PBS+Tween 80 solution used to clean sclerotia was also used to inoculate media, and 24 hours post inoculation there were clear signs of mycelia on ACM, and within 48 hours clear evidence of conidiophore development. This was evident in all 9 replicates on ACM agar as well as PDA and CYA agar, although there was a delay in development of approximately 12-hour delay in both mycelial growth and conidiophore development on the latter media (results not shown).

Figure 4.34 shows a tri-inoculated plate of sclerotia cleaned by 70 % ethanol on ACM Agar after 72 hours. The sclerotia clearly show outgrowth of mycelia that then subsequently undergoes conidiation. By contrast, no growth was seen on plates inoculated with 10 µL of the residual ethanol wash solution. Therefore, these results showed that the use of 70% ethanol washing provided an effective means to inactivate conidia on the surface of sclerotia, whilst maintaining viability of sclerotia.

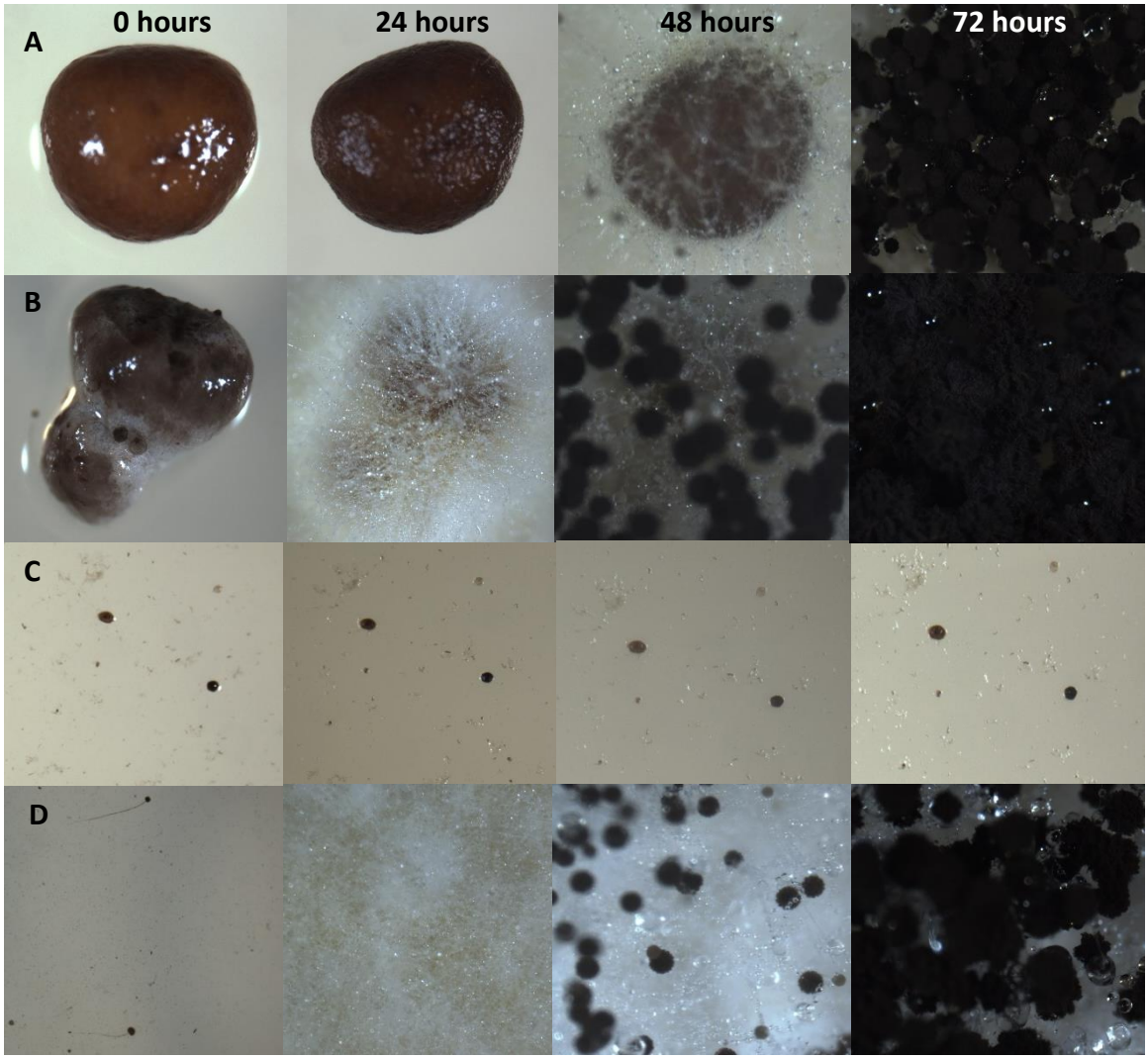


Figure 4.33. Germination of *A. niger* over 4 days under 4 conditions grown on ACM. Images are representative of 9 replicates, 3 replicates on each of three plates. A) Ethanol washed sclerotia. B) PBS+Tween washed sclerotia. C) The ethanol wash solution from A as a control. D) The PBS+Tween wash solution from B as a control. Not shown were replicates grown on PDA and CYA, showing similar results but with an approximately 12-16 hour delay of germination.

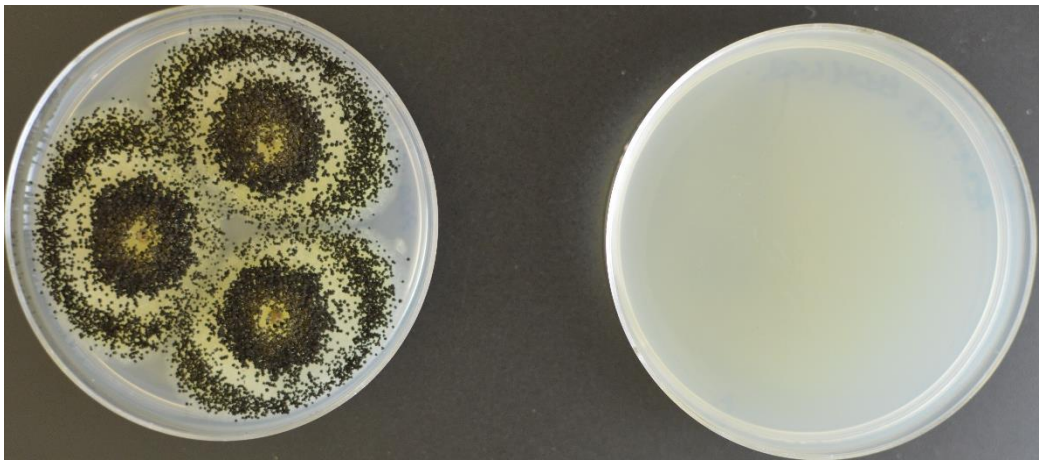


Figure 4.34. ACM plates inoculated in triple with either ethanol cleaned sclerotia (left) or 10 µL of the ethanol wash solution. Plates were incubated for 72 hours and show both mycelial outgrowth of sclerotia and further to conidiation, whilst also confirming the complete deactivation of conidia when washed with 70 % ethanol. Similar results were seen on both PDA and CYA media.

4.3.4 Ascospore Isolation using Cell Cytometry

Conidia of *A. niger* and ascospores of *A. fumigatus* were prepared and analysed as described by cell cytometry as described in **Section 4.2.2.7**. Single spores were recorded as events on a 2D plot utilising FSC (forward scatter) and SSC (side scatter). Events were counted on a pseudocolour scale with white referring to zero events, and increasing through red, yellow, green, pale blue, dark blue and to purple for the highest intensity of events. Each event is then defined by its characteristics (FSC and SSC). Lines can be seen on the 2D plots representing gating functions that allow for separation of events that contain those within defined characteristics from those that do not. In this way, populations can be defined according to the width or level of cellular complexity.

Initial attempts were therefore made to define the characteristics of single-spore type populations. *Aspergillus niger* conidia are easily produced (**Sections 2.2.1** and **4.2.2.7**) and could be mapped as events to 2D plots. An arbitrary line divided events with low width (FSC <60) from events with a width including those of the principal population as indicated by the pseudocolour scale – this was marked as Gate A (as illustrated in **Figure 4.35**). Gate B is an oval gate defined around the principal population of single conidia, defining it against the background of aggregates or conidia with extremes of width or complexity. The population of Gate B made up 68 % of total events despite its relatively small range across width and complexity and indicates the standardisation of *A. niger* conidia.

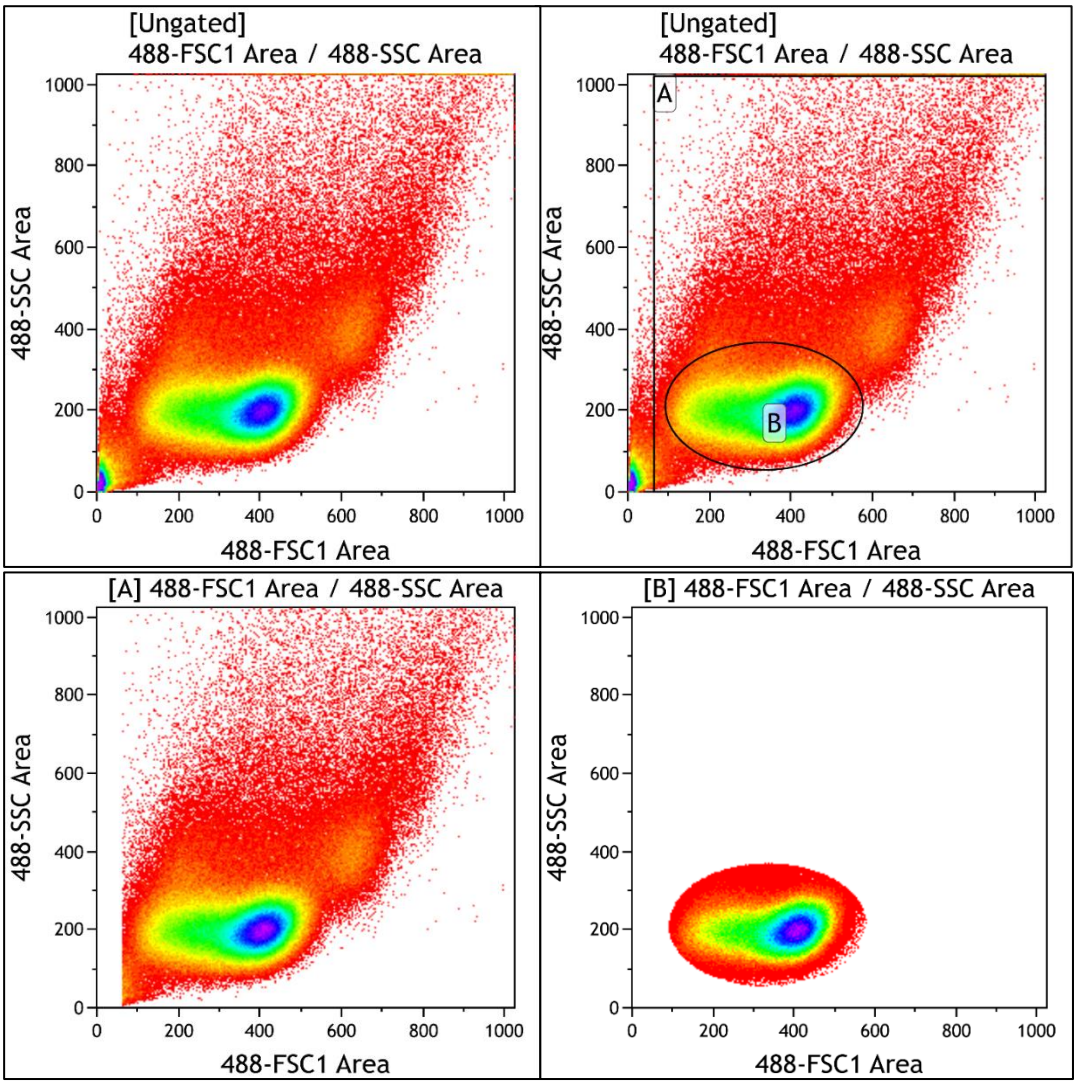


Figure 4.35. 2D pseudocolour plots mapping events using FSC against SSC for *A.niger* conidia. Square gate A drawn excludes cellular debris with little width on the low of the X axis. Circular gate A roughly defines the majority of *A. niger* conidia but excludes most aggregates and individual spore with high width or complexity. This data could then be used to set gates for cell sorting a population of mixed spores.

A. fumigatus ascospores were produced using conventional techniques of selecting cleistothecia, cleaning the attached conidia from the cleistothecial wall and rupturing to release ascospores (Todd *et al*., 2007). However, this only reduces conidial contamination and would never fully remove conidia. Therefore, any spore suspension prepared this way will not consist wholly of ascospores. Two populations of cells were detected, one with a small FSC1 (<350) and small SSC area, and a second population consisting of a larger FSC1 (650-800) and broader range of SSC area values. **Gate C** of **Figure 4.36** shows the second population. The initial gated population could be further defined by adding FSC1 width in place of FSC1 area. This change allows doublet differentiation in one dimension. **Gate D** of **Figure 4.36** shows the new principal population against the backdrop of further debris and doublets (those events with high FSC1 width). Finally, by altering the SSC area to FSC1 height, further doublets in the other dimensions could be identified and removed from the analysis. This complex system of gates allowed for a purer sample of ascospores to be selected against a greater range of debris and doublets. This multiple gated population of ascospores was 38% of the total events.

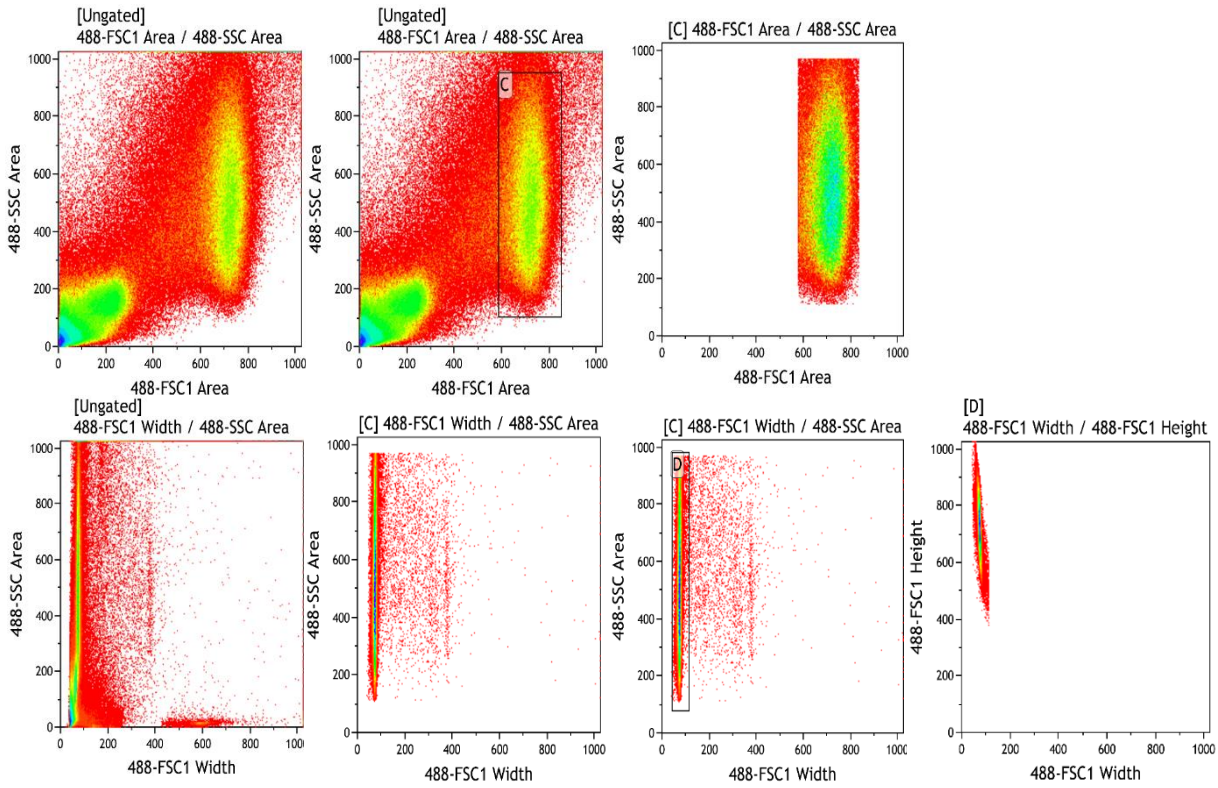


Figure 4.36. 2D pseudocolour plot mapping events using a range of conditions for *A. fumigatus* ascospores. Gate C shows the population of ascospores. This population could then be further defined by altering FSC1 area to FSC1 width and subsequently SSC Area to FSC1 height. This enables the population in Gate C to be separated from any potential conidial doublets and further debris.

A mixture of both *A. niger* conidia and *A. fumigatus* ascospores was then subject to cell sorting against the gated characteristics which were predicted to separate the two described populations (gates) against the background of undefined cells and debris. The results of this cell sorting can be seen in **Figure 4.37**. An additional use of the gates defining one population is that they can be used to overlay a separate population and mark differences in the populations. **Figure 4.38** shows the gate for the *A. niger* principal population and the same gate overlaid over *A. fumigatus* conidia. This highlights that the *A. niger* and *A. fumigatus* conidia both have a large range of conidial sizes but that the principal population of *A. niger* is larger on average, with the heat of events centered on a FSC1 area value of 400, which for *A. fumigatus* is around 200. Conidial sizes for these two species have been defined as 3-5 µm for *A. niger* and 2-3.5 µm for *A. fumigatus* (Raper and Fennell., 1965) and this is in agreement with the data presented. *A. niger* conidia also have a marginally higher complexity according to SSC Area.

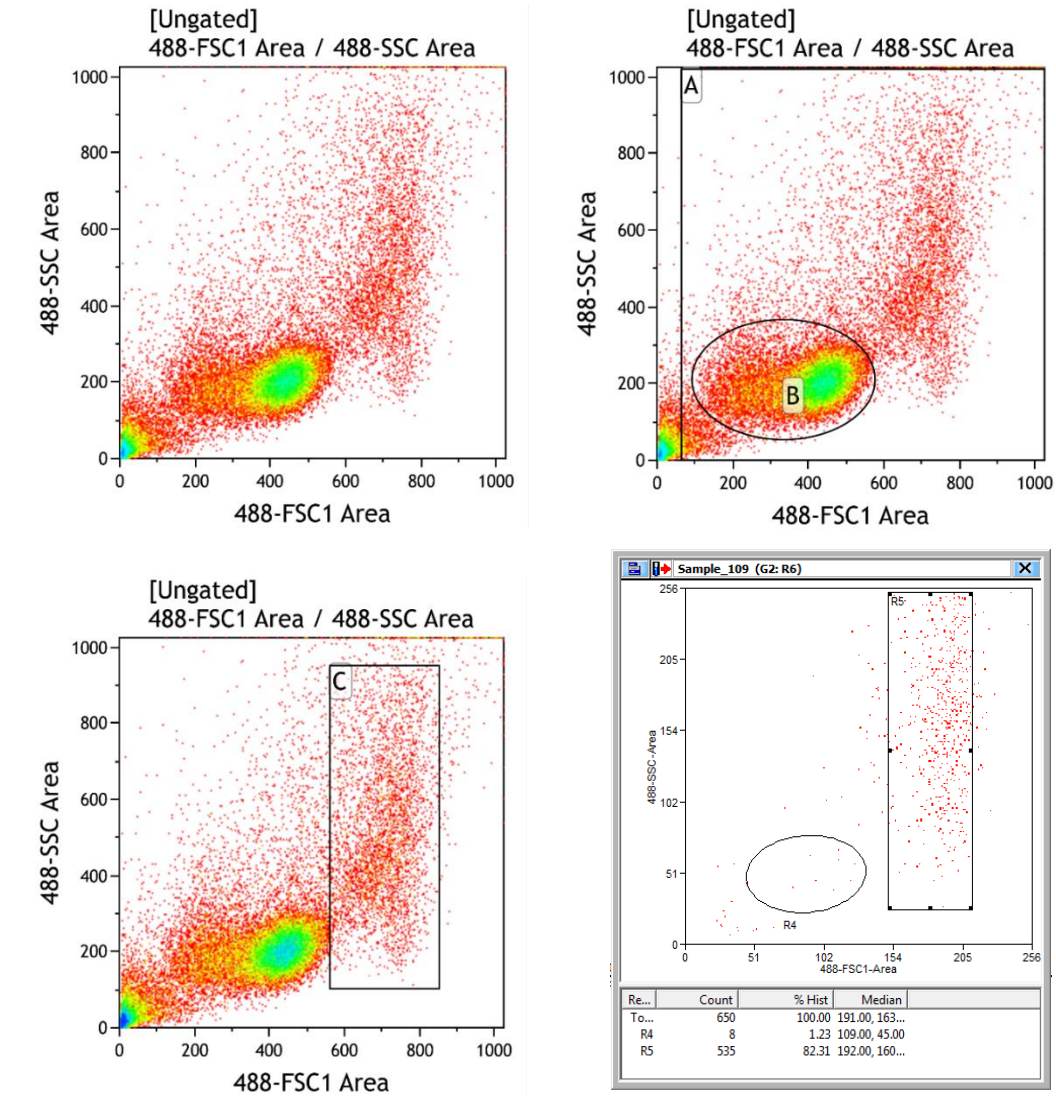


Figure 4.37. 2D pseudocolour plots mapping the cell sorting of a mixture of spore types. *A. fumigatus* ascospores are gated as population C and *A. niger* conidia are gated as population B. Data in image D illustrates the relative success and hit proportion for each of the cell types accordingly.

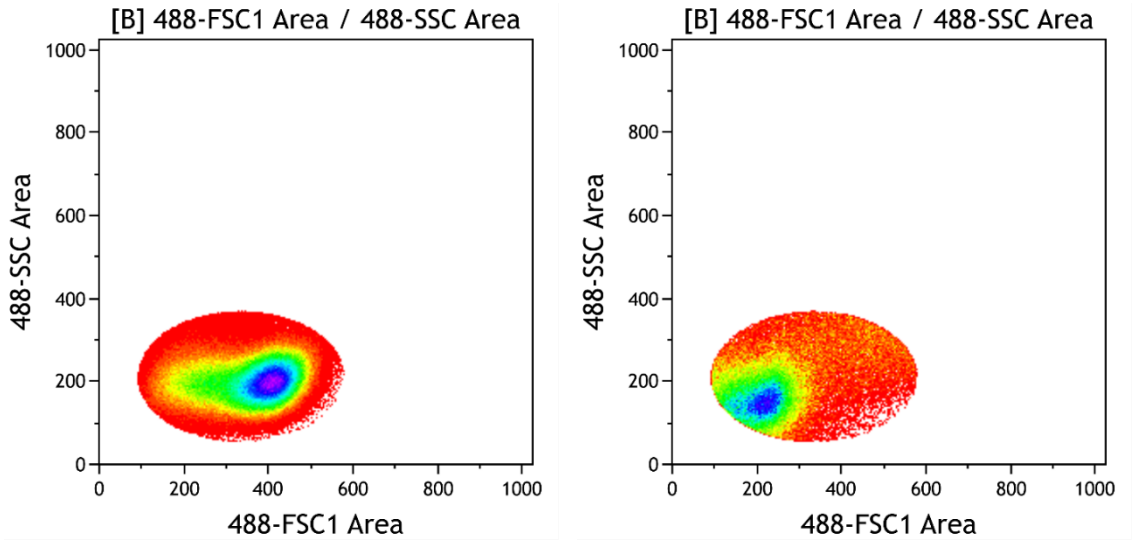


Figure 4.38. 2D pseudocolour plot mapping events using FSC1 area against SSC area. On the left is a gated population showing the principal population of *A. niger* conidia. On the right is the same gate overlaid on events of *A.fumigatus* conidia. *A. fumigatus* conidia can be seen to have marginal reduction in complexity but a stark difference in area. *A. niger* conidia are 3-5 µm and *A. fumigatus* are 2-3.5 µm.

4.3.7 Microbiological Confirmation of Ascospore Recovery

For microbiological validation of cell sorting, five spore suspensions were made. The first was an exclusively *A. niger* conidial suspension. The second was a primarily *A. fumigatus* ascospore suspension, although this would contain some conidia due to the failings of the preparation method. The third solution was a suspension of both *A. niger* conidia and *A. fumigatus* ascospores (prepared 50:50 from the first two solutions). The fourth was a spore suspension of *A. niger* putative conidia that had been collected following cell sorting of the third suspension that had been through the gates defined by flow cytometry for *A. niger* conidia (**Section 4.3.4**). The fifth was a putative spore suspension of *A. fumigatus* ascospores that had been collected following cell sorting of the third suspension that had been through the gates defined by flow cytometry for *A. fumigatus* ascospores (**Section 4.3.4**). These five suspensions were either plated directly or following 30 minutes in a heat block at 80 °C. Suspensions were then chilled and added to ACM using 10 µL and subsequent spreading using a sterile spreader. These cultures were then incubated in the light at 28 °C for 5 days.

The results of growth of these various cultures can be seen in **Figure 4.39**. Solution one and solution four were both *A. niger* conidia. Both spore suspensions grew on ACM without heat shock and produced characteristic black colonies. Both suspensions were totally deactivated by the heat shock treatment of 30 minutes at 80 °C. Solution three containing both *A. niger* conidia and *A. fumigatus* ascospores, and growth was dominated by *A. niger* conidia prior to heat shock but these conidia were totally deactivated by heat shock and allowed for germination and visualisation of the ascospores of *A. fumigatus*, which produced characteristic green colonies (**Figure 4.39**). This was expected as conidia are regularly reported to be deactivated by this method of heat shock (Swilaiman *et al*., 2020; Zhang *et al*., 2022). The results for suspension two and five required more thought. Qualitative assessment of colonies of suspension five, which are theoretically ascospores only, showed germination only after heat shock whereas suspension two showed some growth even prior to heat shock. This suggested the presence of conidial contamination in suspension two, which had been removed in suspension five following the cell sorting.

The final qualitative assessment was that there was a complete absence of *A. niger* colonies in suspension five (**Figure 4.39**). This shows that the flow cytometry gating and cell sorting had proved efficient in separating the two populations and had purified *A. fumigatus* ascospores from a background of *A. niger* conidia.

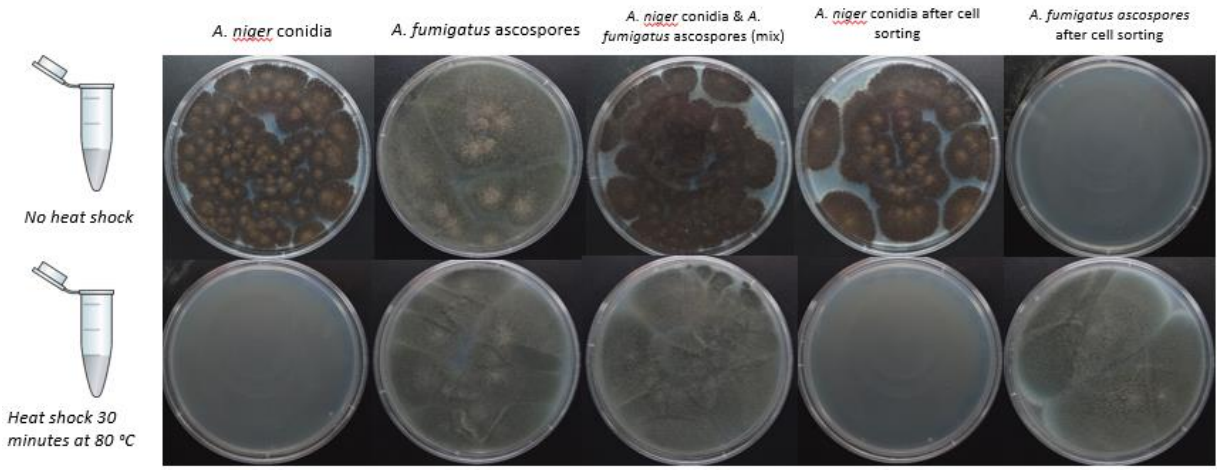


Figure 4.39. Photographs of cultures of *A. niger* and *A. fumigatus* before and after heat shock of 80 °C at 30 minutes. *Aspergillus niger* conidia are completely deactivated by heat shock. Of particular interest is that *A. fumigatus* ascospores sorted by cell sorting do not germinate unless they have undergone heat shock.

4.4. Discussion

The overall aim of the work of this chapter was to assess whether it was possible to induce sexual reproduction in *Aspergillus niger* under laboratory conditions. This required the use of conditions shown to promote the production of sclerotia as described in **Chapter 3**, given that sclerotial formation is a pre-requisite for sex in aspergilli with a petromyces or saitoa sexual morph. Other species such as *A. nidulans*, *A. fumigatus*, *A. sclerotiicarbonarius* and *A. tubingensis* were used as models to verify the applicability of the methods used to induce and characterise sexual development. In addition, techniques were developed to promote the deactivation of conidia and allow for outgrowth of sclerotia, as well as to separate putative ascospore progeny from conidia using flow cytometry and cell sorting.

4.4.1. Sexual Reproduction in the Black Aspergilli

The most disappointing finding from this PhD was the failure to induce sexual reproduction in *Aspergillus niger* as per the pilot work of Ashton (2018), or repeat previous work where sexual cycles of *A. sclerotiicarbonarius* (Darbyshir, 2014) and *A. tubingensis* (Horn *et al*., 2013) had been induced under laboratory conditions. This was despite numerous crosses between several partners of supposed compatible mating type on various media and crossing conditions. The ability to induce sexual reproduction in *A. nidulans* and *A. fumigatus* provided evidence that suitable methods were being used to induce sex in the aspergilli. Furthermore, the use of SEM which identified and provided evidence of the ornamentation of ascospores, for both *A. nidulans* and *A. fumigatus,* provided conclusive evidence that the approaches used allowed for imaging of ascospores at high magnification were suitable and provided good resolution. Therefore, the absence of ascospores in the sclerotia of the target species *A. niger*, *A. sclerotiicarbonarius* and *A. tubingensis* could not be attributed to faults in the preparation methods. Nevertheless, some positive findings and contributions to new knowledge could be taken from results of the present study as discussed below.

Sexual reproduction was successfully induced in both *A. nidulans* and *A. fumigatus.* In the case of *A. nidulans* this is a well described process (Paoletti *et al*., 2007, Kim *et al*., 2002, Pontecorvo *et al*., 1953), and Hülle cells, cleistothecia and ascospores were induced *in vitro*. Hülle cells have a recognised role in the rearing of cleistothecia, with links between their number and cleistothecia size and ascosporogenesis made (Ellis, Reynolds and Alexopoulous, 1973; Hermann *et al.,* 1983). Although the size of Hülle cells is not often explicitly stated, studies on the subject provide evidence that they are often around ~10 µm in width (Kim *et al*., 2002; Paoletti *et al.,* 2007). This matched the size of the

Hülle cells seen in the present study (**Figure 4.7**). Cleistothecia were covered in flattened, smooth cells consistent with the descriptions given for this sexual morph of emericella (Dyer and O'Gorman., 2011). Ascospores were seen to form a dark red pigmented mass as previously described due to a pigment named as ascoquinone A (Brown and Salvo, 1994). More recently a specific pigment asperthecin has been identified which is hypothesised to help protect developing ascospores from UV damage (Palmer *et al.,* 2021). Meanwhile, the sexual cycle in *A. fumigatus* was first described in 2009 by O'Gorman *et al*. The conditions for sex have been described as 'fastidious' (Kwon-Chung and Sugui, 2009) and the same set of conditions broadly used in all subsequent laboratory studies of sex in *A. fumigatus* (Sugui *et al*., 2011; Lim and Park, 2019). The successful induction of sex in this species during this thesis, highlights that protocol could be followed correctly to demonstrate sex. The notoriously fastidious nature of sex in this species required exacting standards and those could be met reliably.

As demonstrated by the induction of sexual reproduction in species from section *Flavi* and *Nigri* (both from subgenus *Circumdati*) the induction of sclerotia is an important precursor and site for the internal development of ascocarps. This has previously been seen in *A. flavus* (Horn *et al*., 2009b), *A. parasiticus* (Horn *et al*., 2009a), *A. tubingensis* (Horn *et al*., 2013) and *A. sclerotiicarbonarius* (Darbyshir, 2014). Mixed cereal agar (MCA) and oatmeal agar (OA) were used as the media for these crosses, just as oatmeal agar had been used to induce sex in *A. fumigatus* (O'Gorman *et al*., 2009). By contrast, this study showed that OA and MCA were poor at inducing sclerotia in *A. niger* (**Chapter 3**) but was in agreement with other research that showed that *A. niger* could reliably produce sclerotia on CYAR (Frisvad *et al.,* 2014). Results gained also suggested that there was no need for an additional 'pre-freezing' step, saving some time over the previous study of Frisvad *et al.* (2014).

Other recent research by Luis *et al*. (2020) has shown the interaction of hyphae from one mating type infiltrating the sclerotia of an opposing mating type in *A. flavus*, possibly linked to the fertilisation of sclerotia and subsequent sexual development. The study utilised fluorescent proteins to mark the opposite mating types and provided evidence that channels exist within sclerotia that could be utilised for ingrowth and spermatization (Luis *et al*., 2020). Similar findings have been demonstrated in *S. minor* (Young and Ashford, 1992) as well as between different species such as *Penicillium ochrosalmoneum* forming ascocarps within the sclerotia of *A. flavus* (Horn *et al*., 2014). The present work demonstrated that similar interactions can occur within sclerotia of *A. niger*. Strains expressing eGFP or Tdtomato were incubated in conditions that allowed for the ingrowth of hyphae from germinating conidia into the sclerotial matrix of sclerotia of an opposite mating type. This additionally highlighted the potential of 'spermatising' pre-formed sclerotia on media inducive to sex in other closely related species, given that such spermatisation methods have been used to successfully induce sex in other fungal taxa (Houbraken and Dyer, 2015). Thus, further work might involve generating sclerotia on media such as CYAR, but then spermatization and induction of sex on alternative media

such as MCA or OA, which was only attempted to a limited degree in this study due to limited time frame.

Extensive use of SEM to examine the sectioned sclerotia produced in this study revealed certain features that have been described or hypothesised in other studies, as well as agreeing with other reported descriptions of sclerotia of *A. niger*. Red pigmented structures were observed inside sclerotia in certain 'crosses', which were never seen in axenic cultures or control 'crosses' between strains of the same mating type. This was particularly common amongst a small subset of *A. niger* strains. If these structures are related to sexual production, this could be akin to the presence of 'supermaters' of *A. fumigatus* which show elevated levels of sexual fertility (Sugui *et al*., 2011; Swilaiman *et al.,* 2021; Korfanty *et al*., 2021). Similar red structures have been described in the closely related *A. tubingensis* (Horn *et al*., 2013). In the latter study, the structure was postulated to be a sterile ascocarp produced by crosses on an alternative media (CZA) to that which induced functional sex (MCA).

The presence of 'channels' through the matrix of sclerotia that might allow ingress of hyphae of a mating partner has been hypothesised in *A. flavus* and supported by secondary data, but not directly demonstrated. Sclerotia of *A. flavus* have been shown to be invaginated by germinating conidia from the exterior of the sclerotia (Luis *et al*., 2020). Additionally, *A. flavus* sclerotia have been shown to rarely be inhabited by ascocarps from other species (Horn *et al*., 2014), through growth through a hitherto undescribed channel. Channels have been described in other more distantly related sclerotia forming species such as *S. sclerotiorum* (Colotelo, 1974), which demonstrated a number of 'channels' from the central cavity often formed, although these were rarely seen on the surface of the sclerotia. This infers that there may be some degree of permeability in the sclerotial rind, and this has been previously described in *S. minor* where the ability to allow certain unpigmented cells from the internal sclerotium to commute to the exterior exists (Willetts and Bullock, 1992; Young and Ashford, 1992). Similarly, work by Young and Ashford (1992) showed that sclerotia were permeable to the tracer sulporhodamine (SR), but that this permeability decreased as the rind of sclerotia of *S. minor* differentiated and matured. It is suggested that further work could be undertaken to look into the factors that cause the degradation of the rind in *A. niger* and might encourage the growth of hyphae through such 'channels' into an area that it seems evident is primed for sexual reproduction.

An additional reason for the lack of sexual reproduction in *A. niger* in this study, may be the role of *het* or *vic* genes. Heterokaryon incompatibility (het) genes and vegetative incompatibility genes have been proposed to govern self/nonself-recognition systems (Anwar *et al*., 1993., Ehrlich *et al*., 2007). It has been proposed that vegetative incompatibility may even promote sexual reproduction as a result of nonself-recognition (Dyer *et al*., 1992). Heterokaryon incompatibility is proposed to be widespread amongst the aspergilli, and particularly amongst *A. niger* in which it blocks the transfer of mycoviruses

and prevents the formation of heterokaryons (Pal *et al*., 2007, van Diepeningen *et al*., 1997). The reason for the prevention of formation of heterokaryons, and therefore sexual recombination, may be the presence of a larger number of HET domain genes in *Aspergillus niger* (Pal *et al*., 2007).

Aspergillus niger, as with the other *Aspergillus* species with described petromyces sexual morphs, produces sclerotia that in sexual species, house ascocarps. The sclerotia may therefore be viewed as a form of female structure, spermatised by conidia of an opposite mating type. This anisogamy, whilst rarely proposed for the aspergilli has been widely reported in other genera and species such as *N. crassa* (Zimmerman *et al.,* 2016). It may explain why there has been reported uniparental mitochondrial inheritance from sclerotia in *A. flavus* (Gell, 2019., Horn *et al*., 2016) as well as reciprocal hermaphroditism. Although uniparental mitochondrial inheritance is generally common (Ni *et al*., 2012), this feature of inheritance from the sclerotium forming parent is notable. It may be that there are species that are non-hermaphroditic but exhibiting anisogamy, which would be overlaid on MAT-type determination of heterothallism, thereby adding an extra layer of complexity to the sexual cycle.

4.4.2 Use of Flow Cytometry/Cell Sorting as a High Throughput Means to Separate Spore Types

Methods for flow cytometry and cell sorting were developed with the initial aim of separating *A. niger* ascospores from conidia if sex were to have been discovered. This would have allowed for a high throughput method upstream of then providing DNA based evidence of recombination. Traditionally ascospores are separated from adhering mycelia and conidia by cleaning on agar media as described by Todd *et al*. (2007). Spore types would then be incubated and after germination, colonies would be selected and grown for further DNA extraction. However, the use of such traditional techniques raises the risk that many of the selected colonies would arise from conidial or mycelial contamination unless other methods such as heat de-activation of conidia are employed. The absence of production of *A. niger* ascospores in this study prevented collection of evidence of the separation of these spore types. However, the flow-cytometry based protocol still demonstrated success. Firstly, it was conclusively shown that this method could be used to separate *A. niger* conidia from *A. fumigatus* ascospores when a mixed spore populations was present. This demonstrates the sensitivity and applicability of flow cytometry and cell sorting for this purpose. Secondly, the validation of this process demonstrated that not only does heat shock deactivate conidia but is a necessity for the activation of ascospores in *A. fumigatus*. This has been described in other species and *A. fumigatus* previously (Dijksterhuis, 2007., Swilaiman, 2018). In any future work where a sexual cycle is described, the use of flow cytometry could be a useful tool in separation of spore types and aid with the provision of evidence of genetic recombination. Flow cytometry has elsewhere been used as a powerful tool for sorting mixed

populations of cells into subpopulations based on factors such as cell size, shape, or aggregation. For example, FSC has previously been used in conjunction with FACS (fluorescence-activated cell sorting) to sort microconidia from macroconidia of *Neurospora crassa* (Roca *et al*., 2005). To our knowledge, the application of flow cytometry or FACS to separate a mixed population of conidia and ascospores reported here is the first time that such flow-cytometry based sorting has been attempted. As an aside, the results of cell sorting provided strong confirmatory evidence that *A. fumigatus* ascospores indeed require heat shock in to be activated. This had previously been suggested by Swilaiman *et al.* (2021), who reported that *A. fumigatus* ascospores showed low germination rates of ca. 9 % without heat shock, but this increased to ca. 75% after 5 minutes, 80 % after 10 minutes, and 91 % after 90 minutes at heat exposure of 80ºC or above. Heat activated germination of ascospores has also been reported in other fungal species (Dijksterhuis, 2007).

4.5 Conclusions

This chapter confirmed the presence of two alternative mating types in *A. niger* (MAT1-1 and MAT1-2) within natural populations of the fungus. Insights were also gained into developmental changes within a sclerotium during 'mating' of *A. niger*. The differentiation of tissues, presence, and interaction of different mating types within 'mated' sclerotia and development of pigmented internal structures suggest that mating in *A. niger* might be possible, but perhaps the approaches trialled in this work lacked some nutritional requirement or stressor. Alternatively, the strains used may have degenerated such that they no longer participate in sex due to a previously described 'slow decline' in fertility (Dyer and Paoletti, 2005; Dyer and Kuck, 2017). Moreover, it may be that the strains used were not compatible for a full sexual cycle based on factors beyond mating type, such as vegetative compatibility groups or the presence of *het* genes.

The use of flow cytometry to separate conidia and ascospores was demonstrated and validated with the conidia of *A. niger* and *A. fumigatus*. This was in lieu of the opportunity to explore this methodology for the conidia and ascospores both of *A. niger*. However, it should prove an important tool for future research into fungal sex, allowing high throughput separation of spores and downstream processing.

Chapter 5: Presence and Functionality of *mating*-type Genes and Meiosis Genes in the Aspergilli.

5.1 Introduction

As previously discussed in the introduction, a 'meiosis detection toolkit' was proposed by Schurko and Logsdon Jr (2008). In this paper and others, a core set of multiple genes required for meiosis have been described to be a positive indicator of sexual reproduction (Villeneuve and Hillers, 2001). This set of meiosis genes have homologs that have been identified across several species that have been described at some point as asexual (although some have recently had their sexual cycle described). These include several medically important pathogens such as *Giardia intestinalis* and *Trichomonas vaginalis* as well as several microsporidia, *Candida spp*., and *Aspergillus spp* (Halary *et al.,* 2011). These homologs encode a set of proteins that have been shown to function specifically during meiosis, based on studies from heavily researched model organisms spanning the animal, fungal and plant kingdoms. As well as meiosis, these genes may be involved in other DNA repair processes that are required for proper completion of meiotic recombination. The genes described include *spo11*, *dmc1*, *hop2*, *rec8* and *msh5* and these genes and their protein function in relation to meiosis and DNA repair processes will now be discussed in detail.

***spo11**:*

Double strand breaks (DSBs) are a double break in the same location of both strands of DNA on a chromosome. They can arise by exposure to radiation or by a mechanism of DNA recombination during meiosis. This latter mechanism causes and repairs the breaks in a process known as homologous recombination (or HR) (Holmes and Haber, 1999, Chen *et al*., 2019). DSBs during HR are initiated and catalysed by a protein called Spo11. Spo11 acts like a topoisomerase and endonuclease to relax the structure of DNA as well as briefly breaking the DNA during a transesterification reaction (Panizza *et al*., 2011) and creating a covalent protein-DNA complex. The removal of Spo11 and subsequent repair of the DSB can yield reciprocal exchange of chromosome flanking the break (a crossover event) or no exchange (non-crossover) (Keeney 2007).

Homology between archaeal topoisomerase TopoVI, known to be involved in DSBs, and Spo11 in the yeast *Saccharomyces cerevisiae* has been shown (Bergerat *et al*., 1997). At the same time DSB complexes were purified from yeast cells undergoing meiosis, and the protein component and catalytic subunit was shown to be Spo11 (Keeney, 1997). The coding sequence of *spo11* is highly conserved, although surprisingly its functional domain has been hypothesised to be less conserved

than the nucleotide sequence as a whole (Grishaeva and Bogdanov, 2018). Orthologs of *spo11* have been described from mice (Romanienko and Camerini-Otero, 2000) and *Drosophila* (McKim and Hayashi-Hagihara, 1998) as well as the fungi *Neurospora crassa* (Bowring *et al*., 2006) and *Schizosaccharomyces pombe* (Steiner, Schreckhise and Smith, 2002). Indeed, it has been said that *spo11* appears to be present in all genome sequenced eukaryotes, including some thought to be asexual (Ramesh, 2005). Its presence in the genome of these asexual species therefore allows speculation over possible cryptic sexuality in these species, unless it has become adapted to another role or mechanism unrelated to meiotic recombination, or there has been insufficient time since the species became asexual for the gene to have become lost or non-functional (Keeney 2007).

Despite its near universality, the loss of *spo11* has been detected in the sexual and meiotic eukaryote *Dictyostelium* (Goodenough and Heitman 2014) and therefore its necessity for meiosis may not hold true as other genes may have 'stepped into the shoes' of a lost meiotic gene such as *spo11*. Similarly, *spo11* was required for homologous recombination within the parasexual cycle of *C. albicans* (Forche *et al.,* 2008) and therefore is not strictly meiosis specific, and may be expressed during mitosis or parasexuality, despite the latter being a rare event.

Regarding *Aspergillus* species, specific homologues of *spo11* have been described from various genome sequencing projects. For example in *A. nidulans*: *spo11* XM_676436.1., and in *A. niger*: *spo11* ANI_1_1528084 / An09g06100 (Galagan *et al.,* 2005; Pel *et al.,* 2008).

***dmc1*:**

An understanding of DSBs came primarily from work with bacteria, and the key recombination protein involved in bacteria is RecA. The *recA* gene has several homologs in eukaryotes including a pair of well characterised homologs called *rad51* and *dmc1* (Lin *et al.,* 2006). The gene *rad51* is part of the *rad50-57* series of genes all linked to the repair of DSBs in both meiotic and mitotic cells. Another homolog, expressed solely in meiotic cells and involved in meiotic recombination mechanisms, is *dmc1* (*d*isrupted *m*eiotic *c*DNA 1) (Bishop *et al*., 1992, Villeneuve and Hillers, 2001). In bacterial cells lacking *recA*, nearly all recombination is eliminated, and eukaryotes lacking functional Dmc1 typically exhibit severe defects in meiotic recombination.

Dmc1 has the role of assembling on the ssDNA ends of DSB's and subsequently searching for homologous dsDNA and completing strand exchange between its ssDNA and the complementary strand of the dsDNA (Chan *et al*., 2019). This exchange is also known as strand invasion, and gives rise to crossover events, and occurs during the formation of the synaptonemal complex (SC) (Tsubouchi

and Roeder, 2002). Dmc1 works with Rad51 to serve this function strictly during meiosis (Tsubouchi and Roeder, 2003).

Trichoderma reesei belongs to a group of organisms that contain a *rad51* homolog but lack a meiosis specific *dmc1* homolog (Li *et al*., 2021). Despite the absence of *dmc1*, meiosis was still viable due to partial functionality within Rad51 that appeared to make up for the loss of meiosis specific Dmc1 function (Li *et al*., 2021). Indeed, *rad51* homologs have been shown to be ubiquitous in the eukaryota and expressed during both mitosis and meiosis. Additionally, *dmc1* genes have been shown to be absent from other species that can complete meiosis, such as *C. elegans* and *D. melanogaster* (Villeneuve and Hillers, 2001). It seems that *rad51* homologs again exhibit some Dmc1 signature-like activity similar to that seen in *T. reesei*.

Regarding *Aspergillus* species, specific homologues of *dmc1* have been described from various genome sequencing projects. For example in *A. nidulans*: *dmc1* XM_677269.1. and in *A. niger*: *dmc1* ANI_1_1312104 / An12g00460 (Galagan *et al.,* 2005; Pel *et al.,* 2008). Furthermore, in *A. nidulans* a *rad51* homolog called *uvsC* was identified, with a null mutant showing meiotic defects. Early stages of cleistothecial development were evident, with formation of croziers with binucleate penultimate cells, but ascospores (i.e. the result of functional meiosis) were absent (van Heemst *et al*., 1997).

***rec8*:** Rec8 belongs to a subfamily of proteins involved in sister chromatid cohesion (Halary *et al*., 2011). Rec8 is defined as an α-kleisin. Kleisins are a subunit of the cohesion complex that is required to hold sister chromatids together (Kudo *et al.,* 2009). Cleavage of the kleisin subunit triggers the release and movement of the sister chromatids during anaphase (Nasmyth and Hearing, 2005). Rec8 is meiosis specific as opposed to the Rad21 subfamily of proteins which fulfil the same role in mitosis (Parisi *et al*., 1999; Halary *et al.*, 2011). It is highly conserved throughout eukaryotes from fission yeasts to humans (Parisi *et al*., 1999). Rec8 was first identified and described in *S. pombe* through the screening of mutants that had reduced meiotic recombination (Ponticelli and Smith, 1989). Rec7 and Rec8 in *S. pombe* were shown to have the highest levels of detectable expression within 2-3 hours of the induction of meiosis. The expression was non-detectable both before induction of meiosis and by 4 hours post induction, and demonstrated both the transient role of these transcripts and the role of the proteins in early meiotic recombination (Lin *et al.*, 1992).

Arbuscular mycorrhizal fungi (*Glomus spp*.) have been described as containing *rec8* (as well as the other meiosis specific genes) despite their supposed asexuality. The presence of these genes and their expression is consistent with the notion that they are capable of traditional meiosis (Halary *et al*., 2011). However, analysis of Δ*rec8* mutants from the supposed asexual *F. oxysporum* have

demonstrated a possible role beyond meiosis within the filamentous fungi (Pareek *et al*., 2019). Sporulation and radial growth of the mutants was not different to the wild type. However, the impact of chromosomal stressors was greater on the Δ*rec8* mutants than the wild type, with reduced germination of spores of the mutant under hydroxyurea stress. This suggested an additional role for Rec8 in homologous recombination repair (Pareek *et al*., 2019).

Regarding *Aspergillus* species, specific homologues of *rec8* have been described from various genome sequencing projects. For example, in *A. nidulans*: *rec8* XM_676948.1 and *A. niger*: *rec8* ANI_1_2110104 / An12g07590 (Galagan *et al.,* 2005; Pel *et al.,* 2008).

***hop2*:**

Hop2 (*ho*mologous *p*airing 2) was described in *S. cerevisiae* as having a function in discriminating between interactions between homologous chromosomes and non-homologous chromosomes (Leu *et al*., 1998). This is necessary due to the many dispersed repeated sequences (such as transposable elements) that might otherwise interact (Tsubouchi and Roeder, 2002). Δ*hop2* mutants undergo wild-type levels of synaptonemal complex (SC) formation, but with increased levels of these complexes occurring between non-homologous chromosomes. This evidence suggests that the role of Hop2 is to facilitate the pairing of homologous chromosomes or prevent the pairing of non-homologous chromosomes (Leu *et al.,* 1998). Later studies provide evidenced of an interplay between Dmc1 and the Hop2/Mnd1 complex in controlling correct implementation of the recombination machinery within the proteinaceous core of the SC (Tsubouchi and Roeder, 2002., Tsubouchi and Roeder, 2003). The Hop2/Mnd1 complex is believed to bind to the DNA and stimulates the recombinase activity of the Dmc1 (Chi *et al.,* 2007; Chan *et al*., 2014).

In the same study that identified Hop2 in *S. cerevisiae*, the expression of *hop2* was shown to be meiosis specific through the construction of a *hop2*::*lacz* fusion protein. The fusion protein demonstrated no expression in vegetative cells. However, after exposure to sporulation medium necessary for normal budding of this yeast, the β-galactosidase activity increased and peaked at around 8 hours post induction (Leu *et al*., 1998). As well as the described role in *S. cerevisiae*, similar conclusions have come from studies on mice, demonstrating the conserved nature of *hop2* even outside of the fungal kingdom (Pezza *et al*., 2007).

Regarding *Aspergillus* species, specific homologues of *hop2* have been described from various genome sequencing projects. For example in *A. nidulans*: *hop2* XM_655958.1. and *A. niger*: *hop2* CBS 513.88 coordinates 2656571-2657815 / An11g11060 (Galagan *et al.,* 2005; Pel *et al.,* 2008).

***msh5*:**

The gene *msh5* is one of six *mutS* homologs (Msh1-Msh6 are named after *M*ut*S* *H*omolog) (Hollingsworth *et al*., 1995; Kolodner and Marsischky, 1999). MutS has a role in mismatch repair, but this seems to be absent in the Msh4 and Msh5 homologs showing divergent evolution (Hollingsworth *et al*., 1995). Msh4 and Msh5 form heterodimers to promote crossing over during meiosis and prevent non-disjunction at the first meiotic division, a role that is conserved in mammalian Msh5 (Hollingsworth, 1995; Harfe and Jinks-Robertson, 2000).

msh5 expression has been demonstrated to be meiosis specific, being highly expressed in in human meiotic tissues. Mutations in mouse Msh5 also cause meiotic defects, providing further evidence that Msh5 is required for normal meiotic recombination (Kolodner and Marsischky, 1999). By contrast, *Drosophila melanogaster* lacks *msh5* (as well as *msh4*, *msh1*, *msh3* and a number of homologs of *mutL*, a similar bacterial gene to the *mutS*; Harfe and Jinks-Robertson, 2000) so the gene is not essential for meiosis in all eukaryotes.

A search of Eurotiales fungi revealed that *msh5* was absent from the genome of *A. nidulans* but present and intact amongst all others, including both *A. niger* (*msh5* ANI_1_1838074 and An08g03470) and *A. fumigatus* (Savelkoul *et al.,* 2019).

5.1.1 Aims of chapter work

Earlier work by Ashton (2019) had shown the presence of putative ascospores in a very limited number of crosses of *A. niger* (**Section 4.1.5**). However, it was not possible to germinate the putative ascospores under a range of environmental conditions tested i.e. the ascospores appeared to be sterile. Work in the present study failed to provide evidence of the presence of ascospores in attempted crosses of *A. niger* but did provide other indications of sexual interaction such as the formation of red pigmented structure within sclerotia and ingress of hyphae into sclerotia (**Figure 4.27** and **4.32**). This chapter therefore aimed to use alternative methods to assess whether there was evidence for the presence of meiosis and sexual recombination, despite sexual reproduction seeming incomplete.

Primers for a suite of genes that are meiosis specific were designed (**Section 2.2.5.4**) and used to investigate expression of these genes in mated cultures grown under putative sex inducing conditions against control cultures grown under the same conditions, but which were not mated cultures. It was postulated that that induction and increased expression of the meiosis-specific genes in mated cultures could both qualitatively and quantitatively provide evidence that some stages of meiosis was occurring. As a further control, expression of these meiosis-specific genes was monitored in *Aspergillus* species with known sexual states as they underwent sexual reproduction.

In addition, the mating-type genes from a series of study isolates of *A. niger* were sequenced to check for any possible mutational changes that might render the arising MAT protein non-functional.

5.2 Materials and Methods

5.2.1 Sequencing of mating-type genes

Genome sequencing of six strains of *A. niger* was completed as described in **Section 2.2.2.10**. The six *A. niger* strains used were 8-160, 8-161, 8-162, 8-166, 8-169 and 8-175. Genomic DNA (gDNA) of each *A. niger* strain was harvested as described previously (**Section 2.2.2.10**). Genomic sequencing necessitates pure DNA without contamination by proteins or RNA, as this disrupts the sequencing process. To ensure that DNA preparations were sufficiently pure, the DNeasy Plant kit (Qiagen) was used to manufacturer's instructions. DNA concentration was verified by Qubit analysis performed by DeepSeq (University of Nottingham).

Library construction and Illumina sequencing was performed by Novogene Co. Ltd (**Figure 5.1**). This raw data was then mapped to a (MAT1-1) reference *A. niger* genome CBS 513.88 (GenBank assembly accession: GCA_000002855.2; Pel *et al*., 2007).

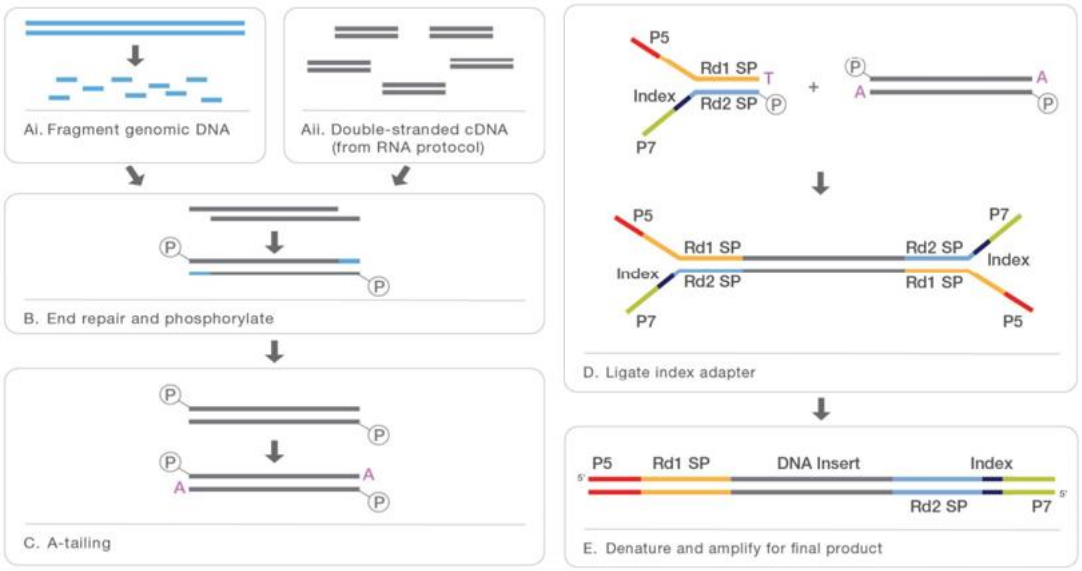


Figure 5.1. Library construction and Illumina sequencing protocol as performed by Novogene Co. Ltd.

Once mapping was complete, several bioinformatic analyses were performed by Novogene Co. Ltd., examining the differences between the query genome and the reference. These included single nucleotide polymorphism (SNP), insertion/deletion (InDel), structural variants (SVs) and copy number variation (CNVs).

For in-depth analysis of sequence variation, genome assemblies were prepared. Raw reads prepared by Illumina sequencing were assembled using SPAdes (thread value 3; Bankevich *et al*., 2012). The resulting contigs were then used in later analyses.

5.2.2 Expression of Meiosis Genes: Primer Design

To assess whether meiosis is occurring it is possible to design PCR primers specific to gene targets, the expression of which are meiosis specific, as described by Schurko and Logsdon Jr (2008). Primers were designed according to criteria described in **Section 2.2.6.1.** PCR was designed such that arising products spanned across an intron as shown in **Figure 5.2**. All introns were of the canonical GT/AG form (Wang *et al*., 2009). The design of primers spanning an intron was such that any product from processed mRNA would lack the intron and the product size would therefore be predicted to be shorter than that of a product from PCR of gDNA and thus easily recognisable through gel electrophoresis analysis. Furthermore, amplicons could be sequenced and compared to reference gDNA products (as shown in **Section 2.2.11**) to provide further evidence that RNA expression is occurring. A complete list of the primers designed, and their sequences is given in **Appendix 13.** The annealing temperature in the PCR protocol used was specific to the primer pairs. **Table 5.1** lists the conditions used for PCR of the various meiosis genes in *A. nidulans* and **Table 5.2** lists the conditions used for these genes in *A. niger.*

Reference sequences for genes for *A. nidulans* were taken from NCBI strain FGSC A4 (available online at https://www.ncbi.nlm.nih.gov/genome/17?genome_assembly_id=360969), whilst sequences for genes for *A. niger* were also taken from NCGI strain 513.88 (available online at https://www.ncbi.nlm.nih.gov/genome/429?genome_assembly_id=54477).

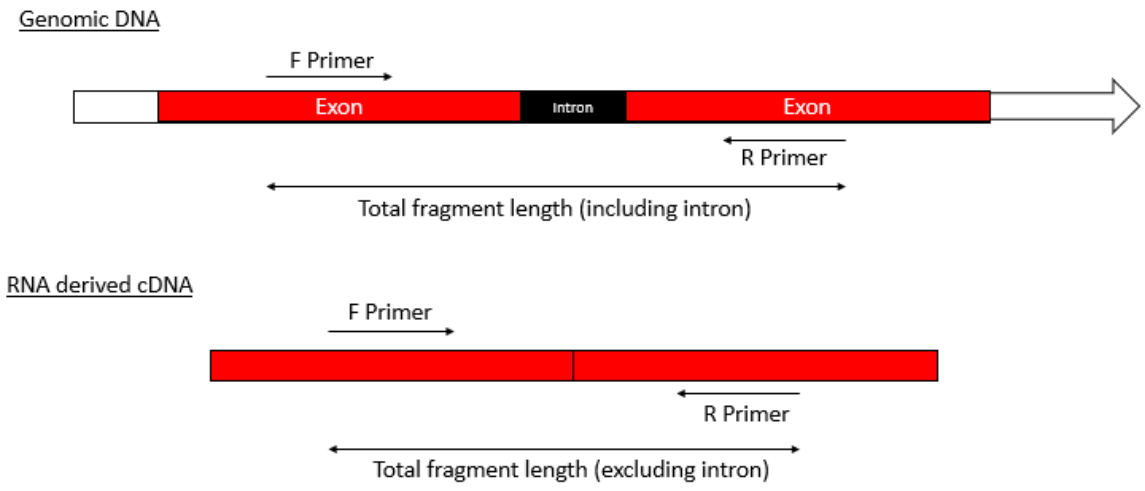


Figure 5.2. Diagram showing the target sites of primers designed for RT-PCR analysis of meiosis gene expression. The primers create a product that spans at least 1 intron. Genomic DNA (gDNA) in any sample will retain an intron and would therefore be distinguishable from RNA derived cDNA which would normally lack any introns after splicing.

5.2.3 Meiosis Gene Expression in *A. nidulans*

The homothallic species *A. nidulans* was selected as a model organism for this experiment due to its established and easy to replicate conditions for sex. Two isolates (2-3 and 2-258; **Appendix 5**) from the University of Nottingham BDUN culture collection were used for experimentation. Spore suspensions (5,000 spores in 50 µL as described in **Section 2.2.2**) were spread over the surface of a fresh 5 cm ACM plate using a sterile spreader. The conditions used to promote asexuality were light and allowing gas exchange (reducing CO_2 accumulation) by leaving the plate unsealed at 28 °C. By contrast, sex inducing conditions were incubation at 32 °C in the dark and sealing of the plate with parafilm 16-24 hours post-inoculation. Incubation was then for an additional minimum four days at 32˚C in the dark. Isolate 2-3 was a prime candidate for study of gene expression during asexual growth as it showed abundant asexual differentiation yet only formed limited numbers of sexual structures even under sex inducing conditions. Meanwhile, isolate 2-258 was well suited to study gene expression during sexual development as it showed abundant formation of cleistothecia under suitable conditions.

DNA from the isolates was extracted using the protocol outlined in **Section 2.2.4.** RNA was extracted from colonies grown on agar plates and exhibiting either sexual or asexual differentiation using either a phenol/chloroform or CTAB protocol as outlined in **Section 2.2.5.2.** RNA from liquid culture was extracted as described in **Section 2.2.5.1.** RNA was tested for the absence of DNA**.** Pure, DNA-free RNA was converted into cDNA using the protocol outlined in **Section 2.2.5.3.** Standardisation was completed at the RNA stage as there is only an assumption that cDNA synthesis is 100% effective. Standardisation of RNA was to a concentration of 500 ng/µl. Following cDNA synthesis, all PCR amplifications were made from the same template cDNA simultaneously. All amplifications were completed in triplicate for comparison.

gDNA was used as template to first optimise conditions for each primer pair. PCR was completed using Phusion® DNA polymerase as described in **Section 2.2.6.** Parameters for PCR were 30 cycles and 65 °C for annealing temperature, which appeared to work efficiently for all primer pairs used.

An assay to determine the appropriate mass of DNA and cDNA to be used in PCR reactions indicated that 10 ng of DNA and 250 ng of total cDNA was optimal (data not shown). Since two primer pairs had been designed for each meiosis gene, this assay also allowed for the qualitative assessment of which pair was more suitable. The whole list of primers and sequences is given in **Appendix 13**. The list of final primers utilised for the assays is given in **Table 5.1**.

Table 5.1. Primer sequences and PCR conditions for the suite of meiosis genes in *A. nidulans*.

Primer names	Primer sequences	Primer pair conditions
Spo11.1	ATCGAGTGACAACGGTGAGG (F) CGGCGAGGATATAGAAGGGG (R)	30 cycles, annealing 65 ˚C
Hop2.1	TCACTTGCTGCTCGCTTGAT (F) CAAAGCCAGGTACAGCTGAGA (R)	30 cycles, annealing 65 ˚C
Rec8.1	CGCTACCCTGAGTTTCTTTCC (F) CGGAGCTGCAGGATCGATAA (R)	30 cycles, annealing 65 ˚C
Dmc1.1	ACATTGCTTATGCCCGTGCT (F) TGAGCAAGAACATGCCCACC (R)	30 cycles, annealing 65 ˚C
Beta tubulin	GTTTGCCCCTTTGACTAGCC (F) GAGACGCGGTTGAAGAGTTC (R)	30 cycles, annealing 65 ˚C

It is noted that an earlier attempts to use a Qiagen one-step RT-PCR kit (Fisher, U.K) for total cDNA conversion and amplification of target sequences rarely yielded products, so the two-step RT-PCR system GoScript^{TM} Reverse Transcription Mix (**Section 2.2.5.3**) was therefore adopted for routine work.

5.2.4 Meiosis Gene Expression in *A. niger*

Aspergillus niger exhibits a heterothallic organism of *MAT* loci (i.e. it is protoheterothallic) and as such crosses between opposite mating types had to be set up for mating to be possible and to evaluate possible expression of meiosis-related genes. Crosses were set up according to the protocols in **Section 4.2.2.5.** Raisins were placed on CYA, resulting in CYAR with the configuration of raisins and spore points (5000 spores) shown in **Figure 5.3**. A cross of isolates 8-160 and 8-166 from the University of Nottingham BDUN culture collection (**Appendix 9**) had previously been shown to both form abundant sclerotia and had also yielded sclerotia containing putative ascospores (Ashton, 2019). Control axenic cultures of each isolate alone with the same inoculation configuration as the mated crosses were also prepared.

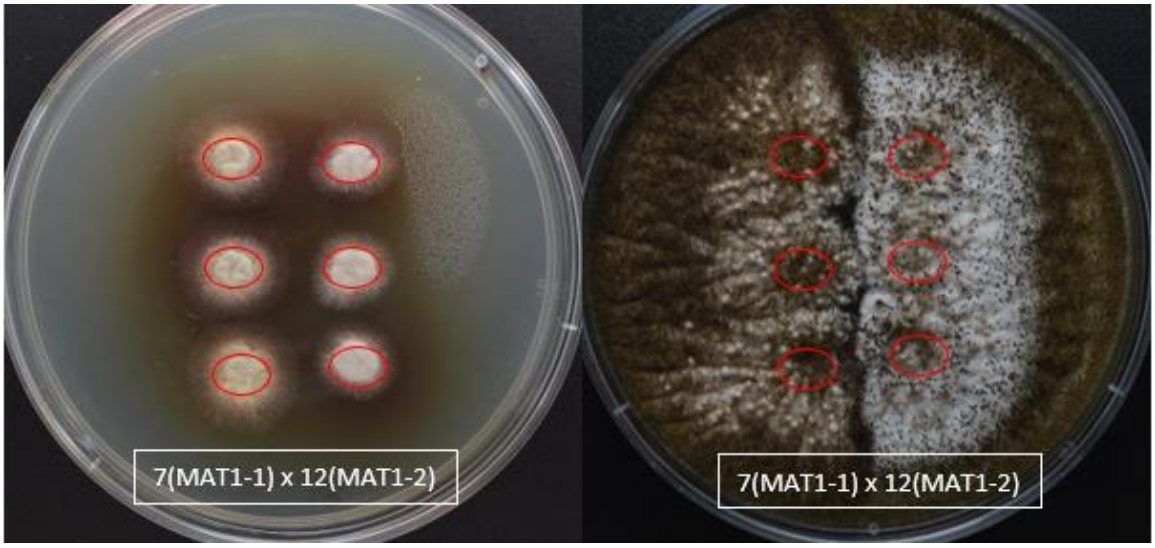


Figure 5.3. Annotated photograph showing the position of raisins and spore inoculation points (here both at the same point) on a 9cm **CYAR** plate. Cultures were sealed with parafilm after 10 days to prevent desiccation. 1) Mated culture 2 days post inoculation. 2) Mated culture 90 days post inoculation. *A. niger* BDUN 8-160 (12 MAT1-2) and 8-166 (7 MAT1-1).

Cultures were incubated at 28°C and 30 ˚C in the dark for 10 days before sealing with parafilm. They were then incubated under the same conditions (CYAR prepared by removing raisins after 24 hours, 28 °C as determined in **Chapter 3**) for a further 80 days before harvesting.

DNA from cultures was extracted according to **Section 2.2.4.** RNA from sexual and asexual cultures was harvested according to **Section 2.2.5.2** and converted to cDNA according to the protocol in **Section 2.2.5.3.** It was found that the CTAB method consistently produced higher quality products than the phenol/chloroform protocol. Standardisation of the RNA concentration was completed prior to cDNA synthesis and set at 1,000 ng/µl. This was preferred over standardisation post cDNA synthesis,

as it could not be ensured that 100 % of RNA would be converted. Any concentration values determined post cDNA synthesis would include unconverted RNA and would therefore bring a higher degree of error from assumptions made.

End-point PCR was performed using both sets of primers for each target and using standard conditions and parameters as stated in **Section 2.2.6**. 10 ng of DNA was used as a template for PCR based on the success of *A. nidulans* assays described above (**Section 5.2.3**). For both isolates used, some primers pairs produced non-specific products (**Figure 5.4**). Therefore, another set of primers were designed, and the preferred set of primers used. The preferred set of primers is listed in **Table 5.2** and primer sequences listed in **Appendix 14**. Additionally, heat gradient PCR assays were used to determine the optimimum annealing temperature of each of the preferred primer pairs. A gradient was used from 61 °C - 67 °C with approximate 1 °C intervals, which allowed for visualisation of conditions that favoured the primer pair for each amplification. An example is shown in **Figure 5.5** where secondary bands were formed for *hop2.1* (in strain 8-160) at temperatures between 62 °C and 65 °C (the standard protocol temperature) but were greatly reduced at 66 °C and 67 °C. Gel electrophoresis of products was visualised as described in **Section 2.2.7**.

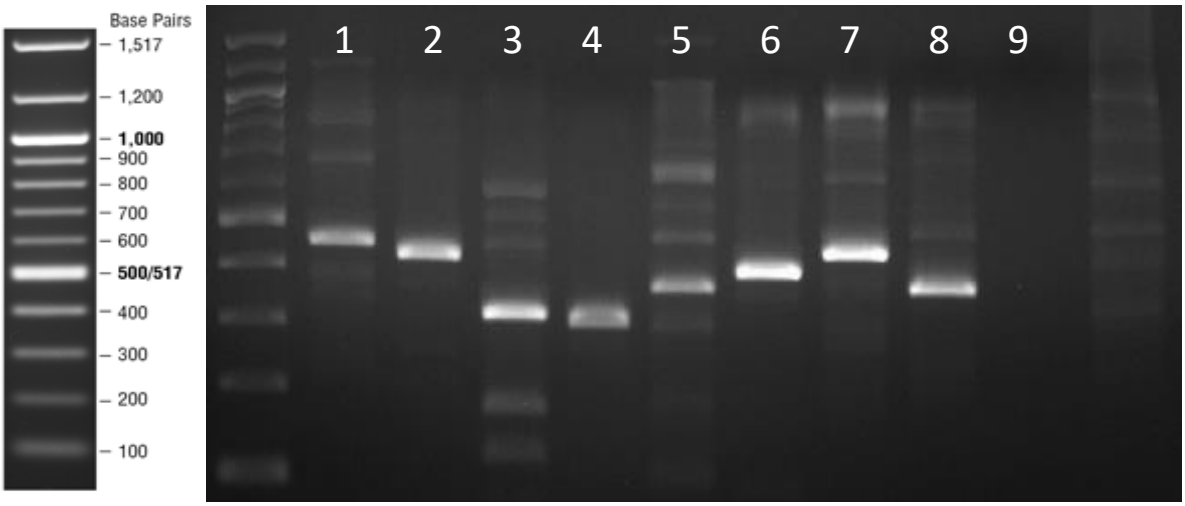


Figure 5.4. Gel electrophoresis image showing PCR products from gDNA of strain 8-160. Standard protocol and parameters were used (**Section 2.2.6**) with the first set of primers for each meiosis gene and all three *sclR* primer pairs. Lane 1: β-tubulin. Lane 2: Spo11.1. Lane 3: Hop2.1. Lane 4: Rec8.1. Lane 5: Dmc1.1. Lane 6: Msh4.1. Lane 7: Msh5.1. Lane 8: SclR.1. Lane 9: SclR.2. Lane 10: SclR.3. (NEB 100 bp ladder used).

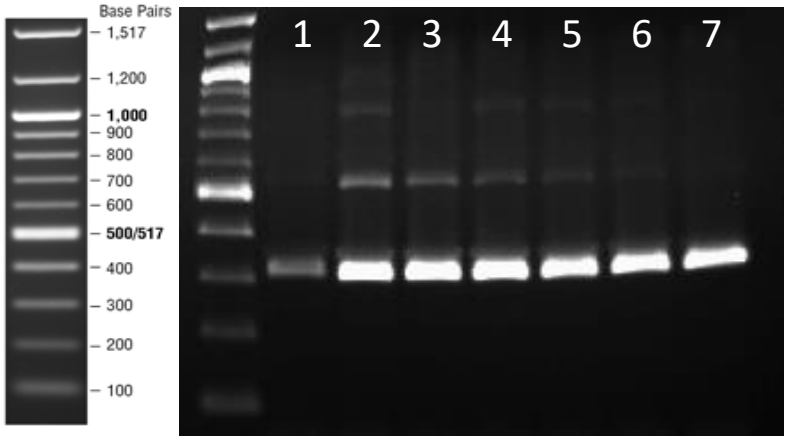


Figure 5.5. Gel electrophoresis image of a heat gradient PCR experiment. The gene primers used here are Hop2.1 FandR. Lane 1 is 61 ˚C-Lane 7 67 ˚C at ~1 ˚C intervals. Non-specific bands can be seen at lower temperatures between 62 ˚C and 65 ˚C but decrease and show a reduced amplification of non-targeted products from 66 ˚C. NEB 1 kb ladder was used as size marker.

Table 5.2. The optimised conditions for PCR amplification of desired meiosis gene product in *A. niger*. A full list of primers can be found in **Appendix 2.** The optimised temperatures were determined by heat gradient PCR against gDNA.

Gene name	8-160	8-166
B-tubulin	Primer pair 1, 30 cycles, 65°C	Primer pair 1, 30 cycles, 65°C
Spo11	Primer pair 1, 30 cycles, 65°C	Primer pair 1, 30 cycles, 65°C
Hop2	Primer pair 2, 30 cycles, 70°C (increase of annealing time to 20 seconds)	Primer pair 2, 30 cycles, 65°C
Dmc1	Primer pair 1, 30 cycles, 65°C	Primer pair 1, 30 cycles, 65°C
Msh4	Primer pair 1, 30 cycles, 65°C	Primer pair 1, 30 cycles, 65°C
SclR	Primer pair 1, 30 cycles, 65°C	Primer pair 1, 30 cycles, 65°C
Rec8	Primer pair 2, 30 cycles, 65°C	Primer pair 2, 30 cycles, 66°C

5.3 Results

5.3.1 *MAT1-1* analysis and SNP detection

Six of the *A. niger* strains used in **Chapter 3** and **Chapter 4** (8-160, 8-161, 8-162, 8-166, 8-169 and 8-175) were genome sequenced using the CBS 513.88 strain as a reference sequence for comparison as described in **Section 5.2.1**. CBS 513.88 has previously been identified as a MAT1-1 strain (Pel *et al*., 2007). Multiple sequence alignments of the CBS 513.88 *MAT1-1* gene against the genomes of the six *A. niger* strains could be used to validate the effectiveness of the MAT-type assays, as well as investigate the possible presence of SNPs within the identified *MAT* genes. Strains 8-161, 8-166 and 8-175 were all determined to be MAT1-1 using the multiplex PCR diagnostic (**Sections 2.2.6.3** and **4.3.2**). The presence of MAT1-1 was confirmed for these strains by multiple sequence alignments against the CBS 513.88 strain (**Appendix 10**). Strains 8-161 and 8-175 had 100 % similarity (at the nucleotide level) with the sequence of CBS 513.88. By contrast, 3 SNPs were found in strain 8-166. The SNP at 331 bp was within the intron of the gene. The SNP at 501 bp was an **A>G** AC**A**>AC**G Thr>Thr.** The SNP at 517 bp was **A>G A**CC**>G**CC **Thr>Ala** (see **Appendix 10**)**.** The SNP at 517 bp is an amino acid change at AA 156 and is indicated in **Appendix 10.** This amino acid substitution is non-synonymous but is outside of the predicted α-box so was likely to have no functional implications. Meanwhile, the strains that had been determined by the MAT diagnostic to be MAT1-2 (8-160, 8-162, and 8-169) had no sequence similarity with the *MAT1-1* gene from CBS 513.88. Perhaps surprisingly, *MAT1-2-1* and *MAT1-2-4* gene sequences of *A. niger* described by Darbyshir (2014) showed no apparent sequence similarity to any of the six genomes sequenced. This was thought to be due to the reference genome being MAT1-1 and reads of the idiomorph genes of MAT1-2 would therefore not map to the reference genome correctly and might have therefore been excluded from assemblies. At the time that this sequence analysis was completed, no public annotated genome of the MAT1-2 was available. However, after this sequencing work had been completed, a sequenced MAT1-2 genome was published for CBS 554.65 (Ellena *et al.*, 2021b).

5.3.2 Expression of meiosis-specific genes in *A. nidulans*

Representative morphologies of cultures used for gDNA and RNA extraction are shown in **Figure 5.6.**

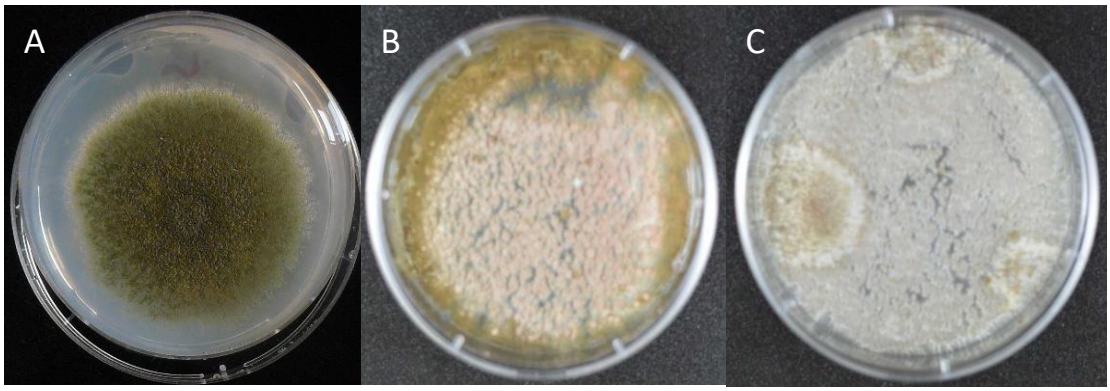


Figure 5.6. Photographs of *Aspergillus nidulans* (*emericella* sexualmorph) isolates showing typical sexual and asexual morphologies prior to gDNA and RNA extaction. A) Isolate 2-3 showing asexual morphology of green conidiation after 4 days growth. B) Isolate 2-258 (a *ΔpyrG* mutant that consistently exhibits sexual morphology) showing both asexual (green conidiation edge of plate) and sexual morphology (beige appearance of mycelia and cleistothecia) after 14 days growth. C) Strain 2-258 grown under sex inducing conditions and exhibiting sexual morphology only after 14 days growth.

In initial work tests were made to determine optimum amounts for use of DNA and RNA templates. Results of the DNA template concentration assay are shown in **Figure 5.7**. The use of 10 ng gDNA gave clear bands, whereas 50 ng gave brighter bands but occasionally yielded no product as can be seen in Lane 4 of the image B in **Figure 5.7**. The absence of bands was amplified when 150 ng of template was used as can be seen in image C of **Figure 5.7**. Therefore 10 ng gDNA was utilised for the assays as the gDNA positive control.

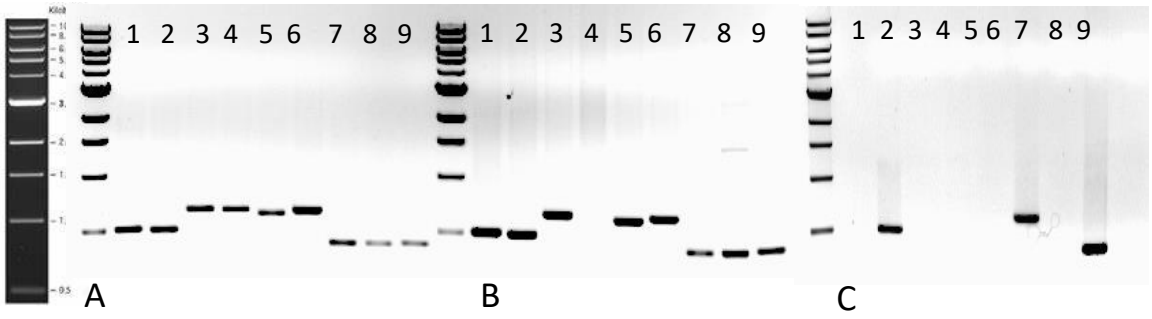


Figure 5.7. Gel electrophoresis images showing the results of template concentration assays for *A. nidulans*. A) The results for each primer set with gDNA at 10 ng. B) The results for each primer pair set with gDNA at 50 ng. C) The results for each primer set with gDNA at 150 ng. For each image, Lane 1 is Spo11.1, Lane 2 is Spo11.2, Lane 3 is Hop2.1, Lane 4 is Hop2.2, Lane 5 is Rec8.1, Lane 6 is Rec8.2, Lane 7 is Dmc1.1, Lane 8 is Dmc1.2 and Lane 9 is beta tubulin. NEB 1 kb ladder used.

Figure 5.8 shows that there was no difference in the quality of the product between either of each set of primers for each gene. Equally, there was no discernible size difference in the PCR products between the two strains. Therefore, the first set of primers for each gene was arbitrarily used for future experiments (Lane 1, 3, 5 and 7), these primers are listed in **Appendix 13.**

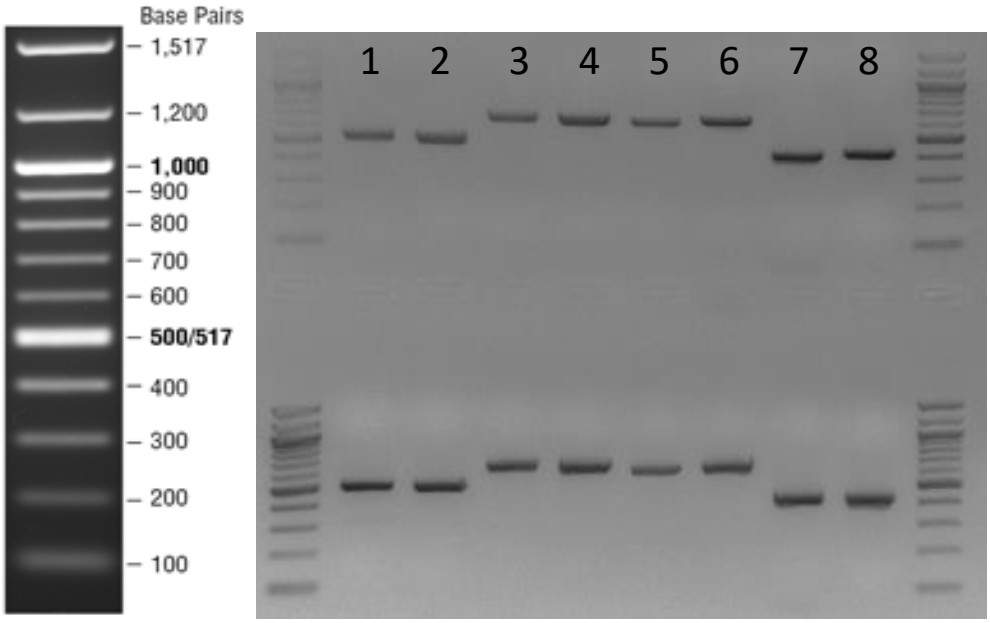


Figure 5.8. Gel electrophoresis image showing the result of *A. nidulans* primer test for both strain 2-3 (top of image) and 2-258 (bottom of image). Lane 1 Spo11.1, Lane 2 Spo11.2, Lane 3 Hop2.1, Lane 4 Hop2.2, Lane 5 Rec8.1, Lane 6 Rec8.2, Lane 7 Dmc1.1, Lane 8 Dmc1.2. NEB 100 bp ladder used.

Once optimisation of the primers was completed, the experiment to investigate expression of meiosis gene levels in *A. nidulans* in both sexual and asexual cultures (obtained from solid agar) was undertaken, using aliquots of gDNA as a positive control (**Section 2.2.5**). Importantly, it did indeed prove possible to show induction of expression of a series of meiotic genes as shown in **Figure 5.9**. For each gene, 10 ng of gDNA was used as a positive control in PCR with the gDNA products expected to show an increase in size due to the retention of introns relative to any amplification from the cDNA templates (this also provided evidence that the DNase treatment step during RMA extraction was functional).

Expression of the 'house-keeping' gene β-tubulin was used as a positive control. The β-tubulin primers amplified a gDNA 427 bp product (including a single 88 bp intron), whereas 339 bp cDNA products, consistent with editing of an intron, were visible in amplification of RNA from asexual and sexual cultures (**Figure 5.9**). This provided evidence that β-tubulin was expressed in both sexual and asexual

conditions to a level that is detected within 30 cycles. β-tubulin is a non-meiosis gene and was expected to be expressed equally in both conditions.

Regarding *dmc1,* a 419 bp product was produced from gDNA (the gDNA product was predicted to contain two introns of 48 and 49 bp). By contrast, a smaller (ca. 322 bp) product was generated from mRNA derived cDNA transcripts from extracts form sexual cultures whilst the cDNA derived from asexual culture did not produce any apparent product after 30 PCR cycles (**Figure 5.9**). This provided key evidence that *dmc1* is expressed at greater levels under sexual conditions than asexual conditions.

Regarding *rec8,* a 620bp product was produced from gDNA (the gDNA product was predicted to contain two introns of 199 bp and 72 bp). By contrast, a smaller (ca. 349 bp) product was generated from mRNA derived cDNA transcripts from extracts form sexual cultures whilst the cDNA derived from asexual culture did not produce any apparent product after 30 PCR cycles (**Figure 5.9**). This provided key evidence that *rec8* was expressed at a level far higher under sexual conditions than asexual conditions for *A. nidulans*.

Regarding *hop2,* a ca. 658 bp product was produced from gDNA (the gDNA product was predicted to contain two introns of 181 bp and 189 bp). By contrast, a smaller ca. 450 bp product was generated from mRNA derived cDNA transcripts from extracts form sexual cultures. This was larger than the 288 bp predicted but in line with the size expected if one of the two predicted introns had been retained. Meanwhile, cDNA derived from asexual culture did not produce any apparent product after 30 PCR cycles (**Figure 5.9**). This provided key evidence that *hop2* was expressed at a level far higher under sexual conditions than asexual conditions for *A. nidulans*.

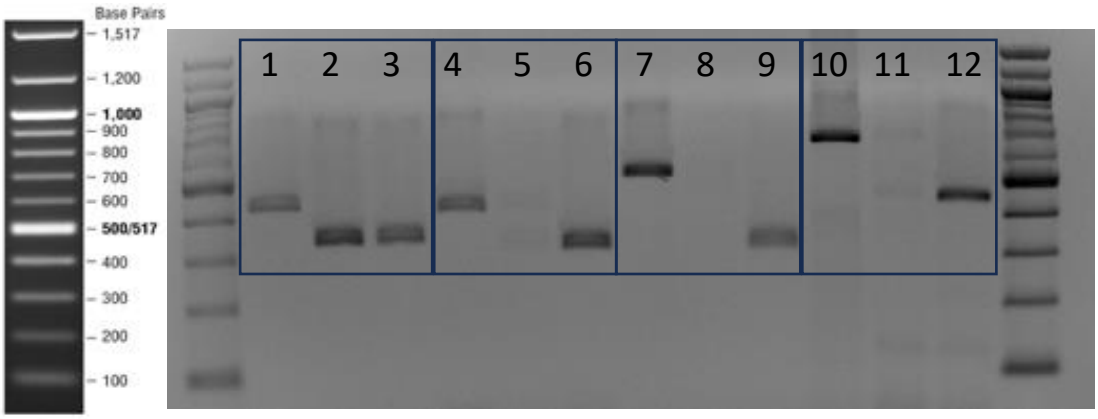


Figure 5.9. Gel electrophoresis image showing the levels of expression of meiosis genes in *A. nidulans* under sexual and asexual conditions with gDNA as control. Lanes 1-3 β-tubulin (gDNA, asexual and sexual respectively). Lanes 4-6 Dmc1 (gDNA, asexual and sexual respectively). Lanes 7-9 Rec8 (gDNA, sexual and asexual respectively). Lanes 10-12 Spo11 (gDNA, asexual and sexual respectively). NEB 100 bp ladder used.

5.3.3 Expression of Meiosis Specific Genes in *A. niger*

For each of the optimised primer pairs from **Table 5.2**, end point PCR using 34 cycles was completed with both gDNA and cDNA. cDNA was derived from axenic cultures of each isolate used in mating, as well as from both sclerotial and mycelial tissue from the 8-160x8-166 crosses. The results of two of these end-point PCR reactions can be seen in **Figure 5.10** and **Figure 5.11.** 34 cycles were used for the endpoint PCR**.** β-tubulin yielded a product in each lane with the lanes for gDNA being larger products as expected due to the obvious inclusion of the introns. A similar, but unexpected result was seen for Dmc1, although the reduced brightness of the bands of axenic culture cDNA suggests that expression is lower in axenic cultures than in sexual cultures. There was expression of both *rec8* and *sclR* (**Figure 5.11**) target transcripts in the cDNA of 'mated' cultures where there were no visible products in the cDNA from axenic cultures. Since the incubation conditions between the axenic cultures and mated cultures are the same, this provides qualitative evidence that there is increased expression of these genes in the conditions used for 'mated' cultures only when paired with its mating partner.

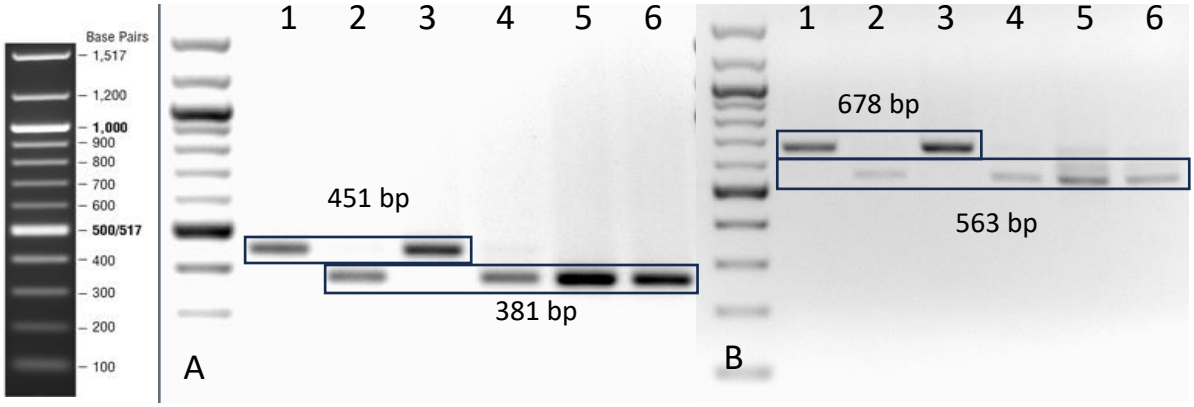


Figure 5.10. Gel electrophoresis image of the products of β-tubulin primers and Dmc1.1 primers. End-point PCR. A) β-tubulin. B) Dmc1.1. For each target gene - Lane 1 is the gDNA of isolate 8-160. Lane 2 is the cDNA of an axenic culture of 8-160 grown on solid media. Lane 3 is gDNA of isolate of 8-166. Lane 4 is cDNA from an axenic culture of strain 8-166 grown on solid media. Lane 5 is cDNA derived from sclerotial tissue of an 8-160x8-166 cross. Lane 6 is cDNA derived from mycelial tissue from an 8-160x8-166 cross. For β-tubulin the gDNA was predicted to produce a 451 bp product inclusion of a single intron. For β-tubulin, the excision of the intron would yield a 381 bp product.For Dmc1, the gDNA was predicted to produce a 678 bp product. Excision of two introns would yield a product og 563 bp.

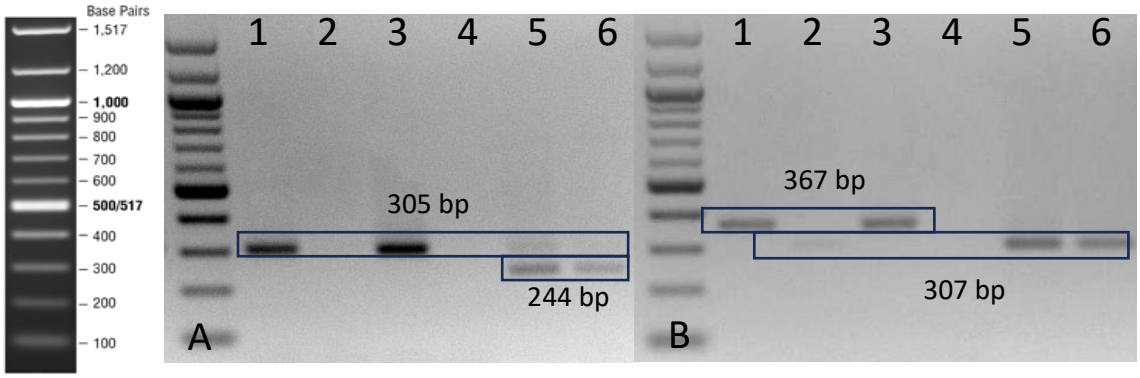


Figure 5.11. Gel electrophoresis image of the products of Rec8 and SclR primers. A) Rec8. B) SclR. For each target gene – Lane 1: gDNA of isolate 8-160. Lane 2: cDNA derived from an axenic culture of 8-160 grown on solid media. Lane 3 gDNA from isolate 8-166. Lane 4 is cDNA derived from an axenic culture of 8-166 grown on solid media. Lane 5 is cDNA derived from sclerotial tissue of a cross between 8-160x8-166. Lane 6 is cDNA derived from mycelial tissue of a cross between isolates 8-160x8-166. Genomic DNA of sclR estimated to be 367 bp inclusive of intron and 307 with intron excised. Genomic DNA of rec8 estimated to be 305 bp inclusive of intron and 244 bp with intron excised.

To assess the relative expression of transcripts of each of the meiosis genes under the different growth conditions (i.e. sexual and asexual), total cDNA was subjected to varying numbers of cycles in a semi-quantitative PCR in a more thorough analysis of all the putative meiosis-specific genes. Other than the change in cycle number, the parameters for each primer pair matched those given in **Table 5.2** and **Section 2.2.6**. This approach allowed for determination of the cycle number required to see a product for each target gene and make partial assumptions about the fold increase in expression between sexual and asexual conditions.

The use of semi-quantitative PCR over the more widely used qPCR was that issues were encountered with qPCR when examining black aspergilli. Reads were wildly erratic and not reproducible the way that a similar methodology was for non black aspergilli such as A. nidulans. These errors were assumed to be as a result of melanin in the RNA and how this carried over into the qPCR, and despite many attempts to reduce these issues (Jaakola *et al*., 2001), they persisted, and a semi-quantitative approach was used instead.

Importantly, the overall results of this semi-quantitative approach showed that RNA extracted from both mycelial and sclerotial tissues of the 'mated' culture yielded higher levels of transcripts of the genes of interest compared with RNA extracted from the axenic unmated culture controls. For example, **Figure 5.12** demonstrates product bands for the semi-quantitative PCR amplification of *sclR*. Gene expression was detected from mated cultures even after 31 cycles and was particularly pronounced within sclerotial tissue. By contrast, RNA extracts from control unmated cultures required several additional cycles to generate a product, providing a semi-quantitative indicator that expression in the mated cultures was far higher than the unmated controls.

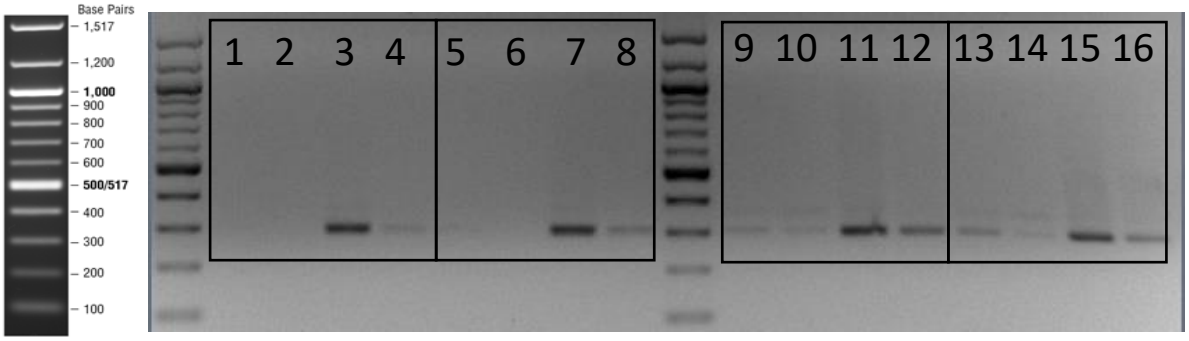


Figure 5.12. Gel electrophoresis images of the results of a semi-quantitative PCR using SclR primers against cDNA. Expected product size is 307 bp and is marked with an asterisk. gDNA inclusive of the intron 367 bp (faint but noticeable). Lanes 1-4 were run for 31 PCR cycles. Lanes 5-8 were run for 33 cycles. Lanes 9-12 were run for 35 cycles. Lanes 13-16 were run for 37 cycles. Lanes 1, 5, 9 and 13 used cDNA derived from an axenic 8-160 culture grown on solid media. Lanes 2, 6, 10 and 14 used cDNA derived from an axenic 8-166 culture grown on solid media. Lanes 3, 7, 11 and 15 used cDNA derived from sclerotia from a 'mated' cross between 8-160x8-166. Lanes 4, 8, 12 and 16 used cDNA derived from mycelial tissue from a 'mated' cross between 8-160x8-166.

The results of Rec8 are shown in **Figure 5.13,** which demonstrates product bands for the semi-quantitative PCR amplification. The expression of 'mated' cultures is limited 31 cycles to cDNA derived from sclerotial tissue. 33 PCR cycles show low but perceivable expression for both mated cultures, but expression of axenic cultures is imperceptible. 35 PCR cycles produced enough product from each condition but was still noticeably stronger expression for cDNA derived from sclerotia at all cycle numbers. A direct comparison can be made between the expression of Rec8 of the 'mated' culture conditions at 33 cycles and the expression of axenic cultures at 37 cycles. This implies a semi-quantitative 4-fold increase in expression for the 'mated' cultures based on the same assumptions as before. Again, at higher cycle numbers a secondary band larger than the target product was visible. This secondary band was perhaps even greater in intensity than the target product for the axenic cultures. The size of the target product was 244 bp and this is indicated by the asterisk. With the retention of the intron within this product, a product of 305 bp would be expected. These product sizes match those demonstrated in **Figure 5.13**.

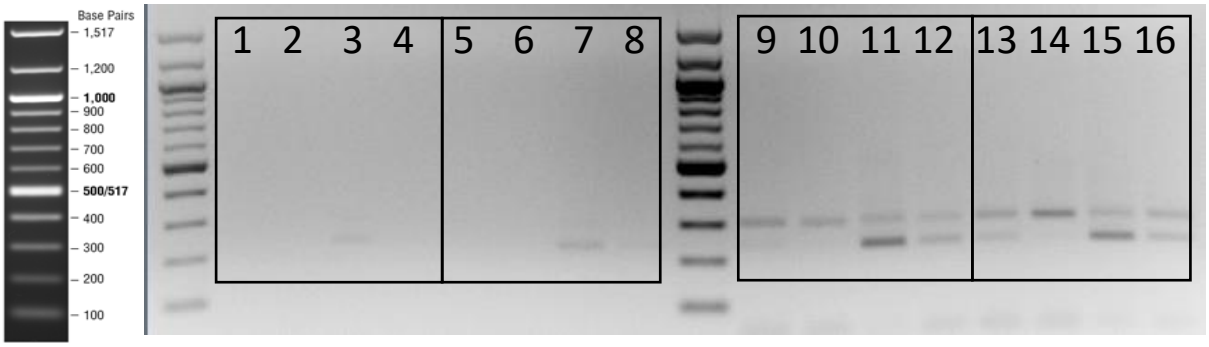


Figure 5.13. Gel electrophoresis image of the results of a semi-quantitative PCR using Rec8 primers against cDNA. Expected product size is 244 bp and is marked with an asterisk. gDNA inclusive of the intron 305 bp. Lanes 1-4 were run for 31 PCR cycles. Lanes 5-8 were run for 33 cycles. Lanes 9-12 were run for 35 cycles. Lanes 13-16 were run for 37 cycles. Lanes 1, 5, 9 and 13 used cDNA derived from an axenic 8-160 culture grown on solid media. Lanes 2, 6, 10 and 14 used cDNA derived from an axenic 8-166 culture grown on solid media. Lanes 3, 7, 11 and 15 used cDNA derived from sclerotia from a 'mated' cross between 8-160x8-166. Lanes 4, 8, 12 and 16 used cDNA derived from mycelial tissue from a 'mated' cross between 8-160x8-166.

The results of Dmc1 are shown in **Figure 5.14 w**hich demonstrates product bands for the semi-quantitative PCR amplification. The expression of 'mated' cultures is limited to the 31 cycles of cDNA derived from sclerotial tissue but is noticeable for both 'mated' cultures by 33 PCR cycles. In addition, 33 cycles were sufficient for perception of product from cDNA derived from axenic 8-160. The axenic culture of 8-166 failed to express sufficient product for visualisation at all cycle numbers. This provides evidence that expression of Dmc1 is greater in 'mated' cultures and particularly within sclerotia but limited to a 2-fold increase compared to the expression within 8-160. Additionally, an interesting novel demonstration is that strain 8-166 failed to express Dmc1 at a level that allowed for detection after even 37 cycles. This demonstrates at least a 16-fold increase in expression between the axenic cultures (based on identified assumptions). Again, at higher cycle numbers, secondary bands larger than the target product were visible - but there were two as opposed to the single band previously detected. The size of the target product for Dmc1 was 563 bp and this is indicated by the asterisk. The two introns are 63 bp and 52 bp, and a gDNA product or cDNA with retained introns would be 678 bp. would be expected. These product sizes match those demonstrated in **Figure 5.14.** The presence of two additional bands in an instance where the product contains two introns provided further evidence that these additional bands were indeed retained introns. The fairly similar size of the two introns would mask whether one or the other of the introns had been retained. The processed Dmc1 product was predicted to have a 69.6% cytoplasm localisation and only a 26.1% nuclear localisation. This was similar to predictions for the product if the two introns had been conserved with 60.9% and 21.7% predicted. However, the prediction for any product retaining all of the introns was less well determined with 30.4% recorded for each of mitochondria, nucleus and cytoplasm. Whilst the retention of the introns we see retained is less not likely to cause a subcellular localisation change, it is possible that the retention of other introns may do so. Perhaps more telling is that the retention of introns may introduce stop codons in the sequence resulting in a truncated protein product. Determination of this is difficult, least of all as we cannot predict whether early introns had been retained and whether this had resulted in a frame shift. However, the introns we can see retained are 63 bp and 52 bp and are in isolation non-frame shifting and frame shifting respectively.

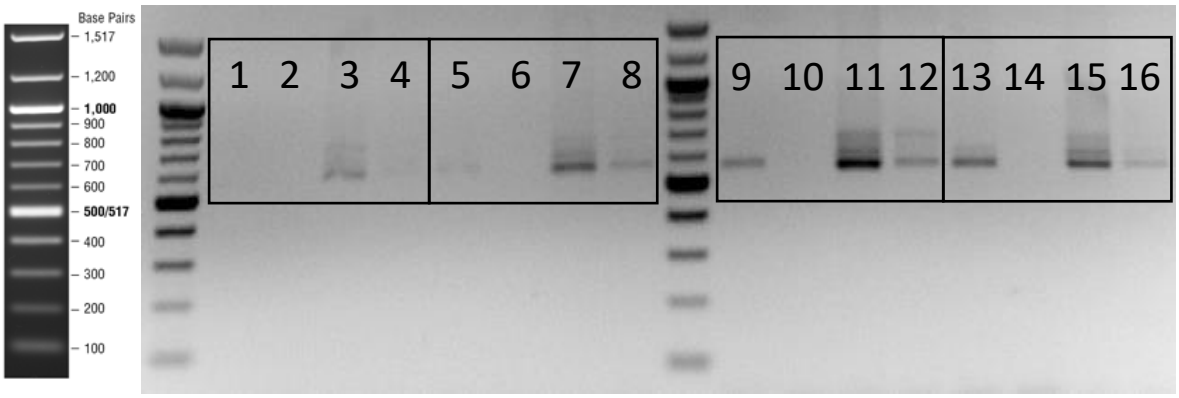


Figure 5.14. Gel electrophoresis image of the results of a semi-quantitative PCR using Dmc1 primers against cDNA. Expected product size is 563 bp and is marked with an asterisk. gDNA is inclusive of two introns 63 bp and 52 bp. Lanes 1-4 were run for 31 PCR cycles. Lanes 5-8 were run for 33 cycles. Lanes 9-12 were run for 35 cycles. Lanes 13-16 were run for 37 cycles. Lanes 1, 5, 9 and 13 used cDNA derived from an axenic 8-160 culture grown on solid media. Lanes 2, 6, 10 and 14 used cDNA derived from an axenic 8-166 culture grown on solid media. Lanes 3, 7, 11 and 15 used cDNA derived from sclerotia from a 'mated' cross between 8-160x8-166. Lanes 4, 8, 12 and 16 used cDNA derived from mycelial tissue from a 'mated' cross between 8-160x8-166.

The final two semi-quantitative PCR results for Hop2 and Msh4 are demonstrated in and respectively**.** The level of expression of Hop2 is greatest for sclerotial derived cDNA, at least 4-fold greater than for either axenic culture. An additional single band greater than the intended product is seen. This is further evidence that introns are being retained. The product sizes of 321 bp (with intron) and 203 bp (without intron) match the product sizes seen in **Figure 5.15**.

The predicted cellular localisation is 60.9% cytoplasm and 26.1% nuclear. This prediction does not alter with the retention of the intron seen in the partial product between the primers used. The intron itself is 118 bp so would result in a frame shift. This intron is at the 3' terminal end of the product.

The level of expression of Msh4 is hard to quantify but is greatest in sclerotial derived cDNA. Again, there was a primary band at the expected size 303 bp and two additional bands which match those of the retention of a single and both introns (54 bp and 44 bp). The size of these introns mask whether there are instances where one intron or the other would be retained. Msh4 fully processed partial transcript was 303 bp, and 401bp with both introns retained. These sizes match those shown in

The processed partial transcript is predicted to have a localisation signal to either nucleus (39.1%) or cytoplasm (39.1%). The retention of either or both of the introns does not change the prediction of the localisation signal. Both introns contain putative early stop codons. The first intron is a non-frame shift inclusion when retained. The second intron is 44 bp and results in a frame shift. Due to the position of these introns towards the 5' end, early stop codons introduced, or frame shifts during the retention will have a considerable impact on the protein.

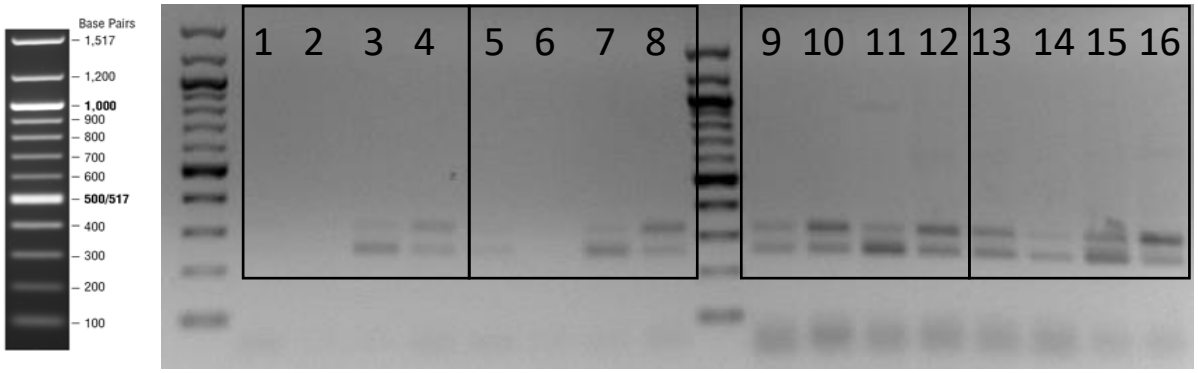


Figure 5.15. Gel electrophoresis image of the results of a semi-quantitative PCR using Hop2 primers against cDNA. Expected product size is 203 bp and is marked with an asterisk. gDNA is inclusive of an intron of 118 (321 bp total). Lanes 1-4 were run for 31 PCR cycles. Lanes 5-8 were run for 33 cycles. Lanes 9-12 were run for 35 cycles. Lanes 13-16 were run for 37 cycles. Lanes 1, 5, 9 and 13 used cDNA derived from an axenic 8-160 culture grown on solid media. Lanes 2, 6, 10 and 14 used cDNA derived from an axenic 8-166 culture grown on solid media. Lanes 3, 7, 11 and 15 used cDNA derived from sclerotia from a 'mated' cross between 8-160x8-166. Lanes 4, 8, 12 and 16 used cDNA derived from mycelial tissue from a 'mated' cross between 8-160x8-166.

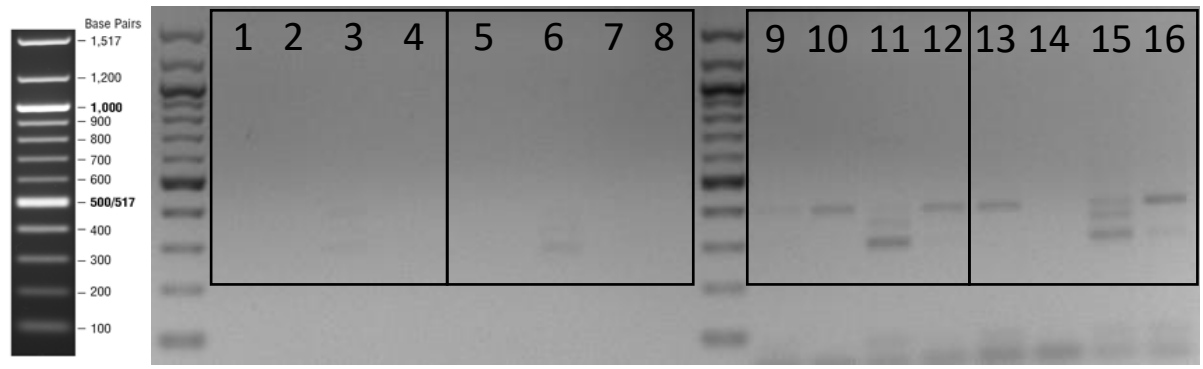


Figure 5.16. Gel electrophoresis image of the results of a semi-quantitative PCR using Msh4 primers against cDNA. Expected product size is 303 bp and is marked with an asterisk. gDNA is inclusive of two introns of 54 and 44 (401 bp total). Lanes 1-4 were run for 31 PCR cycles. Lanes 5-8 were run for 33 cycles. Lanes 9-12 were run for 35 cycles. Lanes 13-16 were run for 37 cycles. Lanes 1, 5, 9 and 13 used cDNA derived from an axenic 8-160 culture grown on solid media. Lanes 2, 6, 10 and 14 used cDNA derived from an axenic 8-166 culture grown on solid media. Lanes 3, 7, 11 and 15 used cDNA derived from sclerotia from a 'mated' cross between 8-160x8-166. Lanes 4, 8, 12 and 16 used cDNA derived from mycelial tissue from a 'mated' cross between 8-160x8-166.

To confirm whether the additional bands that were seen were indeed retained introns as predicted, the products were recovered from the agarose and sequenced. Products from the Semi-Quantitative PCR were recovered from the agarose gel in which they had been visualised as described in **Section 2.2.8.** After recovery of the product, it was sequenced as described in **Section 2.2.11.** Sequence data was processed as described in **Section 2.2.12** and aligned to the sequence data of the gene provided by *A. niger* strain CBS 513.88 from the NCBI using MacVector. A full list of the alignments of sequenced products alongside gene references can be seen in **Appendix 15.** Additionally, the aligned sequences can be visualised using a Pustell DNA Matrix Plot (MacVector) which clearly shows the presence or absence of introns as shown in **Figure 5.17** for Msh4 and a full list is given in **Appendix 16.** The absence

of introns in the alignment against the reference gene show conclusively that the products are cDNA from processed mRNA transcripts.

In cases where there were two or three product bands for a primer set, this method showed conclusively that this was due to splice variants, where the intron (or either one of two introns) had not been spliced, given a range of products that aligned with the reference genes but showed the presence of introns. Earlier PCR verification of DNase treatment had shown that this could not be DNA, since the RNA (pre cDNA conversion) had not shown product when subjected to 40 cycles of PCR with β-tubulin primers which it did after cDNA conversion. The bands thereby seen with an intron are variants of the mRNA that are not yet spliced or where the intron is deliberately retained. In particular, the four isoforms of *dmc1* which resulted in three discernible bands in **Figure 5.14** provide additional evidence that the bands are not caused by the inclusion of gDNA alone, as this could not explain the band matching the retention of a single intron. The sequencing supported that these single intron retention transcripts exist as can be seen in **Appendix 16**.

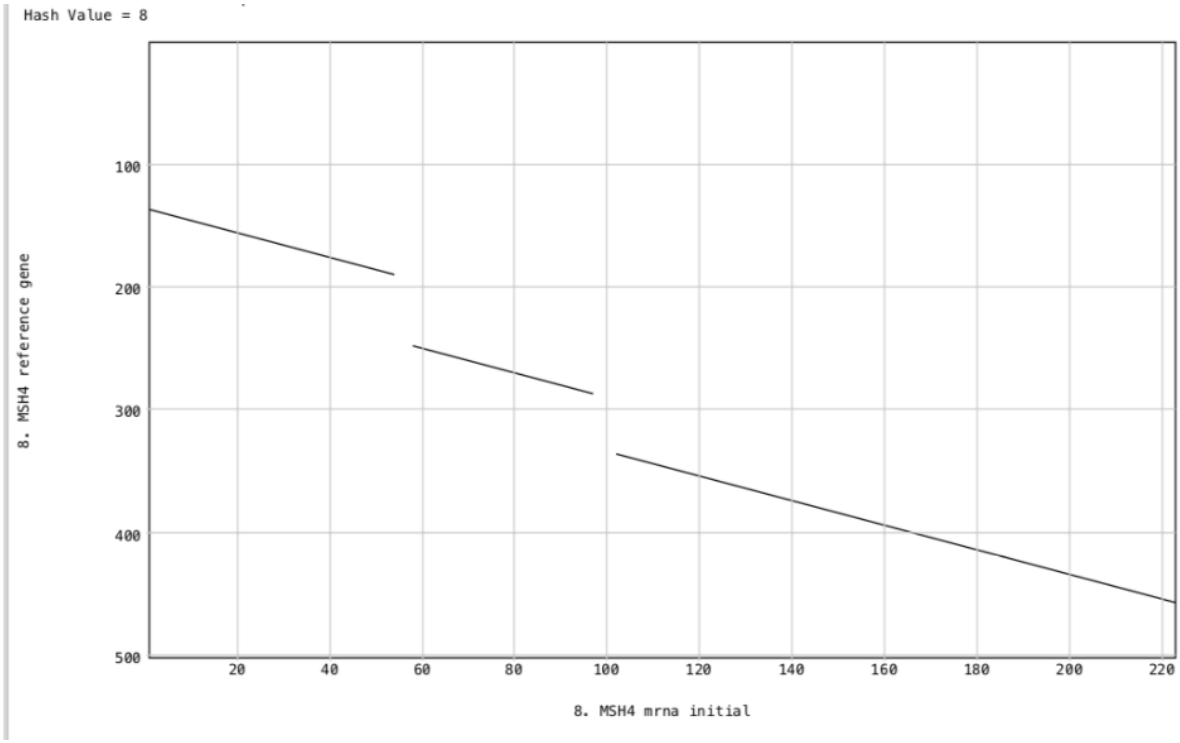


Figure 5.17. A Pustell DNA Matrix Plot (MacVector) showing the alignment of the largest product from Msh4 primers against the reference from CBS 513.88 provided by NCBI. The breaks in the line are the absence of introns in the sequenced product from cDNA that are present in the reference gene.

5.4 Discussion

The discoveries of apparently functional sex-related genes (Pel *et al*., 2007) and a *MAT1-1* locus (Pel *et al*., 2007; Andersen *et al*., 2011) in *A. niger* supported the hypothesis that *A. niger* might indeed be capable of heterothallic sexual recombination. Later, isolates of the complementary *MAT1-2* genotype were identified (Darbyshir, 2014; Ashton 2018) and the genome of a *MAT1-2* strain was made publicly available (Ellena *et al*., 2021b). Until recently, it was considered that section *Nigri* was composed almost entirely of asexual species (Dyer and O'Gorman, 2011), with very few reports of sexual reproduction in this group (Rajendran and Muthappa, 1980; Olarte *et al*., 2016). The absence of direct evidence of sexual recombination in the economically important fungus *A. niger* in this study, despite sclerotia production and identification of opposite mating-type strains was described in **Chapter 4**. Earlier work by Ashton (2019) had shown the production of putative ascospores in *A. niger*. However, these appeared non-viable under the environmental conditions tested for germination. We therefore aimed to seek alternative evidence of meiosis and sexual recombination as well as supporting evidence of the mating type diagnostics described in **Section 2.2.6.2** and **Section 2.2.6.3**. To support these aims, six strains of *A. niger* were subjected to genome sequencing. These comprised three putative strains of each mating type according to the mating-type diagnostics applied earlier in studies. These strains were also chosen as they were of interest due to a combination of sclerotia production, possible sexual compatibility [based on the studies of Ashton (2018)] and production of intriguing red structures within 'mated' sclerotia under certain growth conditions.

5.4.1 Genome sequencing and mating-type genes

Genome sequencing confirmed that the mating-type diagnostics had been accurate in identifying *MAT1-1* containing strains. The three *MAT1-1* strains had sequence similarity at this locus to the CBS 513.88 used as a scaffold. Difficulties associated with the absence of a *MAT1-2* scaffold strain, not published until more recently (Ellena *et al*., 2021b), meant that the sequence data for the likely *MAT1-2* strains did not map at this locus. In either case, the diagnostic appeared functional in determining the mating type in the strains used in this study (**Appendix 9**). Additionally, analysis of the *MAT1-1-1* gene of the strains sequenced showed little variation, congruent with these genes being functional and intact, rather than accruing mutations and forming pseudogenes. This supports the earlier evidence that suggests *A. niger* has functional 'early' sex genes relating to mating pathways (Pel et al., 2007).

5.4.2 Expression of Meiosis genes as evidence of sexual development

Work was then undertaken to assess whether evidence of sexual development in *A. niger* could be found that did not rely on direct observation of sexual ascospores or stroma. A semi-quantitative PCR approach was used to investigate the possible differential expression of genes involved in sclerotia production and meiosis in either mated or unmated axenic cultures of *A. niger*. Of the genes investigated, *sclR* expression was found to be higher in cDNA derived specifically from sclerotia rather than whole cultures. This indicates a tissue specific increase in *sclR* expression. *sclR* has previously been associated with important roles in differentiation, carbon metabolism and sclerotial development in *A. oryzae*. Overexpression of *sclR* promotes abnormal hyphal branching and additionally has been demonstrated to promote heterokaryotic sclerotia formation in *A. oryzae* (Jin *et al*., 2018; Wada *et al*., 2014). This indicates that whilst sclerotia production is *sclR* dependent, it is an unlinked pathway to the process of meiosis itself, as well as the processes upstream of meiosis and *sclR* is independently expressed. Sclerotia production and *sclR* transcription correlate as expected, and this is likely because of nutrient gradients as described in **Section 3.4.1**.

Meanwhile, very significantly, increased expression of a series of supposed meiosis-specific genes were seen in 'mated' cultures as a whole compared to axenic cultures i.e. the process of pairing cultures of *A. niger* of different mating type was correlated with greatly increased expression of meiosis specific genes

We then aimed to provide supporting evidence of meiosis that did not rely on direct observation of sexual ascospores or stroma. Here we used semi-quantitative PCR to show differential expression of genes involved in sclerotia production and meiosis when cultures of *A. niger* were axenic or paired with opposite *mating*-type partner. *sclR* expression was higher in cDNA derived from sclerotia specifically rather than whole cultures. This indicates tissue specific increase in *sclR* expression. *sclR* levels were lower in whole cultures but there was consistency between levels from axenic cultures and those of 'mated' cultures. This contrasts with increased expression of meiosis specific genes which are seen in 'mated' cultures compared to axenic cultures as we will discuss. This indicates that whilst sclerotia production is *sclR* dependent, it is an unlinked pathway to the process of meiosis itself, as well as the processes upstream of meiosis and *sclR* is independently expressed. Sclerotia production and *sclR* transcription correlate as expected, and this is likely because of nutrient gradients as described in **Section 3.4.1**.

The process of pairing cultures of *A. niger* of different *MAT*-type correlated greatly with increased expression of meiosis specific genes. This supports other evidence such as *MAT* locus organisation which support that *A. niger* is a heterothallic organism. Meiosis specific gene expression is

downstream of other potential indicators of sex such as pheromone expression and reception, including MAPK, MAPKK and MAPKKK signal transduction, cell wall reorganisation for plasmogamy and cognizance of post karyogamy meiotic recombination potential.

Semi Quantitative PCR has been used as a tool in studies of sex in the aspergilli with *A. nidulans* (Paoletti *et al*., 2007). In that study, it was demonstrated that expression of genes involved with mating pheromones and signal transduction were expressed in sexual cultures at a level not seen in asexual cultures. This demonstrated that self-fertilisation of a homothallic organism did not circumvent the normal requirements of pheromone production and reception to undergo sex. Therefore, in heterothallic organisms such as *A. niger*, it would certainly be expected that these genes are expressed due to the necessity for mate finding. Indeed, a full complement of functional upstream sexual genes involved with pheromone production and reception and signal transduction have been described from sequencing projects (Pel *et al*., 2007; Hoekstra, 2008). The identification of a complement of apparently functional sexual genes has previously preceded the discovery of sex in both *A. fumigatus* (Poggeler, 2002; Dyer and Paoletti., 2005) and *A. oryzae* (Wada *et al*., 2012).

Evidence of meiosis and sex can come from the presence, maintenance, and expression of meiosis specific genes. Indeed, the use of meiosis as the target of expression studies is one universal to sexual recombination. The use of this approach over conventional direct observation of sex allowed for the determination of the presence of meiosis rather than the perhaps unknown products of sex such as ascospores. The loss of expression of one gene in the suite of genes, even if truly lost rather than not detected, does not disprove that meiosis may occur. Many of the genes used in this study are absent in the sexually reproducing *D. melanogaster* and a DMC1 homolog is absent in many eukaryotic lineages (Schurko and Logsdon, 2008). Therefore, the absence or presence of a single gene would not confirm nor deny the capability for meiosis. Here, we provide evidence of the upregulation of expression of a suite of meiosis specific genes in both *A. nidulans* and *A. niger* and add to the evidence that *A. niger* has retained the ability for heterothallic, sexual reproduction dependent on environmental cues. The reduced expression or absence of expression when cultures are not paired, or when grown under certain conditions not normally conducive to sex in closely related species, support that the genes are functional and expressed under the correct conditions.

In this study, Semi Quantitative PCR was used to determine expression patterns in *A. nidulans* between asexual and sexual forms of reproduction. B-tubulin was used as a control, where expression should be equivalent under both conditions. The expression of three key meiosis genes; *dmc1*, *rec8* and *spo11* showed demonstrable increases in levels of expression under sexual inducing conditions compared to asexual conditions. This demonstrates that sexual differentiation correlates with increased expression of meiosis specific genes without increasing expression of the non-meiosis specific control gene.

The use of *A. nidulans* demonstrated the utility of Semi-Quantitative PCR in determining expression of meiosis genes and was followed with a study using *A. niger*. This assay used Semi-Quantitative PCR at a fixed number of cycles, as well as demonstrating changes over increases in cycle number. At a fixed number of 30 thermocycler cycles, *dmc1* expression was equivalent to the control β-tubulin over each of the conditions. This contrasted with the expected and realized increase in expression of *sclR* and *rec8* under sex inducing conditions compared to asexual conditions. This is interesting as *dmc1* is widely described as being meiosis specific and in the site of production of gametes. This has been recorded in yeast where expression is only detectable during meiosis (Habu *et al*., 1996). Additionally, the expression of *dmc1* in humans is specific to the testes (Jorgensen *et al.,* 2012) and in *A. thaliana*, its expression is specific to the pollen mother cells (Klimyuk and Jones, 1997). That *dmc1* is expressed axenically in both *A. niger* strains used in this study at a level equivalent to that in 'mated' cultures requires further study. The *dmc1* expressed in paired cultures showed a greater variance of splice isoforms than that of the axenically expressed *dmc1*. This may imply that one of the intron retained forms is the meiotic functional form as we will discuss.

It is interesting to note that sclerotia derived cDNA tended to form intron spliced isoforms, whereas mycelial derived cDNA tended to form intron retained products. This is most notable in **Figure 5.15** and **Figure 5.16** which represent *hop2* and *msh4* respectively.

Alternative splicing is common, with many forms. It has been hypothesised that there are five different types of alternative splicing including intron retention, exon cassettes and 5' and 3' alternative splice sites (Kempken, 2013). Alternative splicing can add to changes in protein localisation, enzymatic activity, protein diversity and mRNA stability (Fang *et al*., 2020; Muzafar *et al*., 2021). One of the types of alternative splicing is intron retention (IR). In ascomycetes intron retention appears to be the most common form of alternative splicing (Kempken, 2013). Intron retention may be caused by failure to recognise weak splice sites across introns by the intron definition mechanism (Fang *et al.,* 2020). Alternative splicing is widespread in ascomycota, basidiomycota and mucoromycota. Within these filamentous fungi, 61% of all alternative splicing events have been recorded to be IR (Fang *et al*., 2020).

One of the key reasons for intron retention in the biological context of response to signals, may be the addition or removal of a localisation signal, resulting in differences in subcellular localisation. Indeed, it may result in changes in whether a protein is secreted or not, such as with glycolysis enzymes. *A. nidulans* GAPDH can undergo intron retention resulting in different subcellular localisation to either cytoplasm or peroxisomes (Freitag *et al*., 2012). In *S. cerevisiae*, retention of an intron in *ptc7* gives rise to two isoforms, one localising to mitochondria, the other to the nuclear envelope (Juneau *et al*., 2009). In *A. niger*, intron retention in a β-glucosidase resulted in greater thermostability without change to catalytic function (Zhu *et al*., 2019).

Meiosis specific genes have been recorded as retaining introns to form functional products, whereas functional intron splicing results from mitosis and results in a non-functional form. This has been recorded in MER2 and MER3 in yeast. MER2 and MER3 are a mRNA transport regulator and helicase like protein involved in crossover control respectively (Kempken, 2013; Fang *et al.,* 2020). During meiosis *mer1* is expressed and efficiently splices *mer2* transcripts giving a functional product (Muzafar *et al*., 2021). Many other instances of intron retention include premature stop codons or frame shifts and result in nonsense mRNA. In contrast, examples of alternative splicing where different functional proteins are formed which share 5' exons but differ in their 3' end have been described in filamentous fungi (Kempken and Kuck, 1996). Within the aspergilli, a study of *A. flavus* revealed that 1.6% of 556 proteins examined underwent alternative splicing (Chang *et al*., 2010).

Of interest to this study is the discovery in mammals that both Dmc1 and Msh4, both used in this study, each have two different splice isoforms, of which in Dmc1, the larger splice form is lacking in somatic cells (Venables, 2002).

5.5 Conclusions

This chapter demonstrated that the expression of a suite of meiosis genes is increased in *A. niger* in the 'mating' conditions optimised in previous chapters. The species *A. nidulans* was used as a control organism as it has a well characterized sexual cycle which is easily reproducible. The expression of the suite of meiosis genes was increased under sexual conditions whilst the expression of a control gene (β-tubulin) was comparable between both asexual and sexual conditions as expected. These findings were similar in *A. niger* which again saw increase in expression under sexual conditions for the meiosis genes and comparable for the control gene.

Additionally, sequencing confirmed the utility of mating-type diagnostics developed in **Chapter 3** by confirming the presence of the MAT1-1 gene in all strains that the diagnostics had identified as MAT1-1. Analysis of the MAT1-1 genes revealed conservation amongst the strains sequenced and therefore suggest an extant use for this gene in keeping with a functional sexual cycle rather than degradation into a pseudogene accumulating mutations between diverging strains.

These findings add to the increasing evidence that *A. niger* does have an extant, though cryptic sexual cycle.

Chapter 6: Concluding Discussion

The research in this study focused primarily on attempts to elucidate the sexual cycle in *A. niger* as well as characterizing sclerotia production, both in terms of the environmental signals which govern morphological switching, and some of the transcription factors which govern the process. The primary goal of elucidation of sex in *A. niger* would allow for the strain improvement of this biotechnologically important fungal species.

6.1. Environmental, Metabolomic and Genetic Control of Sclerotia Production

Many exogenous factors are involved in the initiation of sclerotia production. This is reflective of the many factors involved in the morphological switching of fungi to produce other structures such as cleistothecia or conidiophores. This research has shown that optimization of external variables such as temperature, media composition and air exchange are paramount to sclerotia production. The determination of a present diffusible morphogen that governs sclerotia production is less clear. No strategy was found to harness any endogenous metabolite that had impact on sclerotia production as has been reported for *Aspergillus flavus* (Brown *et al*., 2008; Brown *et al*., 2009). Additionally, the use of the conserved endogenous quorum sensing molecule farnesol had no statistical impact on sclerotia production, but this may be linked to variations in the conditions used.

Interaction between the fungus and the external environment is typically mediated by pathways such as the conserved MAPK pathway that receives signal via G protein coupled receptors and sends these downstream to the nucleus where the corresponding response is mediated by relevant transcription factors. Fungi are known to respond to gradient differences in cues such as quorum sensing molecules and pheromones to direct or polarize growth towards a partner. This research supports observations made by other research teams that the presence of raisins on the media to generate sclerotia production, only does such as part of a nutrient concentration gradient. Indeed, sclerotia are rarely seen in the plate where the raisins have been laid on media or are abolished when raisins are macerated on to the media. The production of sclerotia is favoured where raisins are lain overnight on media and removed or left in place. The latter two favourable conditions would present a gradient where maceration would not. Furthermore, attempts at harnessing whichever nutrient cue is responsible for the gradient could not get an adequate sclerotia production response (described in **Section 3.4.3**). Perhaps because the nutrient is not diffusible in the solvents used, or perhaps as any solvent used would not create such as a stark concentration gradient.

The result of the signal cascade that results ultimately in sclerotia production was shown to be under the control of two transcription factors. The first transcription factor, a sclerotia production regulator termed *sclR* was previously described in *A. oryzae*. This study substantiated that previous work, in that *sclR* is responsible for positively producing sclerotia. Knockouts of *sclR* in several strains of *A. niger* resulted in many fold reductions in sclerotia production. Surprisingly, the knockouts of *sclR* were expected to abolish sclerotia production as had been reported but this was not observed here but for a single strain. The second transcription factor *sclB* has been described in *A. niger* (Jorgensen *et al*., 2020) as a repressor of sclerotia production. Indeed, work in this study confirms that *sclB* works to reduce sclerotia production and Δ*sclB* mutants promote additional sclerotia production in strains that already produced sclerotia. In contrast to the earlier work describing *sclB* in *A. niger*, the production of Δ*sclB* mutants in strains that did not already produce sclerotia did not 'awaken' sclerotia production. This is likely the result of blocks in signaling in other genes in those strains. This conclusion is supported by comparative genomics of the *sclB* genes in strains which show very little difference in the coding sequences of *sclB* between strains that do, and strains that do not produce sclerotia. It would be of interest to examine the promoter sequences between the sequenced strains, for variation that may explain the differences in sclerotia production.

6.2. The Fastidious Nature of Sex in the Black Aspergilli.

Advances in research depth of both experimental and molecular data suggest that sex in *A. niger* is likely and heterothallic in nature. Sex has been described in other members of the black aspergilli, such as the closely related *A. tubingensis* (Olarte *et al.,* 2016). The nature of heterothallic reproduction means that axenic cultures would not produce sexual structures, along with the fastidious nutritional and environmental requirements, and the need for prerequisite sclerotia production compound this difficulty in elucidating sex. It may be that the conditions required for sclerotia production are not the same as those required for sex or that an additional step is required to unlock sexual potential in sclerotia. It has been suggested previously that freezing sclerotia of *Sclerotinia* species awakens carpogenesis and this may be seasonally linked, with sexual life cycles being reflective of seasonal changes in temperature, light, or humidity. A previous study has demonstrated a means for foregoing the need for different conditions for sclerotia production and sex. In *A. flavus*, sclerotia of one mating type have been shown to be spermatised by ingrowths of hyphae germinated from conidia of the opposite mating type. The result is the formation of ascocarps or stroma within the sclerotium (Luis *et al*., 2020). Therefore, any conditions necessary for sclerotia production that may limit sexual reproduction can be forgone, as sclerotia can be produced via one set of conditions and spermatised under a separate set of conditions.

In this study, there was no direct physical observation of confirmed stroma or ascospores. Light microscopy enabled observation of a distinct red structure when cultures of opposite mating type were paired, where axenic cultures produced no such structure. To examine whether this was a result of interaction or heterokaryon formation within sclerotia we engineered strains of *A. niger* of different mating types to contain either eGFP or TdTomato. These engineered strains were then paired, and sclerotia produced were examined for the presence of both fluorescent markers. It was determined that germinated hyphae of one mating type can indeed grow invasively into the sclerotium of the opposite mating type. This process was favoured where one sclerotium was already developed as opposed to pairing two growing cultures and examining sclerotia grown at the margins of those cultures. This ingrowth into sclerotia supports the work in *A. flavus* (Luis *et al*., 2020) and suggests some putative attractant. It would be interesting to observe whether these hyphal ingrowths favoured strains of the opposite mating type. An additional experiment may be to place a sclerotium on germinating conidia of both the same strain (or another strain of same mating type) and a strain of the opposite mating type, and score whether ingrowths favour opposite mating types and allude to sexual attraction. This ingrowth of hyphae into a sclerotium of the opposite mating type mirrored the observation under light microscopy that sclerotia produced from pairs of opposite mating type produced a distinct determinable red structure, where axenic cultures did not. However, the absence

of direct physical observation of clear stroma under SEM prevented the visual characterization of these structures and furthermore, did not detect any clear sexual receptor cell type, although distinct and previously undescribed tissue types were observed. A proposed hypothesis for the lack of sex in *A. niger* may be the inversion of the MAT1-1 locus when compared to other (sexual) *Aspergillus* species (Darbyshir, 2014; Ellena *et al*., 2021b).

Additionally, this study advances methods for separation of ascospores and conidia. Flow cytometry was used to separate the spore types of *A. niger* and *A. fumigatus* based on their physical characteristics. This could be developed to separate conidia and ascospores of biotechnologically important species when sex is elucidated. This would provide a high throughput means to generate only cultures with genetic recombination, or at least refine spore suspensions to contain primarily ascospores, aiding strain improvement methodologies. It could additionally reduce the time needed for DNA extraction and subsequent sequencing methods to aid in determining recombination in offspring. This method would involve comparing the genetic sequence of offspring to the parental sequences and observing differences from either parent.

6.3. Transcriptomic Expression of Meiosis Genes in *A. niger*.

Genomic evidence has suggested a proto-heterothallic lifestyle in *A. niger*. MAT1-1 loci have been widely reported and more recently, a MAT1-2 locus has been published (Ellena *et al*., 2021b). This separation of MAT loci between strains is in keeping with the arrangement within other heterothallic species. Sequencing of several strains of *A. niger* in this study revealed high conservation of the MAT1-1-1 gene. This conservation of sequence is in keeping with a functional gene, rather than the accumulation of differences expected within a pseudogene between strains. This suggests an extant (or only recently ceased) sexual life cycle using MAT loci in the conventional sense. However, it may be that the MAT1-1 gene alone is not evidence for an extant sexual cycle as it may have roles beyond sexual reproduction and be required in secondary metabolism. Therefore, an additional suite of 'meiosis' specific genes were identified for transcriptomic analysis. Under the conditions of pairing cultures of opposite mating-type which yielded red structures in sclerotia, many of the meiosis genes were upregulated. This upregulation was in comparison to axenic cultures under the same conditions. These findings support the idea that there may be a functional, extant sexual cycle. The absence of direct observation of ascospores in this study may be due to a block in spore development post meiosis, or a lack of the correct environmental conditions.

6.4. Future Prospects

Whilst the primary objective of this work in elucidating the sexual cycle in *A. niger* was not met, many subsidiary aims were met. The novel findings of *sclB* and *sclR* knockout mutants not awakening sclerotia production in strains that do not produce sclerotia, and not abolishing sclerotia production entirely (respectively) require further investigation. The variation in production of sclerotia between different strains was shown to be unlikely due to limited variation in the coding sequences of the transcription factors, but the promoter sequences regions of these could be explained to explain differences. This could then be harnessed for exploitation of the sclerotia production through engineering.

It seems likely that production of sclerotia and sexual reproduction are under the control of two distinct developmental pathways. In the cases where sex has been elucidated such as *A. tubingensis* and *A. sclerotiicarbonarius* (Olarte *et al*., 2016; Darbyshir, 2014), it is likely that conditions have been met that overlap and allow for both sclerotia production and sclerotia fertilisation to develop sexually. Methodologies to observe changes within sclerotia and development of stroma within A. flavus have been adopted in this study and utilized for *A. niger*. This successfully shows the hyphal ingrowth of one mating type strain into the sclerotium of another mating type strain. The suggestion of an attractant and sexual receptor cell/tissue within sclerotia are obvious and would require further study, perhaps using time course experiments to determine hyphal destination and changes in the novel described tissue types within sclerotia (this study). These tissues could furthermore be examined for the presence of heterokaryon, aided by the creation of fluorescent lab strains of opposite mating type.

The adoption of the technique whereby sclerotia are produced under one set of conditions and subsequently fertilized or spermatised by conidia or germinating hyphae of another mating type strain under another set of conditions could be developed. It may be that sclerotia produced must first undergo some stress, such as freezing or desiccating to mimic seasonality. A similar idea has been described with *A. flavus*, where sclerotia were produced on corn, dried, and shown to be absent ascospores, and later transferred to and incubated on soil at high humidity whereafter sexual ascospores were observed (Horn *et al*., 2014). This growth on host and later dormancy may best mimic natural conditions and seasonal or host variation.

It would be interesting to develop the exploration of functionality of the *A. niger* MAT genes. Heterologous expression of the respective *A. niger* MAT1-1 and MAT1-2 genes at the respective MAT gene sites of a suitable host such as *A. nidulans* and subsequent expression of sex would confirm functionality. A similar experiment has been completed (Sharon *et al*., 1996). A similar experiment

could be completed to explore the impact of the directionality of MAT1-2, although large loci are inherently difficult to transform, this may need to be completed on a gene-by-gene basis.

The finding that a suite of meiosis genes is expressed at higher levels in 'mated' cultures of *A. niger* could be further investigated. Real-time PCR of the same (and expanded) genes used in this study could give insights into respective expression at different time points of the supposed sexual 'mating' occurring. Attempts at this were made in this study, but the inherent nature of sex on solid media includes conidial pigment contamination and this was likely a root cause for the lack of success. However, if this can be overcome, this would be a promising line of investigation.

6.5. Concluding Remarks

Characterization of two transcription factors; *sclB* and *sclR* have suggested that previous findings of abolishment of sclerotia production or awakening of sclerotia production may not be conclusive. This may be due to additional interactions of these transcription factors, perhaps forming homodimers with yet other transcription factors under certain conditions.

The discovery of transcriptomic evidence supporting upregulation of meiosis genes when certain *A. niger* strains of opposite mating type are paired has been shown. This supports other evidence that these pairings generate distinct red structures within sclerotia when paired that are absent when sclerotia are generated axenically. SEM has helped elucidate a potential novel tissue type within the sclerotia produced under paired conditions. Confocal microscopy of fluorescent engineered strains of opposite mating type reveals hyphal ingrowths of one mating type into the sclerotia of the opposite mating type. Sequencing data supports previous evidence of a heterothallic arrangement of mating types. Taken together, these findings are evidence of sexuality within *A. niger*. However, in this study, there has been no direct observation of ascospores, and no recombination has been evidenced. Further studies are required to elucidate the fastidious conditions required for sex to allow for strain improvement in this biotechnologically important fungal species.

7. APPENDICES

Appendix 1. PIP Reflective Statement.

My placement was completed between the start of January and start of April 2021 at the Cambridge Centre for Science and Policy (CSaP). CSaP brings together expertise from the University of Cambridge (as well as other academic institutions) and Policy Fellows from varying government bodies and beyond. The primary projects that I was working on were to attend and document, and finally produce an article of each meeting - of two series of seminars, one of which was the governments 'Levelling Up' policies and the second was 'Being Heard'. The 'Levelling Up' seminars included experts from physical and social infrastructure and policy fellows from streams associated with these policies. The 'Being Heard' seminars revolved around expertise being under attack or how best to disseminate expertise to a broader audience, particularly in areas where expertise is lacking.

In addition, I also worked closely with and helped organised round tables on behalf of CSaP and the DSTL (Defence, Science and Technology Laboratory) bringing together diverse expertise to comment on matters of misinformation and how this intersects with vaccine hesitancy (in light of Covid-19), political polarisation (Brexit, Trump, China) and what can be done to help people with digital literacy and inoculation against misinformation online. This round table, as well as context from other experts and situations was then summarised into an article for dissemination by DSTL.

Further to this, I also arranged Policy Fellowships programmes, by having initial meetings with a new cohort of Policy Fellows, aggregating questions for which they are hoping for expertise to help them answer, then contacting academics, arranging for meetings and round tables with these experts and sitting in for the conversations and how they relate to policy questions from government bodies. I was particularly engaged by contacting a range of academics to bear their expertise on the question of 'How do we roll out the Covid vaccination programs expediently and sufficiently throughout the UK population?'.

In summary, I learnt a lot about how to write for non-scientific audiences and how the intricacies of policy work, particularly at the intersection of academia and government.

Appendix 2: Primers used for *Mating* type Diagnostics

Primer name:	(degenerate) Nucleotide sequence
MAT1F	5'TGGCARAAYGAYCCITTYAARGCN
MAT1R	5'YTIGCCATIGTRTAYTGYTGYTSNYC
MAT1.1F	5'CTTCGCTTCTTGTGGCAGAA
MAT1.1R	5'TGGGCAGCGAARGCCAT
MAT3.5	5'TTICKIGGIKKRWAIYKRTARTYIGG
MAT5.7	5'THSCIMGICCICCIAAYKSITTYAT
Mat1.2for	5'TACAGAGCTGCTTTGGCAGGA
Mat1.2rev	5'CATTTGGTGGWCGAGGRACTTT
ANMAT1F	5'ATTGGCATTATCGAGCCGGA
MAT2amR	5'CTCGGATTCTGCCTTCCAC
ATHYP2	5'TTTCCGTCCCGGTGGCG

Appendix 3: Primers used for Construction of Transformation Cassettes

Primer name:	Nucleotide sequence
AkuB_up_fwd	5'GCCAGTGAATTCGAGCTCGGTACCCGGGTGGAGAAATGATCGGATCG
AkuB_up_rev	5'GTAAGCGGCCGCCTAGTCACTCACCATCTGTC
AkuB_down_fwd	5'GGTGAGTGACTAGGCGGCCGCTTACCGATATGGACGAG
AkuB_down_rev	5'TGCAGGTCGACTCTAGAGGATCCCCGGGATAAGCCTTGTTACTTAG
gpdA_fwd	5'CAGGACAGATGGTGAGTGACTAGGCGGCCGCCTTATTCGTTGACCTAGCTG
gpdA_rev	5'GTCGGCATCTACTCTCGAGCATTGTGATGTCTGCTCAAG tcaccatctcgaGCATTGTGATGTCTGCTC
trpC_fwd	5'ACAATGCTCGAGAGTAGATGCCGACCGGGATC
trpC_rev	5'CCGTAATCAAGCCAGTGTGATGGAATTCGCC
ptrA_fwd	5'TTCCATCACACTGGCTTGATTACGGGATCCC
ptrA_rev	5'CCCGTTCTCGTCCATATCGGTAAGCGGCCGCCGTATTATACTGTCTTTCTTGTTAC AC
TdTomato_fwd	5'GACATCACAATGCTCGAGATGGTCTCCAAGGGTGAG
TdTomato_rev	5'CATCTACTCTCGAGCTACTTGTAGAGCTCGTCC
eGFP-fwd	5'GACATCACAATGCTCGAGATGGTGAGCAAGGGCGAG
eGFP-rev	5'CATCTACTCTCGAGTTACTTGTACAGCTCGTCCATG
SclB_up_P3_fwd	5'GCCAGTGAATTCGAGCTCGGTACCCGGGTGGCCGTTGACAACTTGT AC
SclB_up_P3_rev	5'AAGAGCGGCCGCTTGAAACCTCACGCTTGC
SclB_down_P3_fwd	5'CGTGAGGTTTCAAGCGGCCGCTCTTCGCATAGCACCTCC
SclB_down_P3_rev	5'TGCAGGTCGACTCTAGAGGATCCCCGGGTCTGGGGAAAGGAGATGG
SclR_up_fwd	5'GCCAGTGAATTCGAGCTCGGTACCCGGGCTGGGGTGATCTCTCGGTC
SclR_up_rev	5'GAGGGCCATGAATGCGGCCGCATGTGGATAGGCTCAAGGG

SclR_down_P2_fwd	5'ACATGCGGCCGCATTCATGGCCCTCTCCTG
SclR_down_P2_rev	5'TGCAGGTCGACTCTAGAGGATCCCCGGGACACTCTTCACAGCCCAC

Appendix 4: Primers used for akuB positional PCR

Primer name	Primer sequence 5'-3'	Size of product
AkuB upstr conf F	atttcgccaacaaaggaccc	
ΔAkuB insert conf R	cacaccagcctttccacttc	847 bp
AkuB gene conf R	ttagcggtttctggacgttc	627 bp

Appendix 5. *Aspergillus nidulans* Strains, Point of Origin and Details

Uni Nottingham culture collection accession	Origin	Year	Other culture numbers	Additional information
2-3	John Clutterbuck (Glasgow)		FGSC4	Apparently forms cleistothecia very readily Wild-type prototrophic
2-258	Ozgur Bayram Georg-August-University Goettingen-Germany		AGB551	ΔpyrG (needs uracil and uridine)

Appendix 6. *Aspergillus fumigatus* Strains, Point of Origin and Details

Uni Nottingham culture collection accession	Origin	Year	Other culture numbers	*MAT*-type	Additional information
47-52	Dublin, Ireland	2005	AFIR964	*MAT1-2*	
47-59	Dublin, Ireland	2005	AFRB2	*MAT1-1*	

Appendix 7: *Aspergillus tubingensis* Strains, Point of Origin and *MAT*-type Data

Uni Nottingham culture collection accession	Origin	Year	Other culture numbers	Additional Information	*MAT*-type	Mean sclerotia production score (/cm^2) on CYAR media
76.1	Air Sampling. Dublin, Ireland	2005	AN201		*MAT1-2*	
76.2	Air Sampling. Dublin, Ireland	2005	AN202		*MAT1-2*	
76.3	Air Sampling. Dublin, Ireland	2005	AN204		*MAT1-2*	
76.4	Air Sampling. Dublin, Ireland	2005	AN208	Possible clone of 76.5	*MAT1-2*	
76.5	Air Sampling. Dublin, Ireland	2005	AN205	Possible clone of 76.4	*MAT1-2*	
76.6	Air Sampling. Dublin, Ireland	2005	AN209		*MAT1-2*	
76.7	Air Sampling. Dublin, Ireland	2005	AN210	Possible clone of 76.8	*MAT1-1*	
76.8	Air Sampling. Dublin, Ireland	2005	AN219	Possible clone of 76.7	*MAT1-1*	
76.9	Air Sampling. Dublin, Ireland	2005	AN212		*MAT1-2*	
76.10	Air Sampling. Dublin, Ireland	2005	AN213		*MAT1-2*	
76.11	Air Sampling. Dublin, Ireland	2005	AN214		*MAT1-1*	
76.12	Air Sampling. Dublin, Ireland	2005	AN217		*MAT1-2*	
76.13	Air Sampling. Dublin, Ireland	2005	Rb1			
76.14	Air Sampling. Dublin, Ireland	2005	AN206		*MAT1-2*	
76.15	Air Sampling. Dublin, Ireland	2005	AN207		*MAT1-2*	
76.16	Air Sampling. Dublin, Ireland	2005	AN211		*MAT1-2*	
76.17	Air Sampling. Dublin, Ireland	2005	AN215		*MAT1-1*	
76.18	Air Sampling. Dublin, Ireland	2005	AN221		*MAT1-2*	
76.19	Air Sampling. Dublin, Ireland	2005	AN222		*MAT1-1*	
76.20	Air Sampling. Dublin, Ireland	2005	AN224		*MAT1-2*	
76.21	Air Sampling. Dublin, Ireland	2005	AN225		*MAT1-2*	

76.22	Air Sampling. Dublin, Ireland	2005	AN226		*MAT1-2*	
76.23	Air Sampling. Dublin, Ireland	2005	AN227		*MAT1-2*	
76.24	Air Sampling. Dublin, Ireland	2005	AN228		*MAT1-1*	
76.25	Air Sampling. Dublin, Ireland	2005	AN230		*MAT1-1*	
76.26	Norfolk collection		L9		*MAT1-1*	
76.27	Norfolk collection		CS14		*MAT1-2*	
76.28	Dublin, Ireland	2008	AN801		*MAT1-2*	
76.29	Dublin, Ireland	2008	AN802		*MAT1-1*	
76.30	Dublin, Ireland	2008	AN803		*MAT1-1*	
76.31	Dublin, Ireland	2008	AN804		*MAT1-2*	
76.32	Dublin, Ireland	2008	AN805		*MAT1-2*	
76.33	Dublin, Ireland	2008	AN806		*MAT1-1*	
76.34	Dublin, Ireland	2008	AN807		*MAT1-2*	
76.35	Dublin, Ireland	2008	AN808	(Absent from stocks)		
76.36	Dublin, Ireland	2008	AN809		*MAT1-1*	
76.37	Dublin, Ireland	2008	AN810		*MAT1-2*	
76.38	Dublin, Ireland	2008	AN812	(Absent from stocks)		
76.39	Thailand (Malcolm Stratford)		26			
76.40	Brazil (Malcolm Stratford)		17E		*MAT1-1*	
76.41	Turkey (Malcolm Stratford)		113			
76.42	Turkey (Malcolm Stratford)		39		*MAT1-1*	
76.43	Brazil (Malcolm Stratford)		61A		*MAT1-2*	
76.44	Turkey (Malcolm Stratford)		10B		*MAT1-2*	
76.45	Italy (Grapes) ISPA	2000	Item4208		*MAT1-2*	
76.46	Italy (Grapes) ISPA	2000	Item4210		*MAT1-1*	
76.47	Sicily (Grapes) ISPA	2001	Item 4840		*MAT1-1*	
76.48	Italy (Grapes) ISPA	2001	Item 4496		*MAT1-2*	
76.51	Czech Republic (Sputum-Clinical)		CCF4086		*MAT1-1*	
76.52	Czech Republic (Sputum-Clinical)		CCF4087		*MAT1-1*	
76.53	Czech Republic (Soil)		CCF4080		*MAT1-1*	

76.54	Louny, Czech Republic		CCF2818		*MAT1-1*	
76.55	Environmental (Coal)				*MAT1-1*	
76.56	Environmental (Coal)				*MAT1-1*	
76.57	DSM Foods Industrial Strain	2018			*MAT1-2*	

Appendix 8: *Aspergillus sclerotiicarbonarius*, Point of Origin, and *MAT*-type Data

Uni Nottingham culture collection accession	Origin	Year	Other culture numbers	*MAT*-type	Additional information
80-1	Thailand		CBS121851	*MAT1-1*	
80-2	Thailand		CBS121852	*MAT1-2*	
80-3	Thailand		CBS121853	*MAT1-2*	
80-4	Thailand		CBS121854	*MAT1-2*	
80-5	Thailand		CBS121056	*MAT1-1*	
80-6	Thailand		CBS121057	*MAT1-2*	Type Strain
80-7	Progeny			*MAT1-2*	CBS121056 x CBS121853
80-8	Progeny			*MAT1-1*	CBS121056 x 121853
80-9	Progeny			*MAT1-2*	CBS121056 X 121853
80-10	Progeny			*MAT1-1*	CBS121056 X 121853
80-11	Progeny			*MAT1-2*	CBS121056 X 121853
80-12	Progeny			*MAT1-1*	CBS121056 X 121853
80-13	Progeny			*MAT1-1*	CBS121056 X 121853
80-14	Progeny			*MAT1-1*	CBS121056 X 121853
80-15	Progeny			*MAT1-1*	CBS121056 X 121853
80-16	Progeny			*MAT1-1*	CBS121056 X 121853
80-17	Progeny			*MAT1-2*	CBS121056 X 121853

Appendix 9: *Aspergillus niger*, Point of Origin, and *MAT*-type Data

Strain	Origin	Year	*MAT*-type	Additional names	Other information
8-152	Ronald de Vries (CBS)		*MAT1-1*	CBS113.46	
8-160	Jens Frisvad (ex raisin)	2007	*MAT1-2*	IBT28999	
8-161	Jens Frisvad (ex raisin)	2007	*MAT1-1*	IBT29019	
8-162	Jens Frisvad (ex pepper)	2007	*MAT1-2*	IBT24631	
8-163	Jens Frisvad (ex raisin)	2007	*MAT1-2*	IBT29005	
8-164	Jens Frisvad (ex raisin)	2007	*MAT1-2*	IBT29003	
8-165	Jens Frisvad (ex raisin)	2007	*MAT1-2*	IBT28998	
8-166	Jens Frisvad (ex US citric acid producer)		*MAT1-1*	IBT26389	
8-167	Jens Frisvad (ex raisin)	2007	*MAT1-2*	IBT29006	
8-168	Jens Frisvad (ex raisin)	2007	*MAT1-2*	IBT29020	
8-169	Jens Frisvad (ex raisin)	2007	*MAT1-2*	IBT29001	
8-171	Nottingham (Straw)		*MAT1-1*	W1_5	
8-172	Dublin, Ireland Air Sampling	2005	*MAT1-1*	AN216	
8-173	Brazil		*MAT1-1*	59D	
8-174	Norfolk Collection (Environmental)		*MAT1-1*	L10	
8-175	Norfolk Collection (Environmental)		*MAT1-1*	CS142	
8-176	Norfolk Collection (Environmental)		*MAT1-1*	L26	
8-177	Italy (Grapes)	2004	*MAT1-1*	ITEM7496	
8-178	Italy (Grapes)	2004	*MAT1-1*	ITEM7090	
8-179	Israel (Grapes)	2002	*MAT1-2*	ITEM6292	
8-180	Prague, Czech Republic (Clinical)		*MAT1-2*	SK250	
8-181	Cseke Budejovice, Czech Republic (Clinical)		*MAT1-1*	CB42	
8-182	Prague, Czech Republic (Clinical)		*MAT1-1*	PL8	
8-183	Type Strain (Unknown)	1952	*MAT1-2*	ITEM4501	
8-184	Greece (Grapes)	2001	*MAT1-1*	ITEM5276	
8-185	Italy (Grapes)	2004	*MAT1-1*	ITEM 7091	
8-186	Italy (Grapes)	2004	*MAT1-1*	ITEM7092	
8-187	Pardubice, Czech Republic (Clinical)		*MAT1-1*	CCF3990	

Appendix 10: *MAT1-1* Alignments

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MAT1-1 Gene CBS     1 ATGGATGCCACCACTTCACCTCTTCAGCGTGCTTTCAACGCATTCCTCCTCACAATGCCC   60
MAT1-1 8-166        1 ATGGATGCCACCACTTCACCTCTTCAGCGTGCTTTCAACGCATTCCTCCTCACAATGCCC   60
MAT1-1 8-175        1 ATGGATGCCACCACTTCACCTCTTCAGCGTGCTTTCAACGCATTCCTCCTCACAATGCCC   60
MAT1-1 8-161        1 ATGGATGCCACCACTTCACCTCTTCAGCGTGCTTTCAACGCATTCCTCCTCACAATGCCC   60
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MAT1-1 Gene CBS    61 CCCCAGCAACTCGAAGAGCTCCTCAGGTATCTTCAGGACACCCGAGCCAAGGCCAACAAC  120
MAT1-1 8-166       61 CCCCAGCAACTCGAAGAGCTCCTCAGGTATCTTCAGGACACCCGAGCCAAGGCCAACAAC  120
MAT1-1 8-175       61 CCCCAGCAACTCGAAGAGCTCCTCAGGTATCTTCAGGACACCCGAGCCAAGGCCAACAAC  120
MAT1-1 8-161       61 CCCCAGCAACTCGAAGAGCTCCTCAGGTATCTTCAGGACACCCGAGCCAAGGCCAACAAC  120
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MAT1-1 Gene CBS   121 CAGGTCGCGCACGAAAATGAGCAATCTACTGCAAGATCGAAACCCCCTCTGGACACCAAT  180
MAT1-1 8-166      121 CAGGTCGCGCACGAAAATGAGCAATCTACTGCAAGATCGAAACCCCCTCTGGACACCAAT  180
MAT1-1 8-175      121 CAGGTCGCGCACGAAAATGAGCAATCTACTGCAAGATCGAAACCCCCTCTGGACACCAAT  180
MAT1-1 8-161      121 CAGGTCGCGCACGAAAATGAGCAATCTACTGCAAGATCGAAACCCCCTCTGGACACCAAT  180
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MAT1-1 Gene CBS   181 AATGAGTCGGCCGTTCAGGGCAGTACAAACCAACGTTCTCATCAAACGCGAGGCAAGCGA  240
MAT1-1 8-166      181 AATGAGTCGGCCGTTCAGGGCAGTACAAACCAACGTTCTCATCAAACGCGAGGCAAGCGA  240
MAT1-1 8-175      181 AATGAGTCGGCCGTTCAGGGCAGTACAAACCAACGTTCTCATCAAACGCGAGGCAAGCGA  240
MAT1-1 8-161      181 AATGAGTCGGCCGTTCAGGGCAGTACAAACCAACGTTCTCATCAAACGCGAGGCAAGCGA  240
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MAT1-1 Gene CBS   241 CCTCAGGATGGGAAACGGCGGCCACTGAACAGTTTCATTGCTTTCAGAAGTATTCTATAC  300
MAT1-1 8-166      241 CCTCAGGATGGGAAACGGCGGCCACTGAACAGTTTCATTGCTTTCAGAAGTATTCTATAC  300
MAT1-1 8-175      241 CCTCAGGATGGGAAACGGCGGCCACTGAACAGTTTCATTGCTTTCAGAAGTATTCTATAC  300
MAT1-1 8-161      241 CCTCAGGATGGGAAACGGCGGCCACTGAACAGTTTCATTGCTTTCAGAAGTATTCTATAC  300
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MAT1-1 Gene CBS   301 ACTTCCATCTCATGCATGTACATCGGCTAATGATTATTAGGTTATTATTCAGTCATGTTT  360
MAT1-1 8-166      301 ACTTCCATCTCATGCATGTACATCGGCTAACGATTATTAGGTTATTATTCAGTCATGTTT  360
MAT1-1 8-175      301 ACTTCCATCTCATGCATGTACATCGGCTAATGATTATTAGGTTATTATTCAGTCATGTTT  360
MAT1-1 8-161      301 ACTTCCATCTCATGCATGTACATCGGCTAATGATTATTAGGTTATTATTCAGTCATGTTT  360
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MAT1-1 Gene CBS   361 CCAGACCTTACTCAGAAAGCGAAGTCGGGCATCCTTCGCTTCTTGTGGCAGAACGACCCT  420
MAT1-1 8-166      361 CCAGACCTTACTCAGAAAGCGAAGTCGGGCATCCTTCGCTTCTTGTGGCAGAACGACCCT  420
MAT1-1 8-175      361 CCAGACCTTACTCAGAAAGCGAAGTCGGGCATCCTTCGCTTCTTGTGGCAGAACGACCCT  420
MAT1-1 8-161      361 CCAGACCTTACTCAGAAAGCGAAGTCGGGCATCCTTCGCTTCTTGTGGCAGAACGACCCT  420
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MAT1-1 Gene CBS   421 TTCAAAGCTAAATGGGCCATCGTAGCCAAGGCATACTCCATCATTCGCGATGACCATGAT  480
MAT1-1 8-166      421 TTCAAAGCTAAATGGGCCATCGTAGCCAAGGCATACTCCATCATTCGCGATGACCATGAT  480
MAT1-1 8-175      421 TTCAAAGCTAAATGGGCCATCGTAGCCAAGGCATACTCCATCATTCGCGATGACCATGAT  480
MAT1-1 8-161      421 TTCAAAGCTAAATGGGCCATCGTAGCCAAGGCATACTCCATCATTCGCGATGACCATGAT  480
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MAT1-1 Gene CBS   481 AATGGAGTCTCCCTTGAAACATTTCTGAAACTCAACACCAACCTCATTGGCATTATCGAG  540
MAT1-1 8-166      481 AATGGAGTCTCCCTTGAAACGTTTCTGAAACTCAACGCCAACCTCATTGGCATTATCGAG  540
MAT1-1 8-175      481 AATGGAGTCTCCCTTGAAACATTTCTGAAACTCAACACCAACCTCATTGGCATTATCGAG  540
MAT1-1 8-161      481 AATGGAGTCTCCCTTGAAACATTTCTGAAACTCAACACCAACCTCATTGGCATTATCGAG  540
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MAT1-1 Gene CBS   541 CCGGACCGCTACCTTGACATAATGGGCTGGGAGCTGAATGTCGACGGCCAGCAGCAGTAC  600
MAT1-1 8-166      541 CCGGACCGCTACCTTGACATAATGGGCTGGGAGCTGAATGTCGACGGCCAGCAGCAGTAC  600
MAT1-1 8-175      541 CCGGACCGCTACCTTGACATAATGGGCTGGGAGCTGAATGTCGACGGCCAGCAGCAGTAC  600
MAT1-1 8-161      541 CCGGACCGCTACCTTGACATAATGGGCTGGGAGCTGAATGTCGACGGCCAGCAGCAGTAC  600
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MAT1-1 Gene CBS   601 ACCATGGCTAAGGTTAAGATTACAGCTACTCCTGAAGCTGAGCTCTCAACAAACTATTCA  660
MAT1-1 8-166      601 ACCATGGCTAAGGTTAAGATTACAGCTACTCCTGAAGCTGAGCTCTCAACAAACTATTCA  660
MAT1-1 8-175      601 ACCATGGCTAAGGTTAAGATTACAGCTACTCCTGAAGCTGAGCTCTCAACAAACTATTCA  660
MAT1-1 8-161      601 ACCATGGCTAAGGTTAAGATTACAGCTACTCCTGAAGCTGAGCTCTCAACAAACTATTCA  660
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MAT1-1 Gene CBS   661 GTCGATGATATTGTCAAAAATTGTTACGATACAGGCTATGTATCCCGAGACAAATGCAAG  720
MAT1-1 8-166      661 GTCGATGATATTGTCAAAAATTGTTACGATACAGGCTATGTATCCCGAGACAAATGCAAG  720
MAT1-1 8-175      661 GTCGATGATATTGTCAAAAATTGTTACGATACAGGCTATGTATCCCGAGACAAATGCAAG  720
MAT1-1 8-161      661 GTCGATGATATTGTCAAAAATTGTTACGATACAGGCTATGTATCCCGAGACAAATGCAAG  720
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MAT1-1 Gene CBS  721 AAAAAAACAGACCATAACAAAAACGCGCCTGTTATGGCTTTCGCTGCCCAGCCAACCTTG  780
MAT1-1 8-166     721 AAAAAAACAGACCATAACAAAAACGCGCCTGTTATGGCTTTCGCTGCCCAGCCAACCTTG  780
MAT1-1 8-175     721 AAAAAAACAGACCATAACAAAAACGCGCCTGTTATGGCTTTCGCTGCCCAGCCAACCTTG  780
MAT1-1 8-161     721 AAAAAAACAGACCATAACAAAAACGCGCCTGTTATGGCTTTCGCTGCCCAGCCAACCTTG  780
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MAT1-1 Gene CBS  781 ATTATCCACGAAAATAACAGTATTCAAATTAATGGAAATAATACTGTTGTTACTGGTGAC  840
MAT1-1 8-166     781 ATTATCCACGAAAATAACAGTATTCAAATTAATGGAAATAATACTGTTGTTACTGGTGAC  840
MAT1-1 8-175     781 ATTATCCACGAAAATAACAGTATTCAAATTAATGGAAATAATACTGTTGTTACTGGTGAC  840
MAT1-1 8-161     781 ATTATCCACGAAAATAACAGTATTCAAATTAATGGAAATAATACTGTTGTTACTGGTGAC  840
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MAT1-1 Gene CBS  841 TGCGATGGGAATGCTGCTGTAGAAAGCACATTTCCCCAGAACTTCTCACCAAGTCCTGGT  900
MAT1-1 8-166     841 TGCGATGGGAATGCTGCTGTAGAAAGCACATTTCCCCAGAACTTCTCACCAAGTCCTGGT  900
MAT1-1 8-175     841 TGCGATGGGAATGCTGCTGTAGAAAGCACATTTCCCCAGAACTTCTCACCAAGTCCTGGT  900
MAT1-1 8-161     841 TGCGATGGGAATGCTGCTGTAGAAAGCACATTTCCCCAGAACTTCTCACCAAGTCCTGGT  900
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MAT1-1 Gene CBS  901 GACATGGGAACGATGCTTGCAGAAGCATCGCTTGGTGAATCTGAAGTACTTCACGGCCGT  960
MAT1-1 8-166     901 GACATGGGAACGATGCTTGCAGAAGCATCGCTTGGTGAATCTGAAGTACTTCACGGCCGT  960
MAT1-1 8-175     901 GACATGGGAACGATGCTTGCAGAAGCATCGCTTGGTGAATCTGAAGTACTTCACGGCCGT  960
MAT1-1 8-161     901 GACATGGGAACGATGCTTGCAGAAGCATCGCTTGGTGAATCTGAAGTACTTCACGGCCGT  960
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MAT1-1 Gene CBS  961 GGTTACCAACTCTATGAGCGGGGGCCTATAGTTGCAAACGAATATGAGATTGACGACGGG 1020
MAT1-1 8-166     961 GGTTACCAACTCTATGAGCGGGGGCCTATAGTTGCAAACGAATATGAGATTGACGACGGG 1020
MAT1-1 8-175     961 GGTTACCAACTCTATGAGCGGGGGCCTATAGTTGCAAACGAATATGAGATTGACGACGGG 1020
MAT1-1 8-161     961 GGTTACCAACTCTATGAGCGGGGGCCTATAGTTGCAAACGAATATGAGATTGACGACGGG 1020
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MAT1-1 Gene CBS 1021 ATGCTCAATCTTTGGGGTGAAAACCCAGCTATGTTACCATCATATGTTTCAGCCGGCCAA 1080
MAT1-1 8-166    1021 ATGCTCAATCTTTGGGGTGAAAACCCAGCTATGTTACCATCATATGTTTCAGCCGGCCAA 1080
MAT1-1 8-175    1021 ATGCTCAATCTTTGGGGTGAAAACCCAGCTATGTTACCATCATATGTTTCAGCCGGCCAA 1080
MAT1-1 8-161    1021 ATGCTCAATCTTTGGGGTGAAAACCCAGCTATGTTACCATCATATGTTTCAGCCGGCCAA 1080
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MAT1-1 Gene CBS 1081 TGTACACTTCCCCCGCATGATCCTTGTGTCCCGGATCCGATGGCGAATATCAATATCGAC 1140
MAT1-1 8-166    1081 TGTACACTTCCCCCGCATGATCCTTGTGTCCCGGATCCGATGGCGAATATCAATATCGAC 1140
MAT1-1 8-175    1081 TGTACACTTCCCCCGCATGATCCTTGTGTCCCGGATCCGATGGCGAATATCAATATCGAC 1140
MAT1-1 8-161    1081 TGTACACTTCCCCCGCATGATCCTTGTGTCCCGGATCCGATGGCGAATATCAATATCGAC 1140
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MAT1-1 Gene CBS 1141 CATTACCTCAACCTATGA  1158
MAT1-1 8-166    1141 CATTACCTCAACCTATGAT 1159
MAT1-1 8-175    1141 CATTACCTCAACCTATGA  1158
MAT1-1 8-161    1141 CATTACCTCAACCTATGA  1158
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MAT1-1 no intro   1 MDATTSPLQRAFNAFLLTMPPQQLEELLRYLQDTRAKANNQVAHENEQSTARSKPPLDTN  60
MAT1-1 8-161 no   1 MDATTSPLQRAFNAFLLTMPPQQLEELLRYLQDTRAKANNQVAHENEQSTARSKPPLDTN  60
MAT1-1 8-166 no   1 MDATTSPLQRAFNAFLLTMPPQQLEELLRYLQDTRAKANNQVAHENEQSTARSKPPLDTN  60
MAT1-1 8-175 no   1 MDATTSPLQRAFNAFLLTMPPQQLEELLRYLQDTRAKANNQVAHENEQSTARSKPPLDTN  60
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MAT1-1 no intro  61 NESAVQGSTNQRSHQTRGKRPQDGKRRPLNSFIAFRSYYSVMFPDLTQKAKSGILRFLWQ 120
MAT1-1 8-161 no  61 NESAVQGSTNQRSHQTRGKRPQDGKRRPLNSFIAFRSYYSVMFPDLTQKAKSGILRFLWQ 120
MAT1-1 8-166 no  61 NESAVQGSTNQRSHQTRGKRPQDGKRRPLNSFIAFRSYYSVMFPDLTQKAKSGILRFLWQ 120
MAT1-1 8-175 no  61 NESAVQGSTNQRSHQTRGKRPQDGKRRPLNSFIAFRSYYSVMFPDLTQKAKSGILRFLWQ 120
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MAT1-1 no intro 121 NDPFKAKWAIVAKAYSIIRDDHDNGVSLETFLKLNTNLIGIIEPDRYLDIMGWELNVDGQ 180
MAT1-1 8-161 no 121 NDPFKAKWAIVAKAYSIIRDDHDNGVSLETFLKLNTNLIGIIEPDRYLDIMGWELNVDGQ 180
MAT1-1 8-166 no 121 NDPFKAKWAIVAKAYSIIRDDHDNGVSLETFLKLNANLIGIIEPDRYLDIMGWELNVDGQ 180
MAT1-1 8-175 no 121 NDPFKAKWAIVAKAYSIIRDDHDNGVSLETFLKLNTNLIGIIEPDRYLDIMGWELNVDGQ 180
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MAT1-1 no intro 181 QQYTMAKVKITATPEAELSTNYSVDDIVKNCYDTGYVSRDKCKKKTDHNKNAPVMAFAAQ 240
MAT1-1 8-161 no 181 QQYTMAKVKITATPEAELSTNYSVDDIVKNCYDTGYVSRDKCKKKTDHNKNAPVMAFAAQ 240
MAT1-1 8-166 no 181 QQYTMAKVKITATPEAELSTNYSVDDIVKNCYDTGYVSRDKCKKKTDHNKNAPVMAFAAQ 240
MAT1-1 8-175 no 181 QQYTMAKVKITATPEAELSTNYSVDDIVKNCYDTGYVSRDKCKKKTDHNKNAPVMAFAAQ 240
                    ************************************************************

MAT1-1 no intro 241 PTLIIHENNSIQINGNNTVVTGDCDGNAAVESTFPQNFSPSPGDMGTMLAEASLGESEVL 300
MAT1-1 8-161 no 241 PTLIIHENNSIQINGNNTVVTGDCDGNAAVESTFPQNFSPSPGDMGTMLAEASLGESEVL 300
MAT1-1 8-166 no 241 PTLIIHENNSIQINGNNTVVTGDCDGNAAVESTFPQNFSPSPGDMGTMLAEASLGESEVL 300
MAT1-1 8-175 no 241 PTLIIHENNSIQINGNNTVVTGDCDGNAAVESTFPQNFSPSPGDMGTMLAEASLGESEVL 300
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MAT1-1 no intro 301 HGRGYQLYERGPIVANEYEIDDGMLNLWGENPAMLPSYVSAGQCTLPPHDPCVPDPMANI 360
MAT1-1 8-161 no 301 HGRGYQLYERGPIVANEYEIDDGMLNLWGENPAMLPSYVSAGQCTLPPHDPCVPDPMANI 360
MAT1-1 8-166 no 301 HGRGYQLYERGPIVANEYEIDDGMLNLWGENPAMLPSYVSAGQCTLPPHDPCVPDPMANI 360
MAT1-1 8-175 no 301 HGRGYQLYERGPIVANEYEIDDGMLNLWGENPAMLPSYVSAGQCTLPPHDPCVPDPMANI 360
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MAT1-1 no intro 361 NIDHYLNL  368
MAT1-1 8-161 no 361 NIDHYLNL  368
MAT1-1 8-166 no 361 NIDHYLNL* 369
MAT1-1 8-175 no 361 NIDHYLNL  368
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Appendix 11: *sclB* Multiple Sequence Alignments

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SclB from ATG     1 ATGCAATCATTAGTCCTTCCTCCTTCTTCCTTCATCGCTACAGAGTTTGGACACCCCAGG   60
SclB 8-160        1 ATGCAATCATTAGTCCTTCCTCCTTCTTCCTTCATCGCTACAGAGTTTGGACACCCCAGG   60
SclB 8-161        1 ATGCAATCATTAGTCCTTCCTCCTTCTTCCTTCATCGCTACAGAGTTTGGACACCCCAGG   60
sclB 8-162        1 ATGCAATCATTAGTCCTTCCTCCTTCTTCCTTCATCGCTACAGAGTTTGGACACCCCAGG   60
sclB 8-166        1 ATGCAATCATTAGTCCTTCCTCCTTCTTCCTTCATCGCTACAGAGTTTGGACACCCCAGG   60
sclB 8-169        1 ATGCAATCATTAGTCCTTCCTCCTTCTTCCTTCATCGCTACAGAGTTTGGACACCCCAGG   60
sclB 8-175        1 ATGCAATCATTAGTCCTTCCTCCTTCTTCCTTCATCGCTACAGAGTTTGGACACCCCAGG   60
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SclB from ATG    61 TTTGATCCGGGCCCGGAGCGGCTGTCGTTGAATTTGCCTCGGTCGACAAATGCTCGTCGA  120
SclB 8-160        61 TTTGATCCGGGCCCGGAGCGGCTGTCGTTGAATTTGCCTCGGTCGACAAATGCTCGTCGA  120
SclB 8-161        61 TTTGATCCGGGCCCGGAGCGGCTGTCGTTGAATTTGCCTCGGTCGACAAATGCTCGTCGA  120
sclB 8-162        61 TTTGATCCGGGCCCGGAGCGGCTGTCGTTGAATTTGCCTCGGTCGACAAATGCTCGTCGA  120
sclB 8-166        61 TTTGATCCGGGCCCGGAGCGGCTGTCGTTGAATTTGCCTCGGTCGACAAATGCTCGTCGA  120
sclB 8-169        61 TTTGATCCGGGCCCGGAGCGGCTGTCGTTGAATTTGCCTCGGTCGACAAATGCTCGTCGA  120
sclB 8-175        61 TTTGATCCGGGCCCGGAGCGGCTGTCGTTGAATTTGCCTCGGTCGACAAATGCTCGTCGA  120
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SclB from ATG   121 TATCCCCCGCGGGATCTGCCTCTCCCTCGCTCTATGTCCGGTTCGGTTCCAGCAGACGAC  180
SclB 8-160       121 TATCCCCCGCGGGATCTGCCTCTCCCTCGCTCTATGTCCGGTTCGGTTCCAGCAGACGAC  180
SclB 8-161       121 TATCCCCCGCGGGATCTGCCTCTCCCTCGCTCTATGTCCGGTTCGGTTCCAGCAGACGAC  180
sclB 8-162       121 TATCCCCCGCGGGATCTGCCTCTCCCTCGCTCTATGTCCGGTTCGGTTCCAGCAGACGAC  180
sclB 8-166       121 TATCCCCCGCGGGATCTGCCTCTCCCTCGCTCTATGTCCGGTTCGGTTCCAGCAGACGAC  180
sclB 8-169       121 TATTCCCCGCGGGATCTGCCTCTCCCTCGCTCTATGTCCGGTTCGGTTCCAGCAGACGAC  180
sclB 8-175       121 TATCCCCCGCGGGATCTGCCTCTCCCTCGCTCTATGTCCGGTTCGGTTCCAGCAGACGAC  180
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SclB from ATG   181 CCGCTGGACACTTCTGGACCCGTCAGGCGCCCCGGACATCCAGAGCTGCCCCAAGCCGCG  240
SclB 8-160       181 CCGCTGGACACTTCTGGACCCGTCAGGCGCCCCGGACATCCAGAGCTGCCCCAAGCCGCG  240
SclB 8-161       181 CCGCTGGACACTTCTGGACCCGTCAGGCGCCCCGGACATCCAGAGCTGCCCCAAGCCGCG  240
sclB 8-162       181 CCGCTGGACACTTCTGGACCCGTCAGGCGCCCCGGACATCCAGAGCTGCCCCAAGCCGCG  240
sclB 8-166       181 CCGCTGGACACTTCTGGACCCGTCAGGCGCCCCGGACATCCAGAGCTGCCCCAAGCCGCG  240
sclB 8-169       181 CCGCTGGACACTTCTGGACCCGTCAGGCGCCCCGGACATCCAGAGCTGCCCCAAGCCGCG  240
sclB 8-175       181 CCGCTGGACACTTCTGGACCCGTCAGGCGCCCCGGACATCCAGAGCTGCCCCAAGCCGCG  240
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SclB from ATG   241 ACAACGGTGACCGCTGCAACCTCTGTATCCGCGGGGCTGTCAGGACCGGTCCTGCCTCCA  300
SclB 8-160       241 ACAACGGTGACCGCTGCAACCTCTGTATCCGCGGGGCTGTCAGGACCGGTCCTGCCTCCA  300
SclB 8-161       241 ACAACGGTGACCGCTGCAACCTCTGTATCCGCGGGGCTGTCAGGACCGGTCCTGCCTCCA  300
sclB 8-162       241 ACAACGGTGACCGCTGCAACCTCTGTATCCGCGGGGCTGTCAGGACCGGTCCTGCCTCCA  300
sclB 8-166       241 ACAACGGTGACCGCTGCAACCTCTGTATCCGCGGGGCTGTCAGGACCGGTCCTGCCTCCA  300
sclB 8-169       241 ACAACGGTGACCGCTGCAACCTCTGTATCCGCGGGGCTGTCAGGACCGGTCCTGCCTCCA  300
sclB 8-175       241 ACAACGGTGACCGCTGCAACCTCTGTATCCGCGGGGCTGTCAGGACCGGTCCTGCCTCCA  300
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SclB from ATG   301 GGATCTGCTGGCGCCGTTACCACTCACGAGTCTGTGACCCAACGTGTGGCCCCGGCCTCA  360
SclB 8-160       301 GGATCTGCTGGCGCCGTTACCACTCACGAGTCTGTGACCCAACGTGTGGCCCCGGCCTCA  360
SclB 8-161       301 GGATCTGCTGGCGCCGTTACCACTCACGAGTCTGTGACCCAACGTGTGGCCCCGGCCTCA  360
sclB 8-162       301 GGATCTGCTGGCGCCGTTACCACTCACGAGTCTGTGACCCAACGTGTGGCCCCGGCCTCA  360
sclB 8-166       301 GGATCTGCTGGCGCCGTTACCACTCACGAGTCTGTGACCCAACGTGTGGCCCCGGCCTCA  360
sclB 8-169       301 GGATCTGCTGGCGCCGTTACCACTCACGAGTCTGTGACCCAACGTGTGGCCCCGGCCTCA  360
sclB 8-175       301 GGATCTGCTGGCGCCGTTACCACTCACGAGTCTGTGACCCAACGTGTGGCCCCGGCCTCA  360
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SclB from ATG   361 GCCGACGAAGTGCTGCGACAACCCTTTCCCGTGGGCGATGCCTTCGCCTCATCGCGACTG  420
SclB 8-160       361 GCCGACGAAGTGCTGCGACAACCCTTTCCCGTGGGCGATGCCTTCGCCTCATCGCGACTG  420
SclB 8-161       361 GCCGACGAAGTGCTGCGACAACCCTTTCCCGTGGGCGATGCCTTCGCCTCATCGCGACTG  420
sclB 8-162       361 GCCGACGAAGTGCTGCGACAACCCTTTCCCGTGGGCGATGCCTTCGCCTCATCGCGACTG  420
sclB 8-166       361 GCCGACGAAGTGCTGCGACAACCCTTTCCCGTGGGCGATGCCTTCGCCTCATCGCGACTG  420
sclB 8-169       361 GCCGACGAAGTGCTGCGACAACCCTTTCCCGTGGGCGATGCCTTCGCCTCATCGCGACTG  420
sclB 8-175       361 GCCGACGAAGTGCTGCGACAACCCTTTCCCGTGGGCGATGCCTTCGCCTCATCGCGACTG  420
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SclB from ATG   421 CCACCCTCGCTGGTAGGCCAAGGCATCTCGCAAGCTACCGCTACCGCTTATGCGCAACCT  480
SclB 8-160       421 CCACCCTCGCTGGTAGGCCAAGGCATCCCGCAAGCTACCGCTACCGCTTATGCGCAACCT  480
SclB 8-161       421 CCACCCTCGCTGGTAGGCCAAGGCATCCCGCAAGCTACCGCTACCGCTTATGCGCAACCT  480
sclB 8-162       421 CCACCCTCGCTGGTAGGCCAAGGCATCCCGCAAGCTACCGCTACCGCTTATGCGCAACCT  480
sclB 8-166       421 CCACCCTCGCTGGTAGGCCAAGGCATCCCGCAAGCTACCGCTACCGCTTATGCGCAACCT  480
sclB 8-169       421 CCACCCTCGCTGGTAGGCCAAGGCATCCCGCAAGCTACCGCTACCGCTTATGCGCAACCT  480
sclB 8-175       421 CCACCCTCGCTGGTAGGCCAAGGCATCCCGCAAGCTACCGCTACCGCTTATGCGCAACCT  480
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SclB from ATG   481 TCCTTTGGAACTTCACCTCCCGGTACAACGGCGCGGGCTTTGCCGCAAAAGCCCACCCGA  540
SclB 8-160      481 TCCTTTGGAACTTCACCTCCCGGTACAACGGCGCGGGCTTTGCCGCAAAAGCCCACCCGA  540
SclB 8-161      481 TCCTTTGGAACTTCACCTCCCGGTACAACGGCGCGGGCTTTGCCGCAAAAGCCCACCCGA  540
sclB 8-162      481 TCCTTTGGAACTTCACCTCCCGGTACAACGGCGCGGGCTTTGCCGCAAAAGCCCACCCGA  540
sclB 8-166      481 TCCTTTGGAACTTCACCTCCCGGTACAACGACGCGGGCTTTGCCGCAAAAGCCCACCCGA  540
sclB 8-169      481 TCCTTTGGAACTTCACCTCCCGGTACAACGGCGCGGGCTTTGCCGCAAAAGCCCACCCGA  540
sclB 8-175      481 TCCTTTGGAACTTCACCTCCCGGTACAACGGCGCGGGCTTTGCCGCAAAAGCCCACCCGA  540
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SclB from ATG   541 CGCACCAAAGCTCATGTTGCATCAGCATGCGTTAACTGTAAAAAGAAGCACCTAGGGTGC  600
SclB 8-160      541 CGCACCAAAGCTCATGTTGCATCAGCATGCGTTAACTGTAAAAAGAAGCACCTAGGGTGC  600
SclB 8-161      541 CGCACCAAAGCTCATGTTGCATCAGCATGCGTTAACTGTAAAAAGAAGCACCTAGGGTGC  600
sclB 8-162      541 CGCACCAAAGCTCATGTTGCATCAGCATGCGTTAACTGTAAAAAGAAGCACCTAGGGTGC  600
sclB 8-166      541 CGCACCAAAGCTCATGTTGCATCAGCATGCGTTAACTGTAAAAAGAAGCACCTAGGGTGC  600
sclB 8-169      541 CGCACCAAAGCTCATGTTGCATCAGCATGCGTTAACTGTAAAAAGAAGCACCTAGGGTGC  600
sclB 8-175      541 CGCACCAAAGCTCATGTTGCATCAGCATGCGTTAACTGTAAAAAGAAGCACCTAGGGTGC  600
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SclB from ATG   601 GATCCGGCGCGGCCATGTCGAAGATGCGTTCTGTCAGGGAAAGAAGTGAGTATCATCATA  660
SclB 8-160      601 GATCCGGCGCGGCCATGTCGAAGATGCGTTCTGTCAGGGAAAGAAGTGAGTATCATCATA  660
SclB 8-161      601 GATCCGGCGCGGCCATGTCGAAGATGCGTTCTGTCAGGGAAAGAAGTGAGTATCATCATA  660
sclB 8-162      601 GATCCGGCGCGGCCATGTCGAAGATGCGTTCTGTCAGGGAAAGAAGTGAGTATCATCATA  660
sclB 8-166      601 GATCCGGCGCGGCCATGTCGAAGATGCGTTCTGTCAGGGAAAGAAGTGAGTATCATCATA  660
sclB 8-169      601 GATCCGGCGCGGCCATGTCGAAGATGCGTTCTGTCAGGGAAAGAAGTGAGTATCATCATA  660
sclB 8-175      601 GATCCGGCGCGGCCATGTCGAAGATGCGTTCTGTCAGGGAAAGAAGTGAGTATCATCATA  660
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SclB from ATG   661 TTTCTTTGGTCGCTAGCCTATCTGAACGTGAAGGGGGTGGCATCTGGTCTCTGACTACCC  720
SclB 8-160      661 TTTCTTTGGTCGCTAGCCCATCTGAACGTGAAGGGGGTGGCATCTGGTCTCTGACTACCC  720
SclB 8-161      661 TTTCTTTGGTCGCTAGCCTATCTGAACGTGAAGGGGGTGGCATCTGGTCTCTGACTACCC  720
sclB 8-162      661 TTTCTTTGGTCGCTAGCCTATCTGAACGTGAAGGGGGTGGCATCTGGTCTCTGACTACCC  720
sclB 8-166      661 TTTCTTTGGTCGTTAGCCTATCTGAACGTGAAGGGGGTGGCATCTGGTCTCTGACTACCC  720
sclB 8-169      661 TTTCTTTGGTCGCTAGCCTATCTGAACGTGAAGGGGGTGGCATCTGGTCTCTGACTACCC  720
sclB 8-175      661 TTTCTTTGGTCGCTAGCCTATCTGAACGTGAAGGGGGTGGCATCTGGTCTCTGACTACCC  720
                    *********** ***** ****************************************

SclB from ATG   721 CCAAGAAAAGGCAGAAACTTCATTTTGCACTGCTATTATGAACTATTACTGACATGAACA  780
SclB 8-160      721 CCAAGAAAAGGCAGAAACTTCATTTTGCACTGCTATTATGAACTATTACTGACATGAACA  780
SclB 8-161      721 CCAAGAAAAGGCAGAAACTTCATTTTGCACTGCTATTATGAACTATTACTGACATGAACA  780
sclB 8-162      721 CCAAGAAAAGGCAGAAACTTCATTTTGCACTGCTATTATGAACTATTACTGACATGAACA  780
sclB 8-166      721 CCAGGAAAAGGCAGAAACTTCATTTTGCACTGCTATTATGAACTATTACTGACATGAACA  780
sclB 8-169      721 CCAAGAAAAGGCAGAAACTTCATTTTGCACTGCTATTATGAACTATTACTGACATGAACA  780
sclB 8-175      721 CCAAGAAAAGGCAGAAACTTCATTTTGCACTGCTATTATGAACTATTACTGACATGAACA  780
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SclB from ATG   781 GGCCACCTGTGTTGATGTTACGCACAAGAAGCGAGGAAGGCCGCCATTGAAGGCGGAAGA  840
SclB 8-160      781 GGCCACCTGTGTTGATGTTACGCACAAGAAGCGAGGAAGGCCGCCATTGAAGGCGGAAGA  840
SclB 8-161      781 GGCCACCTGTGTTGATGTTACGCACAAGAAGCGAGGAAGGCCGCCATTGAAGGCGGAAGA  840
sclB 8-162      781 GGCCACCTGTGTTGATGTTACGCACAAGAAGCGAGGAAGGCCGCCATTGAAGGCGGAAGA  840
sclB 8-166      781 GGCCACCTGTGTTGATGTTACGCACAAGAAGCGAGGAAGGCCGCCATTGAAGGCGGAAGA  840
sclB 8-169      781 GGCCACCTGTGTTGATGTTACGCACAAGAAGCGAGGAAGGCCGCCATTGAAGGCGGAAGA  840
sclB 8-175      781 GGCCACCTGTGTTGATGTTACGCACAAGAAGCGAGGAAGGCCGCCATTGAAGGCGGAAGA  840
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SclB from ATG   841 AGCATCGCTTAGAACCTATGCAGCTCATATGGACAATCGAGCGACACAAGGAGAGCAGCA  900
SclB 8-160      841 AGCATCGCTTAGAACCTATGCAGCTCATATGGACAATCGAGCGACACAAGGAGAGCAGCA  900
SclB 8-161      841 AGCATCGCTTAGAACCTATGCAGCTCATATGGACAATCGAGCGACACAAGGAGAGCAGCA  900
sclB 8-162      841 AGCATCGCTTAGAACCTATGCAGCTCATATGGACAATCGAGCGACACAAGGAGAGCAGCA  900
sclB 8-166      841 AGCATCGCTTAGAACCTATGCAGCTCATATGGACAATCGAGCGACACAAGGAGAGCAGCA  900
sclB 8-169      841 AGCATCGCTTAGAACCTATGCAGCTCATATGGACAATCGAGCGACACAAGGAGAGCAGCA  900
sclB 8-175      841 AGCATCGCTTAGAACCTATGCAGCTCATATGGACAATCGAGCGACACAAGGAGAGCAGCA  900
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SclB from ATG   901 CGGCCCCCAATCACGACGCACCCTACACAGAGCTACATCTTCCCGTGAGATTCGCCCCAT  960
SclB 8-160      901 CGGCCCCCAATCACGACGCACCCTACACAGAGCTACATCTTCCCGTGAGATTCGCCCCAT  960
SclB 8-161      901 CGGCCCCCAATCACGACGCACCCTACACAGAGCTACATCTTCCCGTGAGATTCGCCCCAT  960
sclB 8-162      901 CGGCCCCCAATCACGACGCACCCTACACAGAGCTACATCTTCCCGTGAGATTCGCCCCAT  960
sclB 8-166      901 CGGCCCCCAATCACGACGCACCCTACACAGAGCTACATCTTCCCGTGAGATTCGCCCCAT  960
sclB 8-169      901 CGGCCCCCAATCACGACGCACCCTACACAGAGCTACATCTTCCCGTGAGATTCGCCCCAT  960
sclB 8-175      901 CGGCCCCCAATCACGACGCACCCTACACAGAGCTACATCTTCCCGTGAGATTCGCCCCAT  960
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SclB from ATG   961 GACAGATCTTCAAATGCCTGGGGCGCAGACGGGCGCCATGGCCATGAGAGCGTCGGCTGG 1020
SclB 8-160      961 GACAGATCTTCAAATGCCTGGGGCGCAGACGGGCGCCATGGCCATGAGAGCGTCGGCTGG 1020
SclB 8-161      961 GACAGATCTTCAAATGCCTGGGGCGCAGACGGGCGCCATGGCCATGAGAGCGTCGGCTGG 1020
sclB 8-162      961 GACAGATCTTCAAATGCCTGGGGCGCAGACGGGCGCCATGGCCATGAGAGCGTCGGCTGG 1020
sclB 8-166      961 GACAGATCTTCAAATGCCTGGGGCGCAGACGGGCGCCATGGCCATGAGAGCGTCGGCTGG 1020
sclB 8-169      961 GACAGATCTTCAAATGCCTGGGGCGCAGACGGGCGCCATGGCCATGAGAGCGTCGGCTGG 1020
sclB 8-175      961 GACAGATCTTCAAATGCCTGGGGCGCAGACGGGCGCCATGGCCATGAGAGCGTCGGCTGG 1020
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SclB from ATG  1021 GCATCCACAGAGATGGGCCGCTCCCGTATACTCGCAGGCCATAGACCCGTCGATAATGCA 1080
SclB 8-160     1021 GCATCCGCAGAGATGGGCCGCTCCCGTATACTCGCAGGCCATAGACCCGTCGATAATGCA 1080
SclB 8-161     1021 GCATCCGCAGAGATGGGCCGCTCCCGTATACTCGCAGGCCATAGACCCGTCGATAATGCA 1080
sclB 8-162     1021 GCATCCGCAGAGATGGGCCGCTCCCGTATACTCGCAGGCCATAGACCCGTCGATAATGCA 1080
sclB 8-166     1021 GCATCCACAGAGATGGGCCGCTCCCGTATACTCGCAGGCCATAGACCCGTCGATAATGCA 1080
sclB 8-169     1021 GCATCCGCAGAGATGGGCCGCTCCCGTATACTCGCAGGCCATAGACCCGTCGATAATGCA 1080
sclB 8-175     1021 GCATCCGCAGAGATGGGCCGCTCCCGTATACTCGCAGGCCATAGACCCGTCGATAATGCA 1080
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SclB from ATG  1081 GCGCAGTGTTGGGCACAGACGGTTTTCTTCTTCTGGCTCTGCACAGTCCATAACAGCGGC 1140
SclB 8-160     1081 GCGCAGTGTTGGGCACAGACGGTTTTCTTCTTCTGGCTCAGCACAGTCCATAACAGCGGC 1140
SclB 8-161     1081 GCGCAGTGTTGGGCACAGACGGTTTTCTTCTTCTGGCTCAGCACAGTCCATAACAGCGGC 1140
sclB 8-162     1081 GCGCAGTGTTGGGCACAGACGGTTTTCTTCTTCTGGCTCAGCACAGTCCATAACAGCGGC 1140
sclB 8-166     1081 GCGCAGTGTTGGGCACAGACGGTTTTCTTCTTCTGGCTCTGCACAGTCCATAACAGCGGC 1140
sclB 8-169     1081 GCGCAGTGTTGGGCACAGACGGTTTTCTTCTTCTGGCTCAGCACAGTCCATAACAGCGGC 1140
sclB 8-175     1081 GCGCAGTGTTGGGCACAGACGGTTTTCTTCTTCTGGCTCAGCACAGTCCATAACAGCGGC 1140
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SclB from ATG  1141 TTCTCCACCCGGTTATGTCCCGATGCCTGTTGGGTACAACCCAGCCTTGGGAGGGCAACG 1200
SclB 8-160     1141 TTCTCCACCCGGTTATGTCCCGATGCCTGTTGGGTACAACCCAGCCTTGGGAGGGCAACG 1200
SclB 8-161     1141 TTCTCCACCCGGTTATGTCCCGATGCCTGTTGGGTACAACCCAGCCTTGGGAGGGCAACG 1200
sclB 8-162     1141 TTCTCCACCCGGTTATGTCCCGATGCCTGTTGGGTACAACCCAGCCTTGGGAGGGCAACG 1200
sclB 8-166     1141 TTCTCCACCCGGTTATGTCCCGATGCCTGTTGGGTACAACCCAGCCTTGGGAGGGCAACG 1200
sclB 8-169     1141 TTCTCCACCCGGTTATGTCCCGATGCCTGTTGGGTACAACCCAGCCTTGGGAGGGCAACG 1200
sclB 8-175     1141 TTCTCCACCCGGTTATGTCCCGATGCCTGTTGGGTACAACCCAGCCTTGGGAGGGCAACG 1200
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SclB from ATG  1201 GATGCCTATGGGCATGGGAAGACCACTGTCATCCTACACGCATCAAGGCATGAACCCTAC 1260
SclB 8-160     1201 GATGCCTATGGGCATGGGAAGACCACTGTCATCCTACACGCATCAAGGCATGAACCCTAC 1260
SclB 8-161     1201 GATGCCTATGGGCATGGGAAGACCACTGTCATCCTACACGCATCAAGGCATGAACCCTAC 1260
sclB 8-162     1201 GATGCCTATGGGCATGGGAAGACCACTGTCATCCTACACGCATCAAGGCATGAACCCTAC 1260
sclB 8-166     1201 GATGCCTATGGGCATGGGAAGACCACTGTCATCCTACACGCATCAAGGCATGAACCCTAC 1260
sclB 8-169     1201 GATGCCTATGGGCATGGGAAGACCACTGTCATCCTACACGCATCAAGGCATGAACCCTAC 1260
sclB 8-175     1201 GATGCCTATGGGCATGGGAAGACCACTGTCATCCTACACGCATCAAGGCATGAACCCTAC 1260
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SclB from ATG  1261 TACCACGCCGCCCCAGTATCAGCAATCCTTTGTCCCAATTTCGCCCTATCCAGAAAGCGC 1320
SclB 8-160     1261 TACCACGCCGCCCCAGTATCAGCAATCCTTTGTCCCAATTTCGCCCTATCCAGAAAGCGC 1320
SclB 8-161     1261 TACCACGCCGCCCCAGTATCAGCAATCCTTTGTCCCAATTTCGCCCTATCCAGAAAGCGC 1320
sclB 8-162     1261 TACCACGCCGCCCCAGTATCAGCAATCCTTTGTCCCAATTTCGCCCTATCCAGAAAGCGC 1320
sclB 8-166     1261 TACCACGCCGCCCCAGTATCAGCAATCCTTTGTCCCAATTTCGCCCTATCCAGAAAGCGC 1320
sclB 8-169     1261 TACCACGCCGCCCCAGTATCAGCAATCCTTTGTCCCAATTTCGCCCTATCCAGAAAGCGC 1320
sclB 8-175     1261 TACCACGCCGCCCCAGTATCAGCAATCCTTTGTCCCAATTTCGCCCTATCCAGAAAGCGC 1320
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SclB from ATG  1321 CCGGATGTCTAATCGGATGCCAATGGGAGAATCTCCAATGTCTAGGGATCCGCGGGAAGG 1380
SclB 8-160     1321 CCGGATGTCTAATCGGATGCCAATGGGAGAATCTCCAATGTCTAGGGATCCGCGGGAAGG 1380
SclB 8-161     1321 CCGGATGTCTAATCGGATGCCAATGGGAGAATCTCCAATGTCTAGGGATCCGCGGGAAGG 1380
sclB 8-162     1321 CCGGATGTCTAATCGGATGCCTATGGGAGAATCTCCAATGTCTAGGGATCCGCGGGAAGG 1380
sclB 8-166     1321 CCGGATGTCTAATCGGATGCCAATGGGAGAATCTCCAATGTCTAGGGATCCGCGGGAAGG 1380
sclB 8-169     1321 CCGGATGTCTAATCGGATGCCAATGGGAGAATCTCCAATGTCTAGGGATCCGCGGGAAGG 1380
sclB 8-175     1321 CCGGATGTCTAATCGGATGCCAATGGGAGAATCTCCAATGTCTAGGGATCCGCGGGAAGG 1380
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SclB from ATG  1381 CTATTTGGAATCTCCAGTAAGACTTCCGCCCATTTACCCTCCCACGATGGGAACACCGGC 1440
SclB 8-160     1381 CTATTTGGAATCTCCAGTAAGACTTCCGCCCATTTACCCTCCCACGATGGGAACACCGGC 1440
SclB 8-161     1381 CTATTTGGAATCTCCAGTAAGACTTCCGCCCATTTACCCTCCCACGATGGGAACACCGGC 1440
sclB 8-162     1381 CTATTTGGAATCTCCAGTAAGACTTCCGCCCATTTACCCTCCCACGATGGGAACACCGGC 1440
sclB 8-166     1381 CTATTTGGAATCTCCAGTAAGACTTCCGCCCATTTACCCTCCCACGATGGGAACACCGGC 1440
sclB 8-169     1381 CTATTTGGAATCTCCAGTAAGACTTCCGCCCATTTACCCTCCCACGATGGGAACACCGGC 1440
sclB 8-175     1381 CTATTTGGAATCTCCAGTAAGACTTCCGCCCATTTACCCTCCCACGATGGGAACACCGGC 1440
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SclB from ATG  1441 CTCAACGTCGCAAGGGCATCGCCTGAGCGATCCGTACCCAGGAGCTTGGTCGCCGCGGAC 1500
SclB 8-160     1441 CTCAACGTCGCAAGGGCATCGCCTGAGCGATCCGTACCCAGGAGCTTGGTCGCCGCGGAC 1500
SclB 8-161     1441 CTCAACGTCGCAAGGGCATCGCCTGAGCGATCCGTACCCAGGAGCTTGGTCGCCGCGGAC 1500
sclB 8-162     1441 CTCAACGTCGCAAGGGCATCGCCTGAGCGATCCGTACCCAGGAGCTTGGTCGCCGCGGAC 1500
sclB 8-166     1441 CTCAACGTCGCAAGGGCATCGCCTGAGCGATCCGTACCCAGGAGCTTGGTCGCCGCGGAC 1500
sclB 8-169     1441 CTCAACGTCGCAAGGGCATCGCCTGAGCGATCCGTACCCAGGAGCTTGGTCGCCGCGGAC 1500
sclB 8-175     1441 CTCAACGTCGCAAGGGCATCGCCTGAGCGATCCGTACCCAGGAGCTTGGTCGCCGCGGAC 1500
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SclB from ATG  1501 GCGGGAAGAATTTCTTCAGCAGGAGCACCGGCAACAAATGCCCTCGCATGGCTTCATCGA 1560
SclB 8-160     1501 GCGGGAAGAATTTCTTCAGCAGGAGCACCGGCAACAAATGCCCTCGCATGGCTTCATCGA 1560
SclB 8-161     1501 GCGGGAAGAATTTCTTCAGCAGGAGCACCGGCAACAAATGCCCTCGCATGGCTTCATCGA 1560
sclB 8-162     1501 GCGGGAAGAATTTCTTCAGCAGGAGCACCGGCAACAAATGCCCTCGCATGGCTTCATCGA 1560
sclB 8-166     1501 GCGGGAAGAATTTCTTCAGCAGGAGCACCGGCAACAAATGCCCTCGCATGGCTTCATCGA 1560
sclB 8-169     1501 GCGGGAAGAATTTCTTCAGCAGGAGCACCGGCAACAAATGCCCTCGCATGGCTTCATCGA 1560
sclB 8-175     1501 GCGGGAAGAATTTCTTCAGCAGGAGCACCGGCAACAAATGCCCTCGCATGGCTTCATCGA 1560
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SclB from ATG  1561 CCCACTATCCCCTAGCAGTCAAATGCGACACGCCGCATCTGACATGGGGTATGGGGAGCC 1620
SclB 8-160     1561 CCCACTATCCCCTAGCAGTCAAATGCGACACGCCGCATCTGACATGGGGTATGGGGAGCC 1620
SclB 8-161     1561 CCCACTATCCCCTAGCAGTCAAATGCGACACGCCGCATCTGACATGGGGTATGGGGAGCC 1620
sclB 8-162     1561 CCCACTATCCCCTAGCAGTCAAATGCGACACGCCGCATCTGACATGGGGTATGGGGAGCC 1620
sclB 8-166     1561 CCCACTATCCCCTAGCAGTCAAATGCGACACGCTGCATCTGACATGGGGTATGGGGAGCC 1620
sclB 8-169     1561 CCCACTATCCCCTAGCAGTCAAATGCGACACGCCGCATCTGACATGGGGTATGGGGAGCC 1620
sclB 8-175     1561 CCCACTATCCCCTAGCAGTCAAATGCGACACGCCGCATCTGACATGGGGTATGGGGAGCC 1620
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SclB from ATG  1621 CGTCCCTCGGCAATTAGGACCTGGCTCAACTGCAGAGAGACACGCAATGCACATGTCGCT 1680
SclB 8-160     1621 CGTCCCTCGGCAATTAGGACCTGGCTCAACTGCAGAGAGACACGCAATGCACATGTCGCT 1680
SclB 8-161     1621 CGTCCCTCGGCAATTAGGACCTGGCTCAACTGCAGAGAGACACGCAATGCACATGTCGCT 1680
sclB 8-162     1621 CGTCCCTCGGCAATTAGGACCTGGCTCAACTGCAGAGAGACACGCAATGCACATGTCGCT 1680
sclB 8-166     1621 CGTCCCTCGGCAATTTGGACCTGGCTCAACTGCAGAGAGACACGCAATGCACATGTCGCT 1680
sclB 8-169     1621 CGTCCCTCGGCAATTAGGACCTGGCTCAACTGCAGAGAGACACGCAATGCACATGTCGCT 1680
sclB 8-175     1621 CGTCCCTCGGCAATTAGGACCTGGCTCAACTGCAGAGAGACACGCAATGCACATGTCGCT 1680
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SclB from ATG  1681 TGTACCTGCCCCCGATGAACCACCAACGACCGAGGCGGACACTGAGGGCAGCAGGCCAGC 1740
SclB 8-160     1681 TGTACCTGCCCCCGATGAACCACCAACGACCGAGGCGGACACTGAGGGCAGCAGGCCAGC 1740
SclB 8-161     1681 TGTACCTGCCCCCGATGAACCACCAACGACCGAGGCGGACACTGAGGGCAGCAGGCCAGC 1740
sclB 8-162     1681 TGTACCTGCCCCCGATGAACCACCAACGACCGAGGCGGACACTGAGGGCAGCAGGCCAGC 1740
sclB 8-166     1681 TGTACCTGCCCCCGATGAACCACCAACGACCGAGGCGGACACTGAGGGCAGCAGGCCAGC 1740
sclB 8-169     1681 TGTACCTGCCCCCGATGAACCACCAACGACCGAGGCGGACACTGAGGGCAGCAGGCCAGC 1740
sclB 8-175     1681 TGTACCTGCCCCCGATGAACCACCAACGACCGAGGCGGACACTGAGGGCAGCAGGCCAGC 1740
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SclB from ATG  1741 CAAGCGGCGCAAAATGGCCTTGGACGACATGGTGAACGACTAA 1783
SclB 8-160     1741 CAAGCGGCGCAAAATGGCCTTGGACGACATGGTGAACGACTAA 1783
SclB 8-161     1741 CAAGCGGCGCAAAATGGCCTTGGACGACATGGTGAACGACTAA 1783
sclB 8-162     1741 CAAGCGGCGCAAAATGGCCTTGGACGACATGGTGAACGACTAA 1783
sclB 8-166     1741 CAAGCGGCGCAAAATGGCCTTGGACGACATGGTGAACGACTAA 1783
sclB 8-169     1741 CAAGCGGCGCAAAATGGCCTTGGACGACATGGTGAACGACTAA 1783
sclB 8-175     1741 CAAGCGGCGCAAAATGGCCTTGGACGACATGGTGAACGACTAA 1783
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SclB from ATG n    1 MQSLVLPPSSFIATEFGHPRFDPGPERLSLNLPRSTNARRYPPRDLPLPRSMSGSVPADD  60
sclB 8-166 no i    1 MQSLVLPPSSFIATEFGHPRFDPGPERLSLNLPRSTNARRYPPRDLPLPRSMSGSVPADD  60
sclB 8-169 no i    1 MQSLVLPPSSFIATEFGHPRFDPGPERLSLNLPRSTNARRYSPRDLPLPRSMSGSVPADD  60
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SclB from ATG n   61 PLDTSGPVRRPGHPELPQAATTVTAATSVSAGLSGPVLPPGSAGAVTTHESVTQRVAPAS 120
sclB 8-166 no i   61 PLDTSGPVRRPGHPELPQAATTVTAATSVSAGLSGPVLPPGSAGAVTTHESVTQRVAPAS 120
sclB 8-169 no i   61 PLDTSGPVRRPGHPELPQAATTVTAATSVSAGLSGPVLPPGSAGAVTTHESVTQRVAPAS 120
                     ************************************************************

SclB from ATG n  121 ADEVLRQPFPVGDAFASSRLPPSLVGQGISQATATAYAQPSFGTSPPGTTARALPQKPTR 180
sclB 8-166 no i  121 ADEVLRQPFPVGDAFASSRLPPSLVGQGIPQATATAYAQPSFGTSPPGTTTRALPQKPTR 180
sclB 8-169 no i  121 ADEVLRQPFPVGDAFASSRLPPSLVGQGIPQATATAYAQPSFGTSPPGTTARALPQKPTR 180
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SclB from ATG n  181 RTKAHVASACVNCKKKHLGCDPARPCRRCVLSGKEATCVDVTHKKRGRPPLKAEEASLRT 240
sclB 8-166 no i  181 RTKAHVASACVNCKKKHLGCDPARPCRRCVLSGKEATCVDVTHKKRGRPPLKAEEASLRT 240
sclB 8-169 no i  181 RTKAHVASACVNCKKKHLGCDPARPCRRCVLSGKEATCVDVTHKKRGRPPLKAEEASLRT 240
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SclB from ATG n  241 YAAHMDNRATQGEQHGPQSRRTLHRATSSREIRPMTDLQMPGAQTGAMAMRASAGHPQRW 300
sclB 8-166 no i  241 YAAHMDNRATQGEQHGPQSRRTLHRATSSREIRPMTDLQMPGAQTGAMAMRASAGHPQRW 300
sclB 8-169 no i  241 YAAHMDNRATQGEQHGPQSRRTLHRATSSREIRPMTDLQMPGAQTGAMAMRASAGHPQRW 300
                     ************************************************************

SclB from ATG n  301 AAPVYSQAIDPSIMQRSVGHRRFSSSGSAQSITAASPPGYVPMPVGYNPALGGQRMPMGM 360
sclB 8-166 no i  301 AAPVYSQAIDPSIMQRSVGHRRFSSSGSAQSITAASPPGYVPMPVGYNPALGGQRMPMGM 360
sclB 8-169 no i  301 AAPVYSQAIDPSIMQRSVGHRRFSSSGSAQSITAASPPGYVPMPVGYNPALGGQRMPMGM 360
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SclB from ATG n  361 GRPLSSYTHQGMNPTTTPPQYQQSFVPISPYPESARMSNRMPMGESPMSRDPREGYLESP 420
sclB 8-166 no i  361 GRPLSSYTHQGMNPTTTPPQYQQSFVPISPYPESARMSNRMPMGESPMSRDPREGYLESP 420
sclB 8-169 no i  361 GRPLSSYTHQGMNPTTTPPQYQQSFVPISPYPESARMSNRMPMGESPMSRDPREGYLESP 420
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SclB from ATG n  421 VRLPPIYPPTMGTPASTSQGHRLSDPYPGAWSPRTREEFLQQEHRQQMPSHGFIDPLSPS 480
sclB 8-166 no i  421 VRLPPIYPPTMGTPASTSQGHRLSDPYPGAWSPRTREEFLQQEHRQQMPSHGFIDPLSPS 480
sclB 8-169 no i  421 VRLPPIYPPTMGTPASTSQGHRLSDPYPGAWSPRTREEFLQQEHRQQMPSHGFIDPLSPS 480
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SclB from ATG n  481 SQMRHAASDMGYGEPVPRQLGPGSTAERHAMHMSLVPAPDEPPTTEADTEGSRPAKRRKM 540
sclB 8-166 no i  481 SQMRHAASDMGYGEPVPRQFGPGSTAERHAMHMSLVPAPDEPPTTEADTEGSRPAKRRKM 540
sclB 8-169 no i  481 SQMRHAASDMGYGEPVPRQLGPGSTAERHAMHMSLVPAPDEPPTTEADTEGSRPAKRRKM 540
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SclB from ATG n  541 ALDDMVND* 549
sclB 8-166 no i  541 ALDDMVND* 549
sclB 8-169 no i  541 ALDDMVND* 549
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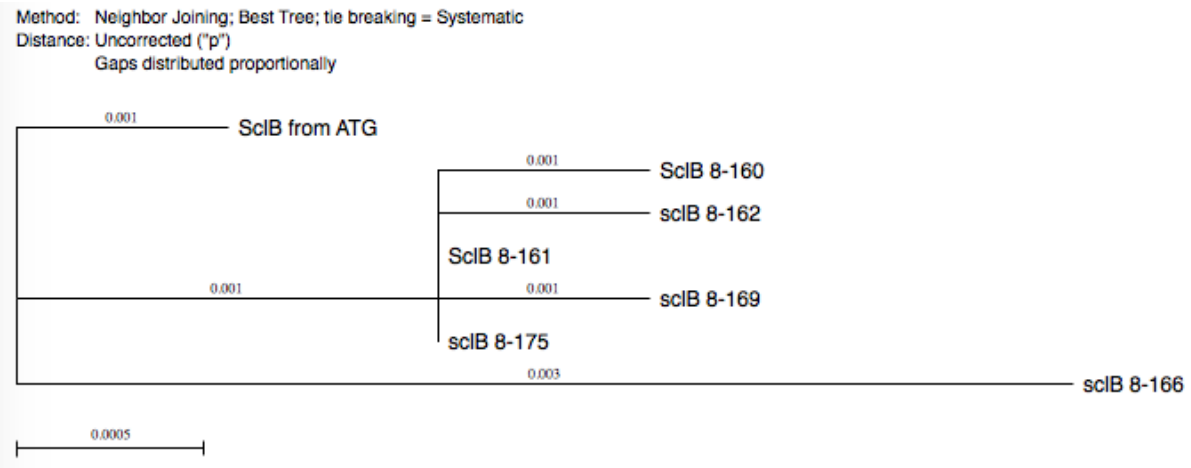

Appendix 12: *sclR* Multiple Sequence Alignments

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SclR CBS w Intr     1 ATGGCATACCCCAGGCCCGATTCCTTCTCGCTCGACGACGAGCGGATGTACAGCATGAGC   60
sclR 8-160          1 ATGGCATACCCCAGGCCCGATTCCTTCTCGCTCGACGACGAGCGGATGTACAGCATGAGC   60
sclR 8-161          1 ATGGCATACCCCAGGCCCGATTCCTTCTCGCTCGACGACGAGCGGATGTACAGCATGAGC   60
sclR 8-162          1 ATGGCATACCCCAGGCCCGATTCCTTCTCGCTCGACGACGAGCGGATGTACAGCATGAGC   60
sclR 8-169          1 ATGGCATACCCCAGGCCCGATTCCTTCTCGCTCGACGACGAGCGGATGTACAGCATGAGC   60
sclR 8-175          1 ATGGCATACCCCAGGCCCGATTCCTTCTCGCTCGACGACGAGCGGATGTACAGCATGAGC   60
sclR 8-166          1 ATGGCATACCCCAGGCCCGATTCCTTCTCGCTCGACGACGAGCGGATGTACAGCATGAGC   60
                      ************************************************************

SclR CBS w Intr    61 CATCCGAGCCCACTCACTCGTCCCAACGATACCTTCGCCAAGGGTCCCGATCCTCTGTCG  120
sclR 8-160         61 CATCCGAGCCCACTCACTCGTCCCAACGATACCTTCGCCAAGGGTCCCGATCCTCTGTCG  120
sclR 8-161         61 CATCCGAGCCCACTCACTCGTCCCAACGATACCTTCGCCAAGGGTCCCGATCCTCTGTCG  120
sclR 8-162         61 CATCCGAGCCCACTCACTCGTCCCAACGATACCTTCGCCAAGGGTCCCGATCCTCTGTCG  120
sclR 8-169         61 CATCCGAGCCCACTCACTCGTCCCAACGATACCTTCGCCAAGGGTCCCGATCCTCTGTCG  120
sclR 8-175         61 CATCCGAGCCCACTCACTCGTCCCAACGATACCTTCGCCAAGGGTCCCGATCCTCTGTCG  120
sclR 8-166         61 CATCCGAGCCCACTCACTCGTCCCAACGATACCTTCGCCAAGGGTCCCGATCCTCTGTCG  120
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SclR CBS w Intr   121 GCTAACTGGAGCTACGACAATGCCATCGATCTCTTCTCCCTCAACACCATGATGCCGGAG  180
sclR 8-160        121 GCTAACTGGAGCTACGACAATGCCATCGATCTCTTCTCCCTCAACACCATGATGCCGGAG  180
sclR 8-161        121 GCTAACTGGAGCTACGACAATGCCATCGATCTCTTCTCCCTCAACACCATGATGCCGGAG  180
sclR 8-162        121 GCTAACTGGAGCTACGACAATGCCATCGATCTCTTCTCCCTCAACACCATGATGCCGGAG  180
sclR 8-169        121 GCTAACTGGAGCTACGACAATGCCATCGATCTCTTCTCCCTCAACACCATGATGCCGGAG  180
sclR 8-175        121 GCTAACTGGAGCTACGACAATGCCATCGATCTCTTCTCCCTCAACACCATGATGCCGGAG  180
sclR 8-166        121 GCTAACTGGAGCTACGACAATGCCATCGATCTCTTCTCCCTCAACACCATGATGCCGGAG  180
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SclR CBS w Intr   181 ACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTTGGACCCTAAGGACTTTCCCGCC  240
sclR 8-160        181 ACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTTGGACCCCAAGGACTTTCCCACC  240
sclR 8-161        181 ACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTTGGACCCTAAGGACTTTCCCGCC  240
sclR 8-162        181 ACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTTGGACCCCAAGGACTTTCCCACC  240
sclR 8-169        181 ACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTTGGACCCCAAGGACTTTCCCACC  240
sclR 8-175        181 ACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTTGGACCCCAAGGACTTTCCCACC  240
sclR 8-166        181 ACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTTGGACCCCAAGGACTTTCCCGCC  240
                      ******************************************* ************ **

SclR CBS w Intr   241 GACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCGGGTGAG  300
sclR 8-160        241 GACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCGGGTGAA  300
sclR 8-161        241 GACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCGGGTGAG  300
sclR 8-162        241 GACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCGGGTGAA  300
sclR 8-169        241 GACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCGGGTGAA  300
sclR 8-175        241 GACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCGGGTGAA  300
sclR 8-166        241 GACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCGGGTGAA  300
                      ************************************************************

SclR CBS w Intr   301 GATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCTATACCCCATTGACTTTGAATGA  360
sclR 8-160        301 GATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCTATACCCCATTGACTTTGAATGA  360
sclR 8-161        301 GATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCTATACCCCATTGACTTTGAATGA  360
sclR 8-162        301 GATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCTATACCCCATTGACTTTGAATGA  360
sclR 8-169        301 GATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCTATACCCCATTGACTTTGAATGA  360
sclR 8-175        301 GATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCTATACCCCATTGACTTTGAATGA  360
sclR 8-166        301 GATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCTATACCCCATTGACTTTGAATGA  360
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SclR CBS w Intr   361 TGAACTATAACTAACATTACACTCAATCAGGACCTGGACAGTGACGACCAATCATGGTCT  420
sclR 8-160        361 TGAACTATAACTAACATTACACTCAATCAGGACCTGGACAGTGACGACCAATCATGGTCT  420
sclR 8-161        361 TGAACTATAACTAACATTACACTCAATCAGGACCTGGACAGTGACGACCAATCATGGTCT  420
sclR 8-162        361 TGAACTATAACTAACATTACACTCAATCAGGACCTGGACAGTGACGACCAATCATGGTCT  420
sclR 8-169        361 TGAACTATAACTAACATTACACTCAATCAGGACCTGGACAGTGACGACCAATCATGGTCT  420
sclR 8-175        361 TGAACTATAACTAACATTACACTCAATCAGGACCTGGACAGTGACGACCAATCATGGTCT  420
sclR 8-166        361 TGAACTATAACTAACATTACACTCAATCAGGACCTGGACAGTGACGACCAATCATGGTCT  420
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SclR CBS w Intr   421 CCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCTGCTGCT  480
sclR 8-160        421 CCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCTGCTGCT  480
sclR 8-161        421 CCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCTGCTGCT  480
sclR 8-162        421 CCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCTGCTGCT  480
sclR 8-169        421 CCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCTGCTGCT  480
sclR 8-175        421 CCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCTGCTGCT  480
sclR 8-166        421 CCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCTGCTGCT  480
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SclR CBS w Intr  481 CGCACTTCCAGAACATCTACCCGTCGCAAGACTGCGTCTCAACCGAAGCCCCGCGAGGTT  540
sclR 8-160       481 CGCACTTCCAAAACATCTACCCGTCGCAAGACTGCGTCTCAACCGAAGCCCCGCGAGGTT  540
sclR 8-161       481 CGCACTTCCAAAACATCTACCCGTCGCAAGACTGCGTCTCAACCGAAGCCCCGCGAGGTT  540
sclR 8-162       481 CGCACTTCCAAAACATCTACCCGTCGCAAGACTGCGTCTCAACCGAAGCCCCGCGAGGTT  540
sclR 8-169       481 CGCACTTCCAAAACATCTACCCGTCGCAAGACTGCGTCTCAACCGAAGCCCCGCGAGGTT  540
sclR 8-175       481 CGCACTTCCAAAACATCTACCCGTCGCAAGACTGCGTCTCAACCGAAGCCCCGCGAGGTT  540
sclR 8-166       481 CGCACTTCCAAAACATCTACCCGTCGCAAGACTGCGTCTCAACCGAAGCCCCGCGAGGTT  540
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SclR CBS w Intr  541 ACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACCTCAGGACTACCCCGCCACCAGT  600
sclR 8-160       541 ACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACCGCAGGACTACCCCGCCACCAGT  600
sclR 8-161       541 ACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACCGCAGGACTACCCCGCCACCAGT  600
sclR 8-162       541 ACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACCGCAGGACTACCCCGCCACCAGT  600
sclR 8-169       541 ACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACCGCAGGACTACCCCGCCACCAGT  600
sclR 8-175       541 ACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACCGCAGGACTACCCCGCCACCAGT  600
sclR 8-166       541 ACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACCGCAGGACTACCCCGCCACCAGT  600
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SclR CBS w Intr  601 GTCTCACCCCCGCCCGCCCCCTCCTCCCCTGCTGCCAACAACACCGCCCGCAAGACCACC  660
sclR 8-160       601 GTCTCACCCCCGCCCGCCCCCTCCTCCCCTGCCGCCAACAGCACCGCCCGCAAGACCACC  660
sclR 8-161       601 GTCTCACCCCCGCCCGCCCCCTCCTCCCCTGCCGCCAACAACACCGCCCGCAAGACCACC  660
sclR 8-162       601 GTCTCACCCCCGCCCGCCCCCTCCTCCCCTGCCGCCAACAACACCGCCCGCAAGACCACC  660
sclR 8-169       601 GTCTCACCCCCGCCCGCCCCCTCCTCCCCTGCCGCCAACAACACCGCCCGCAAGACCACC  660
sclR 8-175       601 GTCTCACCCCCGCCCGCCCCCTCCTCCCCTGCCGCCAACAACACCGCCCGCAAGACCACC  660
sclR 8-166       601 GTCTCACCCCCGCCCGCCCCCTCCTCCCCTGCCGCCAACAACACCGCCCGCAAGACCACC  660
                     ********************************** ******* ********************

SclR CBS w Intr  661 CGTAGTCTCTCCAGCGACTCCAACGCCAGCACGGGTCAGGCCCAGACCACCACCGGCCGC  720
sclR 8-160       661 CGTAGTCTCTCCAGCGACTCCAACGCCAGCACGGGTCAGGCCCAGACCACCACCGGCCGC  720
sclR 8-161       661 CGTAGTCTCTCCAGCGACTCCAACGCCAGCACGGGTCAGGCCCAGACCACCACCGGCCGC  720
sclR 8-162       661 CGTAGTCTCTCCAGCGACTCCAACGCCAGCACGGGTCAGGCCCAGACCACCACCGGCCGC  720
sclR 8-169       661 CGTAGTCTCTCCAGCGACTCCAACGCCAGCACGGGTCAGGCCCAGACCACCACCGGCCGC  720
sclR 8-175       661 CGTAGTCTCTCCAGCGACTCCAACGCCAGCACGGGTCAGGCCCAGACCACCACCGGCCGC  720
sclR 8-166       661 CGCAGTCTCTCCAGCGACTCCAACGCCAGCACGGGTCAGGCCCAGACCACCACCGGCCGC  720
                     ** ************************************************************

SclR CBS w Intr  721 AATGCGGCGAAGCGGGCGGCGCACAACATCATCGAGAAGCGGTACCGCACGAACATGAAC  780
sclR 8-160       721 AATGCGGCGAAGCGGGCGGCGCACAACATCATCGAGAAGCGGTACCGCACGAACATGAAC  780
sclR 8-161       721 AATGCGGCGAAGCGGGCGGCGCACAACATCATCGAGAAGCGGTACCGCACGAACATGAAC  780
sclR 8-162       721 AATGCGGCGAAGCGGGCGGCGCACAACATCATCGAGAAGCGGTACCGCACGAACATGAAC  780
sclR 8-169       721 AATGCGGCGAAGCGGGCGGCGCACAACATCATCGAGAAGCGGTACCGCACGAACATGAAC  780
sclR 8-175       721 AATGCGGCGAAGCGGGCGGCGCACAACATCATCGAGAAGCGGTACCGCACGAACATGAAC  780
sclR 8-166       721 AATGCGGCGAAGCGGGCGGCGCACAACATCATCGAGAAGCGGTACCGCACGAACATGAAC  780
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SclR CBS w Intr  781 GCCAAGTTCGTTGCCTTGGAGAAGGCGATGTGCGGCGGAGTCCAGAAGCCCACCAAGGGG  840
sclR 8-160       781 GCCAAGTTCGTTGCCTTGGAGAAGGCGATGTGCGGCGGAGTCCAGAAGCCCACCAAGGGG  840
sclR 8-161       781 GCCAAGTTCGTTGCCTTGGAGAAGGCGATGTGCGGCGGAGTCCAGAAGCCCACCAAGGGG  840
sclR 8-162       781 GCCAAGTTCGTTGCCTTGGAGAAGGCGATGTGCGGCGGAGTCCAGAAGCCCACCAAGGGG  840
sclR 8-169       781 GCCAAGTTCGTTGCCTTGGAGAAGGCGATGTGCGGCGGAGTCCAGAAGCCCACCAAGGGG  840
sclR 8-175       781 GCCAAGTTCGTTGCCTTGGAGAAGGCGATGTGCGGCGGAGTCCAGAAGCCCACCAAGGGG  840
sclR 8-166       781 GCCAAGTTCGTTGCCTTGGAGAAGGCGATGTGCGGCGGAGTCCAGAAGCCCACCAAGGGG  840
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SclR CBS w Intr  841 GGCTCGGCGTCGCTGAAGAAGTCGGAGATCCTGACCAATGCCATCACCTTCATGCAAGAA  900
sclR 8-160       841 GGCTCAGCGTCGCTGAAGAAGTCGGAGATCCTGACCAATGCCATCACCTTCATGCAAGAA  900
sclR 8-161       841 GGCTCGGCGTCGCTGAAGAAGTCGGAGATCCTGACCAATGCCATCACCTTCATGCAAGAA  900
sclR 8-162       841 GGCTCGGCGTCGCTGAAGAAGTCGGAGATCCTGACCAATGCCATCACCTTCATGCAAGAA  900
sclR 8-169       841 GGCTCGGCGTCGCTGAAGAAGTCGGAGATCCTGACCAATGCCATCACCTTCATGCAAGAA  900
sclR 8-175       841 GGCTCGGCGTCGCTGAAGAAGTCGGAGATCCTGACCAATGCCATCACCTTCATGCAAGAA  900
sclR 8-166       841 GGCTCGGCGTCGCTGAAGAAGTCGGAGATCCTGACCAATGCCATCACCTTCATGCAAGAA  900
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SclR CBS w Intr  901 TTGCAGGAGGAAAACAAGGTGCTCCAGAAGGAGCTTGCCATGCTCAAACAAAGCATGGTC  960
sclR 8-160       901 TTGCAGGAGGAAAACAAGGTGCTTCAGAAGGAGCTTGCCATGCTTAAACAAAGCATGGTC  960
sclR 8-161       901 TTGCAGGAGGAAAACAAGGTGCTCCAGAAGGAGCTTGCCATGCTCAAACAAAGCATGGTC  960
sclR 8-162       901 TTGCAGGAGGAAAACAAGGTGCTCCAGAAGGAGCTTGCCATGCTCAAACAAAGCATGGTC  960
sclR 8-169       901 TTGCAGGAGGAAAACAAGGTGCTCCAGAAGGAGCTTGCCATGCTCAAACAAAGCATGGTC  960
sclR 8-175       901 TTGCAGGAGGAAAACAAGGTGCTCCAGAAGGAGCTTGCCATGCTCAAACAAAGCATGGTC  960
sclR 8-166       901 TTGCAGGAGGAAAACAAGGTGCTCCAGAAGGAGCTTGCCATGCTCAAACAAAGCATGGTC  960
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SclR CBS w Intr  961 CCGAACGGGATGTGGCGACATAGCAAGGG-AGTGAGGCGTTTCACGCTTAA 1010
sclR 8-160       961 CCGAACGGGATGTGGCGACATAGCAAGGGGAGTGAGGCGTTTCACGCTTAA 1011
sclR 8-161       961 CCGAACGGGATGTGGCGACATAGCAAGGGGAGTGAGGCGTTTCACGCTTAA 1011
sclR 8-162       961 CCGAACGGGATGTGGCGACATAGCAAGGGGAGTGAGGCGTTTCACGCTTAA 1011
sclR 8-169       961 CCGAACGGGATGTGGCGACATAGCAAGGGGAGTGAGGCGTTTCACGCTTAA 1011
sclR 8-175       961 CCGAACGGGATGTGGCGACATAGCAAGGGGAGTGAGGCGTTTCACGCTTAA 1011
sclR 8-166       961 CCGAACGGGATGTGGCGACATAGCAAGGGGAGTGAGGCGTTTCACGCTTAA 1011
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Strain	225	238	300	491	579	633	641	663	846	924	945
8-160	CCT>CCC Pro>Pro	GCC>ACC Ala>Thr	GAG>GAA Glu>Glu	AGA>AAA Arg>Lys	CCT>CCG Pro>Pro	GCT>GCC Ala>Ala	AAC>AGC Asn>Ser		TCG>TCA Ser>Ser	CTC>CTT Leu>Leu	CTC>CTT Leu>Leu
8-161				AGA>AAA Arg>Lys	CCT>CCG Pro>Pro	GCT>GCC Ala>Ala					
8-162	CCT>CCC Pro>Pro	GCC>ACC Ala>Thr	GAG>GAA Glu>Glu	AGA>AAA Arg>Lys	CCT>CCG Pro>Pro	GCT>GCC Ala>Ala					
8-166	CCT>CCC Pro>Pro		GAG>GAA Glu>Glu	AGA>AAA Arg>Lys	CCT>CCG Pro>Pro	GCT>GCC Ala>Ala		CGT>CGC **Arg>Arg**			
8-169	CCT>CCC Pro>Pro	GCC>ACC Ala>Thr	GAG>GAA Glu>Glu	AGA>AAA Arg>Lys	CCT>CCG Pro>Pro	GCT>GCC Ala>Ala					
8-175	CCT>CCC Pro>Pro	GCC>ACC Ala>Thr	GAG>GAA Glu>Glu	AGA>AAA Arg>Lys	CCT>CCG Pro>Pro	GCT>GCC Ala>Ala					

Strain	238	491	641
8-160	GCC>ACC Ala>Thr	AGA>AAA Arg>Lys	AAC>AGC Asn>Ser
8-161		AGA>AAA Arg>Lys	
8-162	GCC>ACC Ala>Thr	AGA>AAA Arg>Lys	
8-166		AGA>AAA Arg>Lys	
8-169	GCC>ACC Ala>Thr	AGA>AAA Arg>Lys	
8-175	GCC>ACC Ala>Thr	AGA>AAA Arg>Lys	

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sclR 8-162 no i    1 MAYPRPDSFSLDDERMYSMSHPSPLTRPNDTFAKGPDPLSANWSYDNAIDLFSLNTMMPE  60
sclR 8-169 no i    1 MAYPRPDSFSLDDERMYSMSHPSPLTRPNDTFAKGPDPLSANWSYDNAIDLFSLNTMMPE  60
sclR 8-175 no i    1 MAYPRPDSFSLDDERMYSMSHPSPLTRPNDTFAKGPDPLSANWSYDNAIDLFSLNTMMPE  60
sclR 8-166 no i    1 MAYPRPDSFSLDDERMYSMSHPSPLTRPNDTFAKGPDPLSANWSYDNAIDLFSLNTMMPE  60
sclR CBS no int    1 MAYPRPDSFSLDDERMYSMSHPSPLTRPNDTFAKGPDPLSANWSYDNAIDLFSLNTMMPE  60
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sclR 8-160 no i   61 TFPLEMSNEMMNLDPKDFPTDFFAPPPDISAFTISNHSGEDAASCGSLSSDLDSDDQSWS 120
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sclR 8-162 no i   61 TFPLEMSNEMMNLDPKDFPTDFFAPPPDISAFTISNHSGEDAASCGSLSSDLDSDDQSWS 120
sclR 8-169 no i   61 TFPLEMSNEMMNLDPKDFPTDFFAPPPDISAFTISNHSGEDAASCGSLSSDLDSDDQSWS 120
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sclR 8-166 no i   61 TFPLEMSNEMMNLDPKDFPADFFAPPPDISAFTISNHSGEDAASCGSLSSDLDSDDQSWS 120
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sclR 8-160 no i  121 PTCRVSPLEPIHMELPKPAARTSKTSTRRKTASQPKPREVTATRWSSSPEITPQDYPATS 180
sclR 8-161 no i  121 PTCRVSPLEPIHMELPKPAARTSKTSTRRKTASQPKPREVTATRWSSSPEITPQDYPATS 180
sclR 8-162 no i  121 PTCRVSPLEPIHMELPKPAARTSKTSTRRKTASQPKPREVTATRWSSSPEITPQDYPATS 180
sclR 8-169 no i  121 PTCRVSPLEPIHMELPKPAARTSKTSTRRKTASQPKPREVTATRWSSSPEITPQDYPATS 180
sclR 8-175 no i  121 PTCRVSPLEPIHMELPKPAARTSKTSTRRKTASQPKPREVTATRWSSSPEITPQDYPATS 180
sclR 8-166 no i  121 PTCRVSPLEPIHMELPKPAARTSKTSTRRKTASQPKPREVTATRWSSSPEITPQDYPATS 180
sclR CBS no int  121 PTCRVSPLEPIHMELPKPAARTSRTSTRRKTASQPKPREVTATRWSSSPEITPQDYPATS 180
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sclR 8-160 no i  181 VSPPPAPSSPAANSTARKTTRSLSSDSNASTGQAQTTTGRNAAKRAAHNIIEKRYRTNMN 240
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sclR 8-169 no i  181 VSPPPAPSSPAANNTARKTTRSLSSDSNASTGQAQTTTGRNAAKRAAHNIIEKRYRTNMN 240
sclR 8-175 no i  181 VSPPPAPSSPAANNTARKTTRSLSSDSNASTGQAQTTTGRNAAKRAAHNIIEKRYRTNMN 240
sclR 8-166 no i  181 VSPPPAPSSPAANNTARKTTRSLSSDSNASTGQAQTTTGRNAAKRAAHNIIEKRYRTNMN 240
sclR CBS no int  181 VSPPPAPSSPAANNTARKTTRSLSSDSNASTGQAQTTTGRNAAKRAAHNIIEKRYRTNMN 240
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sclR 8-160 no i  241 AKFVALEKAMCGGVQKPTKGGSASLKKSEILTNAITFMQELQEENKVLQKELAMLKQSMV 300
sclR 8-161 no i  241 AKFVALEKAMCGGVQKPTKGGSASLKKSEILTNAITFMQELQEENKVLQKELAMLKQSMV 300
sclR 8-162 no i  241 AKFVALEKAMCGGVQKPTKGGSASLKKSEILTNAITFMQELQEENKVLQKELAMLKQSMV 300
sclR 8-169 no i  241 AKFVALEKAMCGGVQKPTKGGSASLKKSEILTNAITFMQELQEENKVLQKELAMLKQSMV 300
sclR 8-175 no i  241 AKFVALEKAMCGGVQKPTKGGSASLKKSEILTNAITFMQELQEENKVLQKELAMLKQSMV 300
sclR 8-166 no i  241 AKFVALEKAMCGGVQKPTKGGSASLKKSEILTNAITFMQELQEENKVLQKELAMLKQSMV 300
sclR CBS no int  241 AKFVALEKAMCGGVQKPTKGGSASLKKSEILTNAITFMQELQEENKVLQKELAMLKQSMV 300
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sclR 8-160 no i  301 PNGMWRHSKGSEAFHA* 317
sclR 8-161 no i  301 PNGMWRHSKGSEAFHA* 317
sclR 8-162 no i  301 PNGMWRHSKGSEAFHA* 317
sclR 8-169 no i  301 PNGMWRHSKGSEAFHA* 317
sclR 8-175 no i  301 PNGMWRHSKGSEAFHA* 317
sclR 8-166 no i  301 PNGMWRHSKGSEAFHA* 317
sclR CBS no int  301 PNGMWRHSKGSEAFHA* 317
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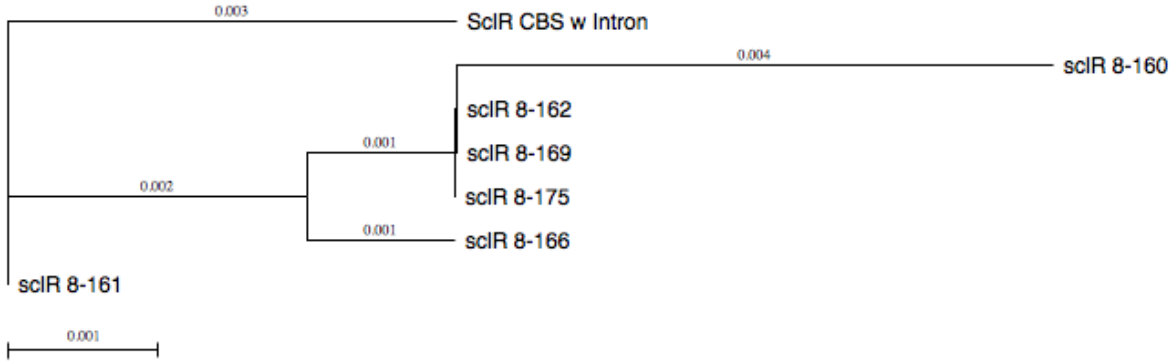
Method: Neighbor Joining; Best Tree; tie breaking = Systematic
Distance: Uncorrected ("p")
Gaps distributed proportionally



Appendix 13: Primers used for Meiosis Gene Expression Analysis in *A. nidulans*:

Name	Sequence	Product length against gDNA (bp)
Beta-tubulin F	GTT TGC CCC TTT GAC TAG CC	474
Beta-tubulin R	GAG ACG CGG TTG AAG AGT TC	
Dmc1.1F A nidulans	GCT TAT GCC CGT GCT TTG AA	414
Dmc1.1R A nidulans	TGA GCA AGA ACA TGC CCA CC	
Dmc1.2F A nidulans	ACA TTG CTT ATG CCC GTG CT	415
Dmc1.2R A nidulans	CAA GAA CAT GCC CAC CGA C	
Hop2.1F A nidulans	TCA CTT GCT GCT CGC TTG AT	658
Hop2.1R A nidulans	CAA AGC CAG GTA CAG CTG AGA	
Hop2.2F A nidulans	TTG ATC AAG GTG TGT GGC GA	651
Hop2.2R A nidulans	CTG CTG TGC AAA GCC AGG TA	
Rec8.1F A nidulans	CGC TAC CCT GAG TTT CTT TCC	620
Rec8.1R A nidulans	CGG AGC TGC AGG ATC GAT AA	
Rec8.2F A nidulans	CGC TAC CCT GAG TTT CTT TCC T	643
Rec8.2R A nidulans	TTG CTC TGG AGT CTG AGC G	
Nidulans SclR1 F	TAT GAT CCC CGA GCC ATT CC	392
Nidulans SclR1 R	TTT GTC GAG GTG GTG GAG AG	
Spo11.1F A nidul	ATC GAG TGA CAA CGG TGA GG	518
Spo11.1R A nidul	CGG CGA GGA TAT AGA AGG GG	
Spo11.2F A nidul	ACG GTG AGG GAC GAG GTT AT	508
Spo11.2R A nidul	TCG GCG AGG ATA TAG AAG GGG	

Appendix 14: Primers used for Meiosis Gene Expression Analysis in *A. niger*

Name	Sequence	Product length against gDNA (bp)
BETA tub niger 1 F	TTT CCA GAT CAC CCA CTC CC	451
BETA tub niger 1 R	GGA ACC ATG TTG ACA GCC AG	
BETA tub niger 2 F	AGT TCC CCG ACC GTA TGA TG	481
BETA tub niger 2 R	CGA ACA TCT GCT GGG TCA AC	
DMC1.1 Niger F PMB	CGG CAA GAC TCA GTT ATC GC	678
DMC1.1 Niger R PMB	GCG AGT CCT GTA TCT TAG CC	
DMC1.2 Niger F PMB	CAT GTC TGT TGT AGC CCA GC	634
DMC1.2 Niger R PMB	ACT CGC TCT TCA CCA CGA C	
Dmc1 F A niger	GACTATTGTGGACGCGGAGA	366
Dmc2 R A niger	TTAGCCACTCGCTCTTCACC	
Niger SclR F 1	TGCCATCGATCTCTTCTCCC	367
Niger SclR R 1	GCGACGGGTAGATGTTTTGG	
Niger SclR F 2	CGTTCACCATCTCCAACCAC	487
Niger SclR R 2	TTCTCGATGATGTTGTGCGC	
Niger SclR F 3 Soad	GAGAACTTCGCTCGATGTGC	~1200
Niger SclR R 3 Soad	CCGAAGTGATAGAACCGGCAT	
Msh4.1 F A niger	ACCTATGGGTGCAAGGCTTC	401
Msh4.1 R A niger	AGACTGCTTTGAGCTGCCAT	
Msh4.2 F A niger	CCTATGGGTGCAAGGCTTCT	401
Msh4.2 R A niger	AAGACTGCTTTGAGCTGCCA	
Msh5 F A niger	AGTGGCAGTGGATGTGAGAG	440
Msh5 R A niger	CTTGATGCGATGCTCATGTCT	
Spo11.1 F A niger	GCAAAAGGACTTGTCGTGGG	427
Spo11.1 R A niger	GACGAATGCCCGAGTACTGA	
Spo11.1 F A niger	AGCAGCAAAAGGACTTGTCG	438
Spo11.1 R A niger	GTTTACGGACGAATGCCCGA	
Hop2.1 F A niger	GAGTGATGCAGGGACTGTGC	318
Hop2.1 R A niger	CAGGATCGTGGACCAGTGTT	
Hop2.2 F A niger	GGGAGTGATGCAGGGACTG	321
Hop2.2 R A niger	TCAGGATCGTGGACCAGTGT	
Rec8.1 F A niger	CGCAAGCGTCTTCATTCATCC	302
Rec8.1 R A niger	AGATGTTCGCAGCAGACGAG	
Rec8.2 F A niger	ACGCAAGCGTCTTCATTCAT	305
Rec8.2 R A niger	AAAGATGTTCGCAGCAGACG	

Appendix 15: Sequence Alignments of Meiosis Genes

```
1.beta tubulin    1 TTTCCAGATCACCCACTCCCTCGGTGGTGGTACCGGTGCCGGTATGGGTACCCTCCTGAT  60
1. Beta tubulin   1                                              ATGGGTACCCTCCTGAT  17
                                                                 ******************

1.beta tubulin   61 CTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCCACCTTCTCCGTTGTTCCCTC 120
1. Beta tubulin  18 CTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCCACCTTCTCCGTTGTTCCCTC  77
                    ************************************************************

1.beta tubulin  121 CCCCAAGGTCTCCGACACCGTTGTTGAGCCTTACAACGCCACTCTCTCCGTCCACCAGCT 180
1. Beta tubulin  78 CCCCAAGGTCTCCGACACCGTTGTTGAGCCTTACAACGCCACTCTCTCCGTCCACCAGCT 137
                    ************************************************************

1.beta tubulin  181 CGTTGAGCACTCCGACGAGACCTTCTGTATCGACAACGAGGTATGATTCCAGACCCAGCA 240
1. Beta tubulin 138 CGTTGAGCACTCCGACGAGACCTTCTGTATCGACAACGAGG------------------- 178
                    ****************************************

1.beta tubulin  241 CAAGTGAGAAGAAGATTGGTGCTCGAGAACTAACGAATTGGCCAATCTAGGCTCTGTACG 300
1. Beta tubulin 179 ----------------------------------------------------CTCTGTACG 187
                                                                         *********

1.beta tubulin  301 ACATCTGCATGCGCACCCTCAAGCTCTCCAACCCCTCTTACGGTGACCTGAACCACCTGG 360
1. Beta tubulin 188 ACATCTGCATGCGCACCCTCAAGCTCTCCAACCCCTCTTACGGTGACCTGAACCACCTGG 247
                    ************************************************************

1.beta tubulin  361 TCTCTGCCGTCATGTCCGGTGTGACCACTTGCCTCCGTTTCCCTGGTCAGCTCAACTCCG 420
1. Beta tubulin 248 TCTCTGCCGTCATGTCCGGTGTGACCACTTGCCTCCGTTTCCCTGGTCAGCTCAACTCCG 307
                    ************************************************************

1.beta tubulin  421 ACCTTCGCAAGCTGGCTGTCAACATGGTTCC   451
1. Beta tubulin 308 ACCTTCGCAAGCTGGCTGTCAACATGGTTCCAA 340
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2_P2_E04 trimme    1                                                       0
REC8 ASPGD      1381 GTCTTCGGTCAAGGCATTGGTTCTGTCGGTGTAGGCGTGGGGGTGTCTCATGTCAAGCAT 1440

2_P2_E04 trimme    1                                                       0
REC8 ASPGD      1441 CCATTGCATTCTTTCTCAGGGGACCAACTATATGAAAATCTCCAAGATAAATCGAATGAT 1500

2_P2_E04 trimme    1                                                       0
REC8 ASPGD      1501 CGAGTACGCAAGCGTCTTCATTCATCCATCGAAGAAGAAGATCCCAATTTCGATACACGA 1560

2_P2_E04 trimme    1                                        GGACGTCACGACGACAGGTTATGG   24
REC8 ASPGD      1561 CGGATACGCCCAAGGGTGGAGGATGATAATCAGATCGGACGTCACGACGACAGGTTATGG 1620
                                                            ************************

2_P2_E04 trimme   25 AATGAGGTTTGTATATGTCGACGTGCTCTCTATGCTTATCGGACCAAAGCTGAAACATTG   84
REC8 ASPGD      1621 AATGAGGTTTGTATAGGTCGACGTGCTCTCTATGCTTATCGGACCAAAGCTGAAATATTG 1680
                    *************** ****************************************** ****

2_P2_E04 trimme   85 TACACAGGACGTCGAATATGGTCGCAATGCACCACCCTCTCTTCGCGATGACCACTCCAT  144
REC8 ASPGD      1681 TACACAGGACGTCGAATATGGTCGCAATGCACCACCCTCTCTTCGCGATGACCACTCCAT 1740
                    ************************************************************

2_P2_E04 trimme  145 CCAAATGCYATGGAACATCACAGCATCTATTCAAAGCTCTCGACAGGGCT            194
REC8 ASPGD      1741 CCAAATGCCATGGAACATCACAGCATCTATTCAAAGCTCTCGACAGGGCTCGTCTGCTGC 1800
                    ******** ****************************************

2_P2_E04 trimme  195                                                       194
REC8 ASPGD      1801 GAACATCTTTCGCGGATTCGGCAGCGCCAGTGACTTCTCATCACATGGAGTTTCAGAGAC 1860

2_P2_E04 trimme  195                                                       194
REC8 ASPGD      1861 TGGATTTGGAAGAGCACGCAGCCGTCTCACAAGCGCAAGTCCCCTTGCTGGCCGTGGGTT 1920

2_P2_E04 trimme  195                                                       194
REC8 ASPGD      1921 CCCTTTTGACGCTGAAGCGCTCAATGTTATCTCGATTCCTGGAAACGAGATGGGTGAGTT 1980

2_P2_E04 trimme  195                                                       194
REC8 ASPGD      1981 AGAGAACCTTGACGACTTTGACATTTCTCAGTATCTACACACCGAGCTTGCAACGGAGAA 2040
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3. Rec8 sequenc   1                                                          0
2. Rec8 referen   1 GAGTACGCAAGCGTCTTCATTCATCCATCGAAGAAGAAGATCCCAATTTCGATACACGAC  60


3. Rec8 sequenc   1                                                 ACAGGTTATGGA  12
2. Rec8 referen  61 GGATACGCCCAAGGGTGGAGGATGATAATCAGATCGGACGTCACGACGACAGGTTATGGA 120
                                                                    ************


3. Rec8 sequenc  13 ATG------------------------------------------------------------  15
2. Rec8 referen 121 ATGAGGTTTGTATAGGTCGACGTGCTCTCTATGCTTATCGGACCAAAGCTGAAATATTGT 180
                    ***


3. Rec8 sequenc  16 ----AGGACGTCGAATATGGTCGCAATGCACCACCCTCTCTTCGCGATGACCACTCCATC  71
2. Rec8 referen 181 ACACAGGACGTCGAATATGGTCGCAATGCACCACCCTCTCTTCGCGATGACCACTCCATC 240
                      ************************************************************


3. Rec8 sequenc  72 CAAATGCCATGGAACATCACAGCATCTATTCAAAGCTCTCGACAGGGCT            120
2. Rec8 referen 241 CAAATGCCATGGAACATCACAGCATCTATTCAAAGCTCTCGACAGGGCTCGTCTGCTGCG 300
                    ***********************************************


3. Rec8 sequenc 121                 120
2. Rec8 referen 301 AACATCTTTCGCGG 314
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4. spo11 refere  661 CTATCAGATGACCACCACAAGCGCGGGAGTAGTGGACGCGCGGTTATCAAACCAGGTCCG  720
4_spo11 trimmed    1                       GCGCGGGAGTAGTGGACGCGCGGTTATCAAACCAGGTCCG   40
                                           ****************************************


4. spo11 refere  721 TTGCAGTCATCACTACTGCACACAGCATGGACTGATGTTCGCAGGATACTCTAGTACCTA  780
4_spo11 trimmed   41 TTGCAGTCATCACTACTGCACACAGCATGGACTGATGTTCGCAGGATACTCTAGTACCTA  100
                     ************************************************************


4. spo11 refere  781 GAATGCAAGACATCCACAAAGTCGACCTTAAAGATGTTGCCTGGGTACTGGTTCTAGAGA  840
4_spo11 trimmed  101 GAATGCAAGACATCCACAAAGTCGACCTGAAAGATGTTGCCTGGGTACTGGTTCTAGAGA  160
                     ***************************** ******************************


4. spo11 refere  841 AGGAGGTCCCTGCTGCCATCAAAAACTTTATATATCAAGCTGGAGTGAACTGACACCCTC  900
4_spo11 trimmed  161 AGGAGGTCCCTGCTGCCATCAAGAACTT-ATATATCAAGCTGGAGTGAACTGACACCCTC  219
                     ********************** ***** ********************************


4. spo11 refere  901 AGGCCGTATACCGTCGACTCGCAAGCAGCAACTACCACATCAGATCTGCAGCAGGCAAGG  960
4_spo11 trimmed  220 AGGCCGTATACCGTCGACTCGCAAGCAGCAACTACCACATCAGATCTGCAGCAGGCAAGG  279
                     ************************************************************


4. spo11 refere  961 GCGTCCTCGTCACTGTAAGTGCTGATACCCCTATTACATTCTAAGAGACATACCAACCAT 1020
4_spo11 trimmed  280 GCGTCCTCGTCACTGTAAGTGCTGATACCCCTATTACATTCTAAGAGGCATACAAACCAT  339
                     ********************************************** ***** ******


4. spo11 refere 1021 TCCAGGGAAAAGGATATCCTGATCTCAGTACTCGGGCATTCGTCCGTAAACTCTTCGACC 1080
4_spo11 trimmed  340 TCCAGGGAAAAGGATATCCTGATC                                  363
                     *************************
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5. SclR trimmed    1                                                    ATGATGAACTT   11
SclR processed   421 CCTCAACACCATGATGCCGGAGACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTT  480
                                                                        **********

5. SclR trimmed   12 GGACCCTAAGGACTTTCCCACCGACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCAC   71
SclR processed   481 GGACCCTAAGGACTTTCCCGCCGACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCAC  540
                     ****************** ****************************************

5. SclR trimmed   72 CATCTCCAACCACTCGGGTGAGGATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCT  131
SclR processed   541 CATCTCCAACCACTCGGGTGAGGATGCTGCCTCCTGCGGCTCTCTCTCTTC---------  591
                     **************************************************

5. SclR trimmed  132 ATACCCCATTGACTTTGAATGATGAACTATAACTAACATTACACTCAATCAGGACCTGGA  191
SclR processed   592 ----------------------------------------------------GGACCTGGA  600
                                                                        *********

5. SclR trimmed  192 CAGTGACSACCAATCATGGTCTCCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACAT  251
SclR processed   601 CAGTGACGACCAATCATGGTCTCCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACAT  660
                     ******* ****************************************************

5. SclR trimmed  252 GGAGCTGCCCAAGCCTGCTGCTCGCACTT                                280
SclR processed   661 GGAGCTGCCCAAGCCTGCTGCTCGCACTTCCAGAACATCTACCCGTCGCAAGACTGCGTC  720
                     ****************************

5. SclR trimmed  281                                                        280
SclR processed   721 TCAACCGAAGCCCCGCGAGGTTACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACC  780
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5. SclR referen 121 CCGGAGACTTTCCCCTTGGAGATGTCCAACGAGATGATGAACTTGGACCCTAAGGACTTT 180
7. Sclr trimmed   1                   GAGATGTCCAACGAGATGATGAACTTGGACCCTAAGGACTTT  42
                                      *******************************************

5. SclR referen 181 CCCGCCGACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCG 240
7. Sclr trimmed  43 CCCGCCGACTTCTTCGCCCCACCCCCAGATATCAGTGCGTTCACCATCTCCAACCACTCG 102
                    ************************************************************

5. SclR referen 241 GGTGAGGATGCTGCCTCCTGCGGCTCTCTCTCTTCGGTATGTCTATACCCCATTGACTTT 300
7. Sclr trimmed 103 GGTGAGGATGCTGCCTCCTGCGGCTCTCTCTCTTCGG----------------------- 139
                    ************************************

5. SclR referen 301 GAATGATGAACTATAACTAACATTACACTCAATCAGGACCTGGACAGTGACGACCAATCA 360
7. Sclr trimmed 140 -------------------------------------ACCTGGACAGTGACGACCAATCA 162
                                                        ***********************

5. SclR referen 361 TGGTCTCCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCT 420
7. Sclr trimmed 163 TGGTCTCCCACCTGCCGTGTCTCTCCCCTTGAGCCTATCCACATGGAGCTGCCCAAGCCT 222
                    ************************************************************

5. SclR referen 421 GCTGCTCGCACTTCCAAAACATCTACCCGTCGCAAGACTGCGTCTCAACCGAAGCCCCGC 480
7. Sclr trimmed 223 GCTGCTCGCACTT                                             235
                    *************

5. SclR referen 481 GAGGTTACGGCGACAAGGTGGTCGTCCAGTCCGGAAATTACACCGCAGGACTACCCCGCC 540
7. Sclr trimmed 236                                                             235
```

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8. MSH4 mrna in   1                                                        0
8. MSH4 referen  61 TCAATGAGACTCTGACACCTATGGGTGCAAGGCTTCTCAGAGCCAACATTCTTCAACCAT 120


8. MSH4 mrna in   1                 GCTRCAAGCACGATACGATGCTGTTGAGGATTTGTCTACAAAGG  44
8. MSH4 referen 121 CAACCGAGGAATCAAAGCTGCAAGCACGATACGATGCTGTTGAGGATTTGTCTACAAAGG 180
                               *** ****************************************


8. MSH4 mrna in  45 AGGACATGTTTGTCTCAGTGAGACAAG---------------------------------  71
8. MSH4 referen 181 AGGACATGTTTGTCTCAGTGAGACAAGGTTCGTTTAGAATCCTGCTATATATGACTCTAT 240
                    **************************


8. MSH4 mrna in  72 ---------------------CYCTCAAAGGCTTTGTTGATGCGGATAAAGTTCTTACTT 110
8. MSH4 referen 241 TCTAACCATACCAAATATTAGCTCTCAAAGGCTTTGTTGATGCGGATAAAGTTCTTACTT 300
                                  * ************************************


8. MSH4 mrna in 111 CT-------------------------------------------CTCATCCTTGTGCC 126
8. MSH4 referen 301 CTGTAAGTGGAGAACTAGCCTTCTTAATAGCTTACTGACAATCCAGCTCATCCTTGTGCC 360
                    **                                          **************


8. MSH4 mrna in 127 CAATAAACGAGCCTTACAATAYGCTGAGCAGTCTGTCAACAATGTCATCATGGTCAAGAC 186
8. MSH4 referen 361 CAATAAACGAGCCTTACAATACGCTGAGCAGTCTGTCAACAATGTCATCATGGTCAAGAC 420
                    ******************** ************************************


8. MSH4 mrna in 187 ATACGTGGGATCAATYAAGTCAGTTTACAAGGCACTG                       223
8. MSH4 referen 421 ATACGTGGGATCAATCAAGTCAGTTTACAAGGCACTGATGGCAGCTCAAAGCAGTCTTCT 480
                    *************** ********************
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9.MSH4 larger b   1                     GCAAGCACGATACGATGCTGTTGAGGATTTGTCTACAAAGG  41
8. MSH4 referen 121 CAACCGAGGAATCAAAGCTGCAAGCACGATACGATGCTGTTGAGGATTTGTCTACAAAGG 180
                               ****************************************


9.MSH4 larger b  42 AGGACATGTTTGTCTCAGTGAGACAAGGTTCGTTTAGAATCCTGCTATATATGACTCTAT 101
8. MSH4 referen 181 AGGACATGTTTGTCTCAGTGAGACAAGGTTCGTTTAGAATCCTGCTATATATGACTCTAT 240
                    ************************************************************


9.MSH4 larger b 102 TCCAACCATACCAAATATTAGCTCTCAAAGGCTTTGTTGATGCGGATAAAGTTCTTACTT 161
8. MSH4 referen 241 TCTAACCATACCAAATATTAGCTCTCAAAGGCTTTGTTGATGCGGATAAAGTTCTTACTT 300
                    ** ********************************************************


9.MSH4 larger b 162 CT-------------------------------------------CTCRTCCTTGTGCC 177
8. MSH4 referen 301 CTGTAAGTGGAGAACTAGCCTTCTTAATAGCTTACTGACAATCCAGCTCATCCTTGTGCC 360
                    **                                          *** **********


9.MSH4 larger b 178 CAATAAACAAGCCTTACAATACGCTGAGCAGTCTGTCRACAATGTCATCATGGTCAAGAC 237
8. MSH4 referen 361 CAATAAACGAGCCTTACAATACGCTGAGCAGTCTGTCAACAATGTCATCATGGTCAAGAC 420
                    ******** *************************** ********************


9.MSH4 larger b 238 ATACGTGGGATCAATCAAGTCAGTTTACAAGGCACTG                       274
8. MSH4 referen 421 ATACGTGGGATCAATCAAGTCAGTTTACAAGGCACTGATGGCAGCTCAAAGCAGTCTTCT 480
                    ************************************
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11. dmc whole r  721 ATGCGGCAAGACTCAGTTATCGCACACCATGTCTGTTGTAGCCCAGCTTCCTAAGGAACT  780
11.DMC removal     1                                ACCTGTTTGAGC--AGCTTCT--AAGGACT   26
                                                      *****  ***  ******   * * ***

11. dmc whole r  781 GGGCGGCGCAGGCGGCAAGGTGGCATATATTGATACCGAGGGAACTTTCAGGCCTGAGCG  840
11.DMC removal    27 GGGCGGCGCATGCGGCAAGGTGGCATATATTGATACCGAGGGAACTTTCAGGCCTGAGCG   86
                  ********** ************************************************

11. dmc whole r  841 CATCGCTCAGATTGCAGAGCGGTTTGGCGTCGATGCTGATGCTGCCCAGGAGAATATTGC  900
11.DMC removal    87 CATCGCTCAGATTGCAGAGCGGTTTGGCGTCGATGCTGATGCTGCCCAGGAGAATATTGC  146
                  ************************************************************

11. dmc whole r  901 TTATGCGCGAGCGTTGAACAGCGAGCATCAGCTGGAGTTGTTGAATACCCTCAGTAAAGA  960
11.DMC removal   147 TTATGCGCGAGCGTTGAACAGCGAGCATCAGCTGGAGTTGTTGAATACCCTCAGTAAAGA  206
                  ************************************************************

11. dmc whole r  961 GTTCGCTGGTGGGGAATACAGGTTGTTGATCATTGATAGCATCATGAATTGCTTCAGGGT 1020
11.DMC removal   207 GTTCGCTGGTGGGGAATACAGGTTGTTGATCATTGATAGCATCATGAATTGCTTCAGAGT  266
                  ************************************************************ **

11. dmc whole r 1021 CGACTATTGTGGACGCGGAGAGCTTGCTGATCGTCAGCAGAAGTTGAATCAGTTCTTGAT 1080
11.DMC removal   267 CGACTATTGTGGACGCGGAGAGCTTGCTGATCGTCAGCAGAAGTTGAATCAGTTCTTGAT  326
                  ************************************************************

11. dmc whole r 1081 GAAGTTGGCTCATATGGCTGAGGGTATATTTTTCACTCCTGATATATATATATCAAGTAC 1140
11.DMC removal   327 GAAGCTGGCTCATATGGCTGAGG-------------------------------------  349
                  **** ******************

11. dmc whole r 1141 TAGAGGGCTGACATTTTCGTTTGCAGAATTCAACATCTGTGTTCTGATGGTCCGTTCCCC 1200
11.DMC removal   350 --------------------------AATTCAACATCTGTGTTCTGATG-----------  372
                                               ***********************

11. dmc whole r 1201 ATCGACATGCAGATAGATAGATATAGTCTAATGCACTGCAGACCAACCAGGTCCAGAGTG 1260
11.DMC removal   373 -----------------------------------------ACCAACCAGGTCCAGAGTG  391
                                                             ******************

11. dmc whole r 1261 ACCCTGGCGCTAGTGCACTCTTCGCTGGAGCCGATGGCCGCAAGCCCGTTGGAGGTCATG 1320
11.DMC removal   392 ACCCTGGCGCTAGTGCACTCTTCGCTGGAGCTGATGGCCGCAAGCCCGTTGGAGGTCATG  451
                  ******************************** ****************************

11. dmc whole r 1321 TCCTGGCACATGCATCGGCGACTCGAGTGCTCCTCCGCAAGGGTCGTGGTGAAGAGCGAG 1380
11.DMC removal   452 TCCTGGCACATGCATCGGCGACTCGAGTGCTCCTCCGCAAGGGTCGT                498
                  ************************************************

11. dmc whole r 1381 TGGCTAAGATACAGGACTCGCCAGGTATGCCACAGTGTATACGTCCCATTCTAGTACTTT 1440
11.DMC removal   499                                                           498
```

```
12. DMC trimmed    1                       GCTTGCTGATCGTCAGCAGAAGTTGAATCAGTTCTTGAT   39
11. dmc whole r 1021 CGACTATTGTGGACGCGGAGAGCTTGCTGATCGTCAGCAGAAGTTGAATCAGTTCTTGAT 1080
                                           ****************************************

12. DMC trimmed   40 GAAGCTGGCTCATATGGCTGAGG-------------------------------------   62
11. dmc whole r 1081 GAAGTTGGCTCATATGGCTGAGGGTATATTTTTCACTCCTGATATATATATATCAAGTAC 1140
                  **** ******************

12. DMC trimmed   63 --------------------------AATTCAACATCTGTGTTCTGATGGCCCGTTCCCC   96
11. dmc whole r 1141 TAGAGGGCTGACATTTTCGTTTGCAGAATTCAACATCTGTGTTCTGATGGTCCGTTCCCC 1200
                                               ************************ *********

12. DMC trimmed   97 ATCGACATG                                                    105
11. dmc whole r 1201 ATCGACATGCAGATAGATAGATATAGTCTAATGCACTGCAGACCAACCAGGTCCAGAGTG 1260
                  *********
```

```
15. mrna trimme   1                                                          0
14. Hop2 ref ge   1 AGGCGCGAGGGAGTGATGCAGGGACTGTGCCCGTGCGGGTGAAGCTGGATGCTCAAAGAG  60


15. mrna trimme   1           TTGGCAGAATCAAGCCAATGTGCGCGGTCGGATTTGTCGCGATCTATGGC  50
14. Hop2 ref ge  61 ACTGGAAACATTGGCAGAATCAAGCCAATGTGCGCGGTCGGATTTGTCGCGATCTATGGC 120
                    **************************************************

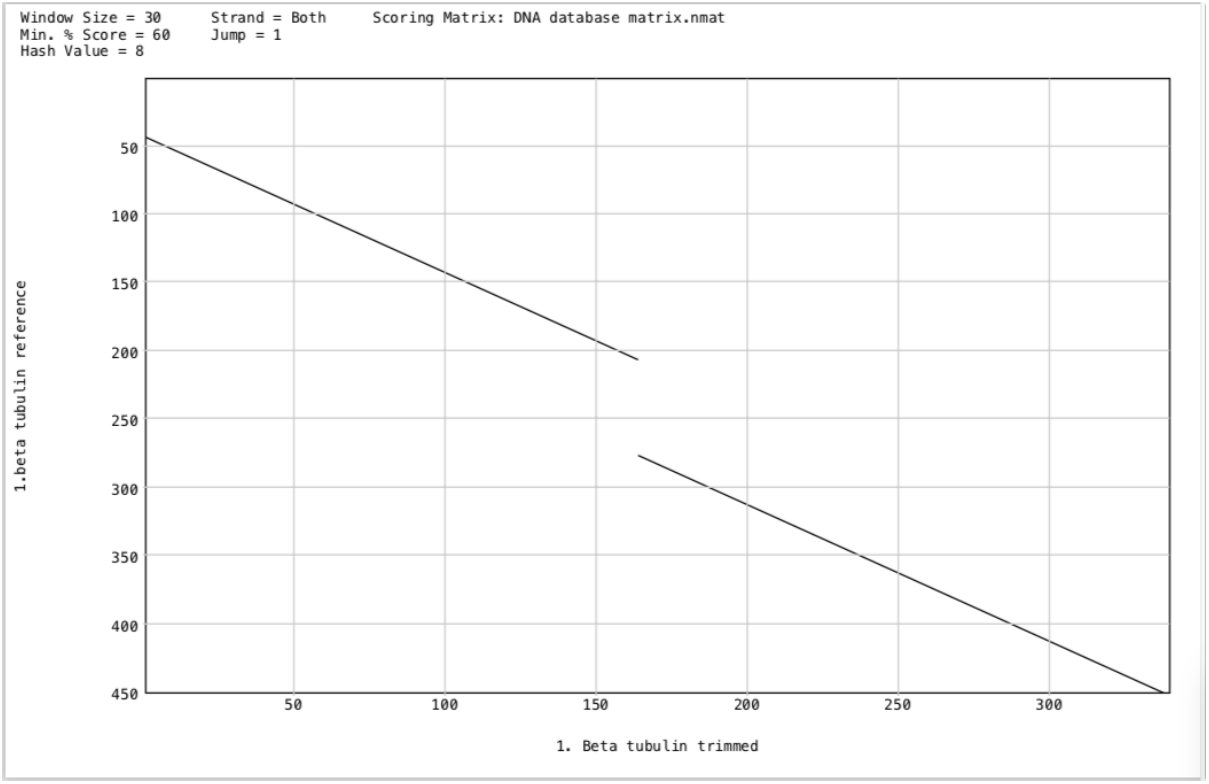
15. mrna trimme  51 GGATGTGTACGGAAGTTGTGCCCGACAACATGGACCGGGAAGAATTATGGG--------- 101
14. Hop2 ref ge 121 GGATGTGTACGGAAGTTGTGCCCGACAACATGGACCGGGAAGAATTATGGGTAAGACATA 180
                    **************************************************

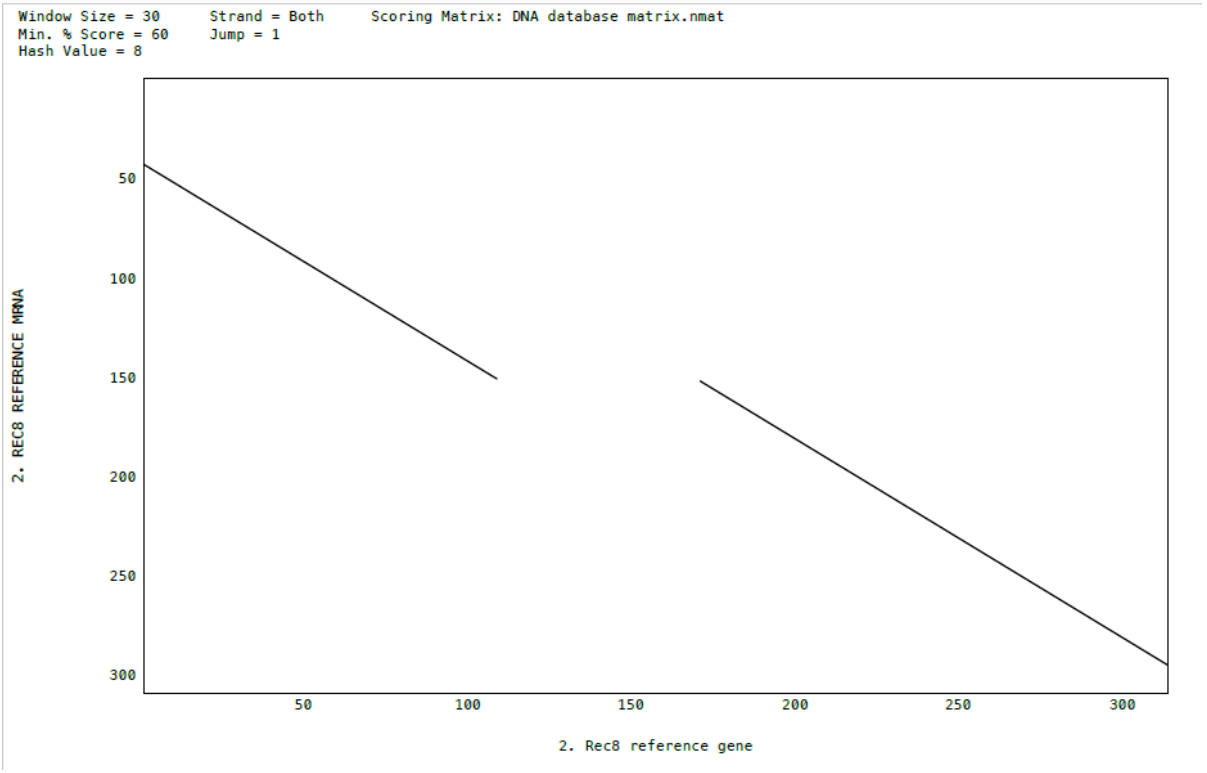
15. mrna trimme 102 ----------------------------------------------------AGAAT 106
14. Hop2 ref ge 181 ACAACCACTAGTCCGTATTGATGAGTGAGGGATGCTGACGATGTCGAGATGAAGGAGAAT 240
                                                                     *****

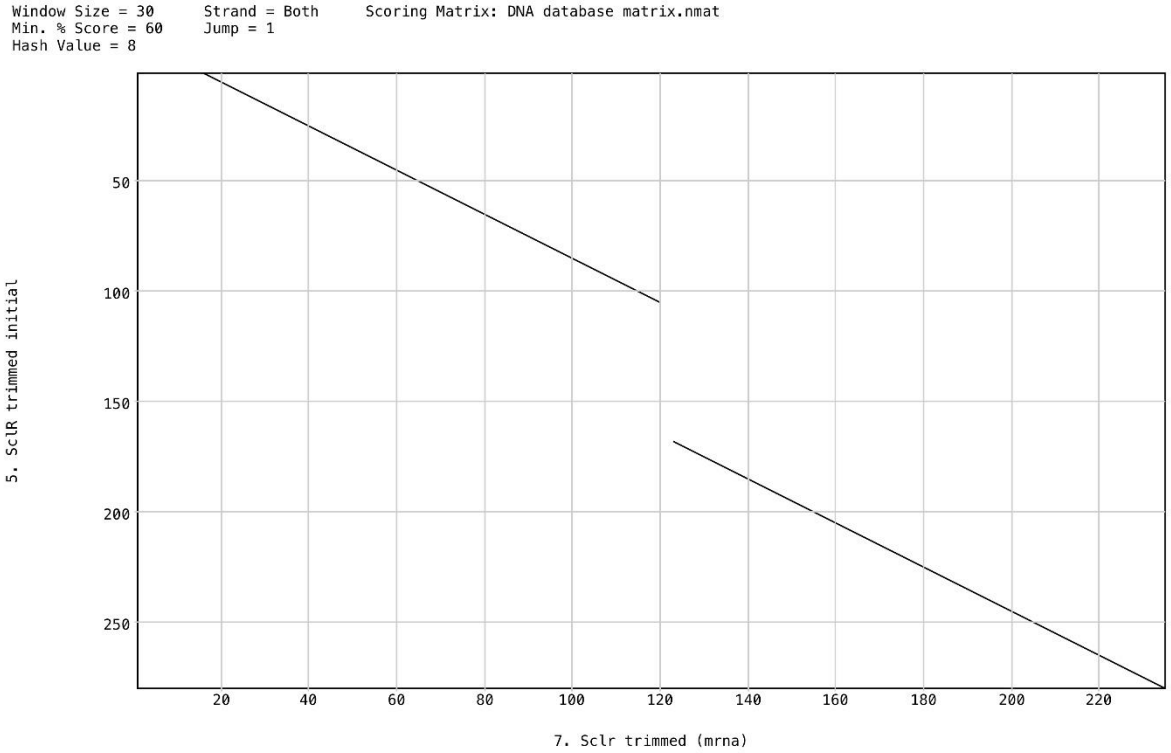
15. mrna trimme 107 CTGGGATTGGAAGGCCCTTTCCCCAAATGATGATGAAATGTGAGCTAGAGGTCTACTGGT 166
14. Hop2 ref ge 241 CTGGGATTGGAAGGCCCTTTCCCCAAATGATGATGAAATGTGAGCTAGAGGTCTACTGGT 300
                    ************************************************************

15. mrna trimme 167 CACAAGCAA                                          175
14. Hop2 ref ge 301 CACAAGCAAACACTGGTCCACGATCCTGAAATCTCCTATTGCCATTGA 348
                    *********
```

Appendix 16: Pustell DNA Matrix Plots (MacVector)



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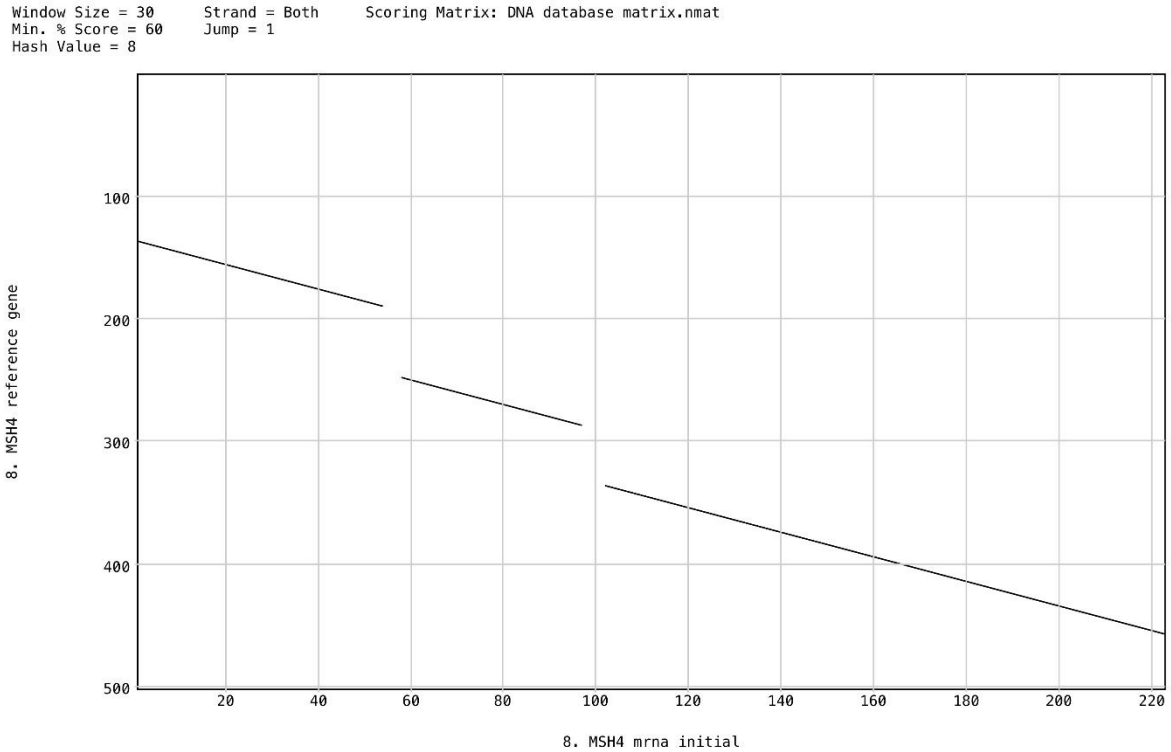
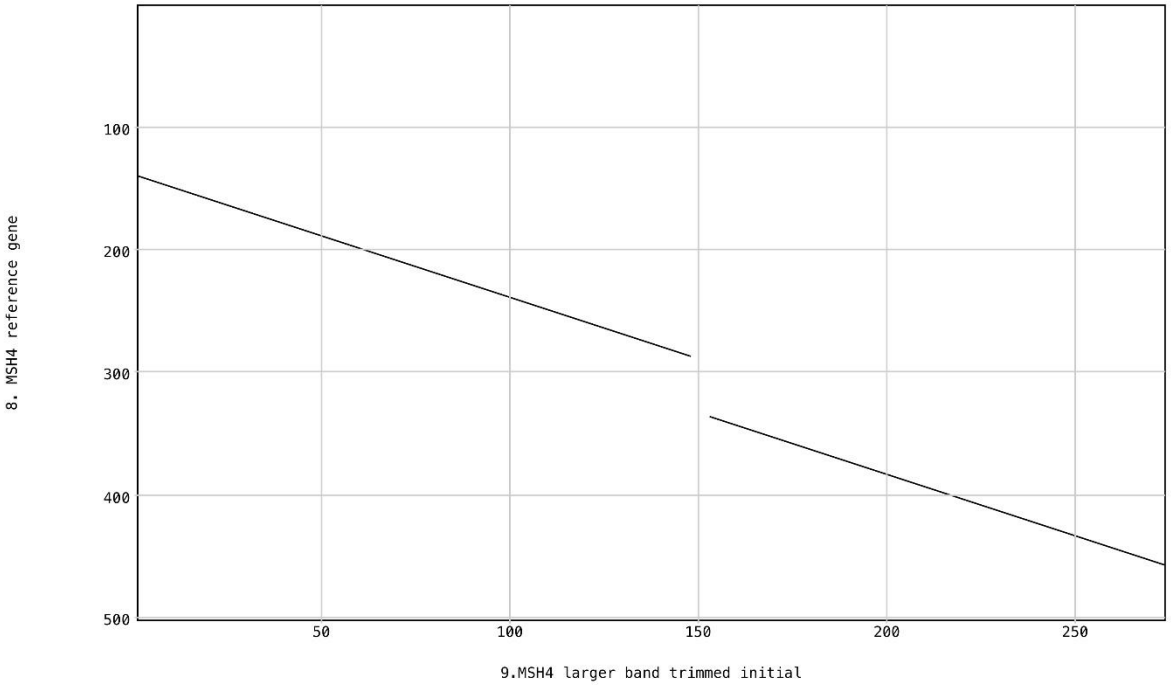
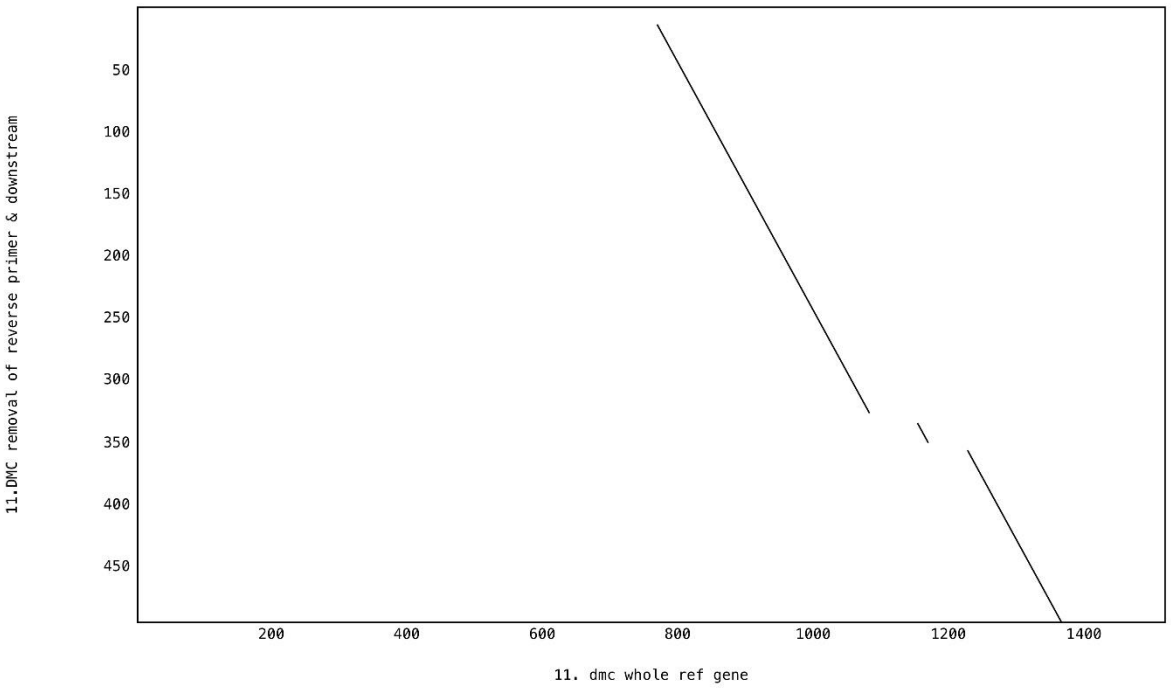
Strand = Both
Scoring Matrix: DNA database matrix.nmat
Min. % Score = 60
Jump = 1
Hash Value = 8
5. SclR trimmed initial
50
100
150
200
250
20
40
60
80
100
120
140
160
180
200
220
7. Sclr trimmed (mrna)

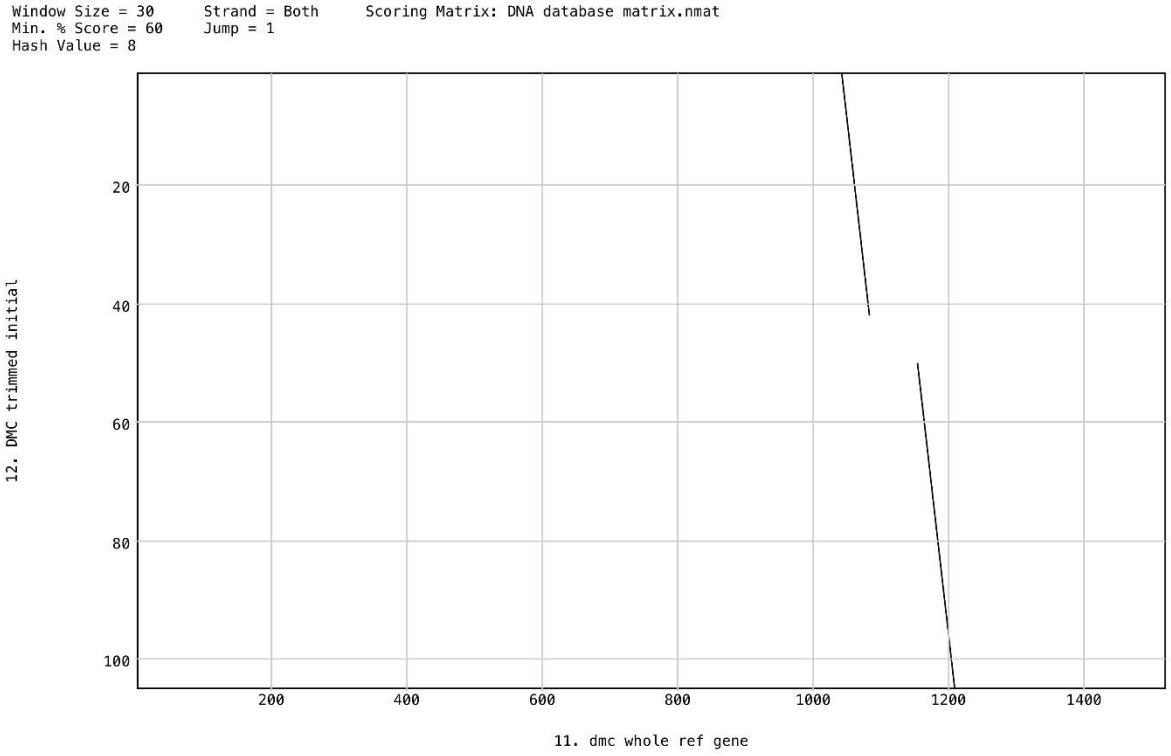

Window Size = 30
Strand = Both
Scoring Matrix: DNA database matrix.nmat
Min. % Score = 60
Jump = 1
Hash Value = 8
8. MSH4 reference gene
100
200
300
400
500
20
40
60
80
100
120
140
160
180
200
220
8. MSH4 mrna initial

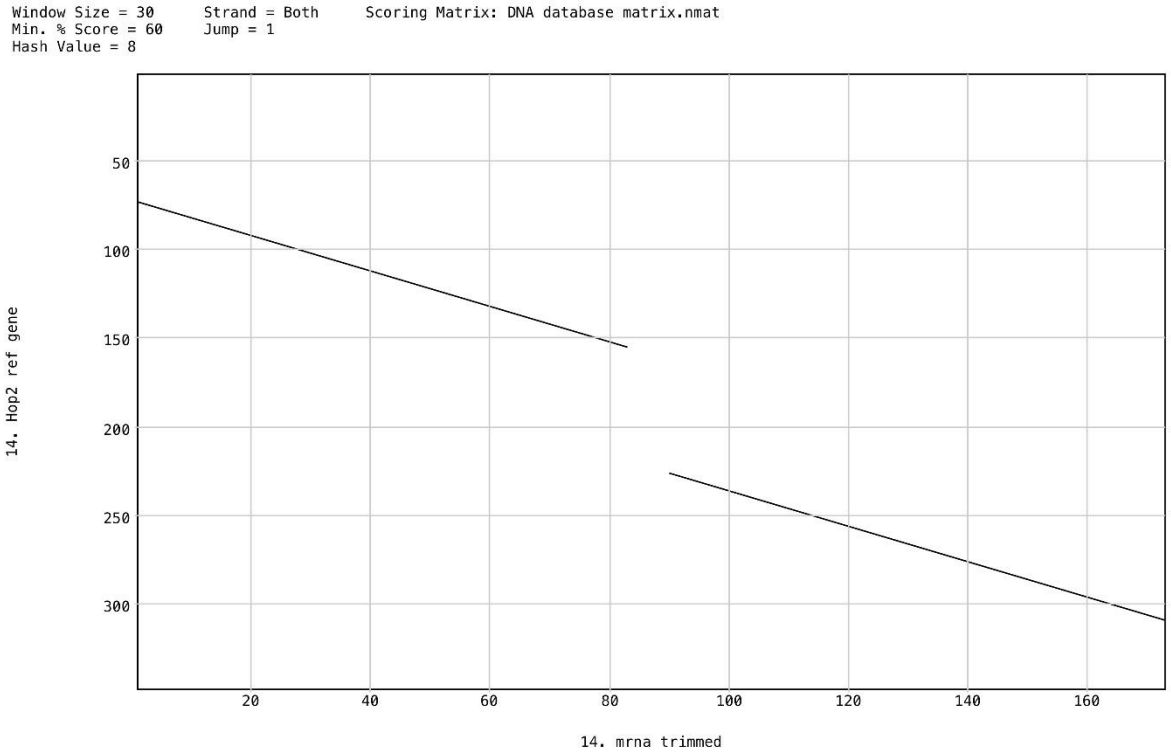
Window Size = 30 Strand = Both Scoring Matrix: DNA database matrix.nmat
Min. % Score = 60 Jump = 1
Hash Value = 8



Window Size = 30 Strand = Both Scoring Matrix: DNA database matrix.nmat
Min. % Score = 60 Jump = 1
Hash Value = 8




Window Size = 30
Strand = Both
Scoring Matrix: DNA database matrix.nmat
Min. % Score = 60
Jump = 1
Hash Value = 8
12. DMC trimmed initial
20
40
60
80
100
200
400
600
800
1000
1200
1400
11. dmc whole ref gene


Window Size = 30
Strand = Both
Scoring Matrix: DNA database matrix.nmat
Min. % Score = 60
Jump = 1
Hash Value = 8
14. Hop2 ref gene
50
100
150
200
250
300
20
40
60
80
100
120
140
160
14. mrna trimmed

Appendix 17: Colony PCR for *akuB/ku80*

AkuB upstr F Primer: atttcgccaacaaaggaccc

AkuB gene conf R Primer: ttagcggtttctggacgttc

Reverse Complement: gaacgtccagaaaccgctaa

627bp product

In red are the exons including the end of the previous upstream gene. Start of first Exon of AkuB/Ku80 marked by ATG*

Gtcaatttgcttctgacagaaaatgcggaggttgatgcatcatgtacactcattgggaccccgttggcggcagtc
atgcctcgtaaatggaagtcctgctgtggtcgttaccatcgaagctgtgctgagcaattaattgcttggggtgca
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gg

Appendix 18: Colony PCR for Δ*akuB_gpdA_trpC_ptrA* cassette

AkuB upstr F Primer: atttcgccaacaaaggaccc
gpdA conf R Primer: cacaccagcctttccacttc Reverse Complement: gaagtggaaaggctggtgtg
SmaI sites CCCGGG
NotI sites GCGGCCGC
XhoI sites CTCGAG
847bp Product

Tcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaagcggat
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Appendix 19: ~1 kb Upstream of *akuB*

Gtcaatttgcttctgacagaaaatgcggaggttgatgcatcatgtacactcattgggaccccgttggcggcagtc
atgcctcgtaaatggaagtcctgctgtggtcgttaccatcgaagctgtgctgagcaattaattgcttggggtgca
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gg

Appendix 20: In Silico Determination of RE for Southern Blot

AkuB WT

BamHI (yellow) 1812 bp

BamHI digestion site marked with * at 5-G*GATTC-3

TCCTCATCGCCGTAGCGCGTTCGGCAAAGTGTTTGTCAAATCTGCAACCCCGAACCATTATTGACTACTGCTGCG
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AkuB knockout with insert (egfp)

BamHI 3180 bp

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