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Exploitation of Fungal Sex for Biotechnology and Disease Control

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I. Abstract

The overall theme of work in the present thesis was to gain knowledge about aspects of the fungal sexual cycle that would allow the practical exploitation of sexual reproduction for use in biotechnology and disease control.

Investigations were first made into the potential sexual cycle in *Aspergillus niger* and *Aspergillus sclerotiicarbonarius*, members of the black aspergilli. Both species are proto-heterothallic, showing distinct *MAT1-1* and *MAT1-2* genotypes. Furthermore, both could be induced to produce sclerotia, a prerequisite for sex in the black aspergilli. In *A. niger* crosses involving one isolate, 8-160, sclerotia were found to contain red-pigmented structures following incubation for 6 months. Putative ascospores were then visualized from these structures through the use of high-resolution imaging techniques. This is the first report of ascospore formation by *A. niger,* indicating a potential sexual cycle in this species. However, it was not possible to induce germination of the putative ascospore progeny or demonstrate that recombination had occurred. In parallel, studies were undertaken to examine possible recombination within progeny from a sexual cross of *A. sclerotiicarbonarius*. SNP analysis provided evidence of recombination, adding new insights to the 'sexual revolution' within supposed asexual fungi.

Secondly, it was assessed whether the technique of bulk segregant analysis (BSA) combined with next-generation sequencing could determine the genetic basis of unknown forms of azole resistance in *A. fumigatus*. This species is the primary agent of aspergillosis, with a mortality rate of >50% and shows increasing resistance to current treatment therapies. Here, the sexual cycle of *A. fumigatus* was exploited for the first time in BSA studies to identify a linked region of the genome containing an itraconazole resistance mutation. The efficiency of BSA was then increased by manipulation of the number of backcrosses in tandem with an increased number of progeny in each DNA pool. This identified smaller linked regions of the genome containing fewer candidate genes. Therefore, BSA may provide a valuable technique to identify novel antifungal resistance genes in *A. fumigatus*.

Thirdly, investigations were made to assess the presence and bioactivity of fungal sex hormones as a potential basis for novel disease control. The homothallic species *Neosartorya* (*Aspergillus*) *fischeri* was used as a model organism. Lipid extractions were made from mating and asexual cultures and bioassayed for activity. Extracts from mating cultures were found to increase production of cleistothecia in a dose-dependent manner. However, the sexual extract had no clear impact on asexual conidiation in *N. fischeri*, and no clear developmental impacts on closely related species. Therefore, it was uncertain whether the extract contained a true sex morphogen.

Finally, the role of oxygenase family *ppo* and *lox* genes in sexual reproduction of *A. fumigatus* was explored. Using deletion and overexpression mutants, it was found that Δ*ppoB* and OE: *loxAB* strains showed increased cleistothecia development in mating cultures, whereas any alteration of expression of *ppoA, ppoC, loxA* or *loxB* genes led to absence of cleistothecia production. This demonstrates for the first time the important role of oxygenase family genes in sexual reproduction of *A. fumigatus,* indicating a possible wider role in regulation of sexual reproduction in heterothallic pezizomycete species.

II. Acknowledgments

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Table of Contents

I. Abstract 2

II. Acknowledgments 4

Chapter 1 – Introduction to Fungal Sex 8

1.1 Introduction 8

1.1.1 Sexual reproduction in fungi 9
1.1.2 Sex in the Ascomycota 10
1.1.3 Sex in the Basidiomycota 20
1.1.4 Early *MAT* genes 21
1.1.5 *MAT* genes of the *MAT1-1* and *MAT1-2* loci in the Ascomycota 23
1.1.6 *MAT* genes as master regulators 27
1.1.7 Other genes involved in sexual reproduction 28
1.1.8 Cryptic sexuality 30
1.1.9 Exploitation of the sexual cycle 31
1.1.10 Fungal sex hormones 34
1.1.11 Aims and objectives 35

Chapter 2 – Sex in the Black Aspergilli 36

2.1 Introduction 36

2.1.1 *Aspergillus sclerotiicarbonarius* 38
2.1.2 *Aspergillus niger* and citric acid production 39
2.1.3 Strain improvement 40
2.1.4 Aims 41

2.2 Methods 42

2.2.1 Strains used 42
2.2.2 Strain maintenance and revival 43
2.2.3 DNA extraction 43
2.2.4 *MAT* type analysis 44
2.2.5 RAPD analysis 45
2.2.6 Genetic similarity 46
2.2.7 Sclerotia production trial 47
2.2.8 Barrage zone trial 48
2.2.9 Sexual crosses 51
2.2.10 Examination of sclerotia 51
2.2.12 Isolation of ascospores 52
2.2.13 Analysis of potential progeny 54
2.2.14 Sexual recombination in *Aspergillus sclerotiicarbonarius* 54
2.2.15 Statistical Analysis 57

2.3 Results 57

2.3.1 Mating-type (*MAT*) Assay 57
2.3.2 RAPD – Genetic similarity between parent strains 59
2.3.3 Sclerotia production trials 60
2.3.4 Barrage zone trial 68
2.3.5 Examination of sclerotia 69
2.3.6 Examination of ascospores 72
2.3.7 Recombination analysis of potential progeny 77
2.3.8 Sexual Recombination in *Aspergillus sclerotiicarbonarius* 79

2.4 Discussion 82

2.4.1 Enhancement of sclerotia production in *A. niger* 82

2.4.2 Discovery of putative sexual reproduction in *A. niger* 85
2.4.3 Sclerotia imaging of *A. niger* 86
2.4.4 Ascospore germination in *A. niger* 87
2.4.5 Sexual recombination and germination of ascospores in *A. niger* 88
2.4.6 Strain improvement of *A. niger* 90
2.4.6 Concluding remarks 92

Chapter 3 – Use of Bulk Segregant Analysis to Investigate Antifungal Resistance in *Aspergillus fumigatus* 93
3.1 Introduction 93
3.1.1 Host immune system 93
3.1.2 C-type lectins 95
3.1.3 Treatment of aspergillosis 95
3.1.4 Drug resistance 97
3.1.5 CYP51A 99
3.1.6 Other mutations 100
3.1.7 Bulk segregant analysis (BSA) 102
3.1.8 Aims 106
3.2 Methods 106
3.2.1 Strain maintenance and revival 106
3.2.2 Itraconazole resistance screening 107
3.2.3 Mating-type (*MAT*) analysis 107
3.2.4 Oatmeal agar preparation 108
3.2.5 Sexual crosses 109
3.2.6 Harvesting cleistothecia 109
3.2.7 Ascospore isolation 110
3.2.8 RAPD-PCR 110
3.2.9 Preparation for bulk segregant analysis 111
3.2.10 DEEP Seq 113
3.2.11 Sequence data analysis 115
3.2.12 Mathematical model for variant prediction 116
3.2.13 Sequencing of *cyp51A* in other resistant strains 116
3.3 Results 118
3.3.1 Mating-type PCR 118
3.3.2 RAPD analysis 118
3.3.3 ETEST - Itraconazole resistance 119
3.3.4 Mathematical model for variant prediction 121
3.3.5 1st round sequencing and analysis 122
3.3.6 2nd round sequencing and Analysis 131
3.3.7 3rd round sequencing and analysis 135
3.3.8 4th round sequencing and analysis 137
3.3.9 Distribution plot – 1st round sequencing JGI 140
3.3.11 Sequencing of *cyp51A* 142
3.4 Discussion 144
3.4.4 Future work 149

Chapter 4 – Investigation of the Role of Hormones in Fungal Sexual Development and Possible Uses for Disease Control 152
4.1 Introduction 152
4.1.1 Sex in the Oomycota 153
4.1.2 Sex in the Chytridiomycota 155
4.1.3 Sex in the Zygomycota 157

4.1.4 Sex in the Ascomycota 159
4.1.5 Aims 165
4.2 Methods 167
4.2.1 Selection of model fungal species for hormone extraction 167
4.2.2 Culture storage, collection and revival 168
4.2.3 Conditions for induction of sexual reproduction 169
4.2.4 Control non-sexual culture conditions 170
4.2.5 Hormone extraction 171
4.2.6 Hormone bioassay 173
4.2.7 Hormonal 'cross talk' 174
4.2.8 *Aspergillus fumigatus* oxygenase mutants - strains used and media supplementation 175
4.2.9 Sexual crosses 176
4.2.10 Scoring of *A. fumigatus* sexual crosses 177
4.2.11 Harvesting cleistothecia and single ascospore progeny 177
4.2.12 RAPD PCR 178
4.2.13 Data analysis 179
4.3 Results 180
4.3.1 Selection of sexual species for hormone extraction 180
4.3.2 Hormone bioassay 181
4.3.3 Hormone 'cross talk' 188
4.3.4 Production of cleistothecia in oxygenase mutants of *A. fumigatus* 191
4.3.5 RAPD PCR recombination 194
4.4 Discussion 196
4.4.1 Fungal sex hormones 196
4.4.2 *Aspergillus fumigatus* oxygenase mutants 200
4.4.3 Concluding remarks 204

5. General Discussion 205
5.1 Exploitation of Fungal Sex for Biotechnology and Disease Control 205
5.2 Concluding Remarks 210

References 212

Appendix – Growth Media 248
1 Growth media (per litre) 248
1.1 *Aspergillus* complete medium (ACM): 248
1.2 *Aspergillus* salt solution: 248
1.3 *Aspergillus* trace element solution: 248
1.4 *Aspergillus* vitamin solution: 248
1.5 Carrot agar: 249
1.6 Cornmeal agar: 249
1.7 Czapek's yeast autolysate: 249
1.8 M40Y: 249
1.9 Malt extract agar (MEA): 249
1.10 Oatmeal agar (OA): 250
1.11 RPMI 1640: 250
1.12 Synthetic growth medium: 250
1.13 Synthetic growth medium trace elements: 251
1.14 Water agar 251

Chapter 1 – Introduction to Fungal Sex

1.1 Introduction

The sexual reproductive pathway is a complex process, which has been widely researched within the fungal kingdom. This is partly due to the large number of fungal species capable of undergoing a sexual cycle, in combination with the vast array of sexual structures that can be formed by different taxonomic groupings of fungi. This has stimulated much interest in this area of research, and has led to a number of studies being performed to understand the environmental factors that control sex, the precise nature of the morphological changes that occur during sex, and subsequently to identify the genetic basis of sexuality in fungi (Metzenberg and Glass, 1990; Yun *et al.*, 2000).

Surprisingly, 20% of fungal species lack a known sexual cycle (Dyer and O'Gorman, 2012). This led to these fungi historically being termed 'imperfect', whilst sexually reproducing species are 'perfect' (Howell, 2003). Of applied significance is that the lack of a sexual reproductive cycle has been a particularly prominent feature in many species that are of industrial and medical importance. Therefore, research investigating the possibility of sexuality, and the induction of a sexual cycle, in these species has been a primary focus (Ropars *et al.*, 2014; Horn *et al.*, 2013; Dahlmann *et al.*, 2015). Discovery of a sexual cycle in such important industrial and medical species would open the door to numerous genetic techniques for strain improvement and trait analysis (Ashton and Dyer, 2016). Research in this area has proved successful in inducing sexual reproduction in a number of species that were previously considered 'asexual', such as *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus fumigatus, Penicillium chrysogenum* and *Penicillium roqueforti* (Horn *et al.,* 2009a; Horn *et al.*, 2009b; O'Gorman *et al.,* 2009; Böhm *et al.*, 2013; Ropars *et al.*, 2014). This topic will be further discussed in more depth later in this introductory chapter.

1.1.1 Sexual reproduction in fungi

Sexual reproduction in fungi can involve a wide range of different sexual strategies or 'breeding systems' according to the specific species involved. Initiation of sex may be from fusion of nuclei from two separate complimentary mating partners, known as 'heterothallic' sex. Heterothallic species are obligate outbreeders and require the presence of two partners of different 'mating type' for sex to occur, such as seen with *Aspergillus fumigatus* (O'Gorman *et al.*, 2009). Alternatively, fungal species may be 'homothallic' where sexual reproduction can occur in monoculture, requiring only one individual to initiate and complete the cycle, as seen in *Fusarium graminearum* (Sikhakolli *et al.*, 2012). However, many homothallic species are not restricted to self-fertility and retain the ability to outcross.

Furthermore, some species are termed 'pseudo-homothallic' and are capable of both self-breeding and out-breeding if certain conditions are met (Grognet and Silar, 2015). The form of breeding that a fungal species adheres to is controlled by the presence of 'mating-type' (abbreviated to '*MAT*') genes, which determine the sexual identity of fungal isolates. Such *MAT* genes, and accompanying sexual processes, have been mainly characterized from the Ascomycota and Basidiomycota, as will be described in the following sections **1.1.2-1.1.3**.

Finally, another form of genetic exchange that can occur between filamentous fungi is through the parasexual cycle. First described in *A. nidulans* (Pontecorvo *et al.,* 1953), parasexuality refers to a process involving first the fusion of vegetative hyphae containing genetically distinct nuclei to form a heterokaryon. Within the heterokaryon nuclear fusion of the genetically distinct nuclei then occurs to form a diploid nucleus. However, unlike regular sexual reproduction, meiosis does not then occur. Instead, aberrant mitosis subsequently occurs alongside recombination or haploidization (Moore *et al.,* 2011), leading to the formation of diploid or haploid recombinant nuclei (**Figure 1.5**). Heterokaryon formation can only occur in the first place when compatible partners are present. The process of heterokaryon formation is regulated by so-called 'het' loci, with fusion

between incompatible hyphae leading to cellular death (Saupe, 2000). This is termed heterokaryon incompatibility. It has been proposed that during aberrant fusion events in certain species, *MAT* genes can act as het loci, whereby *MAT1-1* loci recognize incompatible *MAT1-2* loci, and vice versa (Glass and Kuldau, 1992).

The parasexual cycle has been exploited for a variety of different uses in what were once thought to be asexual species, such as essential gene mapping studies in *A. fumigatus* (Firon *et al.,* 2002) or to enable strain improvement in the penicillin producer *P. chrysogenum* (Ball, 1971).

1.1.2 Sex in the Ascomycota

The genetic factors controlling sexual reproduction in the Ascomycota have been widely studied (Lee *et al.,* 2008; Bobrowicz *et al.*, 2002; Hull *et al.,* 2000; Michaelis and Herskowitz, 1988). This phylum of the fungal kingdom can be subdivided into the Saccharomycotina (the yeasts) and the Pezizomycotina (the filamentous ascomycetes).

1.1.2.1 Sex in the Saccharomycotina

The first in depth studies of mating-type determination were made with the yeast *Saccharomyces cerevisiae.* Study of mating genes in this species has given great insight into homologous recombination, gene regulation and chromosome structure (San Filippo *et al.*, 2008; Aylon and Kupiec, 2004; Hanson and Wolfe, 2017). In *S. cerevisiae*, three types of cell exist that grow vegetatively: **a** mating type cells; α type cells; and the **a**/α cells. The **a** and α exist as haploid cells and upon mating, due to pheromone signaling, form the diploid **a**/α as a result of the fusion of nuclei (Haber, 2012). Pheromone signaling in yeast is described in more detail in section **4.1.4.1.**

The mating type of *S. cerevisiae* is determined by the two mating-type alleles *MAT***a** and *MATα* and sexual reproduction is facilitated in this species due to its ability to switch mating type (Strathern *et al.*, 1980). This is a form of homothallism, as described in section **1.1.1**.

Mating-type switching is achieved through the presence of mating-type cassettes located on chromosome III in the *S. cerevisiae* genome. These cassettes consist of three loci – *HML, HMR* and a *MAT* locus containing either *MAT***a** or *MATα* (Mahoney and Broach, 1989). Furthermore, additional copies of the *MATα* or *MAT***a** genes are found at the *HML* and *HMR* sites (Astell *et al.*, 1981). Generally, laboratory strains normally have a silent *HML*α and *HMR***a** configuration, although the opposite configurations are known to exist (Haber, 2012). In both instances, the genes at HML and HMR are silenced by short regions of heterochromatin (**Figure 1.1)**.

Mating-type switching occurs as a result of homologous recombination due to the formation of double strand breaks by the HO endonuclease (Katz Ezov *et al.*, 2010; Aylon and Kupiec, 2004). This leads to the switching from one mating type to the other using genetic information at the *HML*α or *HMR***a** loci as donors as shown in **Figure 1.1** (Strathern *et al.*, 1982). Further information on this process can be found in the review by Haber (2012).

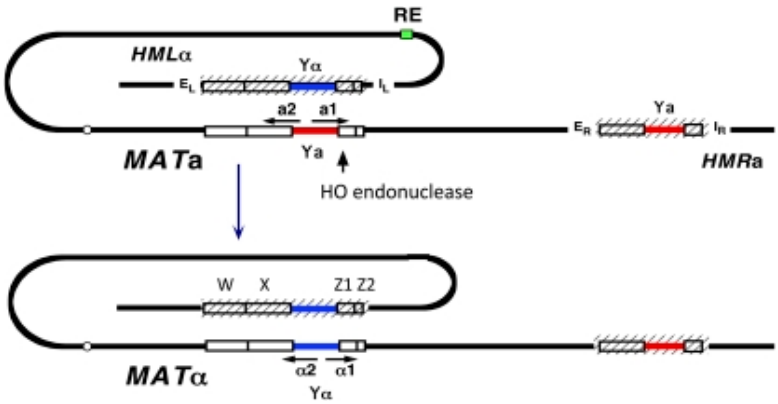


Figure 1.1 *– Representation of the bidirectional MAT locus of* S. cerevisiae*, showing the gene conversion of MAT***a** *to MATα. Here, HMLα and HMR***a** *are silenced by short regions of heterochromatin (hatched line). Through the action of the HO endonuclease, homologous recombination occurs leading to the mating-type switch to MATα. From Haber (2012).*

Each *MAT* allele encodes two mating-type proteins under the control of a bidirectional promoter (**Figure 1.2**). *MAT***a** encodes *MAT***a***1* and *MAT***a***2* homeodomain proteins, whilst *MATα* encodes a *MATα1* alpha domain and *MATα2* homeodomain protein (Herskowitz *et al.,* 1992). The functions of the MAT genes in haploid isolates are summarized in **Table 1.1**.

Table 1.1 *– Genes contained within the* MAT *loci of* S. cerevisiae *and their associated function (if known).*

Allele	**Gene**	**Function**	**Reference**
*MAT***a**	*MAT***a***1*	No effect on **a** specific genes required for mating	Herskowitz, 1989
	*MAT***a***2*	Unknown	Herskowitz *et al.,* 1992
MATα	*MATα1*	α factor expression; *ste2* expression to control formation of reciprocal pheromone **a**	Tan *et al.,* 1988; Grayhack, 1992
	MATα2	Repression of *MF***a***1* and *MF***a***2* (**a** factor pheromone genes); repression of *ste3* (α factor receptor gene)	Herskowitz, 1989

Interestingly, in the diploid *MAT***a**/*MATα* cell, *MAT***a***1* expression coupled with *MATα2* expression leads to the formation of a heterodimer that represses the activity of haploid specific genes, allowing for the induction of a diploid specific gene set (Goutte and Johnson, 1988; Dranginis, 1990). Additionally, this cell type is unable to mate due to the repression of *MATα1* by the **a**1-α2 heterodimer resulting in the inability to produce α factor. Simultaneously, *MATα2* remains expressed which represses the production of **a** factor.

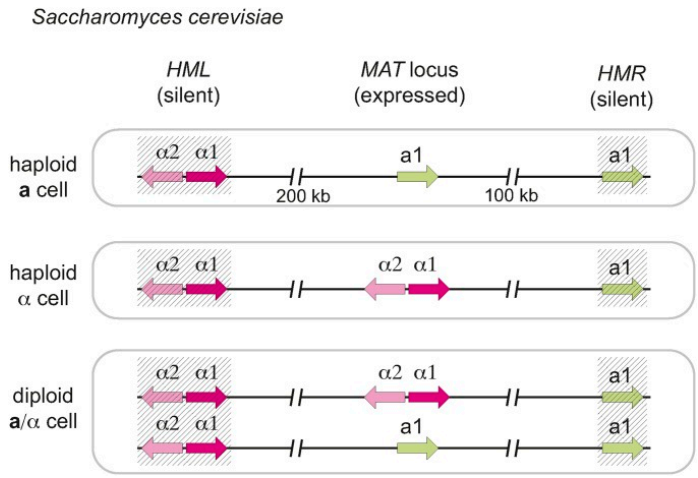


Figure 1.2 *– Representation of the mating type loci of* S. cerevisiae*, showing the haploid* **a** *and α loci, and diploid* **a**/*α cell loci. From* Hanson and Wolfe (2017).

Following the characterization of the mating type system of *S. cerevisiae*, further studies were made to investigate asexuality and sexuality in a number of other members of the Saccharomycotina. A medically important example of this is studies concerning *Candida albicans*, the primary cause of candidiasis infection in immunocompromised patients (Noble and Johnson, 2007). This species is found in nature predominantly in a diploid form and was initially thought to be entirely asexual due to the absence of evidence of sexual recombination in this species (Taylor *et al.*, 1999). However, comparative genomic analysis, using the *S. cerevisiae* mating-type genes as search terms, revealed that the *C. albicans* genome contained many orthologous genes involved in the sexual cycle, such as meiosis genes (Tzung *et al.*, 2001). Indeed, a prior study showed the presence of a mating-type like (MTL) locus in *C. albicans* which was able to express the *MAT* genes *α1* and *α2* on one chromosome and **a*1*** on the other in heterozygous isolates (Hull and Johnson, 1999). Through the production of *MATα* only and *MAT***a** only *C. albicans* strains, it was possible to induce mating of strains when inoculated into a mammalian host (Hull *et al.,* 2000). Later, 95% of clinical isolates were found to exhibit a heterozygous gene profile for the *MAT* types (i.e.

*MAT***a**/*MATα*), which may explain why observation of a sexual cycle was difficult (Lockhart *et al.*, 2002).

An important aspect of pathogenesis in *C. albicans* infections is the ability to switch between two forms – white and opaque – under certain conditions (Slutsky *et al.*, 1987). It was discovered that this switch was tightly regulated by the mating-type genes and that opaque type cells were able to mate 10^6 times more efficiently than their white counterparts (Miller and Johnson, 2002). This feature is distinctly different from that seen in *S. cerevisiae.* Interestingly, use of a chemically synthesised *C. albicans* α factor pheromone induced shmooing in homozygous *MAT***a** opaque isolates, but not in white type isolates (Lockhart *et al.*, 2003). As discussed previously, heterozygous *MAT***a**/*MATα* isolates were unable to mate. This is similar to the mechanism seen in *S. cerevisiae*, where an **a**1-α2 heterodimer represses sexual reproduction. Additionally, the switch from white-opaque is also repressed in heterozygous isolates of *C. albicans* (Bennett and Johnson, 2005).

A key feature to note concerning *C. albicans* is that although mating between strains has been demonstrated, involving fusion of diploid homozygous strains leading to a tetraploid heterozygous form, a complete sexual cycle with subsequent meiosis has not yet been demonstrated. Instead, in *C. albicans* genetic exchange can occur in the tetraploid heterozygous state, and this is followed by chromosome loss to restore the diploid state i.e. a parasexual process (**Figure 1.3**). This is distinctly different from the true sexual cycle in *S. cerevisiae*, which involves fusion of a haploid state to form a diploid heterozygous state, followed by meiosis.

Further information of sexual reproduction within the Saccharomycotina is beyond the scope of this study. However, see a review by Hanson and Wolfe (2017) for further information on sexual reproduction in this fungal group.

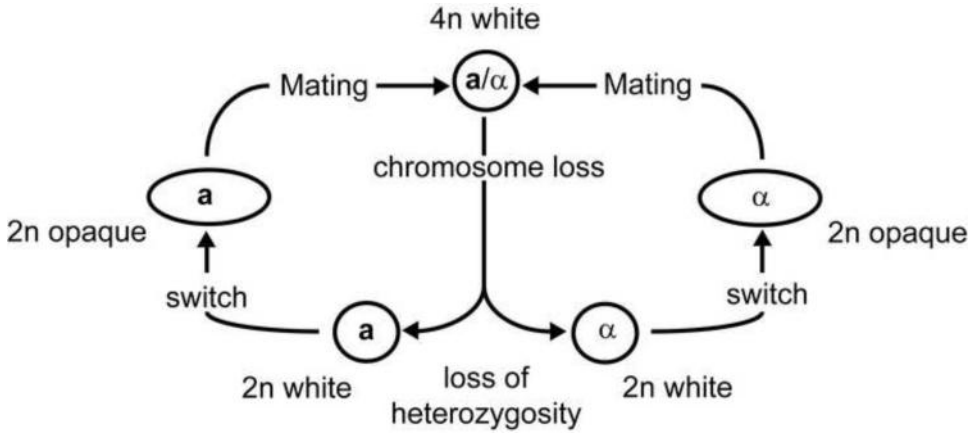


Figure 1.3 *– Representation of the parasexual life cycle of* C. albicans*. Here mating occurs from the fusion of the diploid opaque α and* **a** *cells to form a tetraploid cell. This tetraploid cell may undergo chromosome loss to produce either a heterozygous α/***a** *cell or lose heterozygosity to form a homozygous mating type. From* Lohse and Johnson (2009)*.*

1.1.2.2 Sex in the Pezizomycotina

Work in the present study is primarily focused on the Pezizomycotina, which encompasses a large number of species of economic and medical importance. For example, *Aspergillus niger* used in citric acid production; *Trichoderma reesei*, used for to the production of cellulytic enzymes (Seidl *et al.*, 2009); and *P. chrysogenum*, used in penicillin production (Dahlmann *et al.*, 2015). In heterothallic species, the initiation of the sexual cycle occurs with the fusion of hyphae from the two complimentary mating partners in a process known as plasmogamy. Mate attraction in fungi is governed by the presence of diffusible pheromones (Lee *et al.* 2010). Once this process has been completed, the haploid nuclei from each fungal partner fuse, known as karyogamy, to form a diploid nucleus. Following the formation of a diploid nucleus, meiosis occurs to form four haploid nuclei, followed by at least one round of mitosis to normally form eight ascospores, which are contained within asci (sacs). The number of rounds of mitosis varies according to the fungal species.

In parallel to ascospore formation, developmental programmes occur to form fruiting bodies known as ascomata (also termed ascocarps), which surround, nourish and protect the ascogenous hyphae and developing ascospores. Four different types of ascomata have traditionally been recognised in the Ascomycotina: cleistothecia (which are globular and enclosed); perithecia (flask shaped structures with an open pore to allow ascospore discharge); apothecia (saucer shaped structures which leave the ascospores exposed on the surface); and pseudothecia (resemble the flask structure of perithecia, but with different asci organisation) (Debuchy *et al.* 2010). In the case of cleistothecia, some species produce ascomata that develop within larger structures termed sclerotia, which are similar to cleistothecia in shape, but with a much thicker outer layer. Sclerotia can also act as over-wintering bodies in a number of plant pathogens, independent of sex, so might be considered as secondary sexual structures (Horn *et al.*, 2013; Wicklow, 1991). A summary of the morphologies of different types of ascomata is shown in **Figure 1.4**.

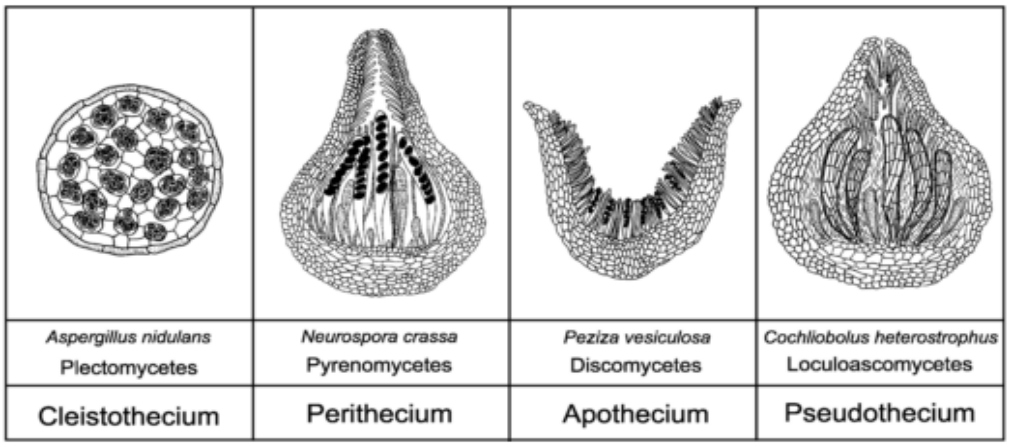


Figure 1.4 – *Representation of the different secondary sexual structures produced by fungi showing the different organization of asci. From* Pöggeler et al. *(2006).*

Once matured, sexual ascospores from fungi are released either actively or passively. The ascospores then germinate when exposed to favorable conditions, initiating an asexual growth cycle. The growth cycle of fungi is represented in **Figure 1.5**. For more information on the parasexual cycle, see section **1.1.1** above.

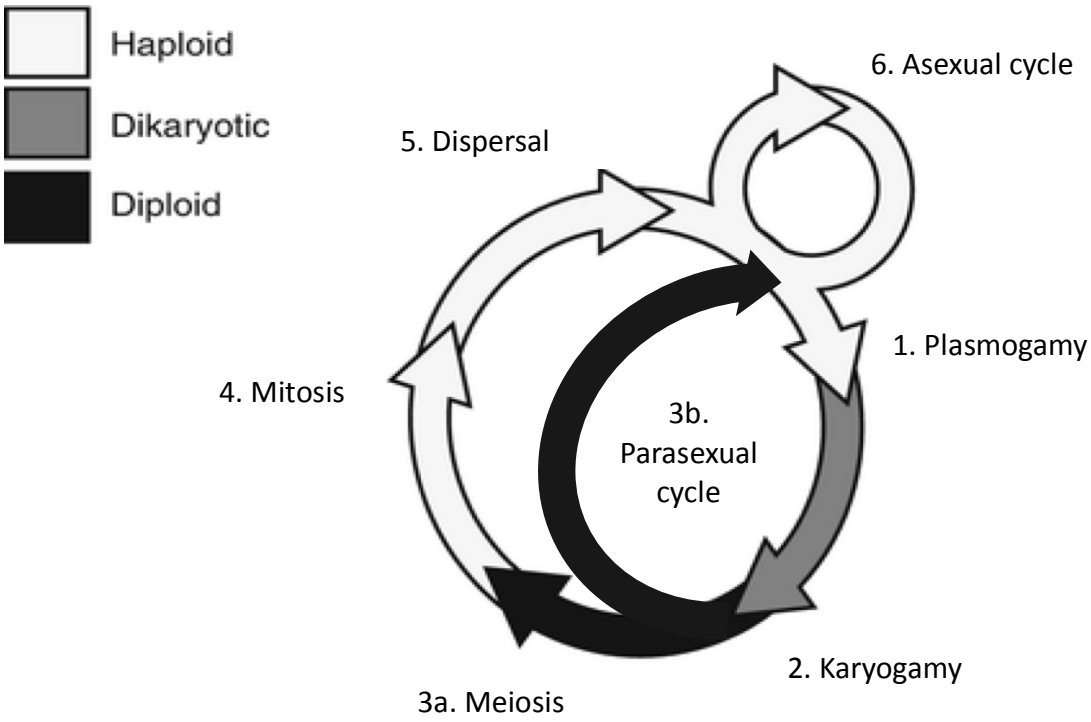


Figure 1.5 *– Life cycle of the Pezizomycete fungi showing the sexual (3a), parasexual (3b) and asexual (6) life cycles. The sexual and parasexual cycle begin when: (1) hyphal fusion occurs between two complimentary mating partners in the sexual cycle, or vegetative hyphae creating a heterokaryon during parasexuality; (2) once hyphal fusion has completed, the two haploid nuclei fuse in a process known as karyogamy; (3a) in the sexual cycle, upon formation of the diploid nucleus meiosis occurs to form four haploid nuclei, or (3b) in the parasexual cycle, aberrant mitosis, recombination and haploidization occur to produce recombinant haploid nuclei, which may re-enter (1) or continue to the asexual cycle (6). In the case of the sexual cycle, (4) a second round of division occurs to form a total of 8 ascospores in a mitotic process; (5) the ascospores are stored within species-specific sexual structures and released either actively or passively; (6) once released, the ascospores then germinate and either undergo an asexual or sexual life cycle dependent on conditions. Modified from Ashton and Dyer (2016).*

Concerning the genetic control of sexual identity in the Pezizomycotina, in heterothallic species two mating types are present, which by convention are now termed *MAT1-1* or *MAT1-2* (Turgeon and Yoder, 2000). Mating-type identity is determined by the presence of *MAT* genes present at a single *MAT* locus within the genome. It is a requirement for heterothallic species that sexually compatible *MAT1-1* and *MAT1-2* mating partners be present for sexual reproduction to occur (Coppin *et al.*, 1997). By contrast, in homothallic species, genes imparting both *MAT1-1* and *MAT1-2* identity are present in the same genome, often closely linked, which therefore enables self-fertility (Paoletti *et al.*, 2007). It is noted that for some species a different mating-type nomenclature is used as these pre-dated the convention of Turgeon and Yoder (2000). For example in *Neurospora crassa* the mating types are known as matA and mat**a**, and in *Podospora anserina* they are termed mat '+' (plus) '-' (minus) (Arnaise *et al.,* 1993; Grognet *et al.*, 2014).

Various *MAT* genes may be present at the *MAT* locus of both heterothallic and homothallic species, as described in more detail in section **1.1.5** below. In the case of heterothallic species, the *MAT* locus contains DNA sequence that is highly dissimilar between isolates of different mating type, and the loci have therefore been termed 'idiomorphs' to recognize the fact that these are not traditional alleles of a single locus (Metzenberg and Glass, 1990). A representation of the *MAT* loci in homothallic and heterothallic fungi is shown below (**Figure 1.6**). Of particular note and importance is that the *MAT* loci invariably include either a *MAT1-1* gene, which encodes a protein with an alpha-box DNA binding domain, or alternatively a *MAT1-2* gene, which encodes for a protein with a high mobility group (HMG) domain (Li *et al.*, 2013). Both types of these MAT proteins act as transcription factors, which are vital for regulation for many processes relating to sexual reproduction, such as sexual structure formation (Doughan and Rollins, 2016). A variety of other genes are encoded within the *MAT* loci and are discussed later on (Section **1.1.5**).

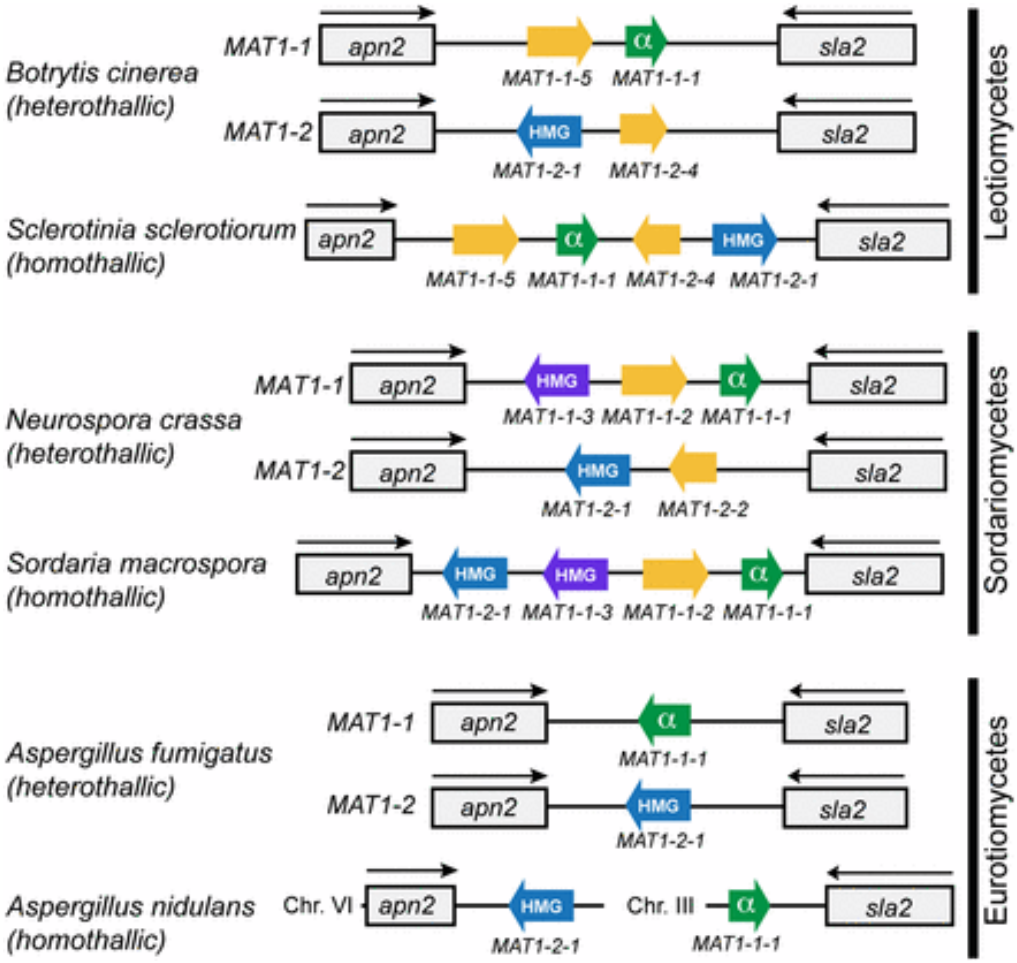


Figure 1.6 – *Representation of the MAT loci, and a selection of genes within, in the Pezizomycotina showing heterothallic and homothallic loci of the Leotiomycetes, Sordariomycetes and Eurotiomycetes. From* Palmer *et al.* (2014). *Further information detailing the function of each MAT gene shown can be found in* ***Tables 1.2*** *and* ***1.3*** *below.*

1.1.2.3 Sex and asexuality in the Pezizomycotina

The discovery and characterization of *MAT* loci in the Pezizomycotina has enabled research over the past decade that has been pivotal in identifying the potential for various species to undergo sexual reproduction (Bihon *et al.*, 2014; Paoletti *et al.*, 2005). In the past, observation of a sexual cycle (i.e. the presence of fruiting bodies containing sexual spores) was the only way to confirm if a species was capable of sex. However, *MAT* genes have been identified in a number of presumed asexual fungi (Bihon *et al.*, 2014; Geng *et al.*, 2014; Arie *et al.*, 2000). Due to their primary role as regulators of sexual

reproduction, it could be argued that their presence indicates a hidden or 'cryptic' sexual cycle. In addition to detecting the presence of *MAT* genes, it has also been possible to demonstrate expression of the *MAT* genes under conditions favorable for sex in a species, again providing an indication of the potential for sex (Dyer and O'Gorman, 2011).

Conversely, although *MAT* loci identification is an important tool in identifying species that have the potential for reproducing sexually, this does not guarantee that a sexual cycle can be induced. There are a number of species in which it has not yet proved possible to induce a sexual cycle under laboratory conditions despite the species containing apparently fully functional *MAT* genes (Geng *et al.*, 2014). Indeed, there is growing evidence that *MAT* genes have additional functional roles beyond purely sexual reproduction. For example, in *Ulocladium botrytis* (thought to be entirely asexual) it has been found that that both *MAT1-1-1* and *MAT1-2-1* genes play roles in regulating asexual sporulation. In gene deletion studies of the *MAT1-1-1* and *MAT1-2-1* genes, conidia size was significantly reduced in comparison to the wild type. However, when the *MAT1-1-1* and *MAT1-2-1* genes were heterologously expressed in *ΔMAT* mutants of the sexual species *Cochliobolus heterostrophus*, a sexual cycle could be partially restored whereby sexual structures (fruiting bodies) were formed, but no asci could be detected (Wang *et al.*, 2017). Meanwhile, heterologous expression of *C. heterostrophus MAT* genes in *U. botrytis MAT* gene deletion mutants led to restoration of asexual reproduction, with an increase in conidial production. This indicates the versatility of *MAT* genes as regulators of both the asexual and sexual reproductive cycles of fungi.

1.1.3 Sex in the Basidiomycota

The second largest sub-group of the fungal kingdom, the phylum Basidiomycota, includes species that exhibit yeast-like, filamentous, or dimorphic forms of growth whereby they are capable of switching growth forms depending on conditions (Fell *et al.*, 2001). Species from all of these

different growth forms can undergo sexual reproduction, involving either heterothallic or homothallic breeding systems.

A rather more complex distribution of *MAT* genes is evident in many basidiomycetes compared to the Ascomycotina. Heterothallic basidiomycetes may contain several *MAT* genes. One group of these genes will encode for pheromone receptors, whilst the other will encode transcription factors required for the sexual cycle to occur (David-Palma *et al.,* 2016). In addition to this, basidiomycetes can be categorized into either tetrapolar or bipolar breeding systems. In tetrapolar species, the two *MAT* genes are unlinked, whereas in bipolar species the *MAT* genes are linked to form a single locus (Fraser and Heitman, 2003). To add further complexity, a number of alleles can occur at each *MAT* locus in certain basidiomycete species, resulting in thousands of different mating types being present in some model species (Bakkeren and Kronstad, 1994). A full discussion of *MAT* gene organization in the Basidiomycota is beyond the scope of the present study.

As well as being required for sexual development, *MAT* genes are also required for pathogenesis in some basidiomycetes. For example, the fungus *Ustilago maydis*, responsible for corn smut, is only capable of becoming virulent once a dikaryotic stage is entered post-sexual reproduction. This was confirmed when its ability to become pathogenic was impaired when the sexual cycle is inhibited (Feldbrügge *et al.*, 2004).

1.1.4 Early *MAT* genes

Although *MAT* loci have been well researched in the Ascomycota and Basidiomycota, the appearance and evolution of mating-type genes in earlier derived lineages of the fungi is poorly understood. However, ongoing research is providing some important insights into how *MAT* genes might have evolved within the fungal kingdom. Of particular note are studies involving the Mucoromycota (formerly encompassed within the Zygomycota) (Spatafora *et al.*, 2016). Although a large amount of research has been performed regarding the morphology and physiology or sexual reproduction

in the Mucoromycota, very little has been understood about the distribution or function of mating-type genes in this group. It was nevertheless understood that members of the Mucorales used trisporic acid as a sex hormone, could be heterothallic and mate if both (+) and (-) mating partners were present (Wetzel *et al.*, 2012; Wurtz and Jockusch, 1975). Using *Phycomyces blakesleeanus* as a model system, Idnurm *et al* (2008) identified ten HMG-domain proteins that were related to HMG-domain proteins in the Basidiomycota and Ascomycota (Idnurm *et al.*, 2008).

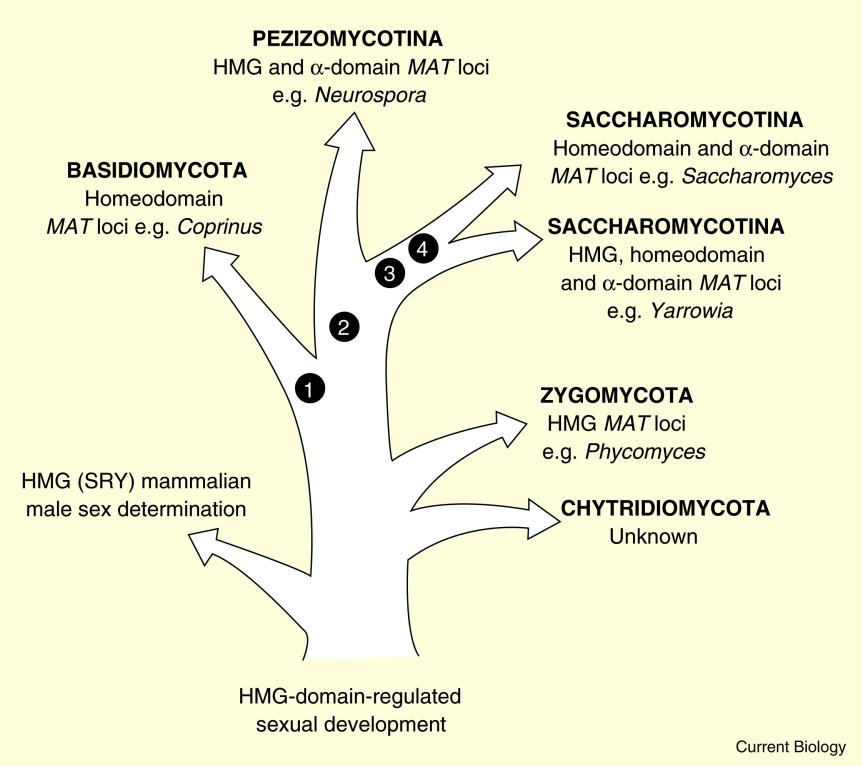


Figure 1.7 – Representation of the proposed evolution of the mating type (MAT) loci within the fungal kingdom from Dyer (2008). Each number represents a genetic change of the fungal sex gene structure: (1) Loss of HMG domains and gain of homeodomain transcription factors in the Basidiomycota; (2) Gain of α-domain transcription factor; (3) Gain of homeodomain transcription factors in the Saccharomycotina; (4) Loss of HMG transcription factors in a subset of Saccharomycotina.

However, no α-box domain proteins could be identified. Further experimental work allowed two of these HMG alleles, termed *sexM* and *sexP*, to be identified as encoding key HMG-box domain sex proteins. This study

suggests that HMG-box domain proteins represent early forms of fungal *MAT* loci and the α-box domain proteins evolved later (Martin *et al.*, 2010). Further evidence for this hypothesis is that HMG-box domain proteins are also involved with sex determination in the animal kingdom, suggesting a shared evolutionary origin in the Opisthokonta (**Figure 1.7**) (Dyer, 2008).

1.1.5 *MAT* genes of the *MAT1-1* and *MAT1-2* loci in the Ascomycota

In later parts of the present study, the presence of *MAT* genes will provide critical insights into possible sexuality in the study species. Therefore, an extended introduction about the various types of *MAT* gene encountered in the Pezizomycotina will now be provided.

Numerous different types of protein have been described as being encoded by *MAT* genes within the *MAT* locus of sexually reproducing fungi. It is important to understand what these genes are and their function in order to understand how they may have evolved within particular groups of fungi, possibly related to their environmental niche. Additionally, it is important to understand which genes are shared between all heterothallic and homothallic species, and which genes are specific to a certain *MAT* locus of particular fungal groupings. Indeed, there have been several reviews detailing the *MAT* genes in detail including the most recent review by Wilken et al. (2017).

The possible genes encoded by the *MAT1-1* locus in heterothallic species are summarized in **Table 1.2**:

Table 1.2 *– Genes associated with the* MAT1-1 *locus and appropriate domain and function if known.*

Gene (gene domain)	Species identified	Function	Reference
MAT1-1-1 (alpha-box DNA	*Neurospora crassa;*	Normal sexual development in	Arnaise *et al.*, 1993;

binding domain)	*Podospora anserina*	homothallic and heterothallic species	Metzenberg and Glass, 1990.
	Penicillium chrysogenum; Ulocladium botrytis	Control of asexual reproduction	Böhm *et al.*, 2013; Wang *et al.*, 2017
	Sordaria macrospora	Regulation of sexual pheromone expression	Klix *et al.*, 2010
MAT1-1-2 (PPF domain)	*Podospora anserina*	Female organ fertilization and fruiting body development	Debuchy *et al.*, 1993
	Sordaria macrospora	Essential gene for sexual reproduction	Klix *et al.*, 2010
	Fusarium graminearum	Hyphal fusion and ascosporogenesis	Zheng *et al.*, 2013
MAT1-1-3 (HMG-box DNA binding domain)	*Neurospora crassa; Podospora anserina*	Required for normal sexual development	Debuchy *et al.*, 2010
	Sordaria macrospora	Non-essential gene for sexual reproduction	Klix *et al.*, 2010
	Fusarium graminearum		Zheng *et al.*, 2013
MAT1-1-4 (metallothionein domain)	*Pyrenopeziza brassicae*	Homology with metallothionein proteins – role in heavy metal acquisition; essential for sexual reproduction; leaf senescence suggested to trigger expression	Ruttkay-Nedecky *et al.*, 2013; Thirumoorthy *et al.*, 2007; Singh *et al.*, 1999; McCartney and Lacey, 1990
MAT1-1-5	*Coccidiodes* spp.	Member of *COX13* gene family; subunit of cytochrome c oxidase –	Mandel *et al.*, 2007

		involved in respiration	
	Botrytis cinerea	Apothecia formation	Amselem *et al.*, 2011; Rodenburg *et al.*, 2018
	Sclerotinia sclerotiorum	Essential gene for sexual development	Doughan and Rollins, 2016
MAT1-1-6	*Coccidiodes* spp.	Member of *APN2* gene family – involved in DNA repair of abasic sites	Mandel *et al.*, 2007
	Pseudogymnoascus destructans	Unknown function	Maggi and Staiger, 2015
MAT1-1-7	*Coccidiodes* spp.	Unknown function	Mandel *et al.*, 2007
MAT1-1-8	*Sphaeropsis sapinea*	Unknown function	Bihon *et al.*, 2014
MAT1-1-9	*Sphaeropsis sapinea*	Unknown function	Bihon *et al.*, 2014

Similarly, the *MAT1-2* locus contains numerous possible genes identified from various species that have been implicated in sexual reproduction. Each known gene encoded by the *MAT1-2* locus are summarized in **Table 1.3**:

Table 1.3 *– Genes associated with the* MAT1-2 *locus and appropriate domain and function if known.*

Gene (gene domain)	Species identified	Function	Reference
MAT1-2-1 (HMG-box DNA binding domain)	*Ascosphaera apis*	Normal sexual development in homothallic and heterothallic species	Aronstein *et al.*, 2007
	Penicillium chrysogenum	Pheromone precursor and pheromone receptor gene	Böhm *et al.*, 2015

		expression	
	Trichoderma reesei	Cellulase production regulation	Zheng *et al.*, 2017
	Fusarium verticilliodes	Associated with photo-inducible carotenoid biosynthesis	Ádám *et al.*, 2011
MAT1-2-2 (HMG-box DNA binding domain)	*Neurospora crassa*	No known function	Pöggeler and Kück, 2001
	Magnoporthe oryzae	No known function; argued to be distinct from other *MAT1-2-2*	Kanamori *et al.*, 2007
MAT1-2-3 (mannosyl transferase domain)	*Coccidiodes* spp.	Encodes a protein containing a mannosyl transferase function; maintains cell membrane integrity and has a role in secretion during *Cryptococcus neoformans* infection	Mandel *et al.*, 2007; Bose *et al.*, 2003
MAT1-2-4	*Coccidiodes* spp.	Unknown function	Mandel *et al.*, 2007
	Aspergillus fumigatus	Promotes cleistothecia production; regulate expression of *preA* and *preB*	Yu *et al.*, 2017; Dyer and O'Gorman, 2012
MAT1-2-5	*Coccidiodes* spp.	Member of *COX13* gene family; subunit of cytochrome c oxidase – involved in respiration	Mandel *et al.*, 2007
	Phyllosticta citricarpa	Unknown function	Bihon *et al.*, 2014
	Sphaeropsis	Unknown function	Wang *et al.*, 2016

	sapinea		
MAT1-2-6	*Coccidiodes* spp.	Member of *APN2* gene family – involved in DNA repair of abasic sites	Mandel *et al.*, 2007
MAT1-2-7	*Huntiella moniliformis*	Unknown function	Wilson *et al.*, 2015
MAT1-2-8	*Villosiclava virens*	Constitutively expressed; may not be directly involved in sexual reproduction	Yu *et al.*, 2015
MAT1-2-10	*Sclerotinia sclerotiorum*	Required for normal ascospore production	Doughan and Rollins, 2016
	Botrytis cinerea	Required for normal apothecia production	Rodenburg *et al.*, 2018

For both *MAT1-1* and *MAT1-2* genes, a consensus has yet to be reached on the nomenclature of *MAT* genes between species. For example, *MAT1-1-6* and *MAT1-2-6* both encode members of the *COX13* gene family. Therefore, it has been argued that these should no longer be included as part of the *MAT* gene loci. Moreover, there have been circumstances where distinct, divergent genes have been identified yet both have been given the same name. An example of this is *MAT1-1-5* described from both *Coccidiodes spp.* and *B. cinerea,* yet these genes are dissimilar in sequence and function (Mandel *et al.*, 2007; Rodenburg *et al.*, 2018). Further information regarding the proposed redefinition of *MAT* genes can be found in a recent review by Wilken *et al.* (2017).

1.1.6 *MAT* genes as master regulators

As discussed earlier, *MAT* genes are known to be essential for virulence in basidiomycetes and have roles in controlling asexual reproduction in certain ascomycete species (Feldbrügge *et al.*, 2004). For example, in *S. macrospora*, deletion of *MAT1-1-2* leads to the downregulaion of 743 genes and upregulation of 111 genes with a range of functions (Klix *et al.*, 2010). Thus,

there is accumulating evidence that *MAT* genes have a much wider range of functions than simply the control of sexual reproduction, and that they additionally act as master regulators of genes throughout the fungal genome (Dyer *et al.*, 2016). Indeed, certain *MAT* genes may play a role in the specific lifestyles of the host fungus. For example, *MAT1-1-4* with a putative function in heavy metal acquisition, due to the metallothionein domain (Ruttkay-Nedecky *et al.*, 2013), may only play a role in sexual reproduction in environments where it is pertinent, such as in *Pyrenopeziza brassicae* (Singh and Ashby, 1998).

Furthermore, it has been found that upwards of 243 target sites in the *Penicllium chrysogenum* genome are targeted by the MAT1-1-1 transcription factor. Moreover, 83.1% of these sites have the general MAT1-1-1 binding motif 'CTATTGAG'. The genes targeted by MAT1-1-1 have diverse roles, such as in iron acquisition, cell defense and primary cell metabolism (Becker *et al.*, 2015). Additionally, *MAT1-1-1* deletion mutants were found to downregulate drug transporter genes compared to the wild type. Furthermore, *MAT1-2-1* gene expression in *P. chrysogenum* was later found to play a role in asexual sporulation and in the germination of conidia (Böhm *et al.*, 2015).

Therefore, there is more than meets the eye with regards to the *MAT* locus in fungi. These genes have significant roles in both sexual and asexual reproduction, but also wider roles in cellular survival and metabolism. The full potential of *MAT* genes remains to be elucidated and further research into their capabilities will be essential for the future of fungal research.

1.1.7 Other genes involved in sexual reproduction

There is a plethora of other genes needed for sexual reproduction in fungi. Indeed, the sexual pathway is an incredibly complex system, as represented in **Figure 1.8**. As such, full discussion of all genes involved in the sexual cycle is beyond the scope of this introduction but more information can be found in the review by Dyer and O'Gorman (2012). However, to give examples of

their wide range of functions, using **Figure 1.8** as a schematic of sexual reproduction in *A. nidulans*, functions can include the following.

Light sensing genes, such as *veA, velB* and *laeA* (Mooney and Yager, 1990; Kim *et al.*, 2002; Bayram *et al.*, 2008; Stinnett *et al.*, 2007). All these genes are involved in the velvet complex, whose role is to activate sexual development in dark conditions whilst acting as repressors of asexual reproduction.

Stress sensing genes, such as *fhbA* and *fhbB* (Baidya *et al.,* 2011). These genes act to detoxify NO present in the environment. Indeed, this appears to have a negative regulatory effect on sexual reproduction, where deletion of *fhbA* led to an increase in sexual development activity. Furthermore, addition of NO generating compounds led to increased sexual development via increased expression of *steA* (Marcos *et al.,* 2016).

MAP kinase cascade genes involved in mating and subsequent sexual development, such as *STE20, steC, STE7, mpkB* and *steA* (Dyer, 2007). This MAP kinase pathway was shown to be essential to the production of cleistothecia, with deletion of any genes involved leading to sterility. Indeed, this MAP kinase pathway was found to be upregulated during the process of sexual development in *A. nidulans* (Paoletti *et al.,* 2007).

Transcription factors vital for sexual reproduction, such as *nsdD*. This transcribes a GATA-type transcription factor and overexpression studies of this protein leads to increased production of cleistothecia production (Han *et al.*, 2001).

As a final example, genes can be involved in **ascospore production**, such as *grrA*. Deletion studies of this gene resulted in full production of cleistothecia in *A. nidulans*, but blockage of meiosis and eventual ascospore formation in *A. nidulans* (Krappman *et al.*, 2006).

To conclude, there is a wide range of functionalities required for both activation and repression of sexual development in fungi. However, it is important to note that many of these gene functions have only been described for the homothallic species *A. nidulans* and their functions may vary widely in heterothallic fungi (Dyer and O'Gorman, 2012).

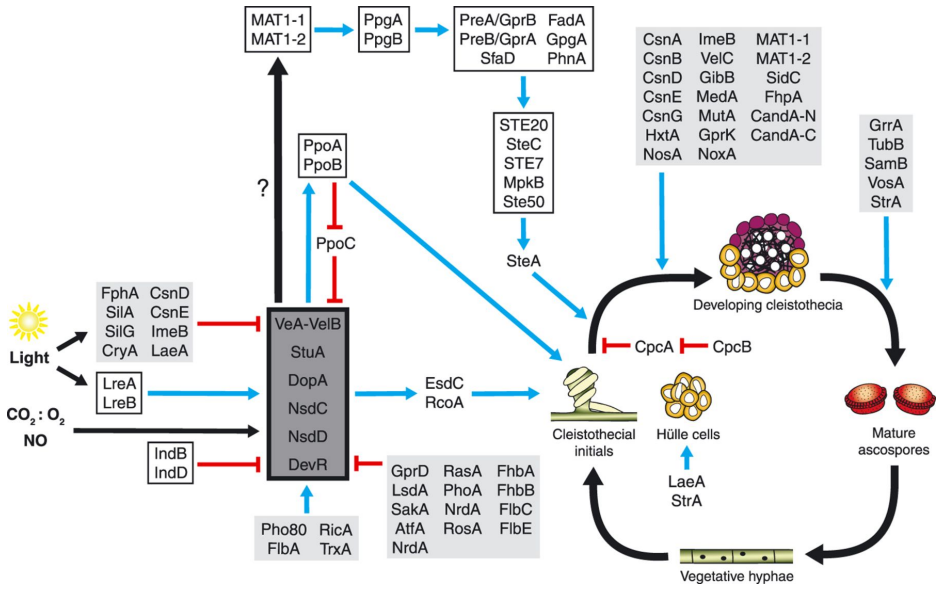


Figure 1.8 *– Schematic to represent the developmental network of genes involved in sexual reproduction within the Aspergilli, primarily based on the homothallic species A. nidulans. Blue lines represent gene activation, whereas red lines represent gene repression. Proteins grouped together in shaded boxes act during similar stages of sexual development, although they may not belong to the same family of proteins. From Dyer and O'Gorman (2012).*

1.1.8 Cryptic sexuality

As already highlighted above (section **1.1**), 20% of known fungal species appear to lack a sexual cycle and these include certain industrially important ascomycete species, such as *A. niger*. However, there has been a concerted effort over the past decade to explore the sexual potential of a number of Pezizomycotina species that have traditionally been dubbed 'imperfect' and therefore asexual (Tzung *et al.*, 2001; O'Gorman *et al.,* 2009). In these supposedly asexual species, it was hypothesized that it might be possible to induce a sexual cycle provided that *MAT* and other sex-related genes were present in the fungal genome, and that compatible *MAT1-1* and *MAT1-2* partners could be detected, and finally that the correct conditions to induce sex could be determined. Pioneering studies using this approach led to the

discovery of a sexual cycle in *A. fumigatus* (O'Gorman *et al.,* 2009). This was later repeated for species such as *A. parasiticus* (Horn *et al.,* 2009b) and *Penicillium roqueforti*, where a complete sexual cycle was discovered when crosses were incubated for three weeks in darkness at 25 °C on biotin supplemented oatmeal agar (OA) (Ropars *et al.*, 2014; Swilaiman 2014). However, if a species had truly evolved to reproduce by entirely asexual means, it would be expected that deleterious mutations would accumulate and lead to the eventual loss of genes vital for sexual reproduction.

The discovery of a sexual cycle in several members of the aspergilli stimulated further research aimed at discovering the sexual cycles of closely related species. Recently, potential sexual structures known as sclerotia were found to be formed in *A. niger* by a limited number of wild isolates when incubated under narrow conditions of 25 °C in darkness in the presence of raisins or other substrates such as white rice (Frisvad *et al.*, 2014). *A. niger* is the primary producer of citric acid in industry, with a predicted worldwide market value of $3.66 billion by 2022 (Sweis and Cressey, 2018). One first key aim of the present PhD program is to build on this discovery, to see if it might be possible to induce a complete sexual cycle in *A. niger* and other members of the aspergilli. Success in this aim would have far reaching implications for the industrial process of citric acid production, enabling more directed strain improvement and increase of yields (Ashton and Dyer, 2016). This will be discussed further in **Chapter 2 – Sex in the Black Aspergilli**.

1.1.9 Exploitation of the sexual cycle

Once a sexual cycle has been described in a species, it provides a gateway to a number of techniques that can be exploited for both fundamental and practical gain. For example, in addition to strain improvement, the sexual cycle can be exploited in order to identify genes of interest and to characterize the genetic basis of traits of interest (Ashton and Dyer 2016). By analysis of sexual progeny from a cross, it is possible to determine whether a trait has a monogenic or polygenic basis (caused by one gene or multiple

genes, respectively). Once a trait has been determined to be mono or polygenic, it can be mapped and identified using classic genetic techniques (Foulongne-Oriol, 2012).

Furthermore, modern techniques such as bulk segregant analysis (BSA) can be used to identify genes responsible for the trait of interest, in situations where cloning or gene manipulation techniques are unsuitable (Michelmore and Paran, 1991). BSA is a technique whereby sexual crosses are set up between one parent expressing a trait of interest and a second parent with the trait absent. The recombinant progeny from this sexual cross are collected and pooled into two groups, those expressing the trait of interest and those that are not. These two pools are sequenced along with the original parents and key differences in the genome sequences are identified. Ideally this will lead to identification of a linked region of the genome containing the gene of interest. However, it is to be expected that there would be a large number of differences in the genome between the two parent strains. Therefore, the use of large numbers of progeny in each pool is required to minimize the background 'noise' within the genomes and highlight the linked region containing the gene of interest responsible for the trait. This technique has been used successfully to identify genes of interest in *Neurospora crassa* (Pomraning *et al.,* 2011).

The second part of the current PhD research is aimed at identifying 'unknown' genes responsible for itraconazole resistance in *A. fumigatus* by exploiting the 2009 discovery of a sexual cycle of this medically important species in conjunction with the use of BSA and sexual backcrossing (O'Gorman *et al.,* 2009). *A. fumigatus* is a species of major medical importance, being the primary cause of invasive aspergillosis in immunocompromised individuals (Toyotome *et al.*, 2018). Cases of aspergillosis within these patients can result in mortality rates of 50% or higher, even when undergoing antifungal drug therapy treatment, and reaching as high as 90% mortality in untreated patients (Dagenais and Keller, 2009). Treatment of this disease involves the use of azoles, echinocandins and amphotericin B antifungals. However, there are an increasing number of

reports showing the evolution of resistance to these drugs in *A. fumigatus* isolates (Arendrup *et al.*, 2010; Chowdhary *et al.*, 2014; Patterson *et al.*, 2016).

Within the azoles, the mutations for resistance have been primarily associated with the gene *cyp51A*, and may be due to point mutations and/or as tandem repeats in the promoter region (Astvad *et al.*, 2014; Chowdhary *et al.*, 2014). However, it is important to note there are several instances of non-*cyp51A* resistant isolates being described. Among these include: mutations in the transcription factor subunit gene *hapE*, leading to increased *cyp51A* expression (Camps *et al.*, 2012); and mutations in the HMG-CoA reductase gene *hmg1*, leading to increased ergosterol synthesis in *A. fumigatus* (Hagiwara *et al.*, 2018). The mutations conferring resistance will be discussed further in **Chapter 3**.

Azole treatment results in deficiencies in ergosterol synthesis. This leads to the buildup of a toxic ergosterol intermediate (14α-methylergosta-8,24(28)-dien-3β,6α-diol) and inhibition of fungal growth (Alcazar-Fuoli *et al.*, 2008; Lelièvre *et al.*, 2013; Ghannoum and Rice, 1999). Following growth inhibition, a fungicidal effect can be observed through the use of azole treatments whereby, azoles trigger the production of cell wall carbohydrate patches (Geißel *et al.*, 2018). Resistance mechanisms reduce the efficacy of this drug treatment and therefore allow fungal growth to continue. Although the genetic basis of azole resistance is known for many isolates, there are increasing numbers of strains of *A. fumigatus* being isolated that are resistant to azole treatment but with no previously known resistance mechanism. Therefore, for the future management of disease control for this disease, it is important to develop a method of identifying these unknown resistance genes, as this will allow molecular diagnostics to be developed to alert clinicians to possible breakdown of drug control - and therefore the need for alternative drug treatment and even drug design.

Using an *A. fumigatus* strain with an unknown resistance mechanism, method development was performed in the current PhD in order to maximize the efficiency of BSA in identifying novel resistant genes of interest. This

research aimed to identify the number of backcrosses and number of progeny in each sequenced pool required that would efficiently identify the genomic region of interest. This will be discussed further in **Chapter 3 – Use of Bulk Segregant Analysis to Investigate Antifungal Resistance in *Aspergillus fumigatus*.**

1.1.10 Fungal sex hormones

Fungal sex hormones can be defined as molecules that act to induce differentiation of gametes and induce morphogenic changes. Fungal sex hormones can be divided into the subgroups of sex pheromones and sex morphogens. An additional third group of sex molecules have been termed 'growth substances' (Siddiq *et al.,* 1992: Dyer *et al.,* 1993). Fungal sex hormones have been studied in many fungal lineages due to their ability to dramatically alter the growth of both neighboring and distant cells. Initial studies of fungal sex hormone were focused on the oomycetes, the Zygomycota and Chytridiomycota (Raper, 1939; Machlis, 1958; Austin *et al.,* 1969). More recent fungal sex hormone research has been primarily focused within the Ascomycota. Two key examples of this have been firstly the discovery of the precocious sex inducer (psi) factors in *A. nidulans* (Champe and El-Zayat, 1989), and secondly the discovery of the SF (sex factor) from *P. brassicae* (Ilott *et al.,* 1986). More details on these hormones will be provided in Chapter **5**. However, very little work has been conducted on the characterization of new fungal sex hormones since the 1990s.

Further research on fungal sex hormones could be of significant practical benefit as they might impact on disease control in plants. This could provide a new line of defence against fungal pathogens, which are responsible for major yield losses in agricultural crops (Godfray *et al.,* 2016). Building on the framework of previous research, the third part of the current PhD research is aimed at identifying possible bioactive sexual hormones from lipid extracts of a member of the aspergilli, *Neosartorya fischeri*. Once a hormonal extract with sexual inducing activity has been detected in this

species, it was aimed to assess any ability to 'cross-talk' to related heterothallic and homothallic species. In addition to this, the effect of oxygenase deletion and overexpression mutants on the sexual cycle of *Aspergillus fumigatus* was determined. This work will be discussed further in **Chapter 4 – Investigation of the Role of Hormones in Fungal Sexual Development and Possible Uses for Disease Control.**

1.1.11 Aims and objectives

The overall theme of work presented in this thesis is to exploit fungal sexual reproduction for use in biotechnology and for disease control. Such research is both of applied and fundamental interest as described above. Specific aims of the study were:

1) To elucidate conditions needed to induce a sexual cycle in the supposedly 'asexual' species *Aspergillus niger.*
2) To confirm that sexual recombination occurs during the putative sexual cycle of *Aspergillus sclerotiicarbornarius*, for use as a model for sexual development within the black aspergilli and provide a framework for further phenotypic analysis of this species.
3) To exploit the sexual cycle of *A. fumigatus* to develop a bulk segregant analysis-based method to identify the genetic basis of unknown mechanisms causing resistance to azole antifungals.
4) To identify bioactive fungal sex hormones from model Pezizomycotina species and then to see if such hormones exhibited cross-species activity.
5) To study oxygenase gene function in sexual reproduction of the heterothallic species A*. fumigatus*.

It was anticipated that work undertaken in this thesis would reveal new insights into the sexual cycles of fungi, and identify novel ways in which the sexual cycle can be exploited in a wide range of important species within the Ascomycota.

Chapter 2 – Sex in the Black Aspergilli

2.1 Introduction

Despite the plethora of advantages of sexual reproduction, approximately 20% of fungal species are only known to reproduce via an asexual life cycle (Dyer and O'Gorman, 2012). Although a sexual cycle has yet to be observed in these fungi, the discovery of suitable mating partners, coupled with the identification of as-yet hidden growth conditions suitable for sex, may unlock their potential as sexually competent species. Indeed, for a number of *Aspergillus* species, sexual cycles have been described for the first time over the past decade, linked to the identification of mating partners and suitable environmental conditions, as will be described later in this chapter. These species include those: (a) of medical importance such as *Aspergillus fumigatus* and *Aspergillus flavus*, both known to cause aspergillosis; (b) mycotoxin producing species such as *Aspergillus parasiticus*; and (c) industrially important species such as *Aspergillus tubingensis* (O'Gorman *et al.,* 2009; Horn *et al.*, 2009a; Horn *et al.,* 2009b; Horn *et al.*, 2013). Furthermore, discoveries of formerly secret sexual cycles have also been seen outside of the *Aspergillus* genus. Examples of this include the primary producer of penicillin, *Penicillium chrysogenum* (Böhm *et al.*, 2013) and *Penicillium roqueforti*, used in the production of blue cheeses (Ropars *et al.*, 2014; Swilaiman 2014).

As discussed in '**Chapter 1 – Introduction to Fungal Sex'**, the ability to identify sexually compatible *MAT1-1* and *MAT1-2* strains of a heterothallic species, via the development of molecular diagnostics to determine mating type, provided the first evidence of the potential sexual cycle in these supposed 'asexual' species (Paoletti *et al.*, 2005). Then, the systematic testing of different growth parameters allowed the identification of specific environmental factors to induce a sexual cycle. These environmental factors were found to be specific to each species of fungus, although some general trends were evident. In the case of *A. flavus*, sclerotia production leading to sexual reproduction was triggered when crosses were set up between

sexually compatible strains on mixed cereal agar (MCA) and incubated in darkness for 14 days at 30 °C. Sclerotia formed during this incubation period were then isolated, air dried and stored in a desiccator over saturated NaCl solution at 25 °C before ascospores were released from the cleistothecia formed within the sclerotia (Horn *et al.,* 2009a). This is in contrast to the case of *P. chrysogenum*, where sex was initiated when compatible *MAT1-1* and *MAT1-2* isolates were grown on oatmeal agar supplemented with biotin for 5 weeks in darkness at 20 °C (Böhm *et al.*, 2013). These diverse methods used to initiate fungal sexual cycles illustrate the challenge of identifying the correct conditions to tempt these species from their typical asexual life cycle. However, with the growing number of sexual cycles being identified in the fungal world, there is promise to continue and apply this work to other species of applied importance.

One such species is *Aspergillus niger* within the section *Nigri*. A key aim of this PhD was to attempt to identify a sexual cycle in *A. niger*, as well as confirm the presence of a sexual cycle in the closely related species *Aspergillus sclerotiicarbonarius*. In recent years, the formation of potential secondary sexual structures known as sclerotia have been observed both in *A. niger* and *A. sclerotiicarbonarius* when cultures were grown under particular conditions. In the case of *A. niger*, this involved growth on Czapek's yeast autolysate (CYA) agar for 10 days at 25 °C in the presence of raisins, whereas in the case of *A. sclerotiicarbonarius* incubation at 28 °C on oatmeal (OA) induced the development of sclerotia (Darbyshir, 2014; Frisvad *et al.*, 2014). This further demonstrates the variation in environmental conditions required for development of secondary sexual structures. Furthermore, both species have had *MAT1-1* and *MAT1-2* isolates described, indicating the ability of these fungi to undergo a heterothallic life cycle (Darbyshir, 2014; Pel *et al.*, 2007). Some further details about these species will now be provided. They are both members of the so-called 'black aspergilli', from the section *Nigri*, which are characterised by the formation of dark brown-black asexual conidia (Abarca *et al.*, 2003).

2.1.1 *Aspergillus sclerotiicarbonarius*

A. sclerotiicarbonarius was first identified in 2008 during the survey of mycobiota of coffee beans from Thailand (Noonim *et al.*, 2008). This species was thought to reproduce exclusively via an asexual life cycle. However, unusually for the black aspergilli, isolates readily formed sclerotia *in vitro.* Given that the formation of sclerotia has been linked to sex in other *Aspergillus* species (**1.1.8**) it was therefore of particular interest to our research group to determine if it was possible to induce a sexual cycle in this species. Identification of a sexual cycle in this species would provide a crucial insight into inducing a sexual cycle in closely related members of the black aspergilli, such as *A. niger*.

Interestingly, the production of sclerotia in *A. sclerotiicarbonarius* is correlated with the production of bioactive compounds which display antifungal activity (Petersen *et al.*, 2015). Indeed, since the species was first identified, it has been important to identify the range of mycotoxins that may be produced during growth. This is because the species is part of the mycobiota of coffee and therefore mycotoxin production could have a serious effect on the coffee industry. Fortunately, it has been suggested that *A. sclerotiicarbonarius*, although growing readily on coffee beans, is unable to produce the mycotoxin ochratoxin A (Akbar and Magan, 2014). However, it is important to note that in the study to determine ochratoxin A production in *A. sclerotiicarbonarius*, only one strain was examined. Additionally, no other toxic metabolites were studied. Therefore, further research of this species to fully understand its secondary metabolite profile is required to elucidate its potential impact on the coffee industry. Moreover, exploitation of the sexual cycle, once confirmed in *A. sclerotiicarbonarius*, could be a powerful tool to identify the full repertoire of toxic metabolites being produced by this newly identified species (see section **1.1.9**).

2.1.2 *Aspergillus niger* and citric acid production

A. niger is used extensively as one of the primary producers of citric acid in the biotechnology industry (Show *et al.*, 2015). This species is also known as a potential pathogen of crop plants. James Currie first detailed the citric acid producing properties of *A. niger* in 1917, demonstrating the capability of this organism to produce large amounts of citric acid at low pH levels (Currie, 1917). The basis of Currie's work eventually led to the industrial fermentation of *A. niger* for citric acid production. Although *A. niger* is primarily thought of as a citric acid producer, this species shows many other desirable metabolic traits and has been exploited for the manufacture of a variety of other products, such as: glucoamylase by AB Enzymes, Germany; catalases by Megazyme, USA; and cellulases by Dyadic, USA (Cairns *et al.,* 2018). It has been granted GRAS (generally regarded as safe) status, important for commercial exploitation, at least for the industrial strains (Sewalt *et al.*, 2016).

Citric acid is primarily produced using submerged fermentation (Shu and Johnson, 1948), growing *A. niger* aerobically in a sugar source. At the end of the fermentation process, *A. niger* is filtered out of the solution and the citrate produced is precipitated out through the use of calcium hydroxide, producing a calcium citrate salt. Finally, citric acid is recovered from the process by treating the salt with sulphuric acid (Cairns *et al.* 2018).

Citric acid has many uses such as food preservation and flavouring, use as a chelating agent in cleaning products, and use in cosmetics (Ciriminna *et al.*, 2017). By 2022, the worldwide manufactured production of citric acid is estimated to reach a total value of $3.66 billion (Sweis and Cressey, 2018). Due to the high value of citric acid production, there have been concerted efforts to produce *A. niger* strains that have improved citric acid yields. Examples of this include the use of x-ray or UV mutagenesis to induce advantageous mutations in industrial strains (Gardner *et al.,* 1956; Schreferl *et al.,* 1989).

2.1.3 Strain improvement

Identification of a sexual cycle in *A. niger* could provide a platform for directed strain improvement without the need for genetic manipulation or mutation via x-ray or UV mutagenesis, which might not be desirable in an industrial setting. Strain improvement using a sexual cycle involves setting up crosses between parents that exhibit two different desirable traits of interest. From this cross, the progeny is screened to select those which express both phenotypic traits linked to changes in expression of the corresponding genes (Ashton and Dyer, 2016). In *A. niger*, strain improvement performed this way could produce novel strains with the ability to produce higher yields of a desired product. Furthermore, the sexual cycle could also be used to restore fitness to heavily mutated industrial strains used by outcrossing the industrial partner with a wild isolate (assuming that the industrial strain still remained fertile). The progeny from this outcross could be screened for the retention of the trait of interest, whilst also increasing its genetic viability (Ashton and Dyer, 2016).

There are several examples where fungal sexual reproduction has been used for strain improvement in industry. One such example relates to the use of the species *P. chrysogenum* for penicillin production. In this study, an industrial precursor strain Q176 was used. This strain showed high yields of penicillin compared to the wild type, but also produced the contaminant chrysogenin, which interferes with penicillin purification. An outcross of Q176 with wild type strain IB 08/921, which produces no chrysogenin but much lower yields of penicillin, resulted in the production of progeny strain AS 1-2-54. This strain was important because it acquired the desirable traits from both parental strains – the ability to produce high yields of penicillin, whilst producing no chrysogenin contaminant (Bohm *et al.,* 2013; Dahlmann *et al.*, 2015).

Additionally, strain improvement techniques using the fungal sexual cycle can have unexpected advantages. Due to genome wide recombination between the two parental strains, novel phenotypes can be observed which are beneficial to the industrial production of the desired product. One of the

consequences of this could be titres of a product above the levels seen in either parental strain, showing vastly improved yields. This process is known as 'transgressive segregation' (Rieseberg *et al.,* 1999). An example of this can be found in *A. flavus*, where sexual recombination generated a strain exhibiting a four-fold increase in production of the mycotoxin cyclopiazonic acid (CPA) when compared to the highest CPA producing parent strain (Olarte *et al.*, 2012).

2.1.4 Aims

Therefore, a key aim of the present PhD thesis work was to try to identify a sexual cycle in the *A. niger*. This would have far reaching implications for industry, allowing for more directed strain improvement for citric acid production and repair of genetic damage caused by mutagenesis techniques in industrial *A. niger* isolates. A precedent for this work had been set, with the description for the first time of a sexual cycle in *A. tubingensis*, a fellow member of the black aspergilli (Horn *et al.,* 2013). Previously a sexual cycle had only been described for one other member of the black aspergilli, namely in *Saitoa japonica* (Rajendran and Muthappa, 1980). To help with this work in this chapter, cutting edge microscopy techniques were used to visualise potential ascospore and cleistothecial structures.

Additionally, a second key aim of the present chapter was to try to confirm the presence of a functional sexual cycle in a related member of the black aspergilli, *A. sclerotiicarbonarius*, for which a potential sexual state has been described by Darbyshir (2014) following studies in the Nottingham fungal biology and genetics group. However, evidence was lacking for sexual recombination in the potential progeny. Therefore, the aim of the current research was to identify unique SNP identifiers from parental isolates and to utilise these to show recombination in the putative ascospore offspring. This could provide insights into unlocking the potential of sex in other *Aspergillus* species within the section *Nigri* including *A. niger.*

2.2 Methods

For media composition, refer to **Appendix**.

2.2.1 Strains used

To screen for sexual production in *A. niger*, ten strains (8-160, 8-161, 8-162, 8-163, 8-164, 8-165, 8-166, 8-167, 8-168, and 8-169) were obtained as a kind gift to our research group from Jens Frisvad (Technical University of Denmark). Each strain was identified as *A. niger* using a polyphasic approach by Frisvad *et al.* (2014), utilising morphological identifiers, ITS sequencing and secondary metabolite profiling (Samson *et al.* 2007). These had all been reported to form sclerotia under suitable conditions *in vitro.* In addition to these, strains 6292, 4501, CS14 and SK256 were used to expand the genetic diversity for use in sexual crosses from the University of Nottingham culture collection. Although previously identified as *A. niger* isolates, β-tubulin DNA sequencing was used to confirm the identity of 6292, 4501, CS14 and SK256. Further strain information can be found in **Table 2.1**.

Table 2.1 – Aspergillus niger *strains used and source isolated from.*

Strains	Isolated
8-160	From raisin, California, USA, 2007
8-161	From raisin, California, USA, 2007
8-162	Black pepper imported to Denmark
8-163	From raisin, California, USA, 2007
8-164	From raisin, California, USA, 2007
8-165	From raisin, California, USA, 2007
8-166	Used by Meyer for citric acid production, USA
8-167	From raisin, California, USA, 2007
8-168	From raisin, California, USA, 2007
8-169	From raisin, California, USA, 2007
CS14	Unspecified
SK256	Clinical isolate, Prague, Czech Republic

4501	Gallic acid fermentation
6292	From grape, Israel

2.2.2 Strain maintenance and revival

A. niger strains used for sexual crosses were maintained as long-term stocks under liquid nitrogen in 20% glycerol (1:5 in sterile distilled water). When required, appropriate strains were thawed and a cotton swab was used to inoculate conidia onto *Aspergillus* complete medium (ACM) agar 9 cm plates (Thermo Scientific, USA) and ACM slopes (STARLAB, Germany) (see **Appendix**) for revival before the stocks were refrozen. The strains, inoculated onto ACM plates were incubated at 28 °C for 7 days before use in further experiments.

2.2.3 DNA extraction

25 ml liquid ACM broth was inoculated with conidia of the appropriate strain via a sterile cotton swab and grown for a period of 72 h at 28 °C under constant shaking of 150 rotations/minute (rpm) (Brunswick Innova 5000 gyratory two-tiered shaker).

Following the growth period, liquid cultures were filtered through a layer of miracloth (Millipore, USA) to isolate mycelial tissue. Mycelia of the fungal sample was then dried and placed in a 50 ml universal container (STARLAB, Germany) where the sample was flash frozen in liquid nitrogen to be freeze dried overnight. Once dried, samples were ground under liquid nitrogen using a sterile pestle and mortar before 500 mg of fungal sample was placed into a 2 ml Eppendorf tube (Sarstedt, Germany). From here, two different extraction methods were performed:

- Phenol:chloroform DNA extraction

To the 2 ml Eppendorf tube containing the fungal sample, 1 ml of DNA extraction buffer was added (0.25 M Tris-HCl pH 8.5; 0.25 M NaCl [Sigma-Aldrich, USA]; 0.025 M EDTA pH 8.0 [Sigma-Aldrich, USA]; and 0.5 % SDS pH

7.2). The tube was inverted several times to mix and was incubated at 65 °C for 30 mins. The sample was then centrifuged at 13,000 rpm (Thermo Scientific, USA) for 10 mins and 600 µl of supernatant was removed and transferred to a 2 ml phase lock tube (Eppendorf, Germany) to which 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, USA) was added. The phase lock tube was then inverted several times to mix and centrifuged at 13,000 rpm for 10 mins. After centrifugation the upper aqueous phase was transferred to a new 1.5 ml Eppendorf tube containing 0.7 volumes of ice cold propan-2-ol (Fisher Scientific, USA), inverted several times and incubated at -20 °C for a minimum of 30 mins. The sample was then centrifuged at 13,000 rpm for 10 mins, the supernatant removed and pellet washed with 700 µl of 70% ice-cold ethanol (Fisher Scientific, USA). The sample was centrifuged again at 13,000 rpm for 10 mins. The ethanol was then removed and pellet left to dry in a laminar flow hood for 20 mins before being suspended in 100 µl of TE buffer. Once resuspended, the sample was left at 4 °C overnight before the DNA concentration was measured using a p330 nanophotometer (Geneflow, UK).

- DNA extraction kit

Following placement of 500 mg of fungal sample into a 2 ml Eppendorf tube, DNA extractions were performed using the Wizard® genomic DNA purification kit (Promega, UK) according to the manufacturer's instructions, adapted from the plant tissue extraction protocol for use with fungal samples.

2.2.4 *MAT* type analysis

Mating-type analysis of *A. niger* strains was performed according to a multiplex PCR method using FastStart High Fidelity (Sigma-Aldrich, USA) developed by Darbyshir during PhD studies at the University of Nottingham (Darbyshir, 2014). Primers used in the multiplex reaction are shown below (Sigma-Aldrich, USA) (**Table 2.2**). The assay is predicted to produce a product of 1.9 kbp if an isolate is of the *MAT1-1* genotype or 3.3 kbp if an isolate is of

the *MAT1-2* genotype. The PCR products were resolved using a 1.5% TAE agarose gel and visualised by ethidium bromide staining (Sigma-Aldrich, USA) and a UV transilluminator (Bio-Rad, USA).

A total reaction volume of 20 μl was used per PCR containing: 1x reaction buffer with $MgCl_2$ to a final concentration of 1.8 mM; 200 μM of dNTPs (NEB, USA); 0.4 μM of each of the three primers; between 10-50 ng of DNA template; 1 U (0.4 μl) of FastStart High Fidelity enzyme; and adjusted to 20 μl with sterile distilled water.

The PCR protocol used for the *MAT* type analysis was as follows: Initial denaturation of 2 mins at 95 °C; followed by 35 cycles of 30 secs at 95 °C, 30 secs at 60.5 °C and 4 mins 20 secs at 68 °C increasing to 7 mins over 35 cycles; and a final extension of 7 mins at 68 °C.

Table 2.2 – *Primers used for* MAT *type analysis of* A. niger *strains*

Primer	**Sequence**
ANMAT1F	5'ATTGGCATTATCGAGCCGGA'3
MAT2amR	5'CTCGGATTCTGCCTTCCAC'3
ATHYP2	5'TTTCCGTCCCGGTGGCG'3

2.2.5 RAPD analysis

To test if sexual recombination had occurred within the progeny of the sexual cross and to identify relative relationships between the parent strains, Random Amplification of Polymorphic DNA (RAPD) PCR was performed, as described previously by Murtagh *et al.* (1999). The primers used to assess recombination in *A. niger* are shown below (**Table 2.3**). The products of the RAPD-PCR were resolved on 1.5% TBE agarose gels and visualised by ethidium bromide (Sigma-Aldrich, USA) staining and a UV transilluminator (Bio-Rad, USA). Resultant bands were then graded on size and intensity and scored accordingly. The RAPD profile-banding pattern for each isolated

progeny was compared to that of the crossing parents and genetic similarity was determined.

A total reaction volume of 25 μl was used per PCR containing: 1x DyNAzyme II DNA polymerase reaction buffer; 500 μM of each dNTP; 50 μmol of a single 10-base pair primer; between 0.1-1 ng of DNA template; 1 U DyNAzyme II DNA polymerase (Thermo Scientific, USA); and adjusted to 25 μl using sterile distilled water.

Table 2.3 *– Primers used for RAPD analysis of* A. niger *strains*

Primer	**Sequence**
Opax16	GTCTGTGCGC
Opa11	CAATCGCCGT
Ubc90	GGGGGTTAGG
Opa10	GTGATCGCAG
OpaJ	ACGGGTCAGA

The PCR protocol used for RAPD analysis was as follows: Initial denaturation of 5 mins at 95 °C; 45 cycles of 30 secs at 93 °C at a ramp rate of 30 °C min^{-1}, 40 secs at 37 °C at a ramp rate of 30 °C min^{-1}, 1 min 20 secs at 72 °C at a ramp rate of 20 °C min^{-1}; followed by a final elongation stage for 5 mins at 72 °C (Murtagh *et al.*, 1999).

2.2.6 Genetic similarity

Genetic relationships of *A. niger* strains 8-160, 8-161, 8-162, 8-163, 8-164, 8-165, 8-167, 8-168 and 8-169, were determined according to their respective RAPD-band profiles. This was in accordance to work using *Giardia intestinalis* Šedinova *et al.* (2005). This was done by assigning each prominent band with a number followed by marking with a '1' or '0' if the band was either present or absent in the *A. niger* strain tested, respectively. A matrix was then formed on the presence or absence of each significant band and relationships

between the strains compared to form an unrooted phylogenetic tree using a neighbour joining method as implemented by Treedraw.

2.2.7 Sclerotia production trial

In preliminary work, the number of sclerotia produced by each *A. niger* strain was scored in order to identify three strains to utilise for further studies to optimise sclerotial production. This would include 'high' (>100), 'medium' (>40) and 'low' (<25) sclerotia producers (described in 1 below).

Studies were then performed to try and increase levels of sclerotial production under a number of different growth conditions using strains 8-160 ('high'), 8-161 ('high') and 8-164 ('low'). Sunmaid raisins used were sterilised at 117 °C before use and placed onto the plates using sterilised forceps. Sclerotial formation was measured by counting the number of visible sclerotia formed on the agar surface after a defined time interval. For inoculations, a 5 μl (500 spore) aliquot of a 1×10^5 spores/ml suspension in 0.05% Tween 80 was used. In all conditions except 3), *A. niger* strains were frozen at -20 °C for two weeks before use, according to the method of sclerotial development by Frisvad *et al.* (2014). Unless otherwise specified, 9 cm CYA agar plates were used in the confirmation dictated by **Figure 2.2** and incubated for 25 °C in darkness for 14 days.

The conditions used are described below:

1) **Sclerotia production in all *A. niger* strains**: To test the ability of each strain to produce sclerotia under the conditions described above, the number of sclerotia on the 9 cm plate were counted.
2) **Dark vs light:** Two different conditions were tested – sclerotial development in light or darkness.
3) **Raisin type and temperature:** Two different types of raisins were tested – California Sunmaid (Tesco, UK) or Organic (Sainsburys, UK). The inoculated plates were incubated at 25 °C, 28 °C or 30 °C in darkness for 14 days.

4) **Freeze treatment**: Tested strains underwent a two-week pre-freeze treatment at -20 °C or no treatment prior to inoculation. The inoculated plates were incubated at 25 °C in darkness for 14 days.
5) **Raisin extract vs removal vs raisin**: 5 g raisin/50 ml distilled water and 10 g raisin/50 ml distilled water extracts were made, heating the raisins in 50 ml of water and simmering for 5 mins. The culture was then autoclaved at 117 °C. The tested CYA agar plates then had raisins placed on them, or 10 µl of the raisin extract was pipetted onto agar plates at the raisin positions seen in **Figure 2.1**. After 24 hours, designated plates had the raisins removed before inoculation.
6) **Media**: Three media types were tested – CYA, mixed cereal agar (MCA) and OA (see Appendix).
7) **Sealed cultures**: Three conditions were tested – the inoculated plates were left unsealed, sealed with Parafilm (Bemis, USA) after 3 days or sealed with Parafilm (Bemis, USA) after 10 days.

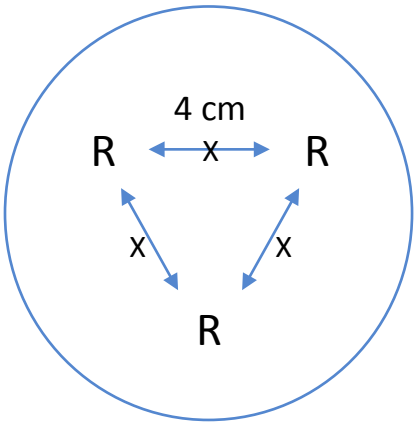


Figure 2.1 *– Conformation of raisin placement (R) and fungal inoculation point (X) on a 9 cm plate for the sclerotial production trials.*

2.2.8 Barrage zone trial

Using the conditions identified in the sclerotia production trials, *A. niger* sexual crosses between *MAT1-1* isolates 8-161 and 8-166 were set up with compatible *MAT1-2* isolate 8-160, which aimed to maximise the potential for

sexual reproduction *in vitro.* 8-160 was chosen due to the relatively high number of sclerotia produced *in vitro* (>100 per 9 cm petri dish). Several different plate confirmations of raisins were assayed in order to maximise potential barrage zone contact between the sclerotia of opposite mating types. Each sexual cross was performed on 9 cm CYA agar plates. All inoculations were done using 5 μl (500 spores) of a 1×10^5 spores/ml suspension in 0.05% Tween 80. Prior to this, appropriate strains were grown on ACM slopes for 10 days before being frozen at – 20 °C for a two-week period. Once inoculated the fungal cultures were then grown at 25 °C, 28 °C and 30 °C in darkness and sealed after 10 days with Parafilm. After 14 days the barrage zone was observed and the conformation that provided the most effective zone was selected for further sexual crosses. The formations that were tested are described below:

a) Three raisin placements along the centre of the agar plate, approximately 1.25 cm apart. Four inoculation points for the corresponding *MAT1-1* and *MAT1-2* strains of *A. niger* were placed 2.5 cm apart from each other and 1.25 cm away from the midpoint where the raisins were placed. This can be seen in **Figure 2.2A**.
b) Four raisin placements 2.5 cm away from each other in a square on to the agar plate. Four inoculation points for the corresponding *MAT1-1* and *MAT1-2* strains of *A. niger* between each raisin placement. This can be seen in **Figure 2.2B**.
c) Five raisin placements, four of which are 2.5 cm away from each other in a square on to the agar plate and one in the centre of this. Four inoculation points for the corresponding *MAT1-1* and *MAT1-2* strains of *A. niger* were placed between each of the four raisin placements in the square, as described in b). This can be seen in **Figure 2.2C**.
d) Four raisin placements, approximately 2.5 cm apart from each other in a square on the agar plate. Four inoculation points for the corresponding *MAT1-1* and *MAT1-2* strains of *A. niger* were placed directly next to each of the placed raisins. This can be seen in **Figure 2.2D**.

e) Six raisin placements through the centre of the agar plate, approximately 2.5 cm apart (north to south) and 1.25 cm apart (east to west). Six inoculation points of the corresponding *MAT1-1* and *MAT1-2* strains of *A. niger* were placed directly next to each raisin. This can be seen in **Figure 2.2E**.

f) Following the barrage zone trial **a**, **b**, **c**, **d** and **e**, it was found that **e** was the most effective sexual cross set up. Attempts to improve the barrage zone were made, by using six raisin placements through the centre of the agar plate, approximately 2.5 cm apart (north to south) and 1.25 cm apart (east to west). After 24 hours, the raisins were removed and six inoculation points of the corresponding *MAT1-1* and *MAT1-2* strains of *A. niger* were placed directly on each raisin point. This can be seen in **Figure 2.2F**.

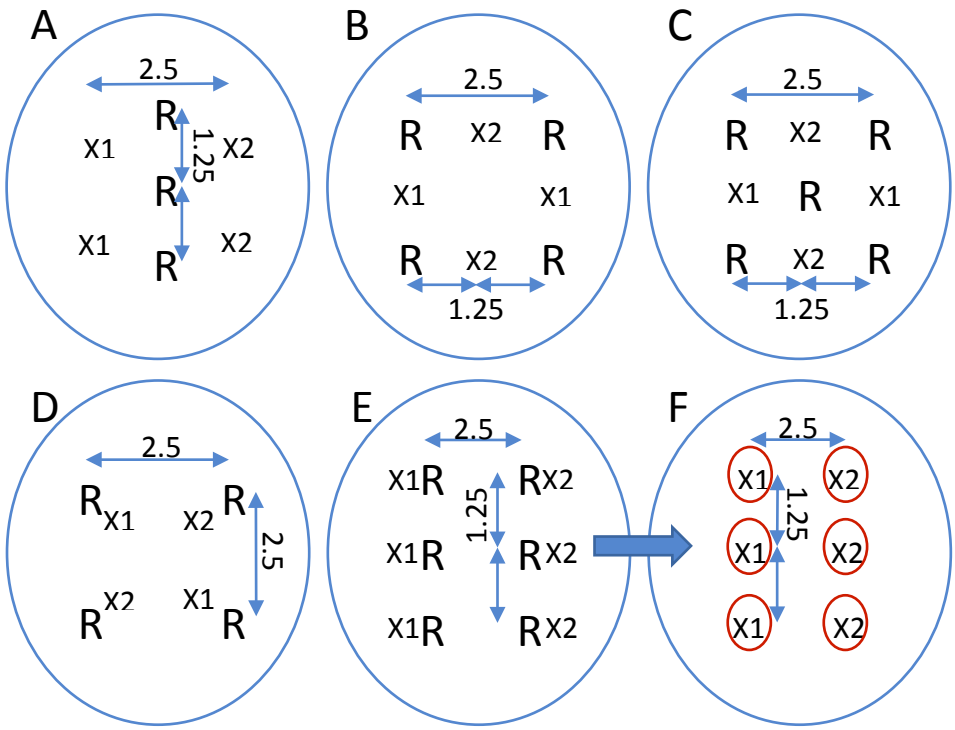


Figure 2.2 *– Conformation of each barrage zone trial, where: R = raisin placement; X1 = MAT1-1 fungal inoculation; X2 = MAT1-2 fungal inoculation; and red circles = raisin removal after 24 hours incubation prior to inoculation.*

2.2.9 Sexual crosses

Following from initial methods developed by Frisvad *et al.* (2014) to induce sclerotia development and the optimisation described above, sexual crosses were set up between *MAT1-1* isolates and *MAT1-2* isolates described in **Table 2.1**. The crossing conditions are described above, dictated by conformation **Figure 2.2F** (see **2.2.8**). The crosses were incubated for a further six months with periodic checking that the Parafilm seal remained intact (plates were resealed where necessary).

2.2.10 Examination of sclerotia

Sclerotia from sexual crosses were isolated after a period of 2 months, 4 months and 6 months to evaluate the possible development of cleistothecia and ascospores. The sclerotia were rolled across water agar (4% w/v) and cut open with a sterile scalpel to observe changes in morphology within the sclerotia. This was performed using a dissection microscope (Nikon SMZ-2B, Japan).

2.2.11 Identification of ascospores

For all imaging work, sclerotia were isolated and either cross-sectioned with a scalpel blade or left whole. This would allow the examination of both the inner and outer layer of the secondary structures. Sclerotia from cross '8-160 × 8-161' were rolled over 4 % (w/v) water agar and cut to reveal the red-pigmented area before preparation for microscopy. A sclerotia sample from another cross was used to compare structures (from cross '8-161 × 8-163').

Three different high-resolution microscopy techniques were used to visualise putative sexual structures: scanning electron microscopy (SEM); cryo-electron microscopy (cryo-EM); and helium ion microscopy. For SEM and cryo-EM, the sclerotia samples had to be prepared in a fixative agent before use.

For SEM imaging, sclerotia samples were inside of a 1.5 ml Eppendorf tube and coated in 20 μl 2 % osmium tetroxide (Sigma-Aldrich, USA) for

approximately 30 mins before being used for imaging. After fixing, the samples were sent to the Nanoscale and Microscale Research Centre (NMRC) (University of Nottingham, UK) and transferred to aluminium stubs with the use of double-sided adhesive tape before being sputter coated with gold. To reduce the damage to the structures using osmium tetroxide, cryo-EM was also performed on the sclerotia. Therefore, no fixative was used prior to sending the samples to the NMRC facility and transferred to aluminium stubs with the use of double-sided adhesive tape before being sputter coated with gold. Imaging for SEM and cryo-EM was performed using a FEI Quanta200 3D DualBeam FIB/SEM.

For helium ion microscopy – sclerotia, both whole and cross-sectioned, were submerged in 2.5% glutaraldehyde diluted in phosphate-buffered saline, before being sent to the Environmental Molecular Sciences Laboratory (EMSL) department of the Pacific Northwest National Laboratory (PNNL) facility (USA) for imaging. From here the samples were prepared according Daly *et al.* (2018), where helium ion microscopy had also been performed. In addition to fixed samples, whole 9 cm Petri dish samples were also sent to the EMSL which bore sexually crossing cultures '8-161 × 8-160'.

2.2.12 Isolation of ascospores

After six months of incubation, *A. niger* sexual crosses were examined for the presence of cleistothecia and ascospore formation within the sclerotia. To test this, sclerotia were isolated from the sexual crosses formed near the barrage zone between the two mating parents. The sclerotia were rolled across water agar (4% w/v) in order to remove external conidia. Sclerotia were then dissected using a scalpel and needles flame sterilised with isopropanol. The red-pigmented sac within was isolated, transferred to a 2 ml Eppendorf tube containing 1 ml 0.05% Tween 80 (Sigma-Aldrich, USA) and crushed to release the putative ascospores, which were vortex mixed. Following agitation, attempts were made to obtain germinating ascospores using a variety of different methods.

a) 100 μl of the ascospore suspension was spread onto ACM, malt extract agar (MEA) and CYA plates and grown at 28 °C for 24 hr. After this growth period, 50 germinating colonies were isolated with a sterile needle and centre inoculated on ACM, MEA or CYA plates and left to grow for 7 days before inoculation of liquid broth for DNA extraction. This method had previously been used by Darbyshir (2016) to successfully isolate putative ascospore progeny of *A. sclerotiicarbonarius*.

b) The 2 ml Eppendorf tube was placed into a heat block at 50 °C, 55 °C or 60 °C for 45 mins. Temperatures were chosen according to the conidial inactivation effect of high temperatures in *A. niger* previously reported (Doyle and Marth, 1975; Fujikawa and Itoh, 1996). From here 100 μl of ascospore suspension was spread on ACM, MEA and CYA plates in accordance with a).

c) The sclerotia were subject to dry heat shock at 50 °C, 55 °C and 60 °C for 45 mins. Post heat shock, the sclerotia was removed from the Eppendorf tube, dissected for the red pigmented structure and placed in a new 2 ml Eppendorf tube containing 1 ml 0.05% Tween 80. The ascospores were crushed with a fine point needle. From here 100 μl of ascospore suspension was spread on ACM, MEA and CYA plates in accordance with a).

d) The 2 ml Eppendorf tube was transferred to a -20 °C freezer for a period of 2 weeks before 100 μl of the ascospore suspension was spread onto ACM, MEA and CYA plates in accordance with (a).

e) The red-pigmented sac was isolated from the sclerotia, transferred to a 2 ml Eppendorf tube containing 1 ml OM/*Trichoderma harzianum* protoplast buffer (1.2 M $MgSO_4$, 10 mM $NaPO_4$ and 1.3% *Trichoderma harzianum* enzyme [Sigma-Aldrich, USA] adjusted to pH 5.8). The 2 ml Eppendorf tube was incubated at 30 °C and shaken at 150 rpm for 4 hours. After 2 hours, 100 μl of the solution was taken every 30 mins and spread onto ACM, MEA and CYA plates supplemented with

sorbitol. The plates were incubated at 28 °C for 24 hours before germinating colonies were isolated in accordance with a).

f) The red-pigmented sac was isolated from the sclerotia and transferred to a 2 ml Eppendorf tube containing 25 μl zymolase-100T (AMS Bio, UK). The 2 ml Eppendorf tube was then incubated at 30 °C for 2 h. Following this, 600 μl sterile distilled water was added and the red structure crushed against the inside of the Eppendorf tube wall with a sterilised fine point needle. 100 μl of the solution was spread on ACM, MEA and CYA plates in accordance with a).

2.2.13 Analysis of potential progeny

RAPD analysis was performed as described above (section **2.2.5**). Additionally, the mating type of all progeny was tested, as also described above (section **2.2.4**).

2.2.14 Sexual recombination in *Aspergillus sclerotiicarbonarius*

Recombination of potential *A. sclerotiicarbonarius* sexual progeny was determined through the use of 30 primer pairs (**Table 2.4**). These had previously been shown to identify single nuclear polymorphisms (SNPs) that differed between the parents of the sexual cross of A. *sclerotiicarbonarius MAT1-1* isolate CBS 121056 and *MAT1-2* isolate CBS 121853 (Darbyshir and Dyer, unpublished results).

Amplification of the SNPs was performed through the use of Phusion® High Fidelity DNA Polymerase (NEB, USA) in a final reaction volume of 50 μl (32.1 μl sterile distilled water, 10 μl 5x buffer, 0.5 μl 10 mM dNTP mix, 3 μl 10 μM forward primer, 3 μl 10 μM reverse primer, 50 ng DNA template and 1 U (0.4 μl) Phusion polymerase).

Using gel electrophoresis, PCR products were separated in a 1.5% w/v agarose gel using TAE buffer (See list of reagents below). If a band was

observed at the correct size a PCR purification was performed. In cases where multiple PCR bands were observed, the band at the corresponding site underwent a gel extraction, cutting out the appropriate band with a scalpel when viewed under the light of a transilluminator (Bio Rad, USA). In both cases, PCR purification and gel extractions were performed using the Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel, UK). Once the correct PCR band was isolated, sequencing was by performed DEEP (QMC, University of Nottingham, UK) using the forward primer of the appropriate SNP primer.

Table 2.4 *– Primer pairs used for SNP amplification of* A. sclerotiicarbonarius *progeny.*

Scaffold	Primer Pair	Sequence F	Sequence R
1	1	5'CGATAAATAAG GTCTTCTCGGC'3	5'TCGCAGTTTCGTC CCAATC'3
1	2	5'CAAGAGTCATCT CAGACGAC'3	5'ATGCGACATCATT GCTGG'3
1	3	5'TTCGCTCACTCC CTTGGCTTTG'3	5'TGTTGTCGTATTT CCTGTCC'3
1	4	5'TCTTTCGCTCAT CAGGCTG'3	5'CGTTCCTACCAGT TGAATCC'3
1	5	5'AGCCCGTCACAA AACATCC'3	5'CATAACCGCATCT GGTATCTTG'3
2	6	5'GCTGTCCGCCAT AGAGATAC'3	5'TAAGCCTCGTCGT CCATTC'3
2	7	5'TTGACAGTTGTG CGTTGC'3	5'CATTGGTGAGGTT TACCGAC'3
2	8	5'TTAGAGCCCTGT TCCTGA'3	5'TAGCATACCGTGA AGACCGC'3
3	9	5'GATGGTGTTAGC CCGAATC'3	5'CAACCCACAACCT GAAAGG'3
3	10	5'TCTATGCGGATG TCTCGTC'3	5'TAGGGTGCCTCCT CCAAATG'3

4	11	5’GCAAAAGACTG GTCAAGCG’3	5’CGTATCCACAACA TCATCACC’3
4	12	5’ATCTCATCGCAT CCATTCTG’3	5’TTGCTCTTCTCAT CCACCGCAC’3
5	13	5’TTCAGGCTTTCA GGACTCC’3	5’GGCTGGATAATA CCAACTTCG’3
5	14	5’ACCCGTATCAAC AGTGCCAC’3	5’TTCACCAGCCCCT ACTTCTG’3
6	15	5’ACTTCACTTCGC TGGAGTC’3	5’CGCAGGTTGTAG AGAGTTC’3
6	16	5’ACCCACCAACTA CTAACCTC’3	5’CCATCAAACTGAA TCACCCC’3
6	17	5’GAAACAGTCCG ATTGGTCG’3	5’ACCCACCTCACTA TCTACTATG’3
7	18	5’TGGCTGGGTTTG TCATACCG’3	5’TTGGTCTTCAACG CAGTC’3
7	19	5’AGAACCAGATG AAGGGGATG’3	5’CCTCCAGTGTATT GCTATGG’3
7	20	5’CCACTGATGAGT CTGTTCTTG’3	5’AAGTTGAAGCCC CAGGTCGTTG’3
8	21	5’GCTTAGTTACTC GTGGAGC’3	5’GGCAGATGCTGG GTTTTTC’3
8	22	5’AGGCTCCTCTTA GAATCCTG’3	5’GTGCTGCTTTCCT CGTTAG’3
9	23	5’TGCGTAGACACA TACAGCC’3	5’AATCCCAGTGACC GAAATG’3
9	24	5’AGCGACCTTGCT GATGTTGC’3	5’AAGCCATTGCCCG TCACTTTGC’3
9	25	5’GTTGAACTGCCT TGCTTTG’3	5’GCTGTAAGAACCT GACTCAC’3
10	26	5’-GGTAGGCGTAGA GAATGTGG-3’	5’AGTTATGGTCGTG TGTTGTG’3
27	28	5’TGTAATCAACAA	5’CGGAAAAGCAGC

		GGTCGGAG'3	CATTGTAG'3
28	29	5'CTTGATACCAGC GATGTCG'3	5'AACGGTGAAACA GTAGCAG'3
4	30	5'CTTTCGTGATGC CATTTGG'3	5'GCTGTCCGCTACA AATACC'3

2.2.15 Statistical Analysis

All statistical analysis was performed using Graphpad Prism 7, using either a 2-way ANOVA statistical test with a Tukey's multiple comparison test and a Bonferoni post-test or multiple T-test – where appropriate.

2.3 Results

2.3.1 Mating-type (*MAT*) Assay

It was necessary to identify the mating type of each isolate before setting up sexual crosses between the different *A. niger* strains. Results for the PCR mating-type diagnostic assay for the *A. niger* strains used are shown below (**Table 2.5**). As predicted, *MAT1-2* isolates produced a 3.3 kb amplicon (highlighted in red; **Figure 2.3**) and *MAT1-1* isolates produced a 1.9 kb amplicon (highlighted in blue; **Figure 2.3**). Given the fact that only two *MAT1-1* strains (8-161 and 8-166) were present that formed sclerotia, it was decided to use these as crossing partners with all remaining *MAT1-2* strains in subsequent sexual crosses.

Table 2.5 *– Mating type of each* A. niger *strain used.*

Strain	MAT Type
8-160	*MAT1-2*
8-161	*MAT1-1*
8-162	*MAT1-2*
8-163	*MAT1-2*

8-164	*MAT1-2*
8-165	*MAT1-2*
8-166	*MAT1-1*
8-167	*MAT1-2*
8-168	*MAT1-2*
8-169	*MAT1-2*
CS14	*MAT1-2*
SK250	*MAT1-2*
4501	*MAT1-2*
6292	*MAT1-2*

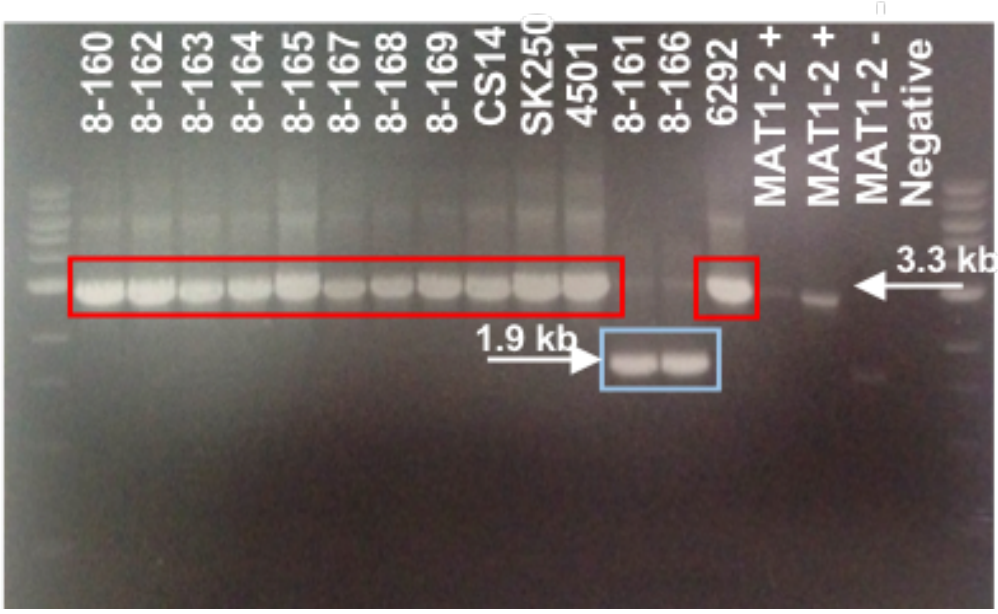


Figure 2.3 *– 0.8% w/v agarose gel showing* A. niger *mating-type diagnostic PCR results. Blue =* MAT1-1 *(1.9kb) Red =* MAT1-2 *(3.3kb) Lanes are as follows: Lane 1, 8-160; lane 2, 8-162; lane 3, 8-163; lane 4, 8-164; lane 5, 8-165; lane 6, 8-167; lane 7, 8-168; lane 8, 8-169; lane 9, CS14; lane 10, SK250; lane 11, 4501; lane 12, 8-161; lane 13, 8-166; lane 14, 6292; lane 15,16* MAT1-2 *positive controls; lane 17,* MAT1-1 *positive control; lane 18, negative control.*

2.3.2 RAPD – Genetic similarity between parent strains

Following RAPD-PCR of all the *MAT1-1* and *MAT1-2* parent strains, prominent DNA bands were selected and scored for their presence or absence in each of the parental isolates. Using this, it was possible to determine genetic relatedness between the isolates before use in sexual crosses (**Figure 2.4**). The results indicate that the two *MAT1-1* (highlighted in blue, **Figure 2.4**) isolates are more closely related in comparison to the *MAT1-2* strains (highlighted in red, **Figure 2.4**). Additionally, CS14 was found to be the most distantly related of the *A. niger* strains.

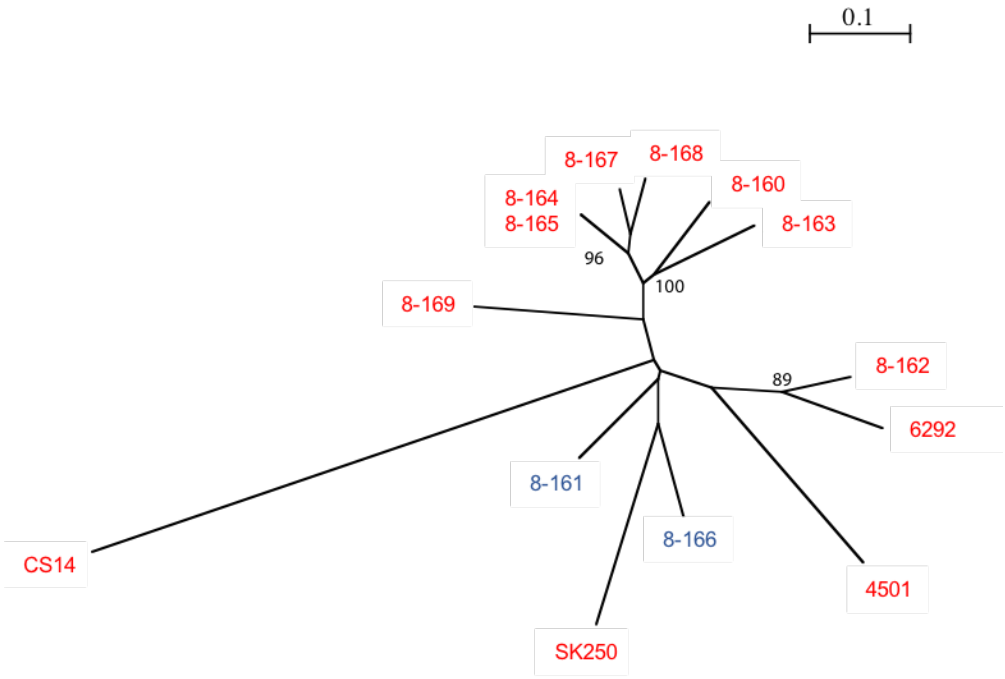


Figure 2.4 *- Unrooted phylogenetic tree to demonstrate the genetic relationships between the parental* A. niger *isolates used in sexual crossing attempts. Branch lengths reflect relative genetic distance between isolates derived from Jaccard's coefficient of RAPD band matching (scale bar: 0.1 = 10% genetic difference). Bootstrap values for certain branches have been added and only those whose value >80% is shown for clarity.* MAT1-1 *and* MAT1-2 *genotypes are shown in blue and red, respectively.*

2.3.3 Sclerotia production trials

Various parameters were assayed in order to try and increase numbers of sclerotia produced, for subsequent use in crossing trials, as described below. In all cases, except where specified, sclerotia production trials used the Petri dish formation shown in **Figure 2.1**. A representation of the sclerotia structure counted in each experiment is shown in **Figure 2.5**.

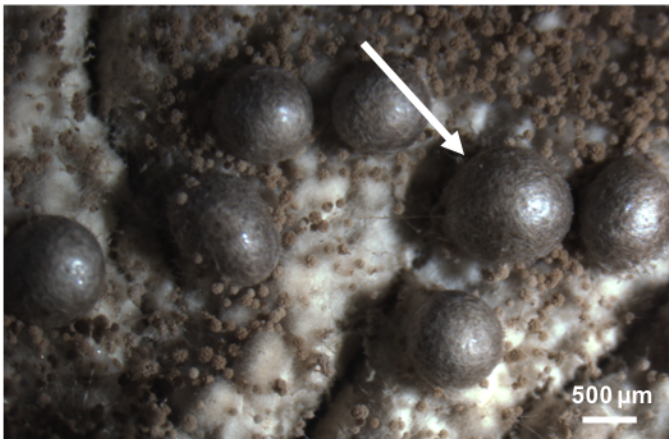


Figure 2.5 *– Representation of the sclerotia structure of* A. niger *counted to measure sclerotia production (indicated by a white arrow).*

1) **Sclerotia production in *A. niger* strains supplied by Jens Frisvad:**

In initial experiments, we wanted to verify that isolates of *A. niger* provided to our research group by Jens Frisvad did indeed form sclerotia, and then ideally, we wanted to identify 'high', 'medium' and 'low' sclerotia producing isolates. Therefore, sclerotia development was evaluated for each of the supplied isolates, using conditions to induce sclerotia development as described by Frisvad *et al.* (2014). All *A. niger* strains tested were indeed found to produce sclerotia after 14 days of growth at 25 °C in darkness on CYA agar (**Figure 2.6**). The highest producing strains were 8-161, 8-162 and 8-165, each producing over 100 sclerotia per test plate. The lowest producing strains were 8-163, 8-164, 8-167, 8-168 and 8-169. These strains produced less than 25 sclerotia on average (**Figure 2.6**). Meanwhile, strains 8-160 and 8-166 produced approximately 50 sclerotia during the trial, suggesting a 'medium' level of sclerotia production. Strains 8-161, 8-160 and 8-164 were selected for use in further optimisations as the 'high', 'medium' and 'low' sclerotia producers, respectively. The reason a 'low' producing strain was chosen was due to our desire to determine optimised conditions that maintained sclerotia production, even in the least productive strains.

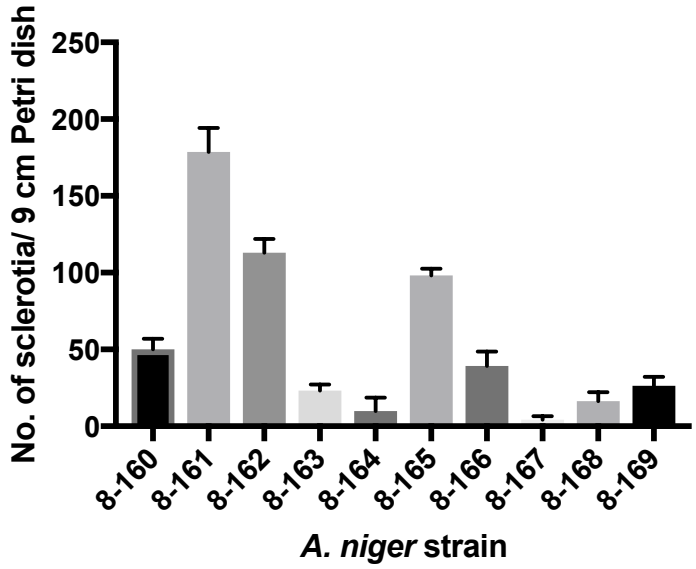


Figure 2.6 *– Analysis of sclerotia production by each strain of* A. niger *supplied by Jens Frisvad after 14 days. All strains were incubated at 25 °C for 14 days in darkness. Numbers indicate the average number of sclerotia formed per 9 cm plate ± SEM. Technical replicates = 3; biological replicates = 2.*

2) **Dark vs light:**

It was first determined whether light influenced development of sclerotia. Fungal cultures were grown according to the method described above to induce sclerotia development in both light and darkness at 25 °C. A significant increase in sclerotia development was observed when strains were grown in darkness for 14 days in comparison to when grown under light in strains 8-160 (multiple t-test; $P<0.05$) and 8-161 (multiple t-test; $P<0.01$). However, strain 8-164 did not show any significant differences in sclerotia development in either condition. These data indicate that sclerotia formation was favoured in darkness (**Figure 2.7**).

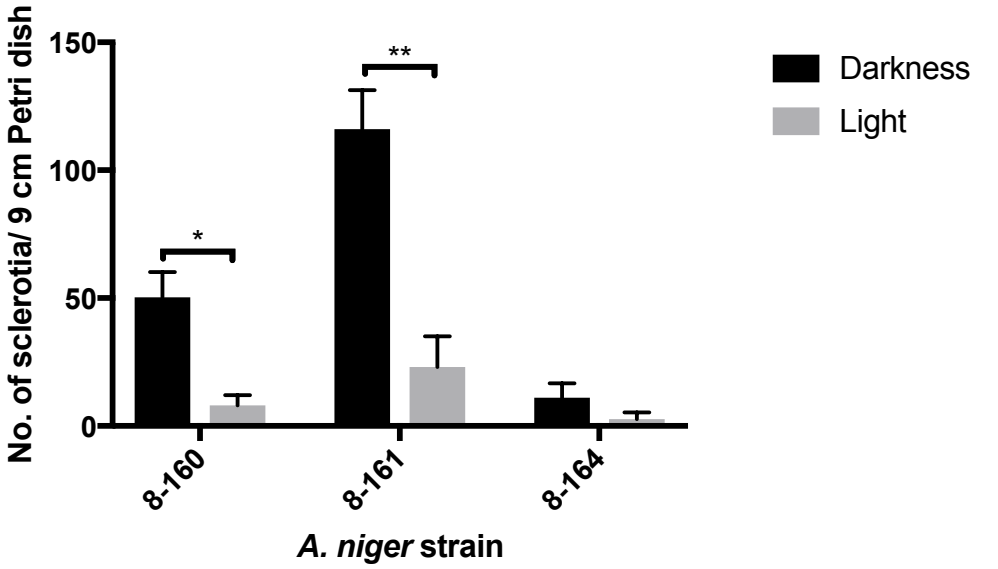


Figure 2.7 *– Analysis sclerotia production in* A. niger *strains 8-160, 8-161 and 8-164 when grown under light and dark conditions. The sclerotia were counted after 14 days of incubation at 25 °C on 9 cm CYA agar plates. Analysis was performed using a multiple t-test - * = p<0.05 and ** = p<0.01 ± SEM. Technical replicates per condition = 3; biological replicates = 2.*

3) **Raisin type and temperature:**

The effect on sclerotia production of using two different types of commercial raisins, as well as three different incubation temperatures was determined. Overall, the type of raisin and temperature used significantly altered the number of sclerotia produced by the 'high' and 'medium' sclerotia producing strains. In particular, the use of Sunmaid raisins produced significantly more sclerotia than the use of organic raisins at 25 °C for both strains 8-160 and 8-161, and although not significant the same effect was seen in the 'low' producing strain 8-164 (**Figure 2.8)**. This was also significant at 28 °C for strain 8-161, with Sunmaid raisins again producing higher numbers of sclerotia than the organic raisins. Although there was not a significant difference for 8-160 at this temperature, the same trend could be observed **(Figure 2.8)**.

Regarding the impact of temperature, the number of sclerotia produced was also significantly different when the temperature was altered. For strain 8-160, the number of sclerotia produced was significantly higher at

25 °C than 30 °C. Whilst not statistically significant, the same negative trend of fewer sclerotia being produced with increasing temperature is also seen at 28 °C with 8-160. Similarly, for strain 8-161, the number of sclerotia produced was significantly higher at 25 °C than 28 °C and 30 °C (two-way ANOVA; F=14.11, P<0.0001) (**Figure 2.8**). Sclerotia were only produced by strain 8-164 at 25 °C.

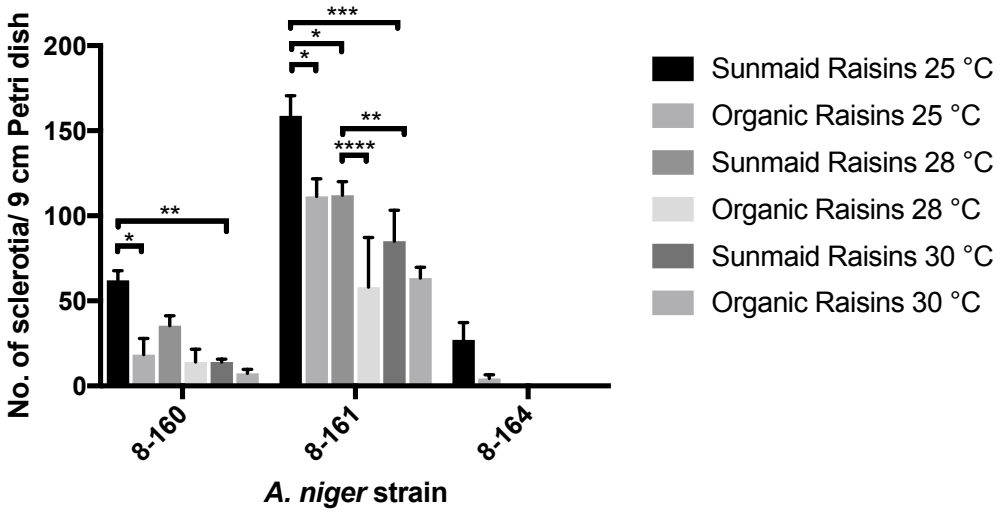


Figure 2.8 *– Analysis of sclerotia production in* A. niger *strains 8-160, 8-161 and 8-164 when incubated at 25 °C, 28 °C or 30 °C using either California Sunmaid raisins or Sainsburys organic raisins. The sclerotia were counted after 14 days of incubation on 9 cm CYA agar plates in darkness. Analysis was performed using a 2-way ANOVA - * = p <0.05, ** = p<0.01, *** = p <0.001 and **** = p<0.0001 ± SEM. Technical replicates per condition = 3; biological replicates = 2.*

4) **Freeze treatment**:

Frisvad *et al.* (2014) reported that sclerotia development in *A. niger* appeared to be promoted when isolates were subjected to freezing treatment at -20 °C prior to inoculation onto CYA agar. We wanted to verify whether this effect occurred. Therefore, the effect of the presence or absence of a pre-freezing treatment on sclerotia production was assayed. It was found that the freeze treatment of fungal cultures at -20 °C for two weeks prior to inoculation did indeed have a significant effect on sclerotia production in strains 8-160

(multiple t-test; P<0.05), 8-161 (multiple t-test; P<0.001) and 8-164 (multiple t-test; P<0.05), with larger numbers of sclerotia produced by the pre-freezing step (**Figure 2.9**). The data indicates the importance of a freezing treatment to promote sclerotia development in *A. niger.*

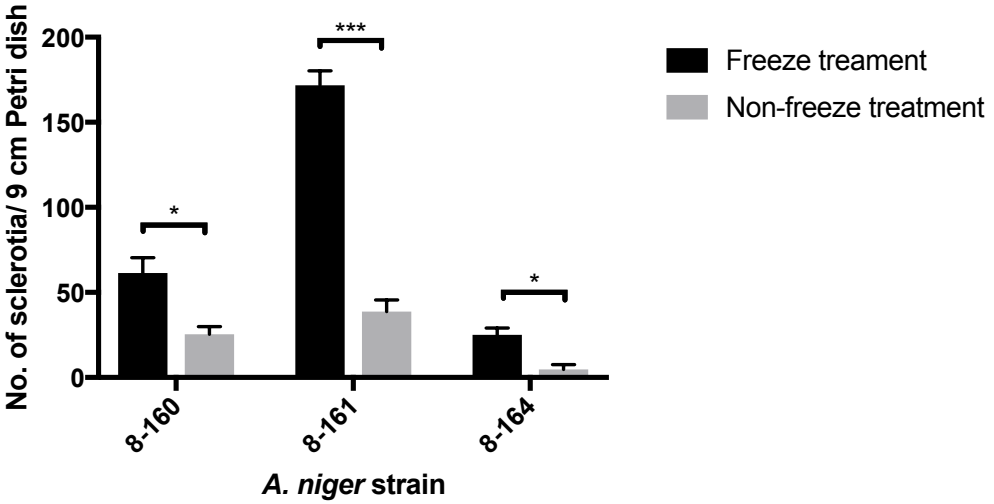


Figure 2.9 *– Analysis of sclerotia production in* A. niger *strains 8-160, 8-161 and 8-164 when exposed to a two week freeze treatment at -20 °C prior to inoculation in comparison to no freeze treatment. The sclerotia were counted after 14 days of incubation at 25 °C on 9 cm CYA agar plates in darkness. Analysis was performed using a multiple t-test - * = p<0.05 and *** = p<0.01 ± SEM. Technical replicates per condition = 3; biological replicates = 2.*

5) **Raisin extract vs removal vs raisin**:

A variety of growth conditions were set up to determine whether maximum numbers of sclerotia could be induced using either raisins direct, initial placement of raisins on agar followed by their removal after 24 hours, or a water extract of the raisins. A significant increase in sclerotia production was observed when raisins were present compared to the raisin water extract or no raisin control for strains 8-160 and 8-161 (**Figure 2.10**). There was also a significant increase in production of sclerotia when the raisin was removed after 24 hours before fungal inoculation compared to the raisin water extract or the no raisin control for strains 8-160 and 8-161 (two-way ANOVA;

F=43.43, P<0.0001). However, no significant difference in production of sclerotia was observed between raisin placement, raisin removal or extract using strain 8-164, although the lowest numbers of sclerotia produced were observed using the raisin water extract (**Figure 2.10**).

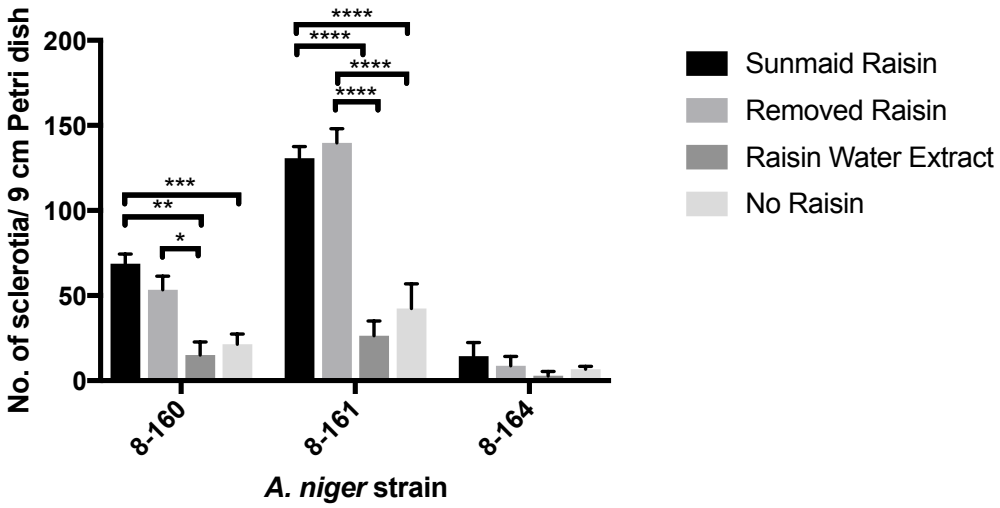


Figure 2.10 *– Analysis of sclerotia production in* A. niger *strains 8-160, 8-161 and 8-164. The different treatment conditions were: Sunmaid raisins left on the agar surface; Sunmaid raisins incubated on the agar surface for 24 hours before removal, prior to fungal inoculation; raisin water extract aliquoted at points where the solid raisins would be placed; and no raisin control. The sclerotia were counted after 14 days of incubation at 25 °C on 9 cm CYA agar plates in darkness. Analysis was performed using a 2-way ANOVA - * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001 ± SEM. Technical replicates per condition = 3; biological replicates = 2.*

6) **Media**:

The effect of different growth media types (CYA, OA and MCA) on sclerotia production was determined. A significant difference in sclerotia production according to growth media used was detected for strains 8-160 and 8-161. In both cases there was significantly higher sclerotia production when incubated on CYA growth media in comparison to OA or MCA (two-way ANOVA; F=112, P<0.0001). Although not significant, the same effect was observed in strain 8-164 (**Figure 2.11**).

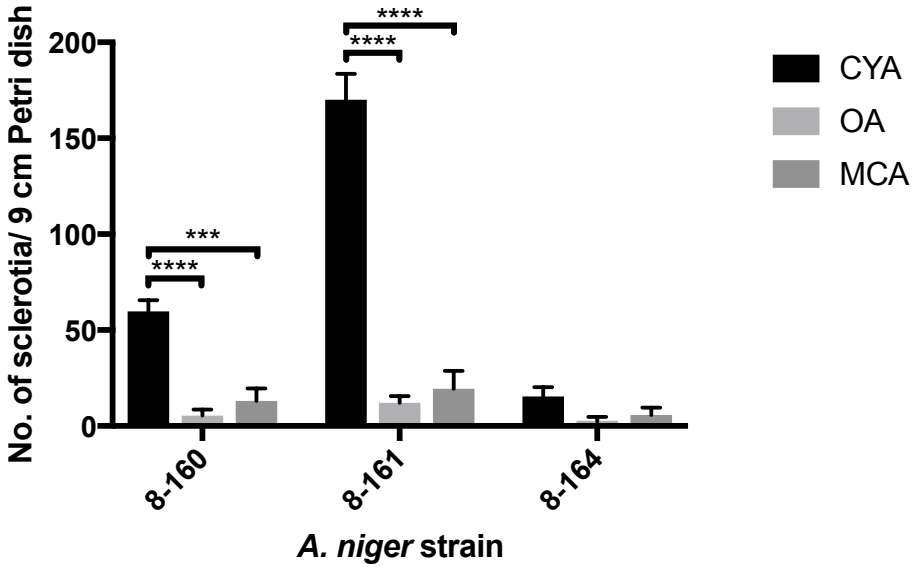


Figure 2.11 *– Analysis of sclerotia production in* A. niger *strains 8-160, 8-161 and 8-164 when grown on CYA, OA or MCA solid media. The sclerotia were counted after 14 days of incubation at 25 °C on 9 cm agar plates in darkness. Analysis was performed using a 2-way ANOVA - *** = p<0.001 and **** = p<0.0001 ± SEM. Technical replicates per condition = 3; biological replicates = 2.*

7) **Sealed cultures**:

Finally, the possible effect of sealing the agar plates with Parafilm was assayed. This was of particular importance given that sexual crossing of *A. niger* might need significant incubation times, and hence plates would ideally be sealed to prevent drying out of the media. Plates were sealed after different time intervals to assess any impact on development of sclerotia. A significant decrease in production of sclerotia was observed when cultures were sealed after 3 days with Parafilm in strain 8-161. However, this effect was not seen when cultures were sealed after 10 days of growth. No significant difference is production of sclerotia was observed in strains 8-160 and 8-164 whether plates were sealed at 3 or 10 days of growth (two-way ANOVA; $F=15.77$, $P<0.0001$) (**Figure 2.12**). The results indicate that a lack of oxygen during the initial stages of incubation may inhibit sclerotia development in *A. niger* for certain, but not all, strains.

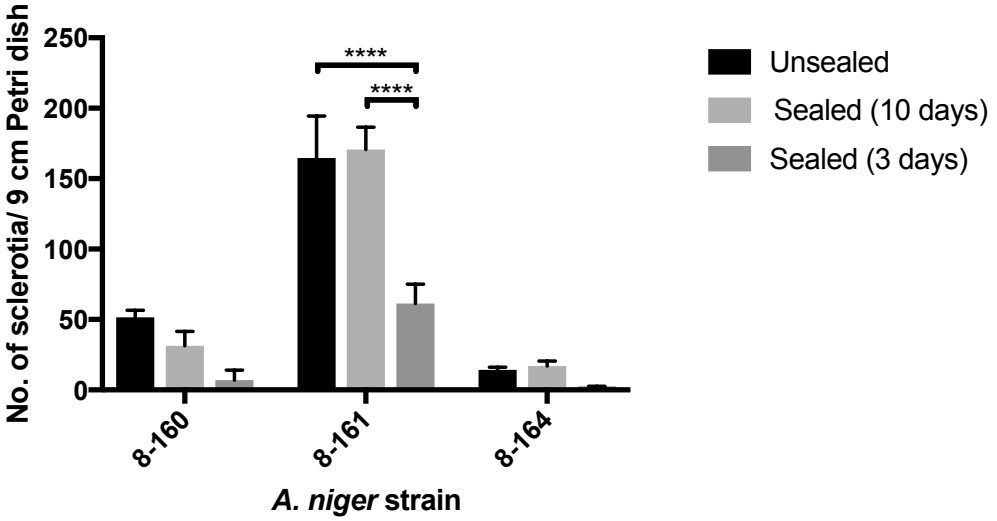


Figure 2.12 *– Analysis of sclerotia production in* A. niger *strains 8-160, 8-161 and 8-164 when inoculated cultures were left unsealed, sealed after 3 days or sealed after 10 days with Parafilm. The sclerotia were counted after 14 days of incubation at 25 °C on 9 cm CYA agar plates in darkness. Analysis was performed using a 2-way ANOVA – **** = p<0.0001 ± SEM. Technical replicates per condition = 3; biological replicates = 2.*

Overall, the conclusion from results (1) – (7) above were that maximum numbers of sclerotia could be produced in *A. niger* by using a pre–freezing step of -20 °C for 2 weeks, followed by incubation at 25 °C on CYA agar, with raisins either present or removed after 24 hours, and with plates sealed after 10 days to prevent drying out of the growth media over the incubation period. These conditions were therefore used in subsequent attempts to induce a sexual cycle.

2.3.4 Barrage zone trial

Sexual crosses were initially set up between *A. niger MAT1-1* isolates 8-161 and 8-166 and *MAT1-2* strain 8-160 using a variety of different barrage methods to allow the mating partners to meet (see **Figure 2.2**). It was found that the conformation described in **Figure 2.2E** appeared to be the most effective for potential sexual production in that this conformation provided a distinct barrage zone between the mating partners that minimised asexual

conidia production between the isolates and had the production of highest numbers of sclerotia (data not shown). It was then tested whether retaining raisins, or removing them after 24 hours of incubation on the growth media before fungal inoculation, might impact on development of sclerotia in the crosses. Results showed that increased numbers of sclerotia were produced using the barrage formation shown in **Figure 2.2F**, which also resulted in a reduced distance between the sclerotia formed by the opposite mating partners, in comparison to arrangement **Figure 2.2E** which retained the raisins. Results of the sclerotia trials of **Figure 2.2D, 2.2E** and **2.2F** are shown below (**Figure 2.13**).

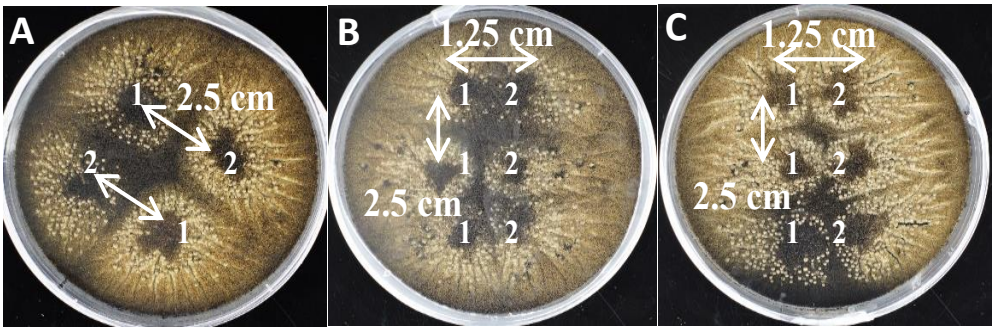


Figure 2.13 – *Results of sclerotia production trials from Figure 2.3D (A), 2.3E (B) and 2.3F (C). In A and B, raisins were left on the CYA Petri dish and inoculated with complimentary* A. niger *isolates. In C, raisins were left on the Petri dish for 24 hours and taken on prior to inoculation with complimentary* A. niger *isolates.*

Therefore, the conformation shown in **Figure 2.2F** was used to set up crosses between the *MAT1-1* strains 8-161 and 8-166 and all remaining compatible *MAT1-2* strains identified in **Table 2.5**. This was in order to maximise the chance of finding a successful sexual cross between the collection of *A. niger* strains.

2.3.5 Examination of sclerotia

It was not known at what time point cleistothecia and ascospores might develop within the sclerotia of the potential sexual crosses of *A. niger*.

Therefore, it was decided to examine the sclerotia at 2 months, 4 months and 6 months post fungal inoculation.

After 2 and 4 months, the sclerotia of each *A. niger* sexual cross was examined under a dissection microscope. The sclerotia structures in all crosses were approximately 1.5 mm in diameter. When cut open using a scalpel, a hollow sphere was observed within i.e. no evidence of cleistothecia or ascospores. The outer colour of the sclerotia was grey (**Figure 2.14A**). By contrast, in crosses '8-161 × 8-160' and '8-161 × 8-168' at six months, the colour of some sclerotia changed to include golden colouration throughout the surface in approximately 50% of sclerotia (**Figure 2.14B**). In addition, these sclerotia had a red spherical structure evident within the hollowed-out interior, when they were cut open. This red structure was approximately 250 μm in diameter (**Figure 2.15**). Furthermore, the inner wall of the sclerotia was distinct from the outer wall in both colour and structural integrity, showing a lighter colouration and softer material on the inner wall. However, the interior of sclerotia from all other attempted crosses remained hollow after six months. In the single isolate 8-161, no golden colouration or red pigment was observed.

Following this, single isolates of 8-160, 8-161 and 8-168 were set up according to **Figure 2.2F**. At each time point, no evidence of golden colouration or red-pigment was present in sclerotia produced by single isolates.

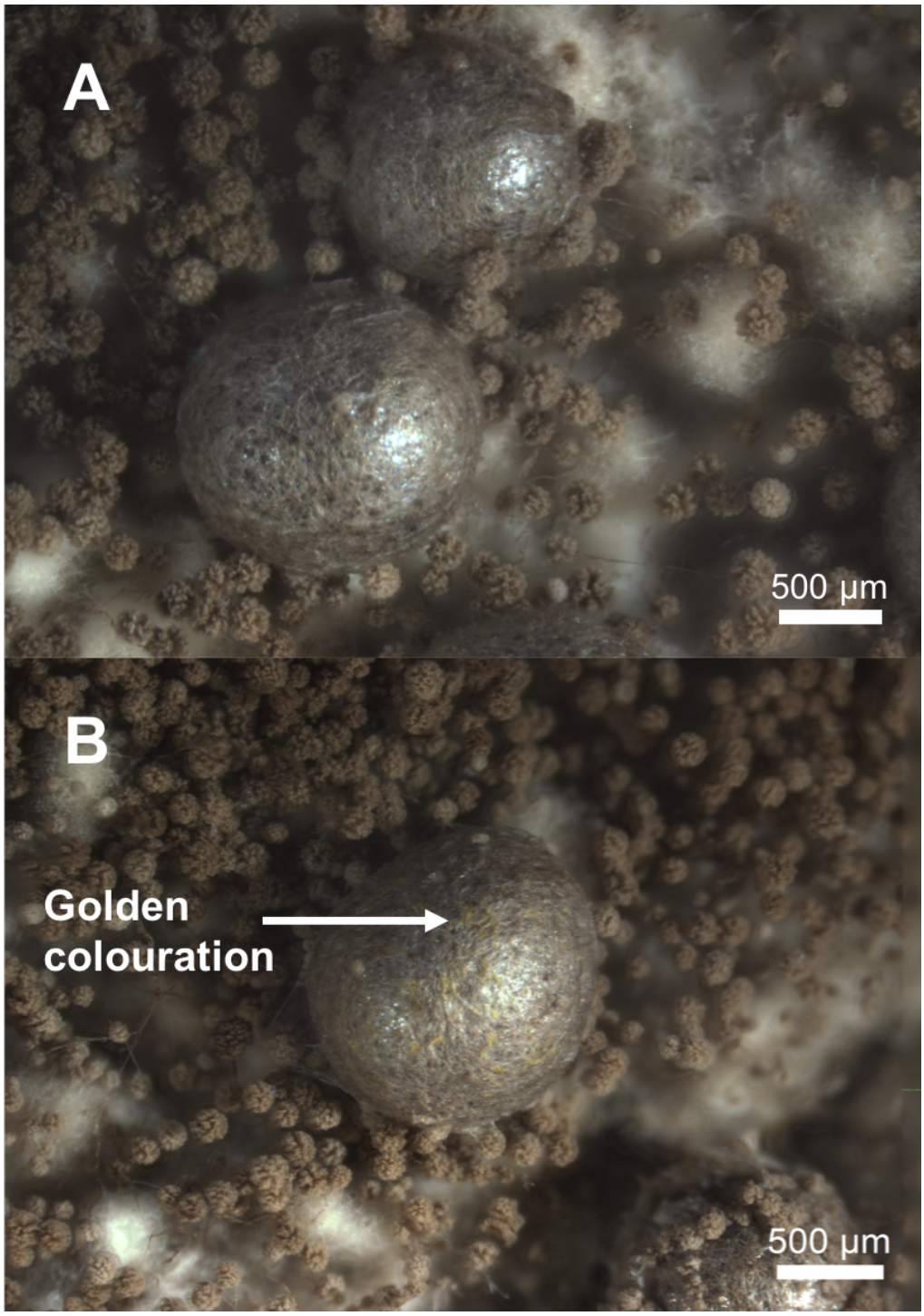


Figure 2.14 *– A) Image showing the structure of sclerotia produced in the* A. niger *cross '8-161 × 8-163' as visualized with a dissection microscope. As can be seen in the image, the outer layer of the sclerotia is grey and much larger than surrounding conidiophores B) Image showing the outer structure of sclerotia produced in the* A. niger *cross '8-161 × 8-160' as visualized with a dissection microscope. As can be seen in the image, the outer layer of the sclerotia is grey/gold and much larger than surrounding conidiophores.*

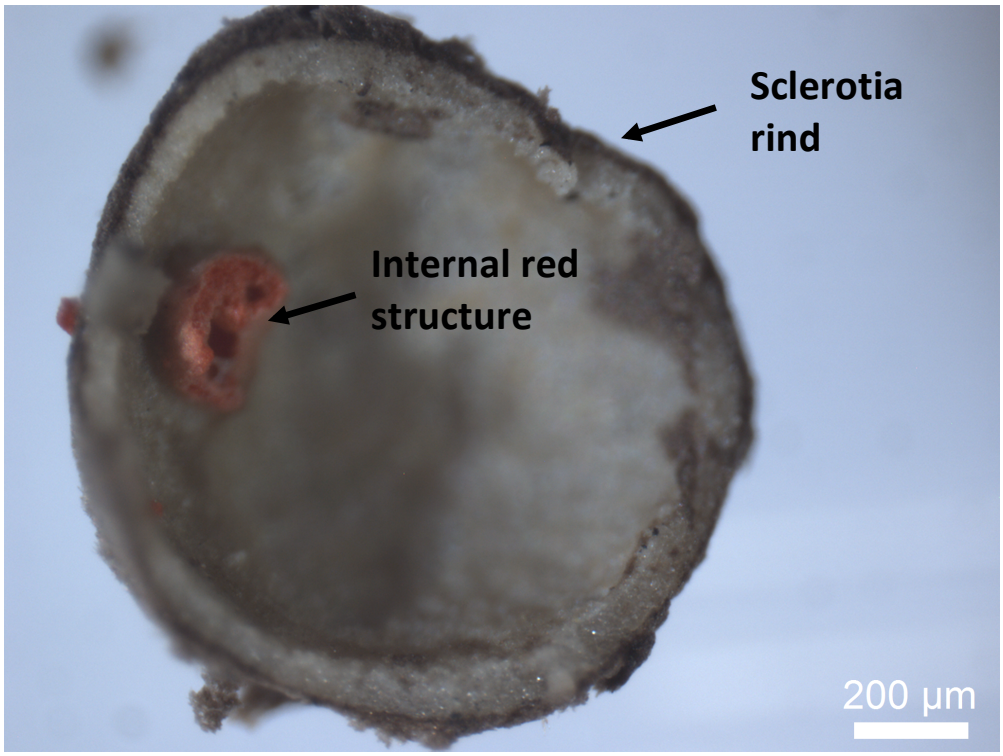


Figure 2.15 *– A sclerotia from the cross '8-161 × 8-160' cut with a scalpel to reveal the inner structure. Within the sclerotia produced by these mating partners is a red spherical structure, absent in sclerotia of other mating partners (excluding '8-161 × 8-168').*

2.3.6 Examination of ascospores

- **Scanning electron microscopy**

To help assess whether the structures seen in the interior of the sclerotia of cross 8-161 × 8-160 might represent genuine cleistothecia with associated ascospores (i.e. a complete sexual cycle), further inspection of the inner structures of the sclerotia using scanning electron microscopy (SEM) was performed in collaboration with the NMRC at the University of Nottingham. From previous studies in the aspergilli, ascospores characteristically have an ornamented 'frilled' appearance, distinct from the relatively smooth-walled conidia (O'Gorman *et al.,* 2009; Horn *et al.*, 2013). At ×700 magnification and above, structures of diameter 5-7 µm similar to ascospores identified in other *Aspergillus* species were observed (**Figure 2.16B**). This provided strong evidence that the mating partners were able to successfully complete a sexual cycle and produce ascospore progeny

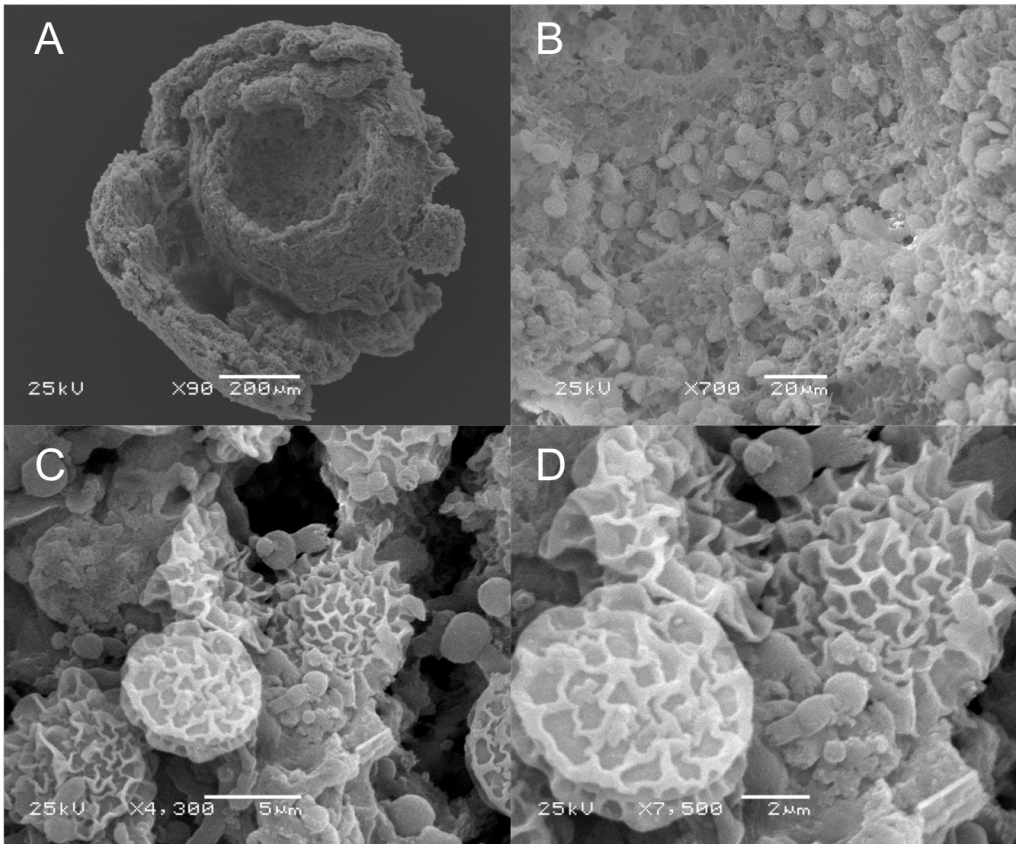


Figure 2.16 *– SEM imaging of a sclerotia cross-section from sexual cross '8-161 × 8-160'. A) ×90 magnification displaying the whole sclerotia cross section, showing the outer sclerotia section and an inner structure; B) ×700 magnification of the inner structure observed in A), revealing smaller spherical structures; C) ×4,300 magnification showing 'frilled' structures embedded into the wall of the inner structure; D) ×7500 magnification showing 'frilled' structures at a greater level of detail.*

In addition, sclerotia with an empty hollow interior (from crosses with other mating partners with strain 8-160) were imaged as a control due to their lack of a red-pigmented structure. It was observed that the 'frilled' ascospore structures were absent (**Figure 2.17**). Therefore, presence of the putative ascospores appeared to be positively correlated with the presence of the red spherical structures.

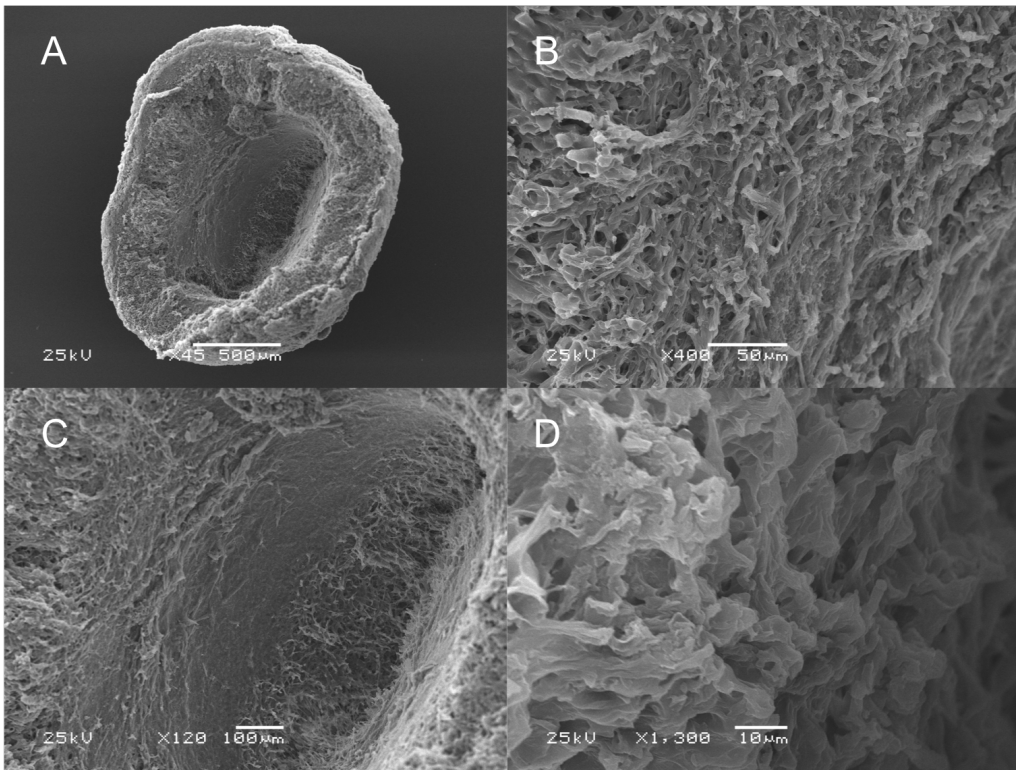


Figure 2.17 *– SEM imaging of a sclerotia cross-section of cross '8-161 × 8-163'. A) ×45 magnification showing the whole sclerotia cross section; B) ×120 magnification displaying the inner sclerotia wall; C) ×400 magnification showing the inner sclerotia wall in greater detail, revealing the mass of mycelia forming the structure; D) ×1300 magnification of the inner sclerotia wall.*

- **Cryo-electron microscopy**

In order to confirm if the structures found during SEM imaging (**Figure 2.16**) were indeed ascospores, cryo-SEM was used in an attempt to provide enhanced definition and overcome possible artefacts of standard SEM practices. However, the low resolution provided at high magnification using cryo-EM meant that the structures seen during SEM imaging could not be observed (images not shown).

- **Helium ion microscopy**

Attempts were then made to visualise the potential ascospores from cross '8-161 × 8-160' at higher definition using helium ion microscopy, in collaboration with the Environmental Molecular Science Laboratory (EMSL)

department of the Pacific Northwest National Laboratory (PNNL). Surprisingly, using this technique, the putative ascospores present within the sclerotia lacked a 'frilled' appearance (**Figures 2.18C** and **2.18D)**. This may indicate an effect the dehydration process of SEM has on the outer structure of ascospores in the aspergilli. Interestingly, the spherical structures identified in **Figure 2.18C** appear to be embedded in the sclerotia mycelial wall, in agreement with observation seen in **Figure 2.16B** using SEM imaging techniques. The spherical structures are also shown to congregate together. However, the putative ascospores seen were also notably different in size, being only 2.5-3.5 µm diameter compared to the previously seen structures of 5-7 µm. It is conceivable that the helium ion microscopy may be showing a more accurate depiction of the ascospores of *A. niger*, with the dehydration process seen in **2.16D** producing a more flattened/collapsed structure due to the loss of water. Therefore, prior to fluid loss in the ascospore structure, a more spherical shape is being observed that is smaller in size.

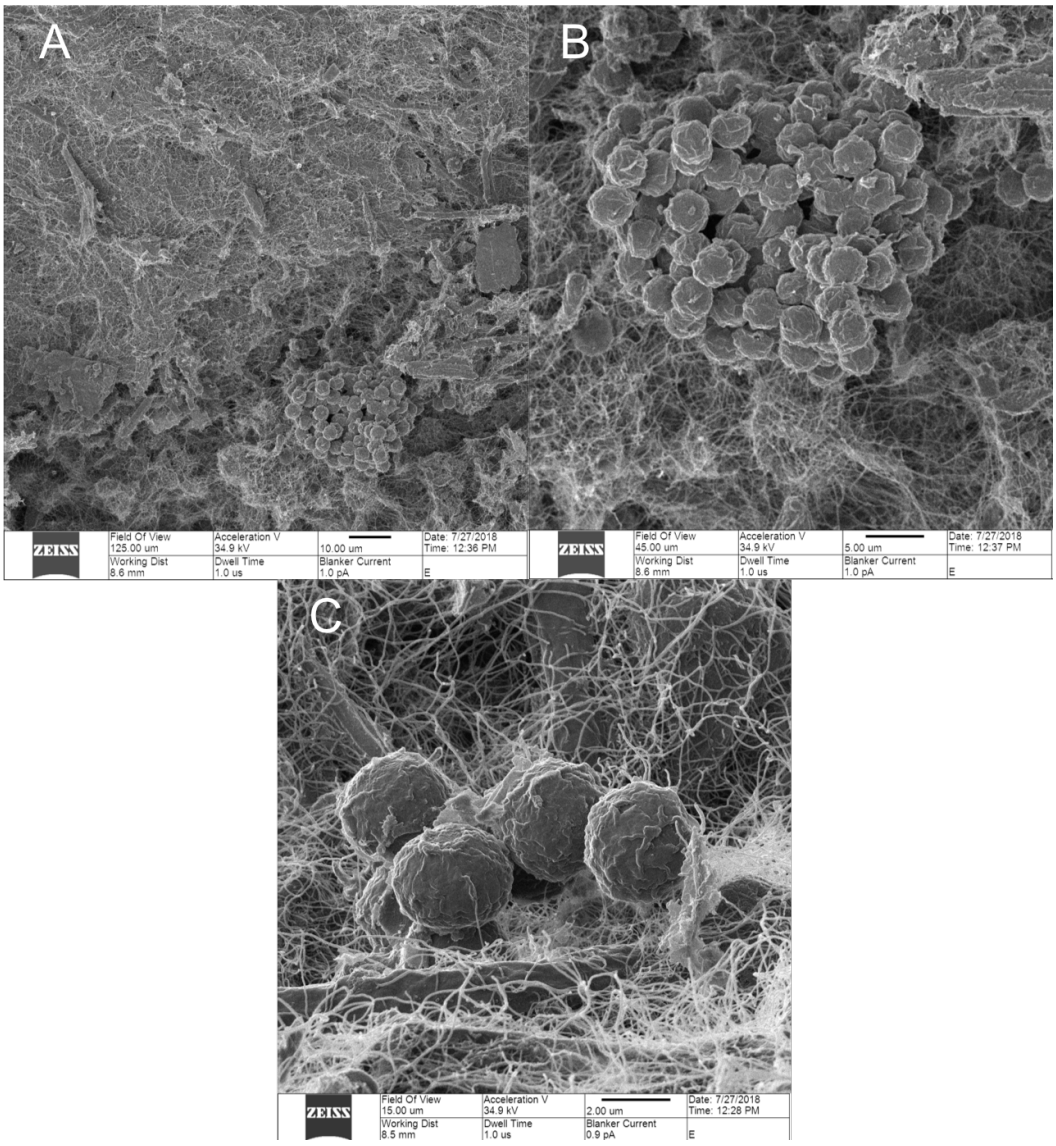


Figure 2.18 *– Helium ion microscopy images of sclerotia contents from cross '8-161 × 8-160'. A) Field of view 125 μm of the inner sclerotia structure, displaying the mycelial matrix of the wall and congregated spherical structures; B) Field of view 45 μm showing higher magnification and detail of the spherical structures and mycelial matrix; C) Field of view 15 μm showing further increased magnification of the spherical structures to reveal finer surface detail, and showing the mycelial matrix at greater resolution.*

Post initial imaging, it was decided that comparisons of the structures seen within the sclerotia should be performed with the conidia of *A. niger*, as there was the possibility that the structures might simply be contaminating conidia. This would allow comparison of shape, size and any other notable features. An image is shown below of a conidiophore and its conidia using helium ion

microscopy (**Figure 2.19**). Although not ideal, certain observations can be made. The conidia of **Figure 2.19B** were approximately size of 2.5 – 3.5 μm in diameter, similar to the structures seen in **Figure 2.18B** and **2.18C**, although the conidia had a 'tyre' shape, in contrast to the more spherical forms seen in **Figure 2.18C**. Therefore, the overall observations from the helium ion microscopy were inconclusive, and might only show contaminating conidia within the sclerotia.

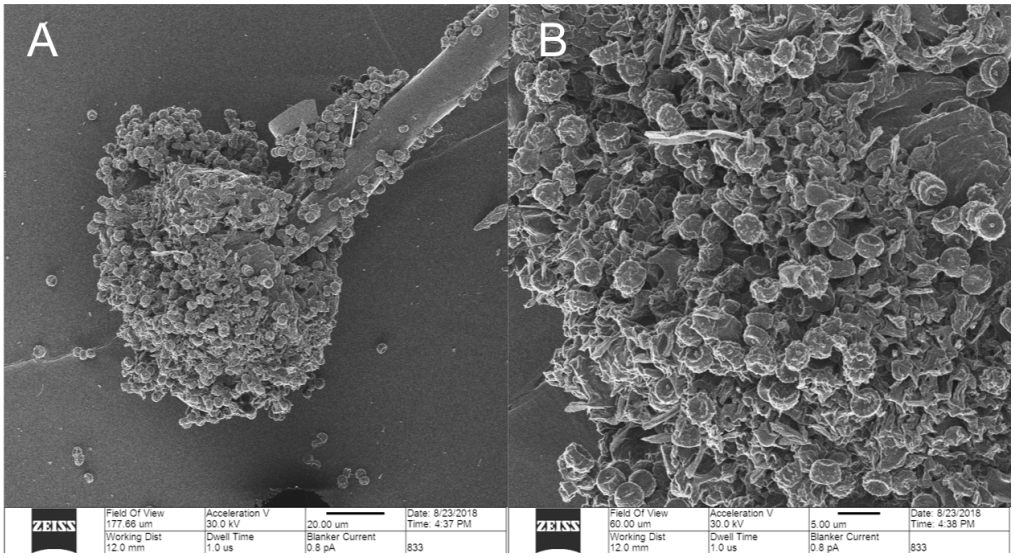


Figure 2.19 *– Helium ion microscopy images of a conidiophore and conidia of* A. niger. *A) Field of view 177.66 μm of the conidiophore of A. niger; B) Field of view 60 μm showing higher magnification and detail of the conidia, highlighting their 'tyre' structure.*

2.3.7 Recombination analysis of potential progeny

Attempts were made to harvest putative ascospores from the sclerotia and then obtain germinating ascospore offspring. Putative progeny were first isolated as described above in section **2.2.12** (a), according to a method successfully used by Darbyshir (2016) to isolate putative ascospore progeny of *A. sclerotiicarbonarius*, and then a RAPD-PCR and mating-type PCR performed in order to assess if genetic recombination had occurred within the potential progeny. RAPD-PCR analysis showed identical RAPD fingerprints

to the parental strain 8-160 in all putative offspring (a representative RAPD using OPA10 is shown in **Figure 2.20**).

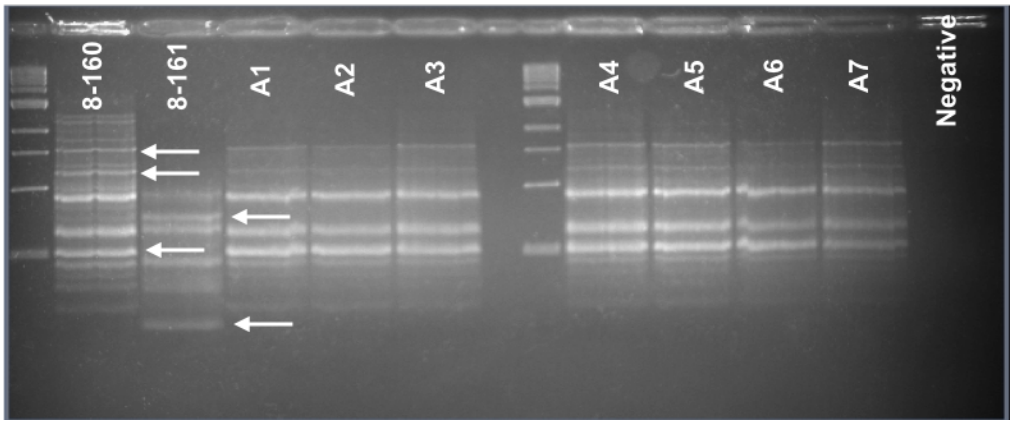


Figure 2.20 *– Representative RAPD using primer OPA10 to assess sexual recombination in potential* A. niger *sexual progeny. The progeny used were isolated using method A) in section* ***2.2.12****. Sexual recombination was identified by presence of unique DNA bands from both parents in the progeny or absence of bands present in both parents. The lanes are as follows: 1) 100 bp DNA ladder; 2) 8-160 parental isolate; 3) 8-161 parental isolate; 4) progeny A1; 5) progeny A2; 6) progeny A3; 7) empty; 8) 100 bp DNA ladder; 9) progeny A4; 10) progeny A5; 11) progeny A6; 12) progeny A7; and 13) negative control.*

Additionally, mating-type analysis identified all 'progeny' as *MAT1-2* (a representative *MAT* assay is shown in **Figure 2.21**), identical to parent strain 8-160. Therefore, the joint RAPD-PCR and *MAT* assays indicated that all the putative progeny were identical to the parent strain 8-160.

This raised the possibility that conditions had not been identified to trigger germination of the putative ascospores, and that the supposed 'progeny' isolated instead arose from contaminating vegetative tissues within the sclerotia – hence were uniformly of the parental strain 8-160 genotype. Therefore, the alternative methods (b) to (f) in section **2.2.12** were used to try and obtain germinating ascospore progeny. For each of these methods at least 16 putative ascospore progeny were obtained and screened for their RAPD-PCR banding pattern and mating type. Unfortunately, in all cases all

putative ascospore progeny were found to have identical banding patterns and be of the same mating type as parent strain 8-160 i.e. there was no evidence of recombination. Curiously, all sclerotia tested appeared to have the 8-160 genotype, when a mixed ratio of parent genotypes might have been expected.

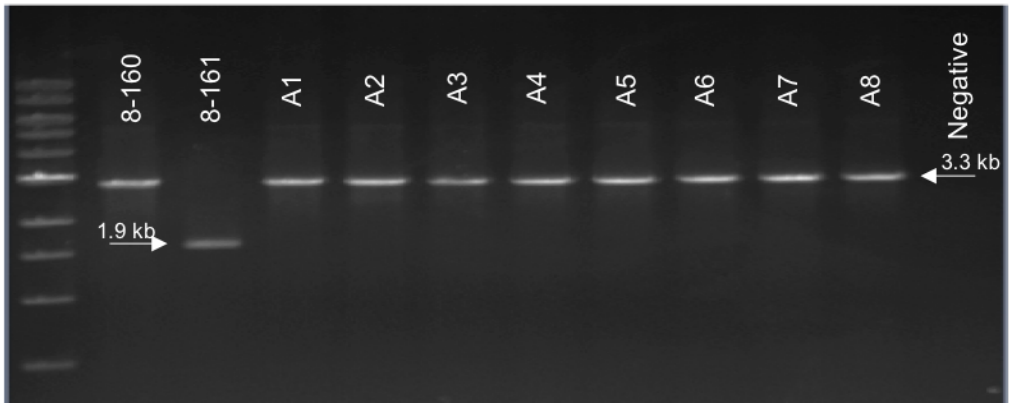


Figure 2.21 – Representative multiplex MAT assay to determine mating type of potential A. niger sexual progeny. The progeny used were isolated using method (A) in section **2.2.12**. MAT1-1 isolates are indicated from a product of 1.9 kb, whilst MAT1-2 isolates are indicated from a product of 3.3 kb. The lanes are as follows: 1) 1 kb DNA ladder; 2) 8-160 parental isolate; 3) 8-161 parental isolate; 4) progeny A1; 5) progeny A2; 6) progeny A3; 7) progeny A4; 8) progeny A5; 9) progeny A6; 10) progeny A7; 11) progeny A8; and 12) negative control.

2.3.8 Sexual Recombination in *Aspergillus sclerotiicarbonarius*

In order to better understand the reproductive cycle and develop accurate diagnostics to determine recombination in the black aspergilli, SNP analysis was performed on potential sexual progeny of an *A. sclerotiicarbonarius* cross between *MAT1-1* isolate CBS 121056 and *MAT1-2* isolate CBS 121853 (Darbyshir and Dyer, unpublished results). This would provide insights into effective methods to investigate sexuality in other members of this group, such as *A. niger*.

To determine whether sexual recombination had occurred, specific primers were used to amplify and sequence specific SNP locations throughout the genome of *A. sclerotiicarbonarius* that differed between the

putative parental isolates. SNP analysis was performed with 38 potential progeny obtained by Darbyshir (2014) during PhD studies. Following amplification of SNPs with all primer pairs, sequence analysis was undertaken to determine the presence of the *MAT1-1* or *MAT1-2* parental SNPs in all progeny. Preliminary data from this analysis indicated the presence of sexual recombination in a number of progeny (**Table 2.6**).

Table 2.6 – *Preliminary data of an* A. sclerotiicarbonarius *sexual cross between "P1 'CBS 121056' × P2 'CBS 121853'", showing the distribution of unique SNP identifiers from each parent - 1, in blue, for P1; and 2, in red, for P2 in progeny strains PrE, PrS, PrU and Pr14.*

SNP	PrE	PrS	PrU	Pr14
1	2	2	1	1
2	2	2	1	2
3	2	2	1	2
4	2	2	1	2
5	2	2	1	2
6	1	1	1	1
7	1	1	1	1
8	1	1	1	1
9	2	1	1	1
10	2	2	1	1
11	1	2	1	2
12	2	2	1	1
13	1	2	1	1
14	1	2	1	1
15	1	2	1	2
16	2	2	2	2
17	2	2	1	2
18	1	2	1	1
19	1	2	1	1
20	1	1	2	1
21	1	1	1	2
22	1	2	1	2

23	2	1	1	1
24	2	2	1	-
25	2	2	1	2
26	1	1	1	2
28	2	-	1	1
29	2	2	1	1

From this preliminary data, we can clearly see recombination between the two mating partners CBS 121056 (P1) x CBS 121853 (P2) in progeny PrE, PrS, PrU and Pr14. Recombination is evidenced by the presence of SNP identifiers from each parental isolate (1 for P1 and 2 for P2) in all of the putative sexual progeny. It was observed that for all crosses SNP 30 did not produce a product and is therefore absent from the data shown above (**Table 2.6**). In addition, it is important to note that the ratio of recombination between each parent genome in the progeny was highly variable e.g. only 7% prevalence of *MAT1-2* markers in progeny PrU, contrasting with 70% prevalence in progeny PrS.

2.4 Discussion

Work was undertaken in the present chapter primarily to determine if it were possible to induce a sexual cycle in *A. niger*. This built on the precedent of the discovery of sexual cycles in species such as *A. fumigatus* and *A. flavus* (O'Gorman *et al.,* 2009; Horn *et al.*, 2009a), and the recent finding that certain strains of *A. niger* exhibited the ability to produce sclerotia. Indeed, it has been shown to be a prerequisite early stage of sexual development in the section *Nigri*, when incubated on a raisin-media (Frisvad, 2014). A secondary aim of work in this chapter was to try to provide evidence for sexual recombination in putative ascospore progeny of *A. sclerotiicarbonarius*, following the detection of a possible sexual cycle in this species (Darbyshir, 2014). This would provide key insights towards identifying sexual cycles in other 'asexual' members of the black aspergilli.

2.4.1 Enhancement of sclerotia production in *A. niger*

A key first part of the work was to identify conditions under which high numbers of sclerotia could be reliably produced, as such structures are an essential pre-requisite for sex in members of the black aspergilli such as *Saitoa japonica and A. tubingensis* (Rajendran and Muthappa, 1980; Horn *et al.*, 2013). A wide variety of conditions were assayed. The most efficient conditions to produce an enhanced number of sclerotia were found to be growth at 25 °C in darkness, a pre-freeze treatment that greatly stimulated the production of sclerotia in most strains and finally the presence of raisins appeared to be critical for sclerotia production. Interestingly, sealing plates with Parafilm after 10 days of culture had a minimal impact on numbers of sclerotia and was essential to prevent desiccation of plates during extended incubation times. These results are in accordance with the studies on carbon and nitrogen sources (Agnihotri, 1968; McAlpin, 2004), pH of growth media (Rai *et al.,* 1967), water availability (Thakur, 1973), and linoleic acid supplementation (Brown *et al.*, 2008) on sexual reproduction – hinting at the impact of these conditions on the sclerotia production.

Although the optimum temperature for sclerotia production was found to be 25 °C, it is worth noting that temperatures below this were not tested. However, preliminary data from our research group indicates an inhibitory effect on sclerotia production in *A. niger* at temperatures <25 °C (data not shown). This is consistent with research in other members of the black aspergilli, where a sexual cycle was initiated at higher temperatures of 30 °C and 28 °C in *A. tubingensis* and *A. sclerotiicarbonarius*, respectively (Horn *et al.,* 2013; Darbyshir, 2014). Indeed, this appears to be a common trend in the black aspergilli and may indicate similar circumstances for other 'asexual' members of this group.

Additional work was then undertaken to see if particular spatial arrangements of crossing partners and methodologies might increase the number of sclerotia formed on crossing plates, again with the assumption that higher numbers of sclerotia would be likely to increase the likelihood of detecting sexual reproduction. It was found that a particular sexual crossing confirmation, combined with temporary placement of raisins before crossing, appeared to optimise the production of sclerotia and minimise the distance between sclerotia from opposite mating partners (as shown in **Figure 2.2F**). These results led to the hypothesis that the presence of raisins was essential for increased sclerotia production. *A. niger* is commonly found as a contaminant mould on vine fruits in the environment (Hocking, 2006). It is therefore interesting that raisins would have such an effect on this species, suggesting that such substrates might provide a site for the sexual cycle in nature. In parallel, the pre-treatment processes involved in the production of raisins from grapes might allow easier access to internal sugars and metabolites that soak into the agar during the 24 h period prior to inoculation. Alternatively, the tannins of the raisin may be leached into the agar. In turn, this would provide *A. niger* with certain nutritional signals for development of sclerotia to occur. It is also worth noting that grapes were not tested in previous research determining sclerotia growth (Frisvad *et al.*, 2014) or in the current thesis. Therefore, it would be interesting to assess the

viability of sclerotia production when grapes are present on the growth media, potentially giving an insight into the *A. niger* sexual cycle in nature. Overall, the rather specific combination of conditions required for production of sclerotia is consistent with the fastidious requirements for sex seen in many other aspergilli and fungi in general (Ropars *et al.*, 2014; O'Gorman *et al.,* 2009; Kwon-Chung and Sugui, 2009).

It is interesting to note, however, that the red-pigmented metabolite did not form within sclerotia of all crosses. Indeed, with regards to sexual crosses with 8-160, only one out of six of the potential mating partners were able to form this pigmented structure. This may suggest that *A. niger* is evolving towards asexuality. Supporting this idea is the distribution of *MAT1-1* isolates to *MAT1-2* isolates in *A. niger.* Recent analysis of *MAT* gene distribution in *A. niger* revealed a 75% dominance of *MAT1-1* isolates in comparison to *MAT1-2* from isolated strains (Mageswari *et al.*, 2016). However, this may be too broad an assumption considering the continuum of sexuality within reproductive species (Paoletti *et al.*, 2005). There is the possibility that the isolates chosen for this study exhibit low fertility and this has reduced their ability to successfully mate.

Of course, further optimisation of sclerotia production of this species might be possible by expansion of the parameters being tested. For example, biotin supplementation has been observed to increase the level of sexuality in a number of species (Molowitz *et al.,* 1976; Böhm *et al.*, 2013). In addition, many strains used in this study were isolated from similar environments. There is therefore potential to widen the screen of sclerotia production outside of this subset of *A. niger* isolates, which as discussed earlier, may exhibit low fertility. Although a number of strains were used from our own culture collection (**Table 2.1**), in the future it will be important to establish and optimise sclerotia production in industrial *A. niger* strains if strain improvement techniques are to be used. One other caveat is that production of large numbers of sclerotia does not necessarily correlate with sexual fertility. For example, study of sclerotia development in *Petromyces alliaceus* revealed that the number of stromata increased depending on the growth

media used. Maximum numbers were produced on Czapek's agar (CZA) and mixed cereal agar (MCA). However, a very low percentage (4%) of sclerotia on CZA contained ascocarps. By contrast, growth on Coons medium produced significantly lower numbers of sclerotia, but approximately 8% were found to contain ascocarps (McAlpin and Wicklow, 2005). In addition, this study showed a similar relationship when varying the nitrogen source on CZA growth media – whereby leucine produced significantly higher numbers of sclerotia than ammonium tartrate, yet had a lower rate of producing ascocarps. Therefore, it would be interesting for us to try to induce sclerotia production of *A. niger* under conditions where higher numbers are not expected, such as lower temperatures <25 °C, or nutritionally supplement the crossing media with different nitrogen sources to assess sclerotia viability.

A final suggestion could be to overexpress genes responsible for sclerotia production in members of the aspergilli in an attempt to induce sexuality in *A. niger*. Indeed, a clear candidate for such a manipulation would be the gene *sclR* (sclerotium regulator). Previous studies have shown that the overexpression of *SclR* results in the induction of sclerotia formation and repression of conidiation in *Aspergillus oryzae* (Jin *et al.*, 2011). This study also reported that deletion of *sclR* resulted in stunted sclerotia formation and increased conidia production. Therefore, overexpression of this gene in *A. niger* may lead to the higher production levels and a faster maturation rate of sclerotia for sexual reproduction.

2.4.2 Discovery of putative sexual reproduction in *A. niger*

Of great excitement, it was discovered that in one particular cross, namely '8-161 × 8-160', that after an incubation period of 6 months that certain sclerotia had a morphology that differed from those in 'failed' crosses, with the former showing a different exterior colouration and the presence of red structures within the sclerotia that represented putative cleistothecia. These observations were similar to those seen of sexual structures in other

members of the section *Nigri*, such as *A. tubingensis* and *Saitoa japonica* which formed distinct cleistothecia within the outer sclerotia shell (Rajendran and Muthappa, 1980; Horn *et al.*, 2013). In particular it was very interesting to observe the red pigmented structure within the sclerotia of *A. niger*, unlike anything seen in this species previously, and to speculate on the genetic factors leading to its production.

Interestingly, both *A. tubingensis* and *A. sclerotiicarbonarius* required extended periods of incubation of 6 months and 12 months, respectively, to undergo a sexual cycle (Horn *et al.*, 2013; Darbyshir, 2014). This is consistent with the observation of the formation of the red structure only after 6 months or more incubation in *A. niger.* This indicates the importance of sealing cultures post sclerotia production due to the risk of media dehydration.

2.4.3 Sclerotia imaging of *A. niger*

Further inspection was then made of the putative sexual structures through the use of both scanning electron microscopy and helium ion microscopy. This allowed the identification of putative ascospores from a cross of mating partners '8-161 × 8-160'. SEM imaging of the internal sclerotia contents revealed structures similar to the ornamented and 'frilly' ascospores seen in the closely related species *A. tubingensis* and *A. parasiticus* (Horn *et al.,* 2009b; Horn *et al.*, 2013). Furthermore, the putative ascospores found associated with the red pigmented structure(s) resembled the size of ascospores seen elsewhere in the black aspergilli e.g. the equatorial ridged ornamented ascospores of *A. tubingensis* (approx. diameter 7 μm) and the bivalve ornamented ascospores of *Saitoa japonica* (approx. diameter 9.5 μm) (Rajendran and Muthappa, 1980; Horn *et al.*, 2013).

In possible future work with *A. niger,* it will be interesting to undertake additional imaging work from sexual crosses. Using helium ion microscopy, imaging of sclerotia that have undergone a dehydration process similar to that used in scanning electron microscopy would confirm if the

structures identified in **Figure 2.16** from SEM were indeed the same structures seen in **Figure 2.18**. If confirmed as the same structures, it would be pertinent to perform helium ion microscopy, where dehydration techniques are not needed, on the ascospores of other fungi such as *A. fumigatus*. Therefore, if the structures are confirmed as the same from **Figure 2.16** and **Figure 2.18**, the ornamentation of ascospores widely reported for other species (O'Gorman *et al.*, 2009) may be confirmed as an artefact of dehydration. However, as discussed **2.3.6** this is unlikely due to the much larger size of the structures in **Figure 2.16** compared to **2.18**.

One theory is that this size difference could be due to the dehydration process of SEM causing structural collapse, flattening the ascospore structure and leading to an increased surface area being observed. Therefore, in an 'untampered' state using helium ion microscopy, the fluid filled and structurally stable ascospore could be a smaller size compared to those seen by conventional SEM preparation.

The observation of putative ascospore was very promising, but alone did not provide sufficient evidence for a complete sexual cycle in *A. niger*. For the sexual cycle of *A. niger* to be confirmed, proof was needed of sexual recombination in the putative ascospore progeny as has been demonstrated for *A. fumigatus, P. chrysogenum* and *A. lentulus* amongst others (O'Gorman *et al.*, 2009; Dahlmann *et al.*, 2015; Swilaiman *et al.*, 2013).

2.4.4 Ascospore germination in *A. niger*

The techniques used during my PhD studies were, unfortunately, unable to induce germination of the putative ascospores of *A. niger*. This was despite the use of methods similar to those that had been used successfully to induce ascospore germination in the related species *A. sclerotiicarbonarius* and *A. tubingensis* (Darbyshir, 2014; Horn *et al.*, 2013), together with the use of a number of other methods that were hoped to trigger ascospore germination based on studies of ascospore germination in *A. fumigatus* and various yeast species (O'Gorman *et al.,* 2009; Bahalul *et al.,* 2010). There could be a variety

of reasons why germination/isolation was unsuccessful in *A. niger*, such as the fastidious nature of sex meaning low numbers of ascospores were present (Dyer and O'Gorman, 2012)*;* or the correct stimulatory growth conditions for ascospore germination had not been identified.

One key reason may be that the putative ascospores were not sufficiently mature when the ascospores were harvested in the present study. Indeed, other closely related heterothallic fungi require lengthy incubation times for successful ascospore isolation, reaching up to 12 months. It may be that delayed harvest of sexual crosses between *A. niger* strains would produce mature ascospores. Furthermore, the fact that all of the putative ascospore offspring were consistently of the same genotype as parent 8-160 raises the possibility that this isolate might have undergone some form of abortive self-fertilisation leading to non-viable ascospores. Nevertheless, the fact that potential ascospores were only found in crosses, rather than single mating-type cultures, suggests a genuine occurrence of a sexual process.

2.4.5 Sexual recombination and germination of ascospores in *A. niger*

There are a number of techniques available that could aid in identifying a functional sexual cycle in *A. niger* in the future. Firstly, it may be possible to identify the expression of meiosis-specific genes in the sclerotia of an *A. niger* sexual cross by end-point PCR (Scherm *et al.*, 2005). In order to do this, it is important to identify genes that have a role solely in meiosis and therefore would expect to be expressed only during the sexual cycle. Indeed, using the species *A. nidulans* as a control, in ongoing studies it has been possible to identify *spo11*, *hop2, mnd1, rec8*, *dmc1*, *msh4* and *msh5* homologues as candidate genes known to be exclusively to be involved meiotic genes (Milgroom *et al.*, 2014; Schurko and Logsdon, 2008). Homologues of these genes have also been identified in *A. niger* using the NCBI BLAST database searches. Most recently, species-specific primer sets have been designed for

both *A. niger* and *A. nidulans* for mRNA expression studies. It is planned to set up sexual and non-sexual cultures from both species and extract RNA at set time points during sexual development. This will entail the extraction of whole cultures for *A. nidulans*, due to the high numbers of cleistothecia produced, whereas extracts will be made from only sclerotia in *A. niger*, due to the restricted numbers of sclerotia produced in this species. Identification of expression of meiotic genes will provide a good indicator of sexual reproduction.

The second suggested method to confirm a sexual cycle in *A. niger* would again use meiosis-specific genes. Here, it may be possible to transform the *A. niger* parent strains with a fluorescent tag (such as GFP) under the control of the promoter of the meiosis gene of interest. Due to the fluorescent reporter being expressed exclusively during sexual reproduction, imaging will confirm the presence of a partial sexual cycle. There are several examples of fluorescent tag being transformed into *A. niger* (Schalén *et al.*, 2016; Bleichrodt *et al.*, 2015).

The techniques described above, will nevertheless only identify if *A. niger* is participating in a partial/complete sexual cycle. Therefore, it will be vital to both isolate and germinate the ascospores of *A. niger* sexual crosses to verify that recombination has occurred. There are a number of additional untested techniques available that could be used to induce ascospore germination in this species, such as: alcohol washing (Lingappa *et al.*, 1970), media supplementation (Molowitz *et al.*, 1976) and pressure (Dijksterhuis and Teunissen, 2004). Of particular promise is the possibility to use flow cytometry techniques in order to isolate fungal cells gated at the 7 μm size indicated from the structures seen in **Figure 2.16**. Once isolated, it may be possible to germinate these structures and identify if genetic recombination between the two parents has occurred by suitable RAPD analysis. However, germination of these potential ascospores may remain problematic. In this instance, it might be possible to perform a single-cell DNA extraction to seek evidence of sexual recombination in the DNA.

Should RAPDs not be suitable for use with *A. niger* progeny, it would also be possible to produce primer pairs based on unique single nucleotide polymorphisms (SNPs) specific to each parent (Darbyshir, 2014). In collaboration with the PNNL, we are currently sequencing the parent strains 8-160 and 8-161. Once the sequencing is completed, it will be possible to determine unique SNPs of each parent and design strain-specific primers. Following this, the presence/absence of a parent specific SNP will be tested, indicating if sexual recombination has occurred. Furthermore, analysis of SNPs to identify the presence of recombination in *A. sclerotiicarbonarius* will inform us of its potential use in *A. niger*.

2.4.6 Strain improvement of *A. niger*

Once, and if, possible to confirm a sexual cycle in *A. niger*, then future research will be to exploit this novel sexual cycle for strain improvement techniques. This would involve setting up crosses between an industrial mating partner and a partner containing a desired trait. However, the potential for sexual reproduction in industrial isolates is thought to be low due to a possible decrease in sexual fertility as a result of numerous sub-cultures, known as the 'slow decline' hypothesis (Dyer and Paoletti, 2005), and accumulation of genetic damage from classical improvement techniques (UV mutagenesis). Therefore, out-crossing of industrial strains with environmental isolates could restore fitness loss, whilst maintaining the trait of interest. Of some promise, studies on heavily mutated industrial strains of *Trichoderma reesei*, RUT-C30 and QM9414, for enhanced cellulase production, were still able to undergo a sexual cycle (Seidl *et al.*, 2009).

The result of strain improvement could have profound effects on citric acid production in industry, leading to greater yields being produced due to sexual recombination of favourable traits between sexually crossing parent strains. As described in the introduction to this chapter, there is also the possibility of producing novel strains with desirable traits above either parent in the phenomenon known as transgressive segregation, such as in *A. flavus*

(Rieseberg *et al.*, 1999). Indeed, a key example of strain improvement utilising the sexual cycle is from *P. chrysogenum*, where progeny of a sexual cross produced an isolate with high penicillin titres and low chrysogenin levels, a contaminant in industrial penicillin purification (Dahlmann *et al.*, 2015).

Furthermore, this research has added to the more general knowledge on sexuality in 'cryptic' fungal species, allowing us to identify and apply such techniques to induce a sexual cycle in closely related members of the aspergilli. A key example of a species with identified *MAT* genes, but unable to undergo sexual reproduction, is *Aspergillus oryzae*. This species is used in the production of soy sauce in industry and sake production (Wada *et al.*, 2012). Although thought to be a domesticated species with low genetic variation (Gibbons *et al.*, 2012), knowledge of a sexual cycle in *A. niger* could aid research in the discovery of a novel sexual cycle in *A oryzae*.

2.4.7 Sexual recombination in *A. sclerotiicarbonarius*

From the SNP analysis above (section **2.3.8**), the progeny produced from a sexual cross between compatible *MAT1-1* and *MAT1-2* strains of *A. sclerotiicarbonarius* exhibit clear signs of recombination. This provides vital evidence, which may aid in the identification of sexual cycles in other 'asexual' members of the black aspergilli. Indeed, as discussed earlier, a proven sexual cycle in this species provides evidence that sexual cycles can occur above 25 °C with extended incubation times in the black aspergilli.

In addition, SNP analysis has been shown to provide an excellent framework for testing the occurrence of sexual recombination in members of the aspergilli. Therefore, use of this technique, depending on genome sequence availability, would become a valuable alternative to RAPD-PCR analysis.

Furthermore, by proving the existence of a sexual cycle in this species, similar crossing techniques (Darbyshir, 2014) could be employed in other 'asexual' members of the aspergilli more generally with the hopes of inducing a sexual cycle – including *A. niger.*

However, final conclusions about the nature of sexual recombination in *A. sclerotiicarbonarius* cannot be made without analysing the sequencing data of further progeny between the sexually crossing parent isolates, particularly given the surprising bias in SNP distribution in certain progeny which diverged from a Mendelian 1:1 ratio.

2.4.6 Concluding remarks

To conclude, a sexual cycle in *A. niger* has possibly been identified through the observation of secondary products (red pigmentation) similar to other members of the aspergilli (Brown and Salvo, 1994), coupled with the presence of previously unseen structures including putative ascospores within the sclerotia of this species (Frisvad *et al.*, 2014). Future work will involve identifying conditions for ascospore isolation and germination; identifying sexual recombination in these progenies; and determination of meiosis gene expression in the sexual structures of this species. Additionally, further analysis of sexual recombination in *A. sclerotiicarbonarius* will confirm the completion of a sexual cycle in a further member of the aspergilli and will establish a novel method for identifying recombination.

Chapter 3 – Use of Bulk Segregant Analysis to Investigate Antifungal Resistance in *Aspergillus fumigatus*

3.1 Introduction

Aspergillus fumigatus is typically found in the environment as a ubiquitous saprophytic fungus with a role in the decomposition of decaying organic matter. For example, it is one of the most commonly encountered fungi in compost heaps. However, *A. fumigatus* is also an opportunistic fungal pathogen, primarily affecting those who are immunocompromised (Dagenais and Keller, 2009; Sugui *et al.*, 2014), causing various forms of aspergillosis following the inhalation of airborne conidia (Latgé, 2001; Mccormick *et al.,* 2010a). Mortality rates can rise to as much as 90% depending on the site of infection, host immune status and the treatment used (Brown *et al.*, 2012).

3.1.1 Host immune system

It has been estimated that most people will inhale hundreds of fungal spores each day. Fortunately, due to the presence of the innate and adaptive immune system we are able to defend ourselves. In healthy individuals there are numerous ways for the host to combat colonization by *Aspergillus* species. However, *A. fumigatus can* employ a number of strategies in order to avoid the host immune system, resulting in infection in immunocompromised individuals. During the establishment of infection, the first point of contact for the conidia of *A. fumigatus* will be the epithelial cells within the lung (Brakhage and Langfelder, 2002). These cells are able to phagocytose the conidia, among many other foreign materials, as they are non-professional phagocytes. Engulfment of the conidia, however, represents the first risk factor of infection because the conidia are able to survive within the epithelial cells (Paris *et al.*, 1997).

Next the invading conidia will encounter host leucocytes, which will act to eliminate the infection. However, in immunocompromised individuals the number of these leucocytes will be significantly decreased (Brakhage, 2005).

Once phagocytosed within the macrophage, the phagosome containing the engulfed conidium is fused with a lysosome to form a phagolysosome, which is then acidified and the conidium is enzymatically killed (Philippe *et al.*, 2003). In the case of *A. fumigatus* infection of immunocompromised hosts, acidification of the phagolysosome is inhibited within the macrophage due to the presence of DHN-melanin in the spore wall, in much the same way as observed in lung epithelial cells (Thywißen *et al.*, 2011).

Alongside the macrophages, neutrophil recruitment and activity is vital in combatting infection by *A. fumigatus*. Once an infectious agent has been recognized, neutrophils are recruited to the site of infection via the production of cytokines from other members of the innate immune response to aid in the proinflammatory response (Geering *et al.*, 2013; Stone *et al.,* 2010). The conidia are then phagocytosed and killed via a variety of mechanisms, such as degranulation (Auvynet and Rosenstein, 2009); neutrophil extracellular trap formation (NETs) (McCormick *et al.*, 2010b); or reactive nitrogen intermediate production (Nathan and Shiloh, 2000) degranulation (Zarember *et al.*, 2007).

Therefore, deficiencies in neutrophil production can severely increase the danger of invasive aspergillosis given the major role of neutrophils in the clearance of *A. fumigatus* infection. Indeed, deficiency in neutrophil production was found to result in high mortality rates in mice infected by *A. fumigatus* conidia (Mircescu *et al.*, 2009). Evasion of the host immune system by *A. fumigatus* then allows infection to persist. This is aided by the ability of *A. fumigatus* to grow readily at 37°C within the lung tissue of the host due to thermotolerance (Kwon-Chung and Sugui, 2013).

3.1.2 C-type lectins

One of the key factors in mediating fungal immunity is the C-type lectin class of receptors (CLRs), which are predominantly expressed in myeloid cells (Zelensky and Gready, 2005). There are four families of receptors involved in pathogen recognition, including: CLRs; Nod-like receptors (NLRs); RIG-I like receptors (RLRs); and Toll-like receptors (TLRs). Although some TLRs have been known to be involved in fungal recognition, such as TLR1 and TLR2 among others (Bourgeois *et al.*, 2011), it is primarily the CLRs that are involved in fungal immunity. Fungal cell walls contain mannan and β-glucan carbohydrates and it is these components that CLRs are able to recognize (Hardison and Brown, 2012).

More recently, a novel melanin-sensing C type lectin receptor (MelLec) that is able to identify the naphthalene-diol unit of 1,8-dihydroxynaphthalene (DHN)-melanin was discovered (Stappers *et al.*, 2018). This CLR was identified via the use of a chimeric protein probe consisting of C-type lectin-like-domain of murine receptors fused with an Fc region of human immunoglobin G1. The recognition of DHN-melanin was found to be required for immunity to *A. fumigatus* infection and led to the induction of a variety of immune responses, such as macrophage stimulation or production of cytokines by neutrophils. In humans, it was found that MelLec was expressed by endothelial and myeloid cells.

3.1.3 Treatment of aspergillosis

It has proved challenging to identify effective treatments for control of *A. fumigatus* infections. This is because of the eukaryotic nature of fungi, meaning that it is difficult to find drugs that specifically target fungi, whilst at the same time exhibiting low toxicity to humans given the similarities in cell biology. Therefore the drugs used to treat invasive aspergillosis such as the polyenes, echinocandins and azoles all have fungal-specific cellular targets such as membrane interaction, chitin biosynthesis and membrane biosynthesis, respectively (Gallis *et al.,* 1990; Lelièvre *et al.*, 2013).

Polyenes, which include amphotericin B, interact with sterols in the fungal cellular membrane. This class of drugs was the first to be used as frontline treatment for systemic fungal infections (Ghannoum and Rice, 1999). Taking amphotericin B as a key example, the mode of action involves the formation of eight amphotericin B molecules within the fungal cell membrane forming an annulus. This annulus links hydrophobically to the sterols present in the cell membrane and form an aqueous pore, through which the fungal cell will release its cytosolic components and die (Holz, 1974). However, although an effective form of treatment, there are a number of detrimental side effects in the host such as nausea, vomiting and most importantly acute renal failure (Bates *et al.*, 2001). Due to these side effects, the frontline treatment method for treating invasive aspergillosis now involves the use of the azole and echinocandin antifungals (Patterson *et al.*, 2016). However, in cases of aspergillosis where the causative agent is resistant to other treatment methods, liposomal amphotericin B may still be recommended (Verweij *et al.*, 2015).

The echinocandins, including micafungin, caspofungin and anidulafungin, target synthesis of the fungal cell wall. This leads to a reduction in treatment toxicity due to mammalian cells not containing certain fungal cell wall components (Balkovec *et al.*, 2014). This class of drug acts as a fungicidal by competitively binding the Fks subunit of the β-(1,3)-D-glucan synthase situated on the fungal cell membrane. The interaction inhibits the production of β-(1,3)-D-glucan, which forms approximately 30-60% of the cell wall (Fesel and Zuccaro, 2016). This leads to the formation of a permeable cell wall and osmotically unstable cell, resulting in death (Cassone *et al.*, 1981). Indeed, echinocandin usage has been shown to be an effective treatment method again many members of the aspergilli (Warn *et al.*, 2003).

The final class, which will form the main focus of this chapter, are the azole antifungals. The first group discovered was the imidazoles, but due to their high toxicity when used for treatment further research then led to the discovery of the triazoles (Shukla *et al.*, 2016). Among the most commonly used triazoles are itraconazole, posaconazole and voriconazole, which all

target cell membrane biosynthesis in fungi. Ergosterol is an essential component of the fungal cell membrane. This sterol is vital to fungal growth, acting much the same way as cholesterol in humans, having important functions in determining membrane fluidity and the distribution of membrane-bound proteins (Bard *et al.*, 1993). A key enzyme in the ergosterol biosynthetic pathway is lanosterol 14α-demethylase (CYP51) (Mellado *et al.*, 2001). There are two CYP51 protein paralogues in *A. fumigatus*, termed CYP51A and CYP51B (Mellado *et al.*, 2001). Azoles act on *A. fumigatus* by binding with the CYP51A protein, specifically the iron protoporphyrin present in the active site of the enzyme (Dawson and Sono, 1987). This binding results in the inhibition of ergosterol biosynthesis and consequently the fungal membrane becomes unstable due to a buildup of 14-methylated sterols, such as lanosterol and eburicol, with a resultant inhibition of fungal growth (Kelly *et al.*, 1995; Alcazar-Fuoli *et al.*, 2008). Azoles can also consequently have a fungicidal effect due to the formation of carbohydrate patches in the cell wall, as discussed earlier (Geißel *et al.*, 2018).

3.1.4 Drug resistance

Over the past 20 years there has been an alarming increase in the number of cases of reported resistance to azole antifungal drugs in *A. fumigatus*. First reported in 1997 (Denning *et al.*, 1997), resistance to azoles antifungals has now spread worldwide (Chowdhary *et al.*, 2014). Although azole use prevents further infection, one of the major drawbacks of azole treatment is their fungistatic nature, relying on the host's immune system for the clearance of the remaining infection (Meletiadis *et al.*, 2007). This can lead to the persistence of infection and selection for resistant isolates in long-term aspergillosis infections.

Worryingly, a number of azole resistant clinical isolates of *A. fumigatus* collected from patients in Europe have been found to share a high genetic relatedness to azole resistant isolates from nearby environmental sources. This indicated that environmental isolates of *A. fumigatus*, selected

in the field by exposure to azole fungicidal sprays, could then exhibit cross resistance to medically used azole treatments and therefore act as a second reservoir for resistance and infection (Verweij *et al.*, 2009). Indeed, it has been reported that azole resistant TR_{34}L98H isolates of *A. fumigatus* are the most common resistance form encountered both in environmental and clinical samples over recent years (Rivero-Menedez *et al.*, 2016). A number of studies have been undertaken to try to elucidate how resistance might evolve in environmental isolates since the proposal that azole resistance may be selected for in the environment, and subsequently lead to cases of invasive aspergillosis via inhalation of azole-resistant spores (Chowdhary *et al.,* 2013; Verweij *et al.,* 2016).

Conversely, it has also been argued that there is not sufficient evidence to claim definitively that selection in the environment for azole resistance is a major cause of resistant aspergillosis in humans (Holloman, 2017). For example, some soil azole-treated *A. fumigatus* strains exhibit a comparably low MIC compared to medical isolates. It is also suggested that azoles remain relatively unavailable to surrounding microflora in the environment due to quick adsorption to soil particulates. Furthermore, although environmental azole resistant *A. fumigatus* appears to be a source of infection, it may only be minimal in comparison to resistance development in the infected patient in hospital. This is suggested from a study of 14 patients infected with itraconazole resistant *A. fumigatus*, where 13 of the isolates evolved resistance during long-term azole treatment (Howard *et al.*, 2009).

However, the risk cannot be ignored that agricultural treatment of crops might lead to incidences of cross-resistance between agricultural and medical azole drugs (Chowdhary and Meis, 2018). This scenario then might lead to a source of *Aspergillus* infection in immunocompromised patients. Indeed, certain studies have shown 64-71% of patients infected with an azole resistant isolate of *A. fumigatus* received no prior azole treatment, highlighting the possible importance of the environmental source of infection (van der Linden *et al.*, 2011).

3.1.5 CYP51A

The majority of resistant isolates of *A. fumigatus* described since 1997 contain mutations in the *cyp51A* gene. There are two subtypes of mutations in *cyp51A* that lead to azole resistance. The first group leads to the structural modification of the CYP51A protein, so the azole can no longer bind efficiently to its target. Key examples of this group are: methionine (M220) substitutions to threonine, valine, isoleucine, lysine, arginine or leucine (Howard *et al.*, 2009; Mellado *et al.*, 2004); and glycine (G54) substitutions to tryptophan, arginine, glutamic acid, valine or alanine (Diaz-Guerra *et al.*, 2003; Xu *et al.*, 2010). Both examples lead to a variety of resistance profiles depending on the substitution involved. For example, M220K produces highly resistant phenotypes to itraconazole, posaconazole and voriconazole (Mellado *et al.*, 2004).

The second common group of *cyp51A* mutations leading to azole resistance are linked with gene overexpression. There are several reported examples and they usually involve a tandem repeat insertion in the promoter region in combination with a point mutation in the coding region. Key examples include: a 34-base pair tandem repeat in the promoter region, in combination with a leucine (L98) to histidine substitution (Morio *et al.*, 2012; Mortensen *et al.*, 2011); a 46-base pair tandem repeat in the promoter region, in combination with a tyrosine (Y121) to phenylalanine substitution and a threonine (T289) to alanine substitution (Astvad *et al.*, 2014; Fischer *et al.*, 2014); and a single 53 base pair tandem repeat in the promoter region (Hodiamont *et al.*, 2009). The presence of a tandem repeat in the promoter region of *cyp51A* leads to enhanced transcription of this gene (Mellado *et al.*, 2007). A representation of *cyp51A* and common mutations is shown below (**Figure 3.1**).

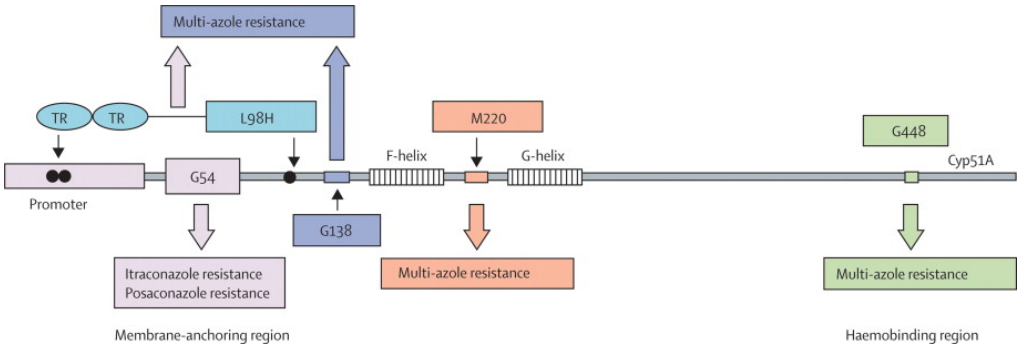


Figure 3.1 *– Schematic representation of the cyp51A gene and common mutations found to induce azole resistance in* A. fumigatus*. From* Verweij *et al.* (2009).

3.1.6 Other mutations

Although mutations in *cyp51A* are attributed as one of the primary causes of azole resistance, a study from Manchester, UK found that approximately 50% of resistant isolates from certain clinical samples did not have a mutation in *cyp51A* (Denning *et al.*, 2011).

Further investigation of one such resistant isolate by genome sequencing revealed that a proline to leucine substitution (P88L) in the *hapE* gene of *A. fumigatus* led to increased mRNA levels of *cyp51A* leading to resistance to azole treatment (Abdolrasouli *et al.*, 2015; Camps *et al.*, 2012). Here resistant and sensitive *A. fumigatus* isolates were obtained from a patient and were genome sequenced. From here, the genome sequences were compared to each other and reference strains Af293 and A1163 (both azole sensitive) to identify key mutations. This represents a similar methodology to the study presented in this thesis, whereby a pool of DNA isolated from sensitive isolates and a pool of DNA from azole resistant isolates with an unknown mutation were sequenced and compared for key differences, as described below in section **3.1.7**.

Furthermore, a recent example of a non-*cyp51A* mutation, which confers azole resistance, can be seen in *hmg1* mutant *A. fumigatus* strains (Hagiwara *et al.*, 2018). The HMG-CoA reductase enzyme encoded by *hmg1* acts during ergosterol biosynthesis, acting as a rate-limiting enzyme. However, a S269F or S269Y mutation in *hmg1* led to increased levels of

internal ergosterol and as a consequence, azole resistance. Indeed, multiple isolates with different genetic backgrounds, but maintaining a mutation in *hmg1* showed increased azole resistance. This highlights the strong impact of this mutation on azole susceptibility.

A series of other studies have also reported the detection of isolates of *A. fumigatus* with non-*cyp51A* forms of resistance to azole drugs e.g. in up to 15% of isolates from a clinical study from the Netherlands (Arendrup *et al*. 2010; van der Linden *et al*. 2015). However, the genetic basis of such mutations remains unaccounted for.

In order for aspergillosis infections to be managed efficiently it is therefore vital to know if a patient may be infected with an azole resistant isolate of *A. fumigatus*, as this will then alert clinicians to the need for alternative control measures and improve patient outcome e.g. through the use of combinational echinocandin and voriconazole therapy or liposomal amphotericin B (Meis *et al.*, 2016). Indeed, with the increasing rate of azole resistance in hospital isolates, it is becoming clear that unless action is taken then the therapeutic use of many azole drugs may become redundant. Therefore, more directed approaches are required to identify the genetic basis of unknown forms of azole resistance in order to survey their emergence and spread throughout both environmental and clinical populations, even if they seldom occur.

Molecular diagnostics have already been developed to identify the presence of mutations in *cyp51A* that can lead to azole resistance (Xu *et al.*, 2010). However, there is a need to develop complementary methods to identify novel, unknown mutations such as those reported in the Manchester and recent Netherlands study (Denning *et al.,* 2011; P. Verweij pers. comm.). Understanding the basis of resistance in such isolates might also allow new targets to be identified for drug development.

3.1.7 Bulk segregant analysis (BSA)

The sexual cycle of fungi provides a valuable tool that can be exploited to identify the genetic basis of traits whose underlying mechanism(s) is unknown (Ashton and Dyer 2016). By crossing two complementary sexual partners that differ in the trait of interest, it is possible to determine whether the trait is either monogenic (i.e. determined by a single gene) or polygenic (i.e. determined by multiple genes acting with each other). Once the nature of the trait has been identified, it is possible to further utilize the sexual cycle to try and identify candidate genes of interest.

For gene identification of monogenic traits, it may be assumed that during a sexual cross the progeny will exhibit a 1:1 ratio of having the gene present (i.e. the allelic form linked to the phenotype) or not, and therefore the trait of interest, or not. However, in examples of polygenic traits the analysis becomes much more complex. In this scenario, the progeny from a sexual cross between the two sexual partners would be expected to exhibit a continuous range of phenotypes as the multiple genes contributing to the trait of interest are spread out among the offspring (Ashton and Dyer 2016).

In the case of traits that have been identified as monogenic, there are various classical genetics methods to identify the region of the genome of the gene of interest or even the individual gene causing it. One such technique involves the use of a genetic map-based method to identify the chromosomal region containing the gene of interest. Using this technique, it is possible to map the segregation of markers linked to the phenotype of interest and thereby identify the region involved (Foulongne-Oriol, 2012). However, there are numerous problems with this technique. At the forefront is the need for a highly saturated genetic map for the species being used, which in the past has been limited to certain model species. However, this has become less of a problem in recent years due to the availability of whole genome sequences, where linked markers may be known.

An alternative method to identify a unknown gene encoding a trait of interest, where detailed genetic information is not known, is the so-called ‘bulk segregant analysis (BSA)’ technique (Michelmore and Paran 1991).

Sexual crosses are performed between complementary mating partners where one of the parents exhibits the phenotype of interest correlated with the presence of the gene (allele) of interest. Once the sexual cycle is complete and fruiting bodies have formed, a number of progeny (approx. 80-120) are separated into two pools: one pool of progeny with the phenotype (gene) of interest (Pool 1); and a second pool without, exhibiting no signs of the desired phenotype (Pool 2). Upon separation into two pools, the DNA is extracted from all the progeny in Pool 1 and Pool 2. The DNA extracted in Pool 1 is then mixed (pooled) together and the same is done for Pool 2. It would then be expected that by pooling the samples in this way, that differences between the parental isolates would be 'evened out' as a result of cross over and recombination in the progeny, except for regions linked to and including the gene of interest itself. The classic use BSA is illustrated in **Figure 3.2.** Current application of BSA employs next generation sequencing to identify SNPs of interest (Niu *et al*., 2015; Kershaw *et al*., 2019). One of the main criticisms of BSA is that due to the large number of genetic differences likely to be present between the two original parents and the unpredictability of the extent of cross over and recombination, that BSA is likely to lead to many false positively identified regions. However, by pooling a large number of recombinant offspring it is predicted that the only differences between the pools would be the in a region linked to the gene of interest.

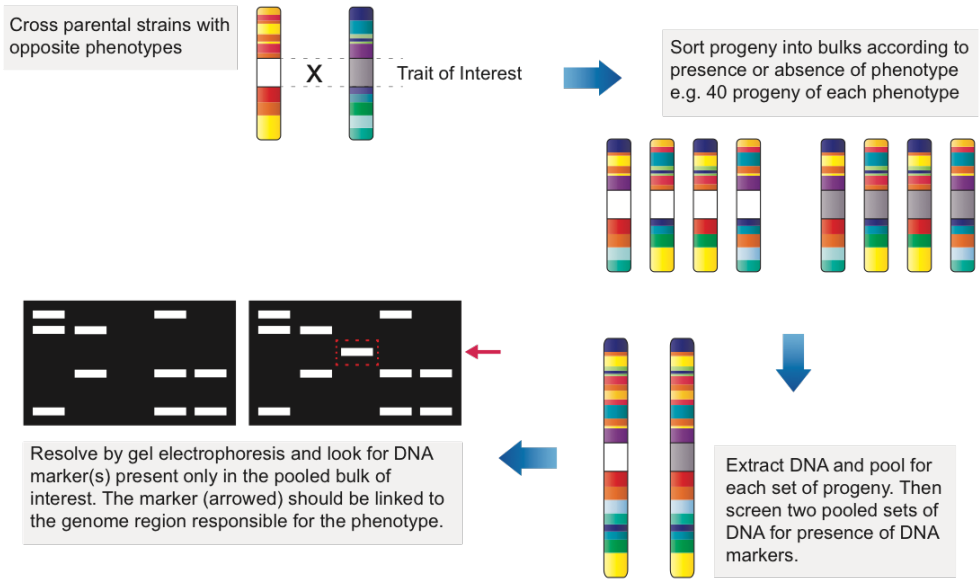


Figure 3.2 *– Representation of the technique bulk segregant analysis (BSA) to identify the region of the genome responsible for the trait of interest. From Darbyshir and Dyer (unpublished).*

Encouragingly, BSA has been employed successfully in the identification of single genes of interest in applications of the technique (Pomraning *et al.,* 2011). For example, BSA has been employed in *A. niger* in order to identify a non-acidifying phenotype, desirable for enzyme production. Here, BSA was used in combination with NGS and the parasexual cycle to isolate haploid recombinants and led to the identification of a point mutation in *laeA* (Niu *et al.*, 2015). BSA has also been used in *A. niger* to provide a novel platform for mutant identification for strain improvement, as described in Arentshorst and Ram (2018). Furthermore, BSA has proven to be a powerful tool in the understanding of plant pathogen interactions, identifying the gene *SMO1* as necessary for septin mediated plant infection in *Magnoporthe oryzae* (Kershaw *et al.*, 2019). Finally, BSA has been employed outside of the filamentous fungi, leading to identification of a gene deletion responsible for a maltose utilization defect in *Schizosaccharomyces pombe* (Hu *et al.*, 2015). These examples highlight the potential use of BSA to identify traits of interest.

The BSA technique was therefore felt to hold promise for gene identification studies with *A. fumigatus* in cases of isolates which exhibited non-CYP51A based forms of azole resistance, with unknown mechanism(s) of resistance. There were three reasons for this. Firstly, although once thought to reproduce entirely by asexual means, O'Gorman *et al.* (2009) discovered a functional sexual cycle for this species, with evidence of a heterothallic breeding system, that could be induced *in vitro* within three months of incubation on oatmeal agar (OA) in darkness. This opened the way for exploitation of the BSA technique because crossing and the production of pools of offspring has now become possible. Secondly, recent advances in next generation sequencing technology have made it possible sequence pools of genomic DNA at relatively low price. This means theoretically that rather than having to develop classical genetic markers for the BSA (a time-consuming task), it should be feasible to use next generation sequencing methods to find single nucleotide polymorphisms (SNPs) linked to the gene of interest, through the use of whole genome sequencing and bioinformatic analysis. Thirdly, it was hypothesised that the classical form of BSA could be improved through the use of backcrosses between selected progeny and one of the original parents, thereby reducing genetic variation present in the offspring except in the linked region of interest. Thus, several rounds of backcrosses would be carried out, selecting for progeny exhibiting the trait of interest that are sexually compatible with the parent without the trait of interest i.e. the rationale behind this would be that the progeny with the trait of interest from multiple backcrosses would become more isogenic compared with the parent without the trait. Thus, performing BSA on the progeny of this backcross (separating the progeny into two pools etc.) would identify the region of the genome or even individual gene of interest much more readily than the use of only one parental F1 sexual cross.

3.1.8 Aims

Therefore, the aim of work in this chapter was to assess whether BSA linked with NGS of progeny pools could be used to identify the genetic basis of azole resistance in isolates of *A. fumigatus* where the resistance mechanism was supposedly unknown, thereby exploiting the newly discovered sexual cycle of *A. fumigatus*.

3.2 Methods

For media composition, refer to **Appendix**.

3.2.1 Strain maintenance and revival

A. fumigatus isolates 47-308 (*MAT1-2*), 47-312 (*MAT1-2*) and 47-316 (*MAT1-1*) exhibiting resistance to azole antifungals were obtained as a gift from P. Verweij and S. Camps (Radbould University, The Netherlands). These were clinical isolates obtained during 2010-2011 and did not exhibit any known mutation in the *cyp51A* gene (S. Camps, personal communication). Preliminary crossing work by Céline O'Gorman in the Nottingham group had established that isolate 47-308 had a monogenic form of resistance to the azole antifungal itraconazole, and that this resistance appeared both stable and of a relatively high level (O'Gorman and Dyer, unpublished results). Therefore, isolate 47-308 was chosen as a representative resistant parent for crosses to the known sensitive parent 47-51 (*MAT1-1*) for subsequent BSA work. Céline O'Gorman obtained 47-51 from air sampling in Dublin, Ireland in 2005. Both parents and any arising backcrossed progeny used for bulk segregant analysis were maintained in 20% glycerol stocks at -80 °C (1:5 in sterile distilled water). When required, appropriate strains were thawed and a cotton swab (SLS, UK) was used to inoculate conidia on ACM agar 9 cm plates (Thermo Scientific, USA) and ACM slopes (STARLAB, Germany) (Paoletti *et al.*, 2005) for revival before the stocks were refrozen.

3.2.2 Itraconazole resistance screening

The ETEST method was used to determine the antifungal sensitivity of isolates of *A. fumigatus* to the azole antifungal itraconazole, which was chosen as a representative azole drug. This method provided an accurate measurement of itraconazole resistance in *A. fumigatus* isolates. In accordance with the ETEST strip manufacturer's instructions (Biomerieux, France), isolates were grown at 37 °C for 48 hr to ensure adequate sporulation. Spore suspensions were made and adjusted to a concentration of $1x10^6$ spores ml^{-1} in 0.05% Tween 80. Media used for the determination of azole resistance with ETEST strips was RPMI 1640 (Sigma Aldrich, UK) supplemented with 2% glucose (Fisher Scientific, USA), 1.5% agar and adjusted to a final pH of 7.0 with morpholinepropanesulphonic acid (MOPS) buffer (Sigma-Aldrich, USA). The RPMI plates were then inoculated with the fungal culture using a sterile cotton swab, which had been submerged in the spore suspension, and streaked across the surface in three different directions. Once the plate had been inoculated and dried, the itraconazole ETEST strip was placed at the centre of the plate and incubated at 35 °C for 48 hr, checking the plate every 24 hr. The minimum inhibitory concentration (MIC) was determined as the maximum point (corresponding with maximum azole concentration that permitted growth) on the ETEST strip that the fungal culture grew to.

When selecting progeny for whole genome sequencing, large numbers of resistant progeny had to be identified. Therefore, to maximise the efficiency of this process, a microbroth dilution method (rather than ETEST) was used to screen progeny according to the EUCAST guidelines (Rodriguez-Tudela, 2008), utilising a range of itraconazole concentrations (2, 4, 8, 16 and 32 mg L^{-1}) (Sigma-Aldrich, USA).

3.2.3 Mating-type (*MAT*) analysis

Mating-type analysis of *A. fumigatus* strains was performed using a multiplex PCR assay as described by Paoletti *et al.* (2005). The primers used for this PCR

are shown below (**Table 3.1**). The assay is predicted to produce a PCR product of 834 bp if an isolate has the *MAT1-1* genotype or 438 bp if an isolate has the *MAT1-2* genotype. The PCR products were resolved using a 1.5% TAE agarose gel and visualised by ethidium bromide staining (Sigma-Aldrich, USA) and a UV transilluminator (Bio-Rad, USA).

A total reaction volume of 25 μl was used per PCR containing: 1x Red Hot DNA polymerase reaction buffer with $MgCl_2$ to a final concentration of 1.8 mM; 200 μM of dNTPs (NEB, USA); 50 ng of primer AFM1; 50 ng of primer AFM2; 100 ng of primer AFM3; between 10-50 ng of DNA template; 1 U Red Hot DNA polymerase (Thermo Fisher, USA); and adjusted to 25 μl using sterile distilled water.

Table 3.1 - MAT *type primers used to identify mating type of isolates of* A. fumigatus

Primer	Sequence	Target Region
AFM1	CCTTGACGCGATGGGGTGG	*MAT1-1* idiomorph
AFM2	CGCTCCTCATCAGAACAACTCG	*MAT1-2* idiomorph
AFM3	CGGAAATCTGATGTCGCCACG	'Common' flanking region

The PCR cycle used for the *MAT* type analysis was as follows: Initial denaturation of 5 mins at 95 °C; 35 cycles of 30 secs at 95 °C, 30 secs at 60 °C, 1 min at 72 °C; and a final extension of 7 mins at 68 °C.

3.2.4 Oatmeal agar preparation

80g of Odlum's Irish pinhead oatmeal (Odlums, Ireland) were added to 2 L of tap water. The water was brought to the boil whilst continuously stirring and simmer for 45 mins. Once boiled sufficiently, filter oatmeal through two layers of cheesecloth. Using the filtered liquid, the volume was adjusted back to 2 L with tap water and mixed thoroughly before adding to 40 g agar (2% w/v).

3.2.5 Sexual crosses

Strains of *A. fumigatus* to be used in sexual crosses were grown for 7 days on ACM. From here, spore suspensions were made and adjusted to a final concentration of 5×10^5 conidia ml^{-1} in 0.05% Tween 80 (Sigma-Aldrich, USA). using a sterile 10 μl loop (Sigma-Aldrich, USA). Two aliquots of 1 μl from the *MAT1-1* isolate were inoculated onto Odlum's Irish oatmeal plates approximately 4 cm apart and perpendicular to aliquots of 1 μl of the *MAT1-2* isolate. The sexual crosses were then sealed with one layer of Parafilm (Bemis, USA) and incubated for three months inverted at 30 °C in darkness as described by O'Gorman *et al.* (2009).

3.2.6 Harvesting cleistothecia

After a three-month incubation period, each cross was examined for the production of cleistothecia. Typically, these would appear at the barrage zones between two complementary mating partners.

Sexual crosses were inspected using a dissection microscope within a laminar flow hood to determine the state of cleistothecia development. Where cleistothecia were found, they were isolated with a sterilised fine point needle. Sterile distilled water was then used to wash the cleistothecia of excess conidia, rolling the cleistothecia through the liquid. 100 μl 0.05% Tween 80 was then aliquoted into sterile 2 ml Eppendorf tubes (Sarstedt, Germany), and cleistothecia were then added to the 2 ml Eppendorf tube (one cleistothecia/Eppendorf tube) and crushed against the side. The volume of the Eppendorf tube was then adjusted to 2 ml, adding 1.9 ml 0.05% Tween 80. The ascospore suspension was vortexed (Vortex Genie 2, Scientific Industries) for 1 min to ensure all the ascospores have been released and asci have been disrupted. Using a heat block at 70 °C (Grant Instruments, UK), the ascospore suspension was heat treated for 1 hour to kill any remaining conidia. Post heat treatment, 100 μl aliquots of the ascospore suspension were spread plated onto 2% w/v malt extract agar (MEA) plates and incubated 36 h at 28 °C.

3.2.7 Ascospore isolation

Once the ascospore suspension has been incubated for 36 hours single germinating ascospores were identified through the use of a dissection microscope within a laminar flow hood. Upon identification, the germinating ascospores were isolated through the use of a sterilised fine point needle. Each isolated ascospore was individually centre inoculated onto a 25 ml 2% w/v MEA plate and incubated at 28 °C until fully grown.

3.2.8 RAPD-PCR

To ensure that recombination had occurred within the progeny of the sexual cross, Random Amplification of Polymorphic DNA (RAPD) PCR was performed, as described previously by Murtagh *et al.* (1999). The primers used to assess recombination in *A. fumigatus* are shown below (**Table 3.2**). The products of the RAPD-PCR were resolved on 1.5% TBE agarose gels and visualised by ethidium bromide staining (Sigma-Aldrich) and UV transilluminator (Bio-Rad, USA). Resultant bands were then graded on size and intensity and scored accordingly. The RAPD profile-banding pattern for each isolated progeny was compared to that of the crossing parents and genetic similarity was determined.

A total reaction volume of 25 μl was used per PCR containing: 1x DyNAzyme II DNA polymerase reaction buffer; 500 μM of each dNTP; 50 μmol of a single 10-base pair primer; between 0.1-1 ng of DNA template; 1 U DyNAzyme II DNA polymerase (Thermo Scientific, USA); and adjusted to 25 μl using sterile distilled water.

The PCR protocol used for RAPD analysis was as follows: Initial denaturation of 5 mins at 95 °C; 45 cycles of 30 secs at 93 °C at a ramp rate of 30 °C min^{-1}, 40 secs at 37 °C at a ramp rate of 30 °C min^{-1}, 1 min 20 secs at 72 °C at a ramp rate of 20 °C min^{-1}; followed by a final elongation stage for 5 mins at 72 °C (Murtagh *et al.*, 1999).

Table 3.2 *- RAPD primers used to produce a genetic profile of* A. fumigatus *strains*

Primer	Sequence
A-05	AGGGGTCTTG
J-01	CCCGGCATAA
W-10	TCGCATCCCT
W-19	CAAAGCGCTC
X-05	CCTTTCCCTC

3.2.9 Preparation for bulk segregant analysis

Progeny were selected for bulk segregant analysis from various different rounds of crossing and backcrossing (**Figure 3.3**). Initially, 120 progeny were isolated from the first F1 round of crossing, and from these a group of 40 sensitive and 40 resistant progeny were identified. Later, 150 progeny were isolated from the 3rd backcross (4th round of crossing total). DNA was extracted and resistance phenotype was determined using the ETEST method as described above. From here, DNA for each progeny pool was adjusted to a final concentration of 50 ng μl^{-1} using a p330 nanophotometer (Geneflow, UK) before being separated into one of two DNA pools: azole resistant, or sensitive. This was similar for the 6th backcross (7th round of crossing total). However, for the 2nd backcross, 200 total progeny were isolated and a group of 80 resistant and 80 sensitive progeny were identified for further sequencing.

For each DNA pool, 2 μl of appropriate DNA sample (resistant or sensitive) was added to create a final DNA pool size of 100 μl at a concentration of 50 ng μl^{-1}. In addition, DNA samples were prepared from *A. fumigatus* isolates 47-51 (sensitive parent) and 47-308 (resistant parent) to the same volume and DNA concentration as the pooled progeny DNA.

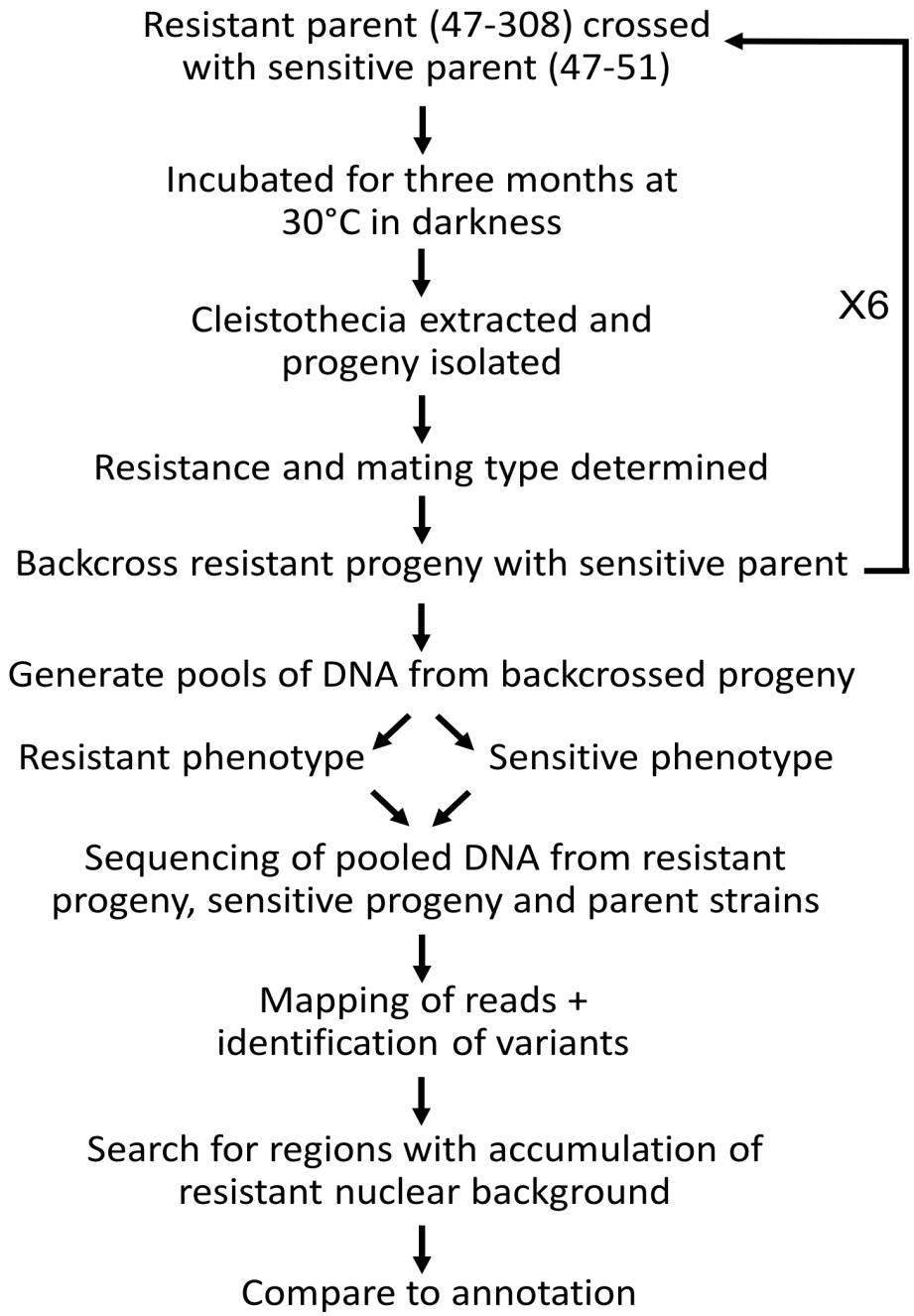


Figure 3.3 – *Methodology of BSA used in this study. An azole resistant isolate (47-308) carrying an unknown mutation is sexually crossed with a sensitive isolate (47-51). From here the progeny are collected and separated into two pools. One pool containing azole resistant progeny and the other contains sensitive progeny. These DNA pools and original parent isolates are subject to next generation sequencing in order to identify genomic regions within the resistant progeny containing the gene of interest. Secondarily, resistant progeny were backcrossed with the original sensitive parent in order to create progeny isogenic to the sensitive parent whilst maintaining the resistance gene. This backcrossing was performed 6 times.*

3.2.10 DEEP Seq

Whole genome sequencing was performed by DEEP Seq in the Queens Medical Centre (Nottingham, UK) using a MiSeq next generation sequencer. Martin Blythe and Daniel Zadik undertook Bioinformatic analysis from the DeepSeq unit, who are acknowledged for their input. An initial run of sequencing of the progeny for BSA was undertaken after one round of sexual crosses (i.e. F1 progeny from the sensitive x resistant cross) and then a second round of sequencing was undertaken from progeny of a 4th round of sexual backcrosses. A series of rules were applied to the arising sequence data following the principles of BSA i.e. that any marker linked to the phenotype of interest should only be present in the resistant pool and resistant parent, and be absent from the sensitive pool and the sensitive parent. Any marker should also be absent from the (sensitive) wild-type reference genomes used (at the NCBI and JGI). Clarification of these rules is illustrated in **Figure 3.4**. The pipeline for the sequencing analysis was as follows. This identified any SNPs evident between the pools, and then data was narrowed down to focus on SNPs present in coding regions that might impact in protein function.

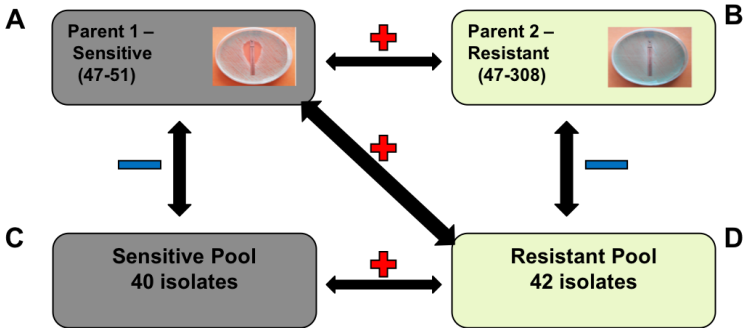


Figure 3.4 – *Representation of the rules used for BSA analysis. Here (+) indicates differences we want to highlight using genome sequencing techniques, whilst (-) indicates similarities to ignore within the analysis. Identifying variants between the resistant and sensitive pool; resistant pool and sensitive parent; and resistant parent and resistant isolates – whilst ignoring similarities between the pool and their appropriate parental phenotype. Adapted from Dyer (unpublished).*

1. **Raw Read Analysis.**

Reads were initially assessed using the FastQC read analysis tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The number and quality of the reads was assessed. FastQC reports were generated for each read file [so 2 files (Forward & Reverse) for each paired end read set].

2. **Read Trimming.**

Raw reads were then adaptor trimmed using Scythe (https://github.com/vsbuffalo/scythe) against the same adaptors used within the FastQC tool. After adaptor trimming the reads were quality trimmed using Sickle (https://github.com/najoshi/sickle). This generated 3 fastq files from each paired set of reads; 2 files representing successfully trimmed paired reads, the third represents orphan single reads where their paired partner has been removed due to extensive trimming.

3. **Read Mapping.**

Reads were aligned using BWA-MEM (http://bio-bwa.sourceforge.net/) version 0.7.4 using default alignment parameters. Paired reads were aligned independently of the single fragment reads (due to BWA requirements). The read alignments were recorded in BAM format. Both sets of alignments from paired and single fragment reads were merged into a single BAM file, sorted, and then read duplicates were identified and marked within the BAM file using Picard Tools (http://picard.sourceforge.net/).

4. **Variant Calling.**

SNPs and InDels (insertions & deletions) were called using the GATK (Genome Analysis Tool Kit version v2.4-9) UnifiedGenotyper program set for a ploidy of 1. These initial variant calls were then filtered using the GATK VariantFiltration program using the same parameters for both SNPs and InDels. Variant calls were recorded in VCF file format. Inspection of the header region of the resulting VCF files revealed the filtering parameters used and their corresponding classification labels seen throughout the VCF file, as

well as the standard explanations of the various other values found within the file. Briefly, variants were filtered according to read mapping quality (MAPQ), the quality scores of the bases detailing each variant, strand bias of the mapped reads and the depth of coverage at the variant call position. Variants that pass the filtering thresholds were labelled "PASS" and those that do not were labelled according to the reason that they did not pass the filter.

5. **Variant Effect Prediction.**

The SNP and InDel filtered variant calls generated by GATK, with a designation of "PASS", were then examined together for their predicted effect against the annotation for the genome using SnpEff (http://snpeff.sourceforge.net).

6. **Short variant distribution plots.**

The positions of the snps and other short variants produced by HaplotypeCaller were then plotted along the lengths of the chromosomes or contigs of the *A. fumigatus* reference genomes [Af293, accession no. NC_007194.1 and JGI database (A1163)]. Black vertical bars represent variant sites more than 10kb from all other sites. Sometimes >1 bars were so close together, that they appear as one bar. Thus, what appears to be a single site may be multiple sites. Therefore, variant sites less than 10kb from all other sites, have been coloured red i.e. what appears to be a single red bar can represent one or more sites.

3.2.11 Sequence data analysis

The arising sequencing data was compared to the reference genomes [NCBI database (Af293), accession no. NC_007194.1 and JGI database (A1163)] by personnel from Deep-Seq (see above). The 1st round of sequencing allowed the identification of gene accession numbers for each variant to be found. Using this and the appropriate database, potential genes were listed and

function identified – including hypothetical genes. Following the second round of sequencing data from the 4th round of sexual crosses, the position of variants and the contig were identified and further narrowed down. From here, using the appropriate database, a list of candidate genes was created – this included hypothetical genes.

3.2.12 Mathematical model for variant prediction

The technique used to predict the number of variants that might be identified during each round of sexual backcrossing using BSA was adapted from Pomraning *et al.* (2011). Here, the ability to identify a region containing the area of interest using BSA was modelled as:

$$S=[C/(R+1)]n^{-1}$$

Where: S is the size of the genomic region predicted to contain the mutation of interest; C is the size of the mutated chromosome in question; R is the number of recombination events during meiosis on the chromosome of interest; and n is the number of progeny sequenced during bulk segregant analysis. This model assumes that the locations for recombination events are random.

This model was applied to predict the size of the linked region of the *A. fumigatus* contig, which might be identified as containing the mutation of interest during the first sexual cross between the resistant and sensitive parents. During each subsequent backcross, a smaller region would be identified as the site of recombination. Additionally, predictions could be made based off the number of progeny used for each round of BSA.

3.2.13 Sequencing of *cyp51A* in other resistant strains

Mutation analyses of *cyp51A* from *A. fumigatus* strains 47-312 and 47-316 were performed using a PCR assay. The assay used two sets of primers with both designed to amplify the *cyp51A* exon region. Additionally, the promoter region was analysed by PCR amplification and sequencing of the amplicon in order to ensure there were no tandem repeat mutations. The primers used

for the amplification of the exon region and promoter region, along with the predicted sizes, are shown below (**Table 3.3**).

Amplification of the *cyp51A* gene and promoter region was performed through the use of Phusion® High Fidelity DNA Polymerase (NEB, USA) in a final reaction volume of 50 μl (34.1 μl sterile distilled water, 10 μl 5x buffer, 0.5 μl 10 mM dNTP mix, 2 μl 10 μM forward primer, 2 μl 10 μM reverse primer, 50 ng DNA template and 1 U (0.4 μl) Phusion polymerase).

Table 3.3 *– Primer pairs used for amplification of the cyp51A gene and promoter region*

Primer Pair	Sequence F	Sequence R	Size (bp)
Cyp1a	CCCCATTGCCGC ATAACAAG	CGAATTTCTCGC CGATGCAG	708
Cyp1b	GATGCTATGGCT TACGGCCT	CTCGCTTCTTGT TATGCGGC	689
Cyp2a	ATGGCTTACGGC CTACATGG	CGCCATACTGCC GGTTAAGA	265
Cyp2b	CATTCATTCCTGG GCGCATT	CGTTGAAAAGT CGCAGGTGG	1284
Cyp51 TR	ATGAGTGAATAA TCGCAGCACC	GTTAGGGTGTA TGGTATGCTGG	123

Once the products were sequenced, the most common *cyp51A* mutations causing azole resistance were checked for. These were: substitution mutation G54/W/R/E/V/A; substitution mutation M220/T/V/I/K/R/L; substitution mutation G448S; substitution mutation G138/C/S; tandem repeat mutation TR_{34} with substitution mutation L98H; tandem repeat mutation TR_{46} with substitution mutation Y121F/T289A; and tandem repeat mutation TR_{53}.

3.3 Results

3.3.1 Mating-type PCR

The mating-type diagnostic of Paoletti et al. (2005) was successfully used to determine the *MAT* genotype of the progeny from each *A. fumigatus* sexual cross, as shown below (**Figure 3.5**). The PCR produced multiple products in each isolate tested. However, a strong band was always observed where the predicted *MAT* products should be located. For example, when testing the original progeny from sexual cross '47-51 × 47-308' both *MAT1-1* and *MAT1-2* genotypes were observed among progeny '1, 2, 3 and 4' (**Figure 3.5**).

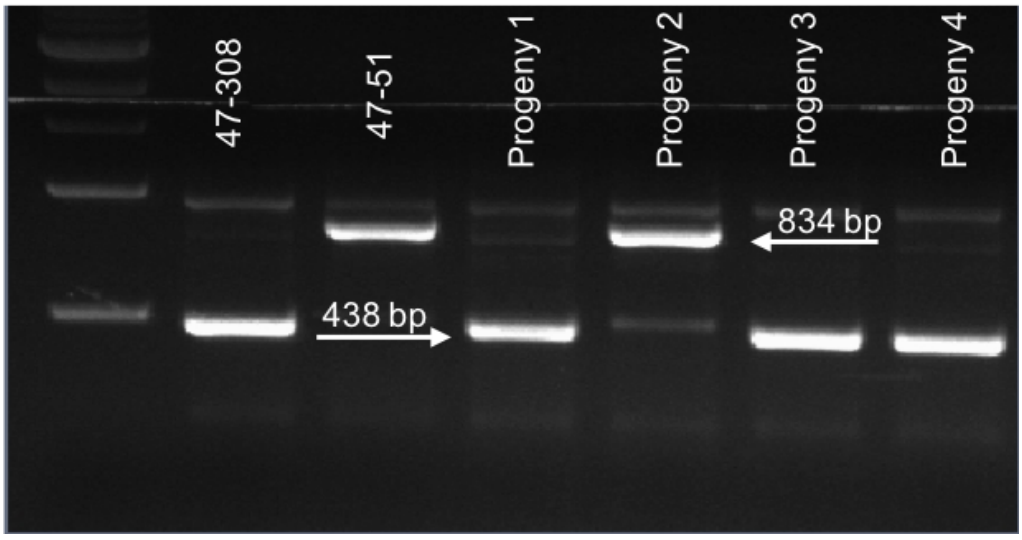


Figure 3.5 – Representative results of the mating- type multiplex PCR diagnostic for A. fumigatus. A product of 438 bp indicates a MAT1-2 isolate and a product of 834 bp indicates a MAT1-1 isolate. The lanes are as follows: 1) 1 kb DNA ladder; 2) strain 47-308; 3) strain 47-51; 4) progeny 1 '47-51 × 47-308'; 5) progeny 2 '47-51 × 47-308'; 6) progeny 3 '47-51 × 47-308'; and 7) progeny 4 '47-51 × 47-308'.

3.3.2 RAPD analysis

RAPD-PCR (using primers A-05, J-01, W-10, W-19 and X-05) was used to successfully produce genetic profiles of the progeny isolated from each *A. fumigatus* sexual cross. Recombination of markers was evident in the offspring (**Figure 3.6**)

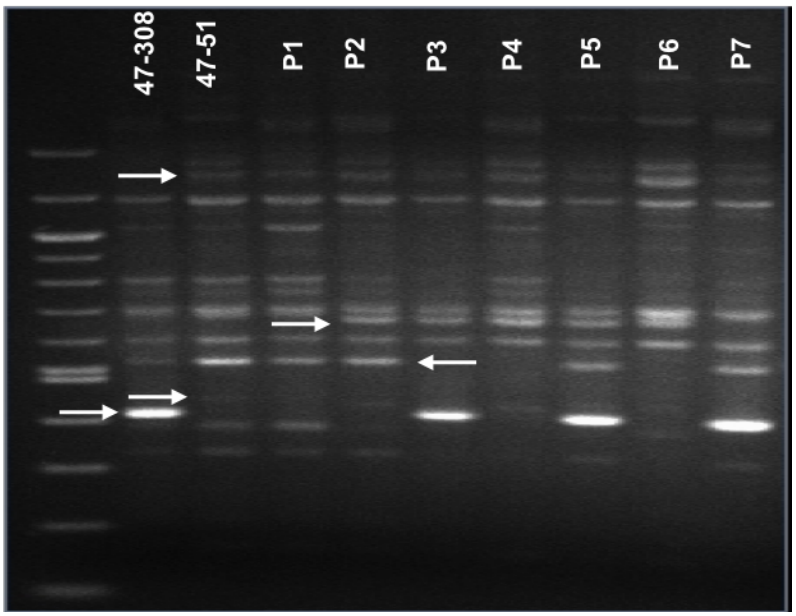


Figure 3.6 *– Representative RAPD-PCR of putative F1 offspring of a sexual cross between* A. fumigatus *47-51 × 47-308 as determined using RAPD primer A-05. The lanes are as follows: 1) 1 kb DNA ladder; 2) strain 47-308; 3) strain 47-51; 4) progeny 1 '47-51 × 47-308'; 5) progeny 2 '47-51 × 47-308'; 6) progeny 3 '47-51 × 47-308'; and 7) progeny 4 '47-51 × 47-308'; 8) progeny 5 '47-51 × 47-308'; 9) progeny 6 '47-51 × 47-308'; and 10) progeny 7 '47-51 × 47-308'. Sexual recombination can be seen due to the sharing of DNA bands between the parent isolates in the progeny and from the absence of bands present in the parents, but not shown in the progeny.*

However, during each subsequent backcross, the RAPD profile of each progeny inevitably shared more traits with the original sensitive parent strain (47-51). This reduced the efficiency of using RAPDs to verify sexual recombination in successfully isolated progeny (data not shown).

3.3.3 ETEST - Itraconazole resistance

The resistant *A. fumigatus* parent strain (47-308) was determined to have a resistance to itraconazole of >32 mg L^{-1} according to the ETEST results, whereas the sensitive parent strain (47-51) had a resistance of 0.75 mg L^{-1} as shown below (**Figure 3.7**).

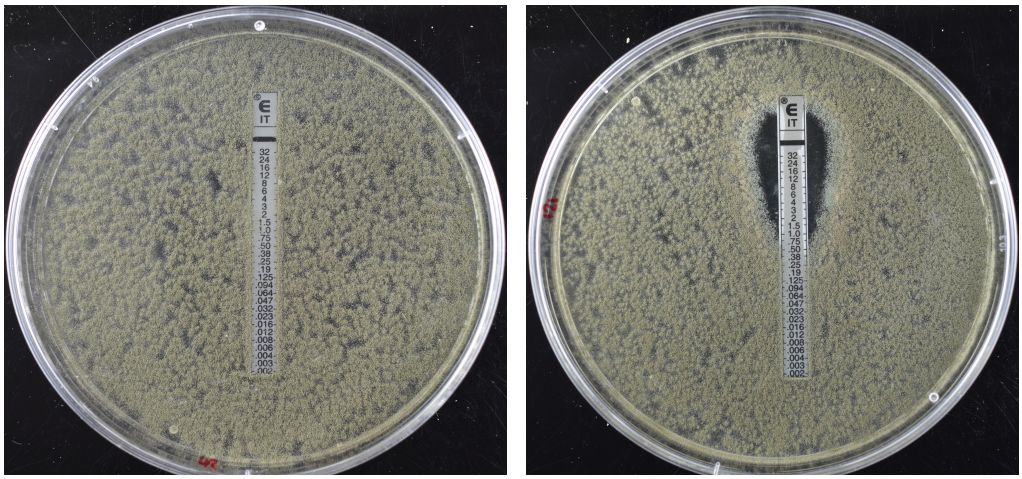


Figure 3.7 *– Itraconazole ETEST of* A. fumigatus *strains 47-308 (left) and 47-51 (right). The ETEST has a concentration of 32 mg* L^{-1} *itraconazole at the top of the strip. This concentration decreases down the strip towards the bottom. 47-308 is showing full growth indicating a resistance of >32 mg* L^{-1} *indicating a resistant phenotype. 47-51 growth is inhibited at 0.75 mg* L^{-1} *indicating a sensitive phenotype.*

The segregation pattern of resistance at each subsequent round of crossing was nearly an exact 1:1 ratio of sensitive and resistant progeny (**Figure 3.8**). For example, 39/80 progeny isolated during the 4th round of sequencing had a resistant background, further confirming the 1:1 ratio. This confirmed that a single gene determines the resistant phenotype. A 1:1 ratio of resistance:sensitivity was also observed in the itraconazole microbroth dilution assay.

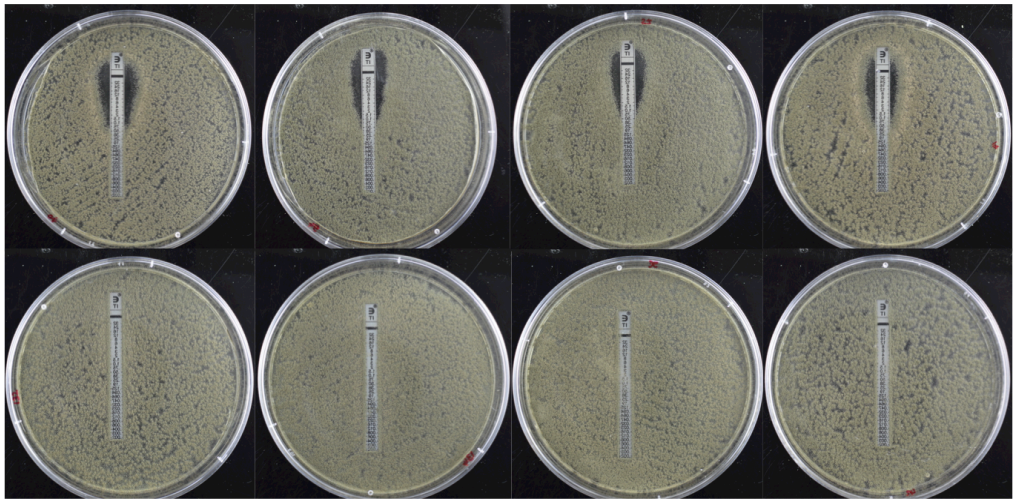


Figure 3.8 *– Representative ETEST of progeny isolated from the* 3^{rd} *backcross between resistant F3 progeny and an itraconazole sensitive isolate 47-51 demonstrating the 1:1 segregation of the resistance phenotype.*

3.3.4 Mathematical model for variant prediction

Using the model described above (**3.2.12**), a prediction was made to determine the relation between the number of progeny sequenced in the DNA pool and the region of the genome that would be identified containing the mutation of interest. In order to accomplish this, genome data (no. of chromosomes and genome size) from *A. fumigatus* reference strain Af293 on the NCBI database was used. The results of this prediction are shown below (**Figure 3.9**).

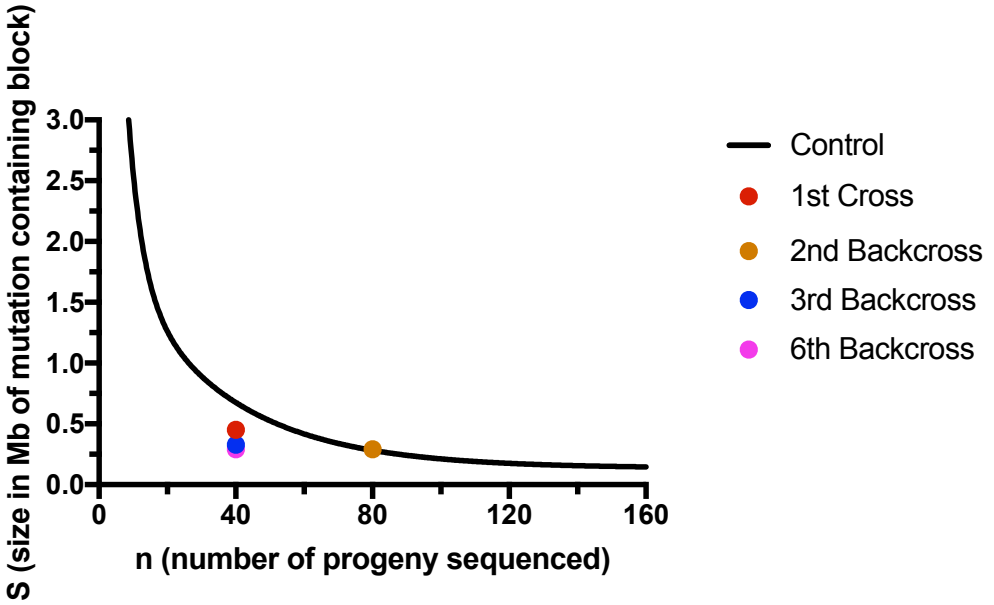


Figure 3.9 *– Mathematical model for variant prediction, showing the exponential decrease of genome size containing the region of interest (S) as the number of progeny in each DNA pool increases (n) using BSA. The actual data points from the initial sexual cross between resistant isolate 47-308 and sensitive isolate 47-51 is shown in red. The data point for the region identified at the 2nd, 3rd and 6th backcross are shown in orange, blue and pink, respectively.*

Once the prediction had been made, data was added to the graph to represent the size of the genome identified from the initial sexual cross between parents 47-51 (sensitive) and 47-308 (resistant), seen in red (**Figure 3.9**). This was done again using the results of the 3rd backcross, seen in blue (**Figure 3.9**). The prediction for 40 progeny from the model showed a region of 0.64 Mb being identified. This was reasonably close to the actual value of

0.452 Mb from the initial cross, with further improvements as the number of backcrosses between resistant progeny and the original sensitive parent increased to three (0.363 Mb) and six (0.292 Mb). Increasing the number of progeny sequenced in each pool to 80, whilst using a reduced number of backcrosses also yielded a region of 0.292 Mb. These results are discussed below in **3.3.5**-**3.3.8**.

3.3.5 1st round sequencing and analysis

From the first round of sequencing using the F1 progeny from the parental sexual cross (47-308 × 47-51), 96.5% of reads were mapped against the NCBI reference genome and 98.6% of reads were mapped to the JGI reference genome used. From this, sample-filtering rules from the DEEP Seq pipeline (see methods section **3.2.10** above) found 1,265 SNPs and 247 indels from the NCBI data set. Of these, 123 genes were affected by these variants. This included 94 non-synonymous, 12 deletions, 2 stop gain/loss and 1 start loss mutation. From the JGI data set, sample-filtering rules found 582 SNPs and 70 indels. Within this, 103 genes were affected by these variants. This included 90 non-synonymous, 8 deletions and 1 stop gain mutation. These genes were spread over 8 chromosomes / supercontigs, with some genes being present as single independent markers, but others present in linked regions – the latter as would be expected from the BSA approach (**Figure 3.10** in **3.3.9**)

The full list of affected genes can be found below from the NCBI and JGI database (**Table 3.4** and **Table 3.5**, respectively). From here the function of each gene was determined using the appropriate database. Any candidate genes that might impact on in azole resistance were then chosen, based on previous knowledge of the genetics of antifungal resistance. This data analysis revealed, surprisingly, that an M220K mutation in *cyp51A* was present.

Table 3.4 *– 150 affected genes from variants identified in the first round of crossing compared to the NCBI* A. fumigatus *reference genome Af293. The chromosome, position, gene ID, gene name, reference and alteration has been listed. Where gene name was not available, function has been listed.*

Chr. No.	Position	Gene ID	Gene/ Function	Reference /Change
6	118389	AFUA_6G00460	Hypothetical Protein	C/T
6	118526	AFUA_6G00460	Hypothetical Protein	G/A
6	693976	AFUA_6G03260	*ap1*	C/T
6	1716890	AFUA_6G07550	Hypothetical Protein	C/T
6	2103554	AFUA_6G08860	*kpsF/gutQ*	T/C
6	2912193	AFUA_6G11700	Hypothetical Protein	C/G
6	3666131	AFUA_6G14420	ADM-A	A/T
1	459218	AFUA_1G01410	Hypothetical Protein	AAATAT deletion
1	2655010	AFUA_1G10300	Hypothetical Protein	G/A
1	2659256	AFUA_1G10320	*fnb3*	C/T
3	122669	AFUA_3G00570	Hypothetical Protein	ATTTCCAA TAC deletion
3	186711	AFUA_3G00830	Pfs domain protein	C/T
3	189680	AFUA_3G00830	Pfs domain protein	T/G
3	189742	AFUA_3G00830	Pfs domain protein	C/G
3	189985	AFUA_3G00830	Pfs domain protein	G/T
3	779587	AFUA_3G02910	Dihydroxy-acetone synthase	G/T
3	855453	AFUA_3G03180	NAD binding Rossmann fold oxidoreductase	A/T
3	865367	AFUA_3G03230	bZIP transcription factor	G/A
3	886841	AFUA_3G03315	C6 transcription factor	C/A
3	886856	AFUA_3G03315	C6 transcription factor	G/A
3	1004504	AFUA_3G03700	MFS sugar transporter	TTC deletion
3	1114931	AFUA_3G03900	C6 transcription factor	A/G
3	1350042	AFUA_3G05570	*scp3*	CAG deletion
3	1538118	AFUA_3G06230	Hypothetical Protein	TCC deletion
3	1573798	AFUA_3G06380	Exosome-associated family protein	T/A
3	1588121	AFUA_3G06430	*sec2p*	GAT deletion
3	1839103	AFUA_3G07330	*yae1*	A/G
3	1991290	AFUA_3G07870	Extracellular serine-rich protein	Deletion
8	5081	AFUA_8G00110	2OG-Fe(II) oxygenase family	G/A
8	16162	AFUA_8G00150	Hypothetical Protein	T/A
8	261170	AFUA_8G01110	Integral membrane protein	T/C
8	408385	AFUA_8G01590	Hypothetical Protein	G/A

8	460449	AFUA_8G01760	Hypothetical Protein	TGC deletion
8	1679577	AFUA_8G06860	Hypothetical Protein	CGC deletion
8	1712974	AFUA_8G06990	Ankyrin repeat protein	G/T
8	1738950	AFUA_8G07090	Extracellular proline-serine rich protein	G/A
8	1745800	AFUA_8G07120	*neg1*	A/C
8	1749139	AFUA_8G07140	Aconitase family protein	G/A
8	1777359	AFUA_8G07235	Integral membrane protein	C/T
8	1801453	AFUA_8G07340	HET domain protein	G/A
8	1807347	AFUA_8G07360	C6 transcription factor	C/A
7	264750	AFUA_7G00970	GPI anchored serine-threonine rich protein	TCCACT deletion
7	1875965	AFUA_7G08310	Hypothetical Protein	A/C
7	1875979	AFUA_7G08310	Hypothetical Protein	C/T
7	1876007	AFUA_7G08310	Hypothetical Protein	A/G
5	51087	AFUA_5G00230	Hypothetical Protein	A/G
5	63988	AFUA_5G00260	Ferric-chelate reductase	A/C
5	214288	AFUA_5G00770	Integral membrane protein	TTT deletion
5	299509	AFUA_5G01190	Hypothetical Protein	C/T
5	861663	AFUA_5G03170	Hypothetical Protein	GGCCTTTC ACGT/GGT
5	942297	AFUA_5G03485	Hypothetical Protein	AAC deletion
5	1942072	AFUA_5G07770	Pentatricopeptide repeat protein	T/C
5	2063669	AFUA_5G08150	ABC bile acid transporter	G/T
5	2356770	AFUA_5G09190	ABC bile acid transporter	A/C
5	2358075	AFUA_5G09190	ABC bile acid transporter	C/T
5	2428768	AFUA_5G09430	RNA directed RNA polymerase	A/G
5	2620351	AFUA_5G10200	Hypothetical Protein	T/C
5	2643923	AFUA_5G10320	*gliH*	T/C
5	2644905	AFUA_5G10340	MFS transporter	T/C
5	2753161	AFUA_5G10770	*pat1*	G/A
5	2772129	AFUA_5G10840	*urb1*	A/G
5	2853907	AFUA_5G11170	*snf2h*	C/G
5	2896439	AFUA_5G11310	MHYT domain signalling protein	C/G
5	2896622	AFUA_5G11310	MHYT domain signalling protein	C/T
5	2956277	AFUA_5G11520	*nrc-2*	C/G
5	3102820	AFUA_5G11985	Eukaryotic translation initiation factor eIF1a-like protein	C/G
5	3184013	AFUA_5G12260	*tigA*	G/C
5	3213331	AFUA_5G12380	Hypothetical Protein	A/G

5	3236652	AFUA_5G12500	Hypothetical Protein	A/G
5	3264752	AFUA_5G12600	*rad5*	G/A
5	3295179	AFUA_5G12680	*cuID*	G/A
5	3782230	AFUA_5G14582	Pfs, NACHT and Ankyrin domain protein	GTT deletion
5	3810826	AFUA_5G14740	*fleA*	GAT deletion
4	382471	AFUA_4G01470	C6 finger domain protein	GCGCAGG CGCAG deletion
4	967296	AFUA_4G03400	Hypothetical Protein	CCT/CCTT
4	974045	AFUA_4G03435	Formate/nitrite transporter family protein	G/A
4	982419	AFUA_4G03460	HLN DNA binding domain protein	G/A
4	992341	AFUA_4G03500	GPI anchored protein	GAC deletion
4	1005592	AFUA_4G03580	Phosphoglycerate mutase family protein	A/T
4	1098996	AFUA_4G03920	MFS drug transporter	C/A
4	1488550	AFUA_4G05840	*nup133*	GAG deletion
4	1617885	AFUA_4G06300	Hypothetical Protein	A/G
4	1626965	AFUA_4G06350	*gar2*	C/G
4	1680374	AFUA_4G06550	*znf1*	C/T
4	1735056	AFUA_4G06730	Hypothetical Protein	C/T
4	1781093	AFUA_4G06890	*cyp51A*	T/A
4	1843907	AFUA_4G07100	Nuclear envelope protein	C/T
4	1853393	AFUA_4G07140	*iki3*	A/G
4	1860388	AFUA_4G07170	*cut8*	G/T
4	1876666	AFUA_4G07240	Pentatricopeptide repeat protein	G/A
4	1878196	AFUA_4G07250	*rsm25*	G/A
4	1879821	AFUA_4G07260	*sog2*	G/T
4	2165630	AFUA_4G08380	Hypothetical Protein	C/T
4	2180123	AFUA_4G08450	Hypothetical Protein	CCCTGGAT CGAAAATC TT deletion
4	2335289	AFUA_4G09010	Prp39	G/A
4	2356790	AFUA_4G09070	ATP binding L-PSP endoribonuclease family protein	GAA deletion
4	2415548	AFUA_4G09240	Hypothetical Protein	A/G
4	2428718	AFUA_4G09270	Ankyrin repeat protein	A/T
4	2429622	AFUA_4G09270	Ankyrin repeat protein	T/A
4	2429707	AFUA_4G09270	Ankyrin repeat protein	A/T
4	2429754	AFUA_4G09270	Ankyrin repeat protein	C/A
4	2430249	AFUA_4G09270	Ankyrin repeat protein	C/T
4	2430282	AFUA_4G09270	Ankyrin repeat protein	G/T
4	2430353	AFUA_4G09270	Ankyrin repeat protein	G/T
4	2430354	AFUA_4G09270	Ankyrin repeat protein	G/T
4	2430361	AFUA_4G09270	Ankyrin repeat protein	T/A

4	2430420	AFUA_4G09270	Ankyrin repeat protein	C/T
4	2430582	AFUA_4G09270	Ankyrin repeat protein	A/G
4	2453271	AFUA_4G09380	Hypothetical Protein	C/T
4	2453589	AFUA_4G09380	Hypothetical Protein	CGCGCCGT AGACGAC ACCCCC deletion
4	2453608	AFUA_4G09380	Hypothetical Protein	G/T
4	2453616	AFUA_4G09380	Hypothetical Protein	AAC deletion
4	2469045	AFUA_4G09460	NACHT domain protein	T/G
4	2498707	AFUA_4G09590	Hypothetical Protein	TAA deletion
4	2498707	AFUA_4G09590	Hypothetical Protein	TAA deletion
4	2609648	AFUA_4G10090	GABA permease	G/A
4	2612301	AFUA_4G10100	FAD binding domain protein	T/C
4	2612775	AFUA_4G10100	FAD binding domain protein	A/G
4	2619219	AFUA_4G10110	Homeobox transcription factor	G/A
4	2801058	AFUA_4G10770	*ppoA*	G/A
4	2869389	AFUA_4G11012	*sap185*	A/G
4	3009150	AFUA_4G11477	Sucrase/ferredoxin-like family protein	CAGCAGC AC deletion
4	3268545	AFUA_4G12450	Conserved lysine-rich protein	AAGAAGG AGGAGAC CGAGGCA deletion
4	3649065	AFUA_4G14000	Tripeptidyl peptidase A	G/C
2	107485	AFUA_2G00490	Glycosyl hydrolase, family 31	GGG/CGG G
2	146059	AFUA_2G00660	*tcsB/sln1*	CCC deletion
2	203122	AFUA_2G00900	PTH11-like integral membrain protein	A/G
2	203126	AFUA_2G00900	PTH11-like integral membrane protein	CTCCACAG TCGCAATG GCTCTTCC TACCGCAT CTTCGCCT CCTCTGGT TTC/CTC
2	204957	AFUA_2G00910	Pfs, NB-ARC and TPR domain protein	A/T
2	214455	AFUA_2G00940	Hypothetical Protein	TAT/TATAT
2	2404776	AFUA_2G09400	Cyclohexanone monooxygenase	A/C
2	2406623	AFUA_2G09410	Alpha-N-acetylglucosaminidase	C/A
2	2409160	AFUA_2G09420	F-box domain protein	T/G
2	2409441	AFUA_2G09420	F-box domain protein	G/T
2	2611342	AFUA_2G10205	Hypothetical Protein	C/T

2	2846055	AFUA_2G11110	*smc2*	G/T
2	2881834	AFUA_2G11210	PHD finger and SET domain protein	A/G
2	2902016	AFUA_2G11270	*ags2*	C/T
2	3010302	AFUA_2G11730	Protein kinase domain-containing protein	ACA deletion
2	3295367	AFUA_2G12850	*gel3*	T/C
2	3345348	AFUA_2G13030	*podG*	G/C
2	3406631	AFUA_2G13250	TRPB	A/G
2	3468440	AFUA_2G13430	Chitin synthase	G/A
2	4368278	AFUA_2G16462	*tfc3*	G/A
2	4370511	AFUA_2G16462	*tfc4*	T/C
2	4408462	AFUA_2G16510	Hypothetical Protein	G/T
2	4414542	AFUA_2G16520	*pld*	A/C
2	4468245	AFUA_2G16740	Hypothetical Protein	G/T
2	4487885	AFUA_2G16810	PHD finger domain protein	G/T
2	4640612	AFUA_2G17360	*mct*	C/T

Table 3.5 *– 123 affected genes from variants identified in the first round of crossing compared to the JGI* A. fumigatus *reference genome A1163. The position, gene ID, gene name, reference and alteration has been listed. Where gene name was not available, function has been listed.*

Contig	**Position**	**Gene ID**	**Gene**	**Reference /Change**
DS_499604	468669	AFUB_102090	Hypothetical protein	G/T
DS_499603	270496	AFUB_099100	MFS drug transporter	C/A
DS_499603	363908	AFUB_099480	Hypothetical protein	A/T
DS_499603	387081	AFUB_099590	HLH DNA binding protein	G/A
DS_499603	395821	AFUB_099620	Hypothetical protein	G/A
DS_499603	444075	AFUB_099820	Hypothetical protein	G/A
DS_499602	609279	AFUB_095040	Hypothetical protein	C/T
DS_499602	1220507	AFUB_097060	Hypothetical protein	G/A
DS_499602	1220644	AFUB_097060	Hypothetical protein	C/T
DS_499601	266350	AFUB_087560	GPI anchored serine-threonine rich protein	TCCACT deletion
DS_499600	212851	AFUB_080380	C6 transcription factor	C/A
DS_499600	218745	AFUB_080400	HET domain protein	G/A
DS_499600	271083	AFUB_080660	Aconitase family	G/A

			protein	
DS_499600	274422	AFUB_080680	neg1	A/C
DS_499600	281269	AFUB_080710	Extracellular proline-serine rich protein	G/A
DS_499600	309249	AFUB_070940	Ankyrin repeat protein	G/T
DS_499600	322867	AFUB_080850	Hypothetical protein	G/A
DS_499600	322945	AFUB_080850	Hypothetical protein	T/G
DS_499600	1546823	AFUB_085020	Hypothetical protein	G/A
DS_499600	1693295	AFUB_085490	Integral membrane protein	T/C
DS_499600	1916581	AFUB_086380	Hypothetical protein	T/A
DS_499599	374298	AFUB_073510	Hypothetical protein	C/T
DS_499599	762987	AFUB_074820	kpsF/gutQ	T/C
DS_499599	1584191	AFUB_077690	Hypothetical protein	C/G
DS_499599	2146426	AFUB_079710	Polyketide synthase	G/A
DS_499599	2184914	AFUB_079870	Hypothetical protein	C/T
DS_499598	39212	AFUB_062920	nup133	GAG deletion
DS_499598	168480	AFUB_063420	Hypothetical protein	G/A
DS_499598	177560	AFUB_063470	gar2	C/G
DS_499598	230981	AFUB_063630	znf1	C/T
DS_499598	285670	AFUB_063800	Hypothetical protein	C/T
DS_499598	331794	AFUB_063960	cyp51A	T/A
DS_499598	359699	AFUB_064070	Hypothetical protein	A/T
DS_499598	394630	AFUB_064190	Hypothetical protein	C/T
DS_499598	404117	AFUB_064230	iki3	A/G
DS_499598	411115	AFUB_064260	sts1	G/T
DS_499598	427401	AFUB_064330	Hypothetical protein	G/A
DS_499598	428931	AFUB_064340	37S ribosomal protein S25, mitochondrial	G/A
DS_499598	430556	AFUB_064350	sog2	G/T
DS_499598	718751	AFUB_065490	Hypothetical protein	C/T
DS_499598	890317	AFUB_066110	prp39	G/A
DS_499598	970429	AFUB_066360	Hypothetical protein	A/G
DS_499598	983586	AFUB_066400	Hypothetical protein	A/T
DS_499598	984490	AFUB_066400	Hypothetical protein	T/G

DS_499598	984575	AFUB_066400	Hypothetical protein	A/T
DS_499598	984622	AFUB_066400	Hypothetical protein	C/A
DS_499598	985117	AFUB_066400	Hypothetical protein	C/T
DS_499598	985150	AFUB_066400	Hypothetical protein	G/T
DS_499598	985221	AFUB_066400	Hypothetical protein	G/T
DS_499598	985222	AFUB_066400	Hypothetical protein	G/T
DS_499598	985229	AFUB_066400	Hypothetical protein	T/A
DS_499598	985288	AFUB_066400	Hypothetical protein	C/T
DS_499598	985450	AFUB_066400	Hypothetical protein	A/G
DS_499598	1008162	AFUB_066500	Hypothetical protein	C/T
DS_499598	1028330	AFUB_066580	Hypothetical protein	T/G
DS_499598	1364769	AFUB_067850	ppoA	G/A
DS_499598	1433077	AFUB_068070	sap185	A/G
DS_499598	2215594	AFUB_070910	Tripeptidyl peptidase A	G/C
DS_499597	140529	AFUB_049240	Integral membrane protein	TTT deletion
DS_499597	225872	AFUB_049660	Hypothetical protein	C/T
DS_499597	793784	AFUB_051680	Hypothetical protein	GGCCTTTC ACGT/GGT
DS_499597	891232	AFUB_052070	Plasma membrane H(+) ATPase	CAAGGT deletion
DS_499597	1841798	AFUB_055300	Pentatricopeptide repeat protein	T/C
DS_499597	1963397	AFUB_055680	ABC bile acid transporter	G/T
DS_499597	2255674	AFUB_056730	ABC bile acid transporter	A/C
DS_499597	2256979	AFUB_056730	ABC bile acid transporter	C/T
DS_499597	2327709	AFUB_056970	RNA directed RNA polymerase	A/G
DS_499597	2517346	AFUB_057800	Hypothetical protein	T/C
DS_499597	2540870	AFUB_057900	gliH	T/C
DS_499597	2541852	AFUB_057910	MFS transporter	T/C
DS_499597	2651997	AFUB_058370	pat1	G/A
DS_499597	2670965	AFUB_058440	urb1	A/G
DS_499597	2757420	AFUB_058740	snf2h	C/G
DS_499597	2799958	AFUB_058880	MHYT domain signalling protein	C/G
DS_499597	2800141	AFUB_058880	MHYT domain signalling protein	C/T

DS_499597	2859798	AFUB_059090	nrc-2	C/G
DS_499597	3006468	AFUB_059560	Eukaryotic initiation factor 1A	C/G
DS_499597	3087676	AFUB_059890	tigA	G/C
DS_499597	3116987	AFUB_060050	Hypothetical protein	A/G
DS_499597	3140307	AFUB_060170	Hypothetical protein	A/G
DS_499597	3168413	AFUB_060260	rad5	G/A
DS_499597	3198842	AFUB_060340	culD	G/A
DS_499597	3691718	AFUB_062260	Pfs, NACHT and Ankyrin domain protein	GTT deletion
DS_499597	3720317	AFUB_062410	fleA	GAT deletion
DS_499596	2210074	AFUB_041750	yae1	G/A
DS_499596	2459060	AFUB_042620	sec2p	GAT deletion
DS_499596	2473394	AFUB_042680	Hypothetical protein	T/A
DS_499596	2610281	AFUB_043150	osh3	ACG deletion
DS_499596	3226332	AFUB_044930	C6 transcription factor	G/A
DS_499596	3226347	AFUB_044930	C6 transcription factor	C/A
DS_499596	3247826	AFUB_045020	bZIP transcription factor	G/A
DS_499596	3257739	AFUB_045070	NAD-binding Rossmann fold oxidoreductase	A/T
DS_499596	3331957	AFUB_045340	Dihydroxy-acetone synthase	G/T
DS_499596	3674310	AFUB_046620	Isoflavone reductase family protein	AAG deletion
DS_499596	3927107	AFUB_047570	Pfs domain protein	G/T
DS_499596	3927351	AFUB_047570	Pfs domain protein	C/G
DS_499596	3927413	AFUB_047570	Pfs domain protein	T/G
DS_499596	3930389	AFUB_047570	Pfs domain protein	C/T
DS_499595	195609	AFUB_017980	PTH11-like integral membrane protein	A/G
DS_499595	195613	AFUB_017980	PTH11-like integral membrane protein	CTCCACAG TCGCAATG GCTCTTCC TACCGCAT CTTCGCCT CCTCTGGT TTC/CTC
DS_499595	198033	AFUB_017990	Pfs, NB-ARC and TPR domain protein	C/T
DS_499595	2387533	AFUB_025260	ChnB	A/C
DS_499595	2389380	AFUB_025270	Alpha-N-acetylglucosaminidase	C/A

DS_499595	2391917	AFUB_025280	F-box domain protein	T/G
DS_499595	2392198	AFUB_025280	F-box domain protein	G/T
DS_499595	2586350	AFUB_026010	Hypothetical protein	C/T
DS_499595	2822835	AFUB_026870	smc2	G/T
DS_499595	2858620	AFUB_026970	PHD finger and SET domain protein	A/G
DS_499595	2878797	AFUB_027030	Alpha-1,3-glucan synthase (ags2)	C/T
DS_499595	2989065	AFUB_027480	Protein kinase domain-containing protein	ACA deletion
DS_499595	3274124	AFUB_028470	gel3	T/C
DS_499595	3381560	AFUB_028880	TRPB	A/G
DS_499595	3441564	AFUB_029070	Chitin synthase	G/A
DS_499595	4342625	AFUB_032140	tfc3	G/A
DS_499595	4344858	AFUB_032140	tfc3	T/C
DS_499595	4357233	AFUB_032190	Hypothetical protein	G/T
DS_499595	4363309	AFUB_032200	PLD	A/C
DS_499595	4417005	AFUB_032410	ATPase synthesis protein 25, mitochondrial	G/T
DS_499595	4436649	AFUB_032480	PHD finger domain protein	G/T
DS_499595	4597806	AFUB_033030	mct	C/T
DS_499594	102517	AFUB_000340	ADM-A	A/T
DS_499594	2728031	AFUB_009710	Hypothetical protein	G/A
DS_499594	2732277	AFUB_009730	fnb3	C/T

3.3.6 2nd round sequencing and Analysis

A further round of genome sequencing was performed using pooled DNA from itraconazole sensitive and resistant progeny from the 3rd backcross between a resistant F3 isolate and sensitive parent strain (47-51). Bioinformatic analysis was then performed to try and identify any SNP or region linked to the resistance phenotype. This identified one clear region of linked genes, as would be expected from the BSA approach, together with some remaining single independent genes throughout the genome (**Figure 3.11** in **3.3.10**)

From the NCBI dataset, 151 variants were found. These variants affected 60 genes. The list of genes affected is found below (**Table 3.6**). From the JGI database, 141 variants were found. Of these variants, 46 genes were

affected. The list of genes affected is also found below (**Table 3.7**). The function of each gene identified from the variants was then determined by interrogating the appropriate database. Any candidate gene(s) that might result in azole resistance were then identified based on previous knowledge of the genetics of antifungal resistance. This data analysis again revealed that an M220K mutation in *cyp51A* was present as the only clear putative change correlated with resistance, although other possible target were present e.g. genes that had a function as MFS drug transporters.

Table 3.6 *– 60 affected genes from variants identified from the second round of bulk segregant analysis, after backcrossing, compared to the NCBI* A. fumigatus *reference genome Af293. The chromosome, position, gene ID, gene name, reference and alteration has been listed. Where gene name was not available, function has been listed.*

Chr. No.	Position	Gene ID	Gene/Function	Reference/ Change
1	453317	AFUA_1G01380	*hoc1*	T/C
3	1094588	AFUA_3G03790	Hypothetical	C/G
3	1309931	AFUA_3G05440	*rad18*	G/T
3	1535605	AFUA_3G06220	*cia30*	G/A
3	3917759	AFUA_3G14760	*fum15*	A/G
4	859349	AFUA_4G03070	DEAD/DEAH box helicase	A/G
4	874519	AFUA_4G03120	Mitochondrial cytochrome b2	A/G
4	875546	AFUA_4G03120	Mitochondrial cytochrome b2	C/A
4	880166	AFUA_4G03140	*sky1*	C/T
4	928229	AFUA_4G03280	Dehydrogenase family protein	C/T
4	938098	AFUA_4G03310	C6 finger domain protein	C/T
4	1061795	AFUA_4G03790	Serine peptidase	T/A
4	1083746	AFUA_4G03860	*EifCc*	T/C
4	1087696	AFUA_4G03880	60s ribosomal protein	G/A
4	1098996	AFUA_4G03920	MFS drug transporter	T/G
4	1124663	AFUA_4G04000	LysM domain protein	G/C
4	1162028	AFUA_4G04130	*afg1*	C/T
4	1555193	AFUA_4G06080	Myo-inositol transporter	T/C
4	1602307	AFUA_4G06230	*apg2*	C/G
4	1612663	AFUA_4G06280	Hypothetical	T/C
4	1617885	AFUA_4G06300	Hypothetical	T/C
4	1625690	AFUA_4G06350	*gar2*	T/C
4	1626965	AFUA_4G06351	*gar2*	C/G
4	1629133	AFUA_4G06370	Hypothetical	C/T

4	1651306	AFUA_4G06480	dDENN domain protein	G/C
4	1660009	AFUA_4G06490	*mlh3*	C/G
4	1664791	AFUA_4G06510	Hypothetical	C/T
4	1664938	AFUA_4G06510	Hypothetical	A/G
4	1664940	AFUA_4G06510	Hypothetical	C/T
4	1679468	AFUA_4G06550	*znf1*	A/G
4	1679469	AFUA_4G06550	*znf1*	G/T
4	1680374	AFUA_4G06550	*znf1*	A/G
4	1709170	AFUA_4G06620	Glutamate/ leucine/ phenylalanine/valine dehydrogenase	A/G
4	1716519	AFUA_4G06640	Acid sphingomyelinase	G/A
4	1721151	AFUA_4G06650	Hypothetical	T/C
4	1731270	AFUA_4G06700	GPI anchored cell wall protein	A/G
4	1735056	AFUA_4G06730	Hypothetical	A/G
4	1765753	AFUA_4G06850	Coenzyme A synthetase	T/C
4	1766192	AFUA_4G06850	Coenzyme A synthetase	T/C
4	1781093	AFUA_4G06890	*cyp51A*	T/A
4	1787082	AFUA_4G06910	Outer mitochondrial membrane porin	A/C
4	1798015	AFUA_4G06960	UBX domain protein	A/T
4	1806558	AFUA_4G06990	Amino acid transporter	C/A
4	1814684	AFUA_4G07020	Mitochondrial cytochrome b2-like	A/T
4	1827647	AFUA_4G07060	Acyl-CoA thioester hydrolase	T/C
4	1843906	AFUA_4G07100	Nuclear envelope protein	A/G
4	1843907	AFUA_4G07100	Nuclear envelope protein	A/G
4	1853393	AFUA_4G07140	*iki3*	C/T
4	1860388	AFUA_4G07170	*cut8*	T/G
4	1870355	AFUA_4G07220	KH domain protein	T/C
4	1876666	AFUA_4G07240	Pentatricopeptide repeat protein	T/C
4	1878196	AFUA_4G07250	*rsm25*	A/G
4	1879821	AFUA_4G07260	*sog2*	T/G
4	1879930	AFUA_4G07260	*sog2*	C/G
4	1884641	AFUA_4G07270	Serine carboxypeptidase	A/T
7	1200543	AFUA_7G05080	C6 transcription factor	T/C
7	1227395	AFUA_7G05200	Hypothetical	A/G
7	1248377	AFUA_7G05250	PHD finger domain protein	AGCT/A
7	1275385	AFUA_7G05330	Hypothetical	A/G
7	1563468	AFUA_7G06370	C6t transcription factor	C/G

Table 3.7 *– 46 affected genes from variants identified in the second round of bulk segregrant analysis, following backcrossing, compared to the JGI* A. fumigatus *reference genome A1163. The chromosome, position, gene ID, gene name, reference and alteration has been listed. Where gene name was not available, function has been listed.*

Contig	Position	Gene ID	Gene	Reference /Change
DS_499603	244828	AFUB_052480	LysM domain protein	C/G
DS_499603	270496	AFUB_099100	MFS drug transporter	A/C
DS_499603	281796	AFUB_099140	60s ribosomal protein	C/T
DS_499601	1270813	AFUB_090890	Splicing factor U2af subunit	C/CTCTCCTCTT
DS_499601	1614835	AFUB_092190	Pth11-like membrane protein	G/C
DS_499598	143724	AFUB_063310	Fungal specific transcription factor	G/A
DS_499598	148327	AFUB_063320	Flavin-binding mono oxygenase	A/G
DS_499598	149261	AFUB_063330	JmjC domain protein	T/C
DS_499598	150061	AFUB_063330	JmjC domain protein	G/C
DS_499598	151616	AFUB_063350	*agp2*	A/G
DS_499598	163325	AFUB_063400	Hypothetical	T/C
DS_499598	168480	AFUB_063420	Hypothetical	T/C
DS_499598	176285	AFUB_063470	*gar2*	T/C
DS_499598	177560	AFUB_063470	*gar2*	C/G
DS_499598	179728	AFUB_063490	Hypothetical	C/T
DS_499598	192943	AFUB_063540	Fungal specific transcription factor	C/T
DS_499598	193095	AFUB_063540	Fungal specific transcription factor	AG/A
DS_499598	201933	AFUB_063560	dDENN domain protein	G/C
DS_499598	210643	AFUB_063570	*mlh3*	C/G
DS_499598	215425	AFUB_063590	FAD dependent oxido-reductase	C/T
DS_499598	230075	AFUB_063630	*znf1*	A/G
DS_499598	230076	AFUB_063630	*znf1*	G/T
DS_499598	230981	AFUB_063630	*znf1*	A/G
DS_499598	267123	AFUB_063720	Acid sphingo-myelinase	G/A

DS_499598	281565	AFUB_063770	GPI anchored cell wall protein	C/A
DS_499598	281881	AFUB_063770	GPI anchored cell wall protein	A/G
DS_499598	285670	AFUB_063800	Hypothetical	A/G
DS_499598	316444	AFUB_063920	Coenzyme A synthetase	T/C
DS_499598	316883	AFUB_063920	Coenzyme A synthetase	T/C
DS_499598	331794	AFUB_063960	*cyp51A*	T/A
DS_499598	337780	AFUB_063980	Mitochondria membrane porin	A/C
DS_499598	348715	AFUB_064030	Hypothetical	A/T
DS_499598	357262	AFUB_064060	Trans-membrane amino acid transporter	C/A
DS_499598	359699	AFUB_064070	Hypothetical	T/A
DS_499598	365406	AFUB_064100	Mitochondrial cytochrome b2-like	A/T
DS_499598	378366	AFUB_064150	Acyl-CoA thioesterase	T/C
DS_499598	394629	AFUB_064190	Hypothetical	A/G
DS_499598	394630	AFUB_064190	Hypothetical	A/G
DS_499598	404117	AFUB_064230	*iki3*	C/T
DS_499598	411115	AFUB_064260	*sts1*	T/G
DS_499598	417160	AFUB_064290	Hypothetical	A/G
DS_499598	427401	AFUB_064330	Hypothetical	T/C
DS_499598	428931	AFUB_064340	Ribsomal protein S25	A/G
DS_499598	430556	AFUB_064350	*sog2*	T/G
DS_499598	430665	AFUB_064350	*sog2*	C/G
DS_499598	435376	AFUB_064360	Serine carboxy-peptidase	A/T

3.3.7 3rd round sequencing and analysis

A further round of genome sequencing was performed using pooled DNA from itraconazole sensitive and resistant progeny from the 6th backcross between a resistant F6 isolate and sensitive parent strain (47-51). Bioinformatic analysis was then performed to try and identify any SNP or region linked to the resistance phenotype. The preliminary data, shown below in **Table 3.8** represents the variants clustered together on contig DS_499598, where we know the *cyp51A* mutation is located. In comparison to the 1st and 2nd round of sequencing, the number of genes identified had

decreased. Indeed, when looking specifically at DS_499598 in the 2nd round of sequencing, 46 genes were identified. By contrast, this number of genes had been reduced in the 6th backcross to 34 candidate genes being identified. However, this was not as large as a reduction as expected from prior results (**Tables 3.7** and **3.6**), although the data suggests that the 292 kb region of the genome containing these variants may be recombining as a whole segment when compared with section **3.3.8** below.

Table 3.8 *– 34 affected genes from variants identified from preliminary results in the third round of bulk segregrant analysis, following backcrossing, compared to the JGI* A. fumigatus *reference genome A1163. The chromosome, position, gene ID, gene name, reference and alteration has been listed. Where gene name was not available, function has been listed.*

Contig	Position	Gene ID	Gene	Ref/Change
DS_499598	143724	AFUB_063310	Fungal specific transcription factor	G/A
DS_499598	148327	AFUB_063320	Flavin-binding mono oxygenase	A/G
DS_499598	149261	AFUB_063330	JmjC domain protein	T/C
DS_499598	151616	AFUB_063350	*agp2*	A/G
DS_499598	163325	AFUB_063400	Hypothetical	T/C
DS_499598	168480	AFUB_063420	Hypothetical	T/C
DS_499598	176285	AFUB_063470	*gar2*	T/C
DS_499598	177560	AFUB_063470	*gar2*	C/G
DS_499598	192943	AFUB_063540	Fungal specific transcription factor	C/T
DS_499598	201933	AFUB_063560	dDENN domain protein	G/C
DS_499598	210643	AFUB_063570	*mlh3*	C/G
DS_499598	215425	AFUB_063570	FAD dependent oxido-reductase	C/T
DS_499598	230981	AFUB_063630	*znf1*	A/G
DS_499598	267123	AFUB_063720	Acid sphingo-myelinase	G/A
DS_499598	281565	AFUB_063770	GPI anchored cell wall protein	C/A
DS_499598	281881	AFUB_063770	GPI anchored cell wall protein	A/G
DS_499598	285670	AFUB_063800	Hypothetical	A/G
DS_499598	316444	AFUB_063920	Coenzyme A synthetase	T/C
DS_499598	316883	AFUB_063920	Coenzyme A synthetase	T/C
DS_499598	331794	AFUB_063960	*cyp51A*	T/A
DS_499598	337780	AFUB_063980	Mitochondria	A/C

			membrane porin	
DS_499598	348715	AFUB_064030	Hypothetical	A/T
DS_499598	359699	AFUB_064070	Hypothetical	T/A
DS_499598	378366	AFUB_064150	Acyl-CoA thioesterase	T/C
DS_499598	394629	AFUB_064190	Hypothetical	A/G
DS_499598	394630	AFUB_064190	Hypothetical	A/G
DS_499598	404117	AFUB_064230	*iki3*	C/T
DS_499598	411115	AFUB_064260	*sts1*	T/G
DS_499598	417160	AFUB_064290	Hypothetical	A/G
DS_499598	427401	AFUB_064330	Hypothetical	T/C
DS_499598	428931	AFUB_064340	Ribsomal protein S25	A/G
DS_499598	430556	AFUB_064350	*sog2*	T/G
DS_499598	430665	AFUB_064350	*sog2*	C/G
DS_499598	435376	AFUB_064360	Serine carboxy-peptidase	A/T

3.3.8 4th round sequencing and analysis

A further and final round of genome sequencing was performed using pooled DNA from itraconazole sensitive and resistant progeny from the 2nd backcross between a resistant F2 isolate and sensitive parent strain (47-51). In this round of sequencing 80 progeny from each phenotypic pool was used, as opposed to 40 used previously. Bioinformatic analysis was then performed to try and identify any SNP or region linked to the resistance phenotype. The preliminary data, shown below in **Table 3.9** illustrates that the variants clustered together on contig DS_499598, where the *cyp51A* mutation is known to be located.

In comparison to the 1st and 2nd round of sequencing, the number of genes identified has notably decreased. Indeed, when looking specifically at DS_499598 in the 2nd round of sequencing, 46 genes were identified. The number of genes had been reduced in the 4th backcross to 34 genes being identified. This is the same number of genes and region identified from the 3rd round of sequencing using the 6th backcross (**Table 3.8**). However, the 4th round of sequencing used an earlier backcross (2nd backcross) involved the use of a larger number of progeny in each phenotypic pool. This shows the beneficial effect that increasing the number in the sequencing pool can have on BSA in terms of reducing the time needed for crossing to produce a

smaller region of the genome for gene identification (9-month total crossing time over 3 sexual reproductive cycles in comparison to 21 months in the 3rd sequencing round using a 6th backcross).

Table 3.9 *– 34 affected genes from variants identified from preliminary results in the fourth round of bulk segregrant analysis, following backcrossing, compared to the JGI* A. fumigatus *reference genome A1163. The chromosome, position, gene ID, gene name, reference and alteration has been listed. Where gene name was not available, function has been listed.*

Contig	Position	Gene ID	Gene	Ref/Change
DS_499598	143724	AFUB_063310	Fungal specific transcription factor	G/A
DS_499598	148327	AFUB_063320	Flavin-binding mono oxygenase	A/G
DS_499598	149261	AFUB_063330	JmjC domain protein	T/C
DS_499598	151616	AFUB_063350	*agp2*	A/G
DS_499598	163325	AFUB_063400	Hypothetical	T/C
DS_499598	168480	AFUB_063420	Hypothetical	T/C
DS_499598	176285	AFUB_063470	*gar2*	T/C
DS_499598	177560	AFUB_063470	*gar2*	C/G
DS_499598	192943	AFUB_063540	Fungal specific transcription factor	C/T
DS_499598	201933	AFUB_063560	dDENN domain protein	G/C
DS_499598	210643	AFUB_063570	*mlh3*	C/G
DS_499598	215425	AFUB_063570	FAD dependent oxido-reductase	C/T
DS_499598	230981	AFUB_063630	*znf1*	A/G
DS_499598	267123	AFUB_063720	Acid sphingo-myelinase	G/A
DS_499598	281565	AFUB_063770	GPI anchored cell wall protein	C/A
DS_499598	281881	AFUB_063770	GPI anchored cell wall protein	A/G
DS_499598	285670	AFUB_063800	Hypothetical	A/G
DS_499598	316444	AFUB_063920	Coenzyme A synthetase	T/C
DS_499598	316883	AFUB_063920	Coenzyme A synthetase	T/C
DS_499598	331794	AFUB_063960	*cyp51A*	T/A

DS_499598	337780	AFUB_063980	Mitochondria membrane porin	A/C
DS_499598	348715	AFUB_064030	Hypothetical	A/T
DS_499598	359699	AFUB_064070	Hypothetical	T/A
DS_499598	378366	AFUB_064150	Acyl-CoA thioesterase	T/C
DS_499598	394629	AFUB_064190	Hypothetical	A/G
DS_499598	394630	AFUB_064190	Hypothetical	A/G
DS_499598	404117	AFUB_064230	*iki3*	C/T
DS_499598	411115	AFUB_064260	*sts1*	T/G
DS_499598	417160	AFUB_064290	Hypothetical	A/G
DS_499598	427401	AFUB_064330	Hypothetical	T/C
DS_499598	428931	AFUB_064340	Ribsomal protein S25	A/G
DS_499598	430556	AFUB_064350	*sog2*	T/G
DS_499598	430665	AFUB_064350	*sog2*	C/G
DS_499598	435376	AFUB_064360	Serine carboxy-peptidase	A/T

3.3.9 Distribution plot – 1st round sequencing JGI

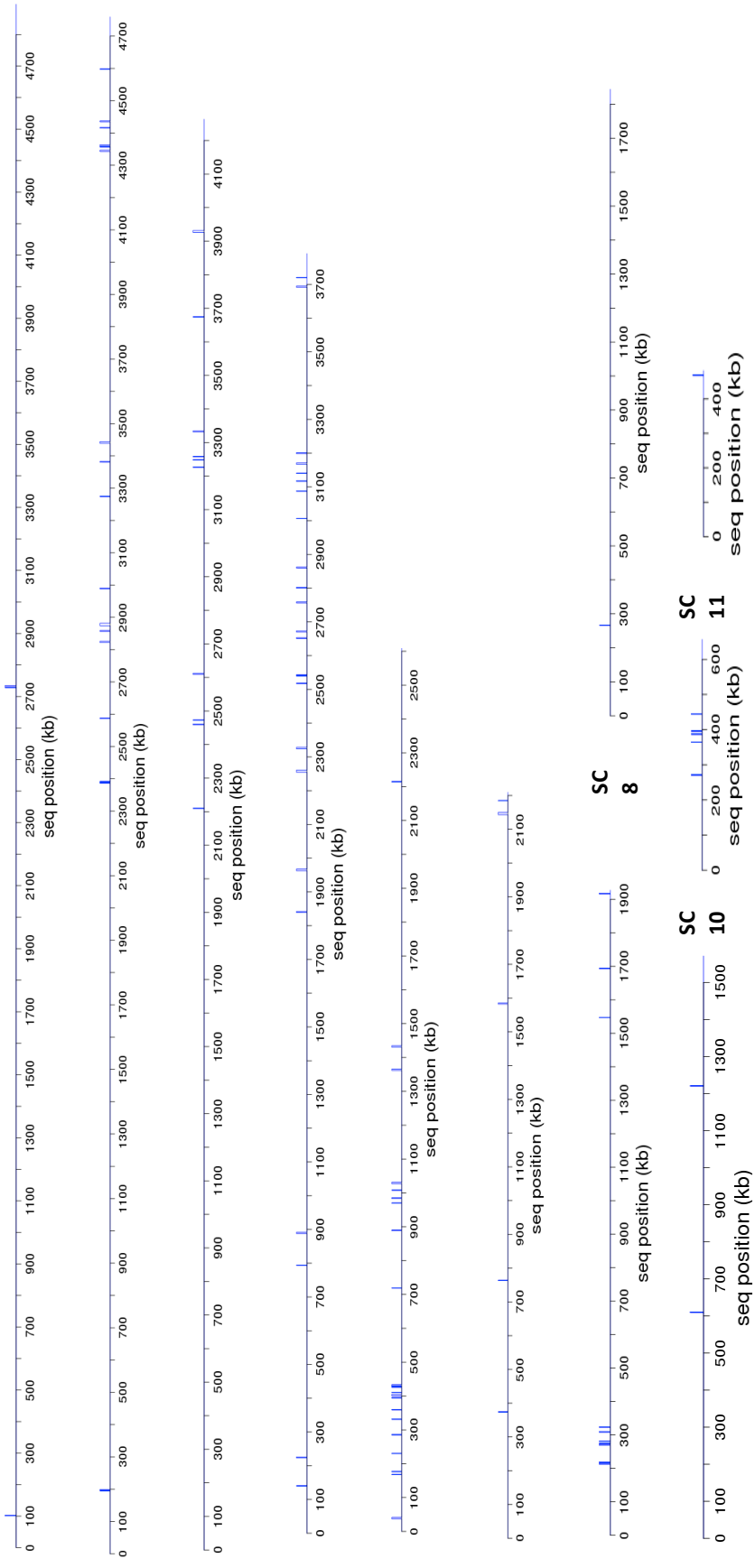


Figure 3.10 *- Distribution plots of identified variants mapped onto the JGI* A. fumigatus *reference genome during the first round of sequencing A1163. Where each blue line identifies an affected gene within the supercontig (SC)*

3.3.10 Distribution plot – 2nd round sequencing JGI

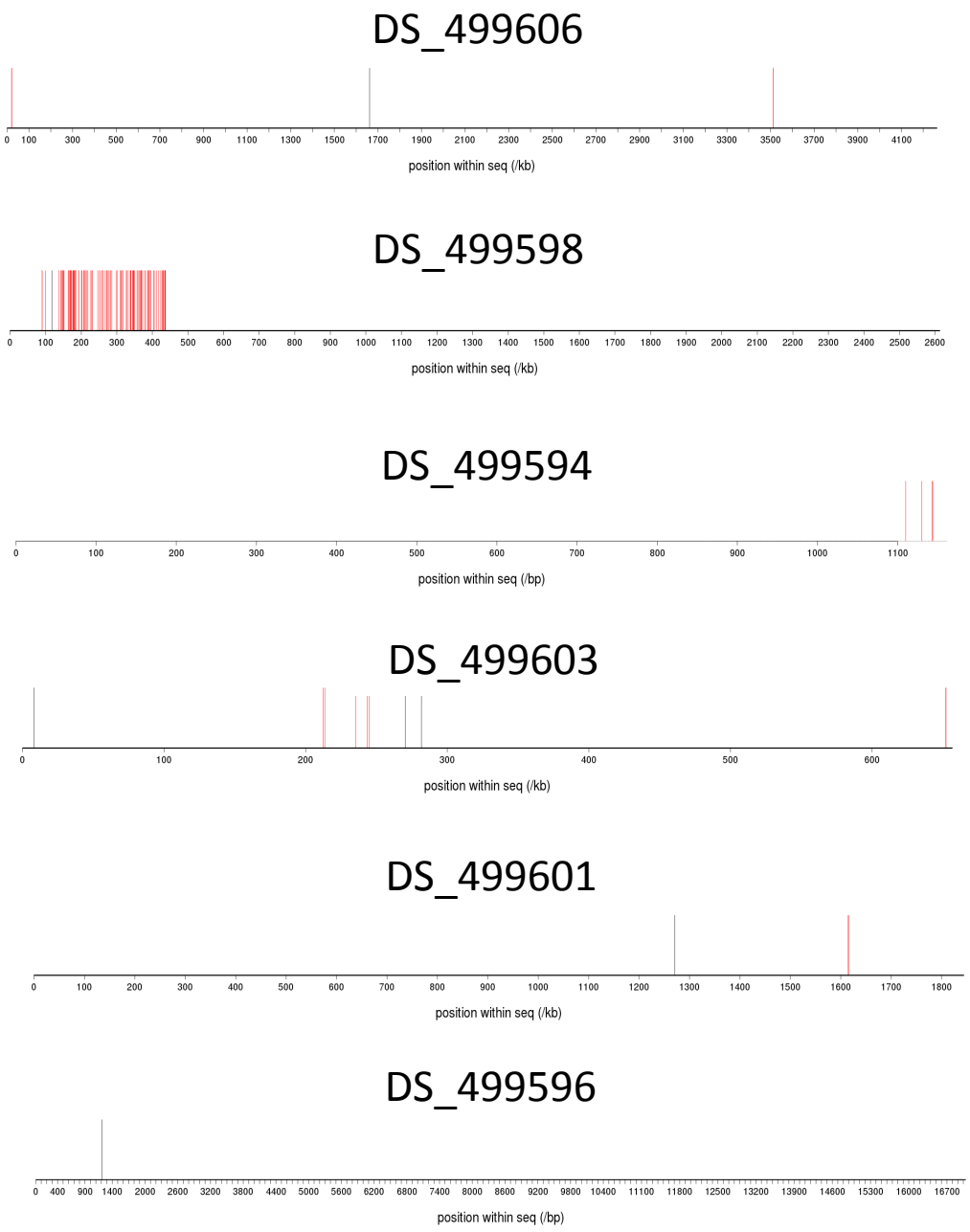


Figure 3.11 *- Distribution plots of identified variants mapped onto the JGI* A. fumigatus *reference genome during the second round of sequencing A1163. Where each blue line identifies an affected gene within the supercontig (SC). Black vertical bars represent variant sites more than 10kb from all other sites. Variant sites less than 10kb from all other sites have been coloured red. What appears to be a single red bar can represent one or more sites.*

Both **Figure 3.10** and **Figure 3.11** show distribution plots representing the areas of the genome, mapped onto the JGI reference genome from *A. fumigatus* isolate A1163. The blue and red columns from **Figure 3.10** and **Figure 3.11**, respectively, show the variant sites identified by the comparison of resistant progeny and sensitive parent isolate 47-51. It was predicted that the gene of interest would be located in a linked region where a large number of variant sites would be located. Additionally, our results show the number of variant sites from **Figure 3.10**, where BSA was performed on the first sexual cross '47-308 × 47-51', is much higher in comparison to the variant sites identified in **Figure 3.11**, where BSA was performed on the third backcross. This gives clear evidence in favor of our hypothesis that backcrossing resistant progeny with the original sensitive parent will improve our ability of identifying the resistance gene of interest.

3.3.11 Sequencing of *cyp51A*

Using the *cyp51A* PCR primers (Cyp1a/Cyp1b and Cyp2a/Cyp2b) described above, sequencing data of itraconazole resistant *A. fumigatus* strains 47-312 and 47-316 showed no substitution mutations at G54/W/R/E/V/A; nor at substitution mutation M220/T/V/I/K/R/L; nor at substitution mutation G448S; and neither at substitution mutation G138/C/S. By contrast, sequencing of *cyp51A* from 47-308 confirmed the presence of the M220K mutation with a change from A to a T at amino acid position 220. The PCR for *cyp51A* amplification of isolates 47-51, 47-308, 47-312 and 47-316 is shown below (**Figure 3.12**).

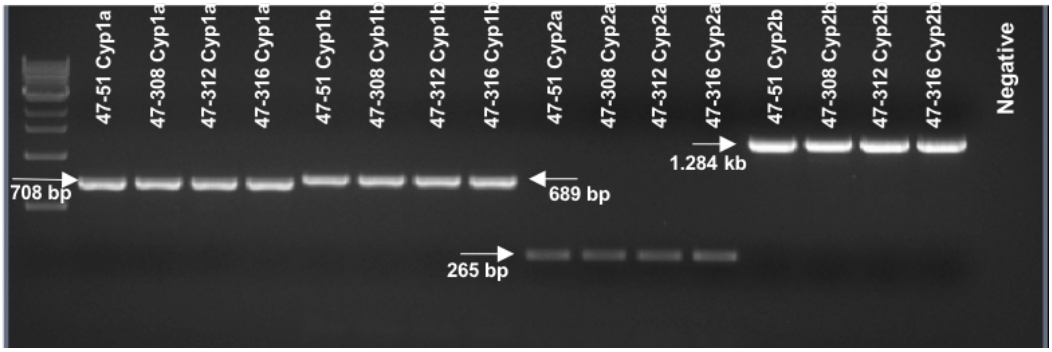


Figure 3.12 *– PCR amplification of cyp51A in* A. fumigatus *isolates 47-51, 47-308, 47-312 and 47-316 using primer sets Cyp1a/Cyp1b (producing a PCR product of 708 and 689 bp, respectively) and Cyp2a/Cyp2b (producing a PCR product of 265 and 1284 bp, respectively). When combined, each primer set covers the coding region of the cyp51A gene. The lanes are as follows: 1) 1 kb DNA ladder; 2) Cyp1a 47-51; 3) Cyp1a 47-308; 4) Cyp1a 47-312; 5) Cyp1a 47-316; 6) Cyp1b 47-51; 7) Cyp1b 47-308; 8) Cyp1b 47-312; 9) Cyp1b 47-316; 10) Cyp2a 47-51; 11) Cyp2a 47-308; 12) Cyp2a 47-312; 13) Cyp2a 47-316; 14) Cyp2b 47-51; 15) Cyp2b 47-308; 16) Cyp2b 47-312; 17) Cyp2b 47-316; and 18) negative control.*

Meanwhile, primers Cyp51-TR were used to amplify the promoter region from the same isolates. Sequence analysis of the PCR products of strains 47-312 and 47-316 showed no: tandem repeat mutation TR_{34} with substitution mutation L98H; no tandem repeat mutation TR_{46} with substitution mutation Y121F/T289A; and no tandem repeat mutation TR_{53}. This was indicated by an amplicon size of approximately 120 bp being produced, as would be predicted with no tandem repeat (data not shown).

3.4 Discussion

Work presented in the present chapter aimed to evaluate whether the classical genetic technique of bulk segregant analysis (BSA) could be adapted for use with next generation sequencing, in order to identify 'unknown' genes of interest – in this case a gene(s) conferring resistance to azole antifungal drugs in *A. fumigatus.* BSA was attempted with F1 progeny from a single round of crosses, and also after three rounds of backcrosses. Some exciting, but unexpected, results were obtained as will now be discussed.

3.4.1 Identification of genomic regions by BSA

Overall, through the use of successive backcrosses it was possible to reduce the number of potential resistance genes in *A. fumigatus* strain 47-308 from ~123 based on analysis of only backcross (using the NCBI and JGI reference genome, respectively) to 34 genes at the 6th backcross between resistant progeny and sensitive parent 47-51.

We successfully showed that each backcross led to the production of more homogenous resistant progenies compared to the sensitive parent strain 47-51, whilst retaining the region of the genome from *A. fumigatus* strain 47-308 that contained the gene responsible for itraconazole resistance as the key difference allowing its identification. However, increasing the number of progeny in each pool to 80 enhanced this result, leading to identification of 34 potential genes by the 2nd backcross between resistant progeny and parental strain 47-51. Interestingly, this was the same number of genes identified when comparing sequence data from the 6th backcross when using a lower number of progeny (40) in each pool. This suggests that this region of ca. 30-35 genes is strongly linked and could be located between two hotspots for sexual recombination. This would result in an inability to reduce the region of the genome identified to contain the gene for resistance further, highlighting a previously unforeseen problem with BSA. Overall, a key conclusion from the 4th round of sequencing (section **3.3.8**) is that increasing the number of progeny being sequenced per respective DNA pool can

alleviate the need for a high number of successive backcrosses, thereby reducing the time of the process.

Regrettably, amongst the genes identified in the resistant pools and the parental strain 47-308 was the *cyp51A* gene, where it was found that a known mutation responsible for itraconazole resistance involving a substitution of A to a T at amino acid position 220 was present. This resulted in an amino acid change of methionine to lysine (M220K). An M220K substitution in *cyp51A* has already been described in 2004, where substitutions of methionine to valine, lysine or threonine at position 220 reduced susceptibility to all triazoles. This substitution gave the *A. fumigatus* isolates resistance to itraconazole >8 mg L^{-1} (Mellado *et al.*, 2004). Due to the pattern of segregation of the resistance phenotype (see itraconazole ETEST), it was concluded that this is the only mutation causing azole resistance in isolate 47-308.

This was in some respects a very unexpected and disappointing finding, as it was hoped to identify a novel genetic form of azole resistance in this isolate. Furthermore, the isolate had supposedly been pre-screened for known *cyp51A* mutations by our Dutch collaborators. However, it became clear that this mutation had somehow been overlooked in their *cyp51A* sequence analysis.

On a more positive note, the work nevertheless showed that the BSA approach, in tandem with NGS, showed great promise as a technique to identify 'unknown' forms of monogenic genetic traits such as those conferring resistance to antifungal drugs. Although the *cyp51A* gene had been identified from the first round of F1 progeny analysis, there were still some reservations about BSA at this point. Rather than a single gene, or one region of linked genes, being identified, instead multiple regions with over 120 genes in total had been identified. As a result of knowing the resistance mechanism, it was therefore easier to assess how the BSA technique might be used more efficiently in the future to identify regions containing a gene of interest such as the azole resistance gene. In combination with the mathematical modelling to predict the variant region, it was possible to

reduce the number of genes identified to 34 – including the M220K *cyp51A* mutation.

From our mathematical model (section **3.3.4**) based on Pomraning *et al.,* 2011) using 40 progeny in each pool, the model predicted a 0.64 Mb DNA region would be identified by BSA from the genome, including the gene of interest, following one round of crossing. In comparison to the prediction of this model, a region of 0.452 Mb was obtained from the first cross. An increased prediction value from the model may be due to using the whole genome size in order to identify an unknown mutation. This finding confirms that this model can inform the efficiency of BSA by predicting which minimum numbers should be used in each pool size. However, the efficiency of the model will increase with more detailed information, such as a prediction of the chromosome the mutation may be located.

The second round of sequencing revealed a 27.3% decrease, to 0.329 Mb, in the region of the genome containing the gene of interest. A subsequent decrease of 11.3% was found between the second round of sequencing to the third and fourth round, to 0.292 Mb and a decreased number of identified genes. This may indicate the maximum effectiveness of BSA in identifying a genomic region of interest in this instance. Therefore, we can confirm that as the number of backcrosses increases, the number of genes identified will reduce. Although it simultaneously reveals that the efficiency of the method is reduced past the 3rd backcross in terms of ability to identify an ever-smaller linked region of the genome.

Therefore, if the whole genome size of the organism of interest is known, or the specific chromosome containing the gene of interest is known, it is possible to apply this variant model to predict the most efficient methodology for identification. Using a combination of backcrosses paired with a large number of progeny in each genetic pool, the region of the genome that can be identified through BSA and NGS will decrease significantly.

Comparing our use of BSA combined with next generation sequencing to other similar studies, the concept of using a higher number of segregants

to maximise BSA efficiency has also been favoured elsewhere (Niu *et al.*, 2015). For example, Niu *et al.* (2015) used a minimum of 62 segregants in a single DNA pool in order to identify the role of *laeA* in citric acid production. They even showed that even numbers of segregants in each DNA pool were not needed in order to identify the gene of interest. By contrast, *SMO1,* a gene responsible for appressorium formation and eventual disease in *Magnoporthe oryzae* was functionally identified using BSA (Kershaw *et al.*, 2018), with only 50 segregants in total being used to identify the genomic region of interest. However, in this study it was possible to enhance the identification of the gene of interest by comparison of the genomic region identified by BSA to whole genome data available from previously reported *SMO1* mutants. Meanwhile, BSA has also been employed successfully to identify single genes of interest, or linked regions of the genome, in parallel studies with *N. crassa* (Pomraning *et al.,* 2011; Nowrousian *et al.,* 2012).

These recent studies using BSA (albeit without backcrosses) illustrate the importance of concepts that should be considered in order to maximise the success of BSA for future experiments – such as use of high progeny number in the respective pools, or comparisons to previous genetic data available from strains showing similar phenotypes.

3.4.2 Drawbacks of BSA

The use of BSA in this study concerned a situation where the trait of interest was known to be monogenic in nature. This was confirmed through the analysis of progeny from sexual backcrosses, where itraconazole resistance segregated in a 1:1 ratio in the progeny. However, in situations where a trait has been determined to be polygenic, BSA may not be as efficient or even applicable to locate the gene of interest. This would be due to the segregation pattern of the trait of interest, with the potential of variable trait penetration according to the number of trait-causing genes within each isolate (Ashton and Dyer, 2016). Therefore, if a trait is determined as polygenic then alternative techniques such quantitative trait locus (QTL) analysis would need to be used (Miles and Wayne, 2013). In addition to

polygenic gene traits, BSA as applied in the current work only focused on variations within gene-coding regions of the genome. Therefore, to improve this technique further, it will be necessary to determine any differences originating from non-coding regions. Indeed, this would be critical for the identification of mutations present in promoter regions, which are known to cause antifungal resistance in *A. fumigatus* (Lelièvre *et al.*, 2013). The BSA can in principle be used to identify such mutations, but this will entail a higher degree of SNP analysis and inspection for possible segmental deletions and/or duplications.

Furthermore, an important aspect to be mindful of is that whilst effective, the time investment to produce an *A. fumigatus* F7 progeny (6 backcrosses) is considerable, reaching up to 21 months. This represents a serious disadvantage of the method if a high number of backcrosses are necessary to identify the region of interest. Although in species where the sexual cycle is completed within a much shorter time period, it may be possible to reliably use BSA in conjunction with backcrosses to effectively identify genomic regions of interest.

RAPD-PCR was used to produce genetic profiles of progeny at various stages of backcrossing. Using this technique, the genetic similarity of the progeny to the parental strains could be assessed to verify the occurrence of recombination. However, after each subsequent backcross the RAPD profile for each progeny becomes increasingly similar to the sensitive parent (47-51). Therefore, the use of RAPDs becomes less relevant for providing evidence of recombination, but did show that the backcrossing was achieving the aim of the progeny becoming more isogenic to the sensitive parent.

An improved method to identify genetic recombination in the sexual progeny of fungi has been discussed in **Chapter 3, 'Sex in the Black Aspergilli'**. By sequencing the original parent strains to be used in BSA and subsequent rounds of backcrossing, it will be possible to identify a number of isolate specific SNPs throughout the genome. Once identified, primers could then be designed to amplify these SNP regions within the progeny of a sexual

cross. The hope would be to more accurately determine if sexual recombination is occurring over the multiple backcrosses used in this study.

3.4.3 JGI vs. NCBI

Using data from the 2nd round of sequencing (involving the 3rd backcross) allowed a comparison to be made between the variations detected using the NCBI Af293 reference genome and the JGI A1163 reference genome. When comparing the 60 genes identified using the NCBI reference to the 46 genes from the JGI reference, 23 genes were shared between the two. The remaining genes from the NCBI and JGI indicate different annotations within the reference genomes. Indeed, this indicates the advantage of using multiple reference genomes when using BSA to ensure the identification of the gene of interest.

3.4.4 Future work

In this study, the efficiency of BSA as a method for gene identification has been evaluated using an *A. fumigatus* resistance isolate with a known mutation. The next step in this research, therefore, is to use an *A. fumigatus* isolate with an unknown resistance mutation. From our research, we have identified that using pools of 80 progeny arising from a 2nd round of backcrossing can provide an effective method for candidate gene identification using the BSA technique coupled with NGS analysis. This led to the identification of a relatively small, linked region of the genome being identified that contained the mutation of interest, in comparison to the other conditions tested and variant model predictions. From the method development of this study, it will be possible to perform BSA on a much larger number of isolates in a reduced time frame. It is anticipated that this will allow the identification of many novel resistance genes of interest as the number of strains with 'unknown' forms of resistance to antifungal drugs has been steadily increasing (Verweij *et al.*, 2016).

Once a region has been identified, it will then be important to locate genes within this region with a high potential of causing drug resistance. Then, using this list of candidate genes, it is anticipated that it will be possible to identify the gene of interest by using a mixture of experimental genetic manipulation experiments in tandem with screening for identified resistance genes identified from other research groups e.g. high basal *cdr1B* expression (Fraczek *et al.*, 2013) or R243Q mutation of *AfCox10* (Wei *et al.*, 2016). In addition, current work is underway at the University of Manchester to generate gene knockouts of all genes of *A. fumigatus* in a CEA10 genetic background using hygromycin as a selective marker (Bromley, unpublished). This could provide significant insights into genes in which mutations may lead to drug resistance in *A. fumigatus*.

Identification of resistance genes in both environmental and medical isolates of *A. fumigatus* will have a number of impacts. Foremost, identification of a novel resistance gene will enable molecular diagnostic techniques to be developed for use in disease management of *A. fumigatus* infections. A key example of how diagnostics can be developed for the detection of resistant microbial strains can be seen from efforts aimed at detecting multi-drug resistant *Mycobacterium tuberculosis.* Here, a critical development, and one that could be used for *A. fumigatus,* is the development and use of line probe assays (LiPa) (Heifets and Cangolosi, 1999). Here, LiPas are used to detect the presence of *M. tuberculosis* and a number of genetic mutations related to resistance, such as in the *rrpoB* gene responsible for rifampicin resistance (Juréen *et al.*, 2004). This technique can be performed from both cultured and direct clinical samples and meta-analyses of LiPa use shows their effectiveness for detection to be greater than 95% (Morgan *et al.*, 2005). Therefore, identification of novel mutations can lead to diagnostics that utilise LiPa methodology to quickly and effectively identify resistance to a number of drugs. In turn, this could pinpoint an effective treatment program and prognosis.

Furthermore, upon detection novel mutations must be characterised and graded in order to understand the resistance mechanism and correct

treatment necessary. To do this, NGS has been highlighted as an effective tool to quickly identify key mutations in the genome associated with drug resistance in *M. tuberculosis* (WHO, 2018). Here, NGS is employed to characterise clinical strains against a database of known mutations. Each mutation has been separated into high, moderate, minimal, 'not associated with resistance', or intermediate categories with regards to resistance risk. Alongside this, MIC data for each drug is also given for each associated mutation. This allows for efficient analysis of mutations, their risk to the patient and the appropriate treatment necessary.

Chapter 4 – Investigation of the Role of Hormones in Fungal Sexual Development and Possible Uses for Disease Control

4.1 Introduction

During fungal sexual reproduction, relatively complex sexual structures are produced from the simple building block of fungal hyphae. Such morphogenesis requires chemical signalling to cause such developmental changes (Dyer *et al.,*, 1992). Such chemical signals may originate from a single individual, in homothallic systems, or involve two compatible mating partners, in heterothallic systems. In particular, fungal sexual development can involve the production of metabolites known as fungal 'sex hormones'. These metabolites are able to have potent effects on cell morphology, acting both internally and externally on surrounding cells (Gooday, 1974; Dyer *et al.,* 1992). As a result, it has been proposed that some types of fungal sex hormone might have potential applications as novel disease control agents, due their ability to suppress fungal asexual reproduction (Siddiq *et al.,* 1992). Work in this chapter involves firstly an investigation into the activity of lipid fungal sex hormones in the ascomycete species *Neosartorya fischeri*. Secondly the activity of lipo-oxygenases, known for their important role in sexual regulation in the homothallic *Aspergillus nidulans* (Tsitsigiannis *et al.*, 2004a; Tsitsigiannis *et al.*, 2005), is explored in the heterothallic species *Aspergillus fumigatus.*

By way of introduction, a survey of current knowledge of fungal sex hormones is first presented below. Key examples include antheridiol, characterised from the water mould oomycete *Achlya* (Raper, 1939); sirenin, characterised from the chytridomycota (Machlis, 1958); trisporic acid, identified as a sexual hormone in *Mucorales* (Austin *et al.,* 1969); peptide pheromones from *Saccharomyces cerevisiae* and other Ascomycota (Stötzler and Duntze, 1976; Betz *et al.,* 1977); psi factors from *A. nidulans* (Champe

and El-Zayat, 1989); and Sex Factor (SF) from *Pyrenopeziza brassicae* (Ilott *et al.,* 1986). The hormones antheridiol, sirenin and trisporic acid are of particular historical interest because structural analysis of these hormones was hotly contested in a race to identify the first fungal sex hormone.

It is important to note that although many molecules have been identified as having an effect on the sexual cycle on fungi, not all of these molecules can be classified as sex hormones. Indeed, these molecules are separated into three subgroups (Siddiq *et al.,* 1992; Dyer *et al.,* 1993): firstly, the sex pheromones, acting to induce the differentiation of gametes and for fusion to occur; secondly, the sex morphogens, which act to induce morphogenic changes and the production of secondary sexual structures; and thirdly, growth substances, which act as precursors or intermediates in the production of either sex pheromones or morphogens and/or promote sexual reproduction through cellular metabolism.

4.1.1 Sex in the Oomycota

4.1.1.1 Antheridiol

Although oomycetes are now reclassified within the kingdom Chromalveolata, John Robert Raper performed seminal work on sexual hormones in 1939 (Raper, 1939). Raper's work led to the subsequent elucidation of sexual hormones in other true fungi. Working on the oomycete species *Achlya bisexualis* and *A. ambisexualis*, Raper demonstrated that female cells produced an active hormone metabolite, which induced hyperbranching in compatible male cells. The hormone was named hormone A, now known as antheridiol. The sexual cycle is completed when the male cells, responding to the antherdiol secreted by the female cells, produce their own complementary hormone (hormone B). Once detected, hormone B induces the production of oogonium by female cells (Raper, 1940). This was later named oogoniol and its structure as a sterol-based hormone elucidated (Barrow and McMorris, 1982).

The active hormone antherdiol was first extracted from the female mating partners late in 1942 (Raper, 1942). The activity was measured and it was found to be effective at very low concentrations, being able to induce an effect in the reciprocating male partner at the pg/µl level (McMorris and Barksdale, 1967). However, due to the limitations in technology, structure elucidation remained unachievable. However, later use of mass spectrometry analysis of antheridiol later revealed a steroid structure. Two isoforms were identified, one of which was more preferred by the male sexual partners (Arsenault *et al.*, 1968). The structure of antheridiol is shown in **Figure 4.1.** This work provided an important precedent, as it demonstrated that sex hormones can be extracted from mating cultures and later applied, post-extraction, to induce sexual reproduction in the species of interest.

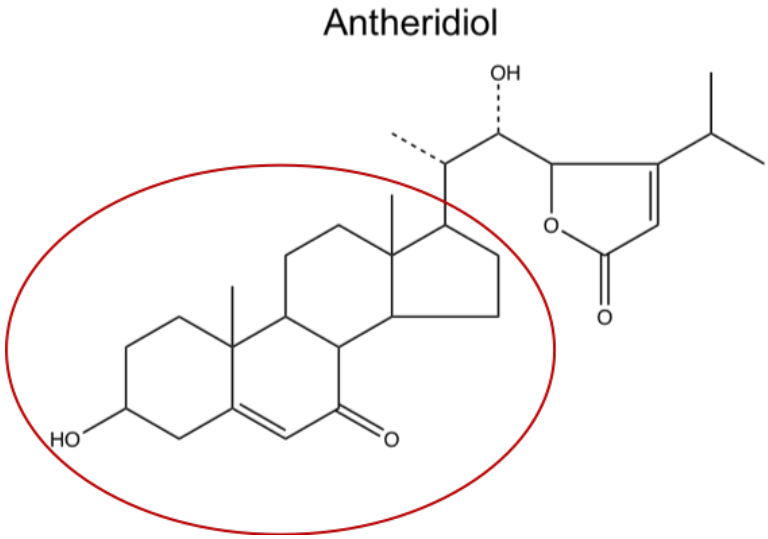


Figure 4.1 *– Chemical structure of antheridiol, the sex hormone of 'female'* Achyla *strains initiating sexual reproduction and growth towards oogonia in 'male' strains ($C_{29}H_{42}O_5$; MW 470 g/mol). The sterol component of antheridiol is highlighted in red.*

Subsequent research on oomycete *Pythium* spp. provided further evidence that sexual reproduction in oomycetes is regulated by sterols (Gall and Elliott, 1985). *Pythium* spp. are biotrophic parasitic plant pathogens and are also responsible for *Pythium*-induced root rot, resulting in the death of germinating seedlings (Kerns and Tredway, 2008). Haskins *et al.* showed that

vegetable oils with a variety of fatty acid compositions could induce sexual reproduction in *Pythium* spp. Isolation of active compounds from sunflower oil suggested that the presence of a phytosterol β-sitosterol, among others, is required for the completion of the fungal sexual cycle (Haskins *et al.,* 1964).
In addition, it has been discovered that in *Pythium* species, sterols capable of inducing a sexual reproductive cycle (and production of sexual structures) are not actually metabolised. Instead, they are adsorbed onto the surface of the oomycete cell, suggesting potential incorporation into the cellular membrane (Sietsma and Haskins, 1967).

4.1.2 Sex in the Chytridiomycota

4.1.2.1 Sirenin

Moving into the world of true fungi, the first fungal sexual hormone identified was within the Chytridiomycota. Leonard Machlis worked on *Allomyces* spp. in the 1950s and identified a fungal sex hormone produced by female cells, which were able to attract male gametes. He termed the hormone 'sirenin' (Machlis, 1958). In *Allomyces* species, sexual reproduction is initiated by motile male gametes of the fungal species that swim towards the female gametangium. Once the female gametes have developed, the female gametangium ruptures allowing the female gametes to escape and fuse with the male gametes. Machlis observed that male gametes of *Allomyces macrogynum* were attracted to female gametangium even when the female recipients were embedded in agar, showing the presence of a diffusible factor (Machlis, 1958). However, researching hormone production in this species remained difficult due to the limited time period in which a haploid state occurred. Therefore, Machlis produced a hybrid strain between *A. macrogynus and A. arbusculus.* Through this, Machlis was able to produce a strain that exclusively developed female gametangium in order to isolate the fungal sex hormone in large quantities. Similarly, Machlis also produced a strain that produced male gametes, enabling a bioassay to test female hormone attractiveness (Machlis, 1968).

Once extracted, sirenin was found to be a potent inducer of male gamete attraction at concentrations as low as 10^{-10} M (Machlis, 1968). However, at concentrations above 10^{-4} M, the ability to attract male gametes by female gametangium was lost due to presumably disproportionate amounts of hormone in the cultures. Indeed, this led to the hypothesis that male gametes are able to breakdown sirenin as a possible feedback mechanism. A full account of the structure of sirenin was finally reported in 1968 (Nutting *et al.,* 1968). Extracts from female only hybrid strains led to both *l* and *d* isomers of sirenin being identified (Plattner and Rapoport, 1971). Later research by Machlis identified that only the *l*-isomer of sirenin was capable of acting as an attractant for male gametes, shown in **Figure 4.2** (Machlis, 1973).

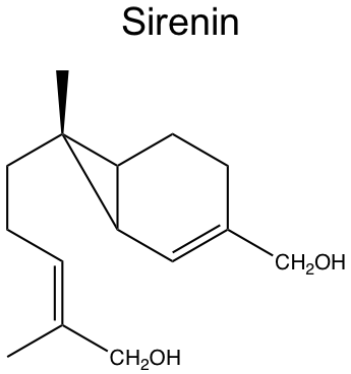


Figure 4.2 *– Chemical structure of sirenin, the sex hormone of female* Allomyces *strains, leading to the attraction of male gametes ($C_{15}H_{24}O_2$; MW 236 g/mol).*

Continuing the theme of research into fungal sex hormones in the Chytridiomycota, a reciprocal male sexual hormone produced by the male gametes of *Allomyces* spp. was subsequently identified, which was named parisin (Pommerville and Olson, 1987). This demonstrates evidence of a male-female fungal mating system where both mating partners produce hormones. Although research on sirenin has diminished, there has been recent work investigating the bioactivity of sirenin on human sperm cells (Syeda *et al.*, 2016).

4.1.3 Sex in the Zygomycota

4.1.3.1 Trisporic Acid

The third fungal sex hormone to be structurally identified in the 'hormone research race of the 1960s' was trisporic acid, a sexual hormone from the zygomycete *Mucor mucedo* (Austin *et al.,* 1969). This species is heterothallic, with complementary (+) and (-) mating partners required for sexual reproduction.

In 1924, Hans Burgeff observed the production of zygophores between (+) and (-) mating cultures when separated by a membrane (Burgeff, 1924). This is an essential step first step in the sexual reproduction of *M. mucedo*, allowing the two mating partners to fuse before undergoing a complete sexual cycle. It was concluded that a diffusible molecule must therefore be involved in the communication between cultures of opposite mating partners for sexual reproduction. Further studies led to the proposal that a series of six separate hormones were required in order to initiate and complete a sexual cycle in *M. mucedo* (Plempel, 1962). Initially, (+) and (-) progamone hormone from each partner were thought to be produced to induce (+) and (-) gamone hormone production in the opposite mating strain. The resulting gamone hormone was then thought to induce zygophore formation in the complimentary mating partner. Subsequently, this leads to the production of (+) and (-) zygotropic factors that induced mating cultures to grow towards each other for fusion.

The Farmitalia pharmaceutical company later isolated the metabolites trisporic acid B and C from mated cultures of the zygomycete fungus *Blakeslea trispora,* a vital step towards the discovery of trisporic acid as a sexual hormone in *Mucor*. *B. trispora* was able to form large amounts of β-carotene (the desired product of Farmitalia) in unmated cultures (Caglioti *et al.*, 1964). Extracts from *B. trispora* mated cultures were found to induce zygophore formation in both mating partners of *M. mucedo* (van den Ende, 1968). This indicated that the sexual hormone of *M. mucedo* was in fact trisporic acid B and C (Austin *et al.,* 1969). Indeed, it was shown that the

artificially synthesised methyl ester of trisporic acid B was able to induce zygophore formation in both mating partners of *M. mucedo* (Edwards *et al.*, 1971). Follow-up research in *M. mucedo* revealed that both (+) and (-) mating partners produce different precursors of trisporic acid, but are unable to complete its biosynthesis without the action of the opposite mating partner. In (+) strains, the trisporic acid precursor methyl-4-dihydrotrisporate is exclusively made into trisporic acid by a (-) strain. Similarly, (-) strains produce the precursor trisporal, which is exclusively converted to trisporic acid by (+) partners (van den Ende, 1976). John Bu'Lock *et al.* (1976) reported that β-carotene acts as a precursor molecular for trisporic acid production, resulting in a positive feedback loop of sexual reproduction (Bu'Lock *et al.,* 1976). The structure of trisporic acid is shown below in **Figure 4.3**.

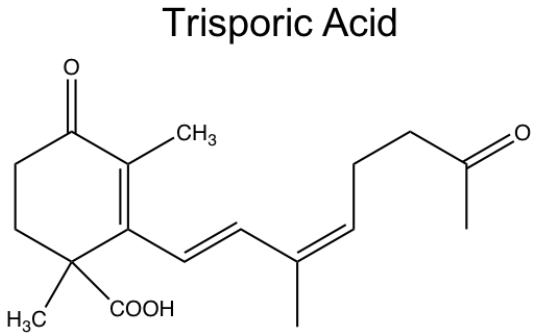


Figure 4.3 *– Chemical structure of trisporic acid, the sex hormone of* Mucor *species, initiating zygophore formation in both mating partners ($C_{18}H_{24}O_4$; MW 304 g/mol).*

In more recent times, research has centred on the molecular-genetic background and control of sex hormone production in *M. mucedo*. Two HMG-domain transcription factor genes, termed *sexM* and *sexP*, have been shown to determine sexual identity in *M. mucedo*, and transcriptional analysis has been undertaken of cultures stimulated by trisporoid pheromones (Wetzel *et al.*, 2012).

4.1.4 Sex in the Ascomycota

4.1.4.1 α and a factor sex pheromones

Within the Ascomycota, fungal sex hormones have been studied particularly within the yeast *S. cerevisiae* (Stötzler and Duntze, 1976; Betz *et al.,* 1977). In *S. cerevisiae*, mating type is determined by the presence of a *MAT* α or *MATa* genes. The yeast can grow in either a haploid or diploid form. The diploid phase is initiated by the fusion of yeast cells of complimentary mating types (Steensels *et al.*, 2014).

The first observations of potential sexual hormones in *S. cerevisiae* were in 1956, when it was shown that a diffusible factor produced from α cells was able to act on **a** cells to initiate a sexual cycle (Levi, 1956). This factor, termed α factor, inhibited cell division in **a** cells at the G1 phase of the cellular cycle (Bücking-Throm *et al.*, 1973). Meanwhile, **a** cells were shown to produce a reciprocal pheromone, termed **a** factor, which has a direct effect on α cells (Strazdis and Mackay, 1983; Merlini *et al.,* 2013). In addition to arrest of the cell cycle these pheromones triggered development of cellular projections (shmooing) to aid in cell fusion, and activated a number of genes necessary for conjugation to occur (Lockhart *et al.*, 2003; O'Reilly *et al.*, 2012).

Isolation of α factor from yeast cultures initially revealed it to be a low molecular weight peptide (Lockhart *et al.*, 2003). Further analysis of extractions from isogenic α yeast cells by Stötzler and Duntze in 1976 revealed that there were actually four structurally related and active α factor isolates, approximately 10-12 amino acids in length (**Figure 4.4**) (Stötzler and Duntze, 1976). It was determined that three of the structures isolated were produced from a tridecapeptide progenitor pheromone. Two pheromone precursor genes were identified as responsible for the production of α factor, termed *MFα1* and *MFα2* (Caplan and Kurjan, 1991).

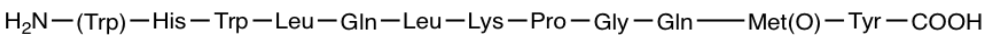


Figure 4.4 *– General amino acid sequence of the α factor peptide from* Saccharomyces cerevisiae.

Similarly, *a* factor hormone was determined to be a hydrophobic dodecapeptide, with the definitive chemical structure identified through the use of mass spectrometry (Betz *et al.,* 1977). This revealed the presence of both a farnesyl and carboxyl methyl ester modification on the oligopeptide, responsible for the hydrophobic properties *a* factor (Anderegg *et al.*, 1988) (**Figure 4.5**). Genetic studies revealed two pheromone precursor genes responsible for the production of *a* factor in yeast cells, *MFa1* and *MFa2* (Michaelis and Herskowitz, 1988).

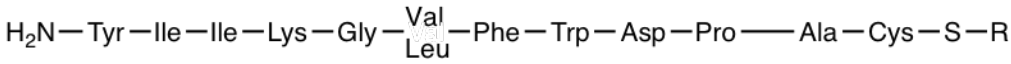


Figure 4.5 *– General amino acid sequence of the* ***a*** *factor peptide from* Saccharomyces cerevisiae.

Subsequent studies into the activity of α and **a** factor in *S. cerevisiae* have provided key insights into the nature of how cells respond to external signals, involving MAPK signalling cascades (Bardwell, 2005). Moreover, studies of the pheromones in *S. cerevisiae* have served as a model to understand how modification and processing of peptides occurs in eukaryotic cells, demonstrating the importance of the discovery of α and **a** factor.

Incredibly, research into α factor has also revealed functional links between the animal and fungal kingdoms, with α factor demonstrated to stimulate the release of luteinizing hormone from gonadotrophs (Loumaye *et al.*, 1982).

Studies of the Pezizomycotina, including genome analysis, have revealed that the model established for *S. cerevisiae* holds true throughout the sub phylum Ascomycotina. Isolates of complimentary *MAT1-1* and *MAT1-*

2 mating type have been shown to produce either α or **a** factor pheromone, respectively, which resemble the α and **a** factors of *S. cerevisiae* albeit with different peptide compositions (Debuchy *et al.,* 2010). Pheromones have even been shown to be necessary for sexual development in homothallic species such as *A. nidulans* (Dyer and O'Gorman 2011). Furthermore, basidiomycete fungal species have been shown to produce 10 to 15 amino acid long lipopeptide pheromones with a similar structure to the identified in *S. cerevisiae*, such as the a1 and a2 factor in the basidiomycete *Ustilago maydis*; and tremerogen A-9291-I in *Tramella brasiliensis* (Bölker *et al.*, 1992; Spellig *et al.*, 1994; Ishibashi *et al.*, 1984).

4.1.4.2 Psi factor

In 1987, research conducted on mutants of *A. nidulans* unable to sporulate asexually, but which retained the ability to sexually reproduce, identified several solvent-extractable compounds (Champe *et al.,* 1987). From these compounds, biochemical purification identified a hormone-like factor able to inhibit asexual sporulation in *A. nidulans* and trigger initiation of the sexual cycle earlier than normal. This factor was named precocious sexual inducer (psi) (Champe and El-Zayat, 1989). Further biochemical analysis of psi factor revealed it to be composed of a mixture of oxylipin molecules, characterised as primarily hydroxylated linoleic (18:2), oleic (18:1) and linolenic (18:3) acid, which were named psiα, psiβ and psiγ, respectively (Mazur *et al.*, 1990; Calvo *et al.,* 2001). The structures of the presumed precursors oleic, linoleic and linolenic acid are shown below (**Figure 4.6**).

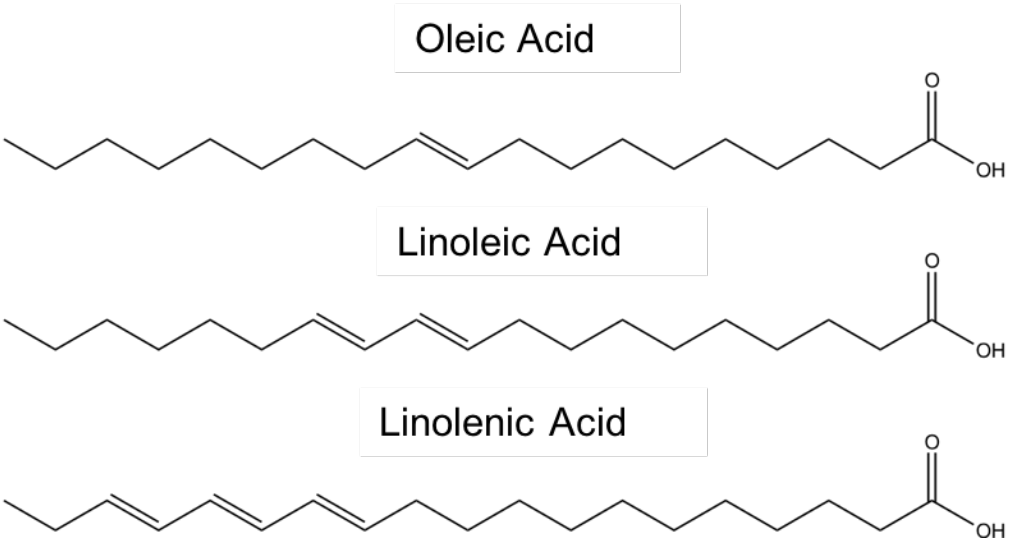


Figure 4.6 – *Chemical structure of fatty acids thought to be Psi factor precursors: psiβ derived from oleic acid ($C_{18}H_{34}O_2$; MW 282.468g/mol); psiα derived from linoleic acid ($C_{18}H_{32}O_2$; MW 280.452 g/mol; and psiγ derived from linolenic acid ($C_{18}H_{30}O_2$; MW 278.436 g/mol).*

Oxylipins are long chain fatty acids that are known to have important biological functions as signalling molecules throughout the eukaryota (Calvo *et al.*, 1999; Funk, 2001; Feussner and Wasternack, 2002). Additionally, psiα, psiβ and psiγ were found to have alternate psiA, psiB and psiC forms, dictated by the position of the hydroxyl group on the fatty acid backbone (Mazur *et al.*, 1991). Furthermore, it was found that psi factor psiAα has a function in inhibiting sexual sporulation, whereas psiBα and psiCα stimulated sexual sporulation (Champe and El-Zayat, 1989; Champe *et al.,* 1987). This led to the proposal that the relative levels of each psi factor in a fungus would determine whether sexual or asexual reproduction was more prominent. The structures of psiAα, psiBα and psiCα are shown below (**Figure 4.7**).

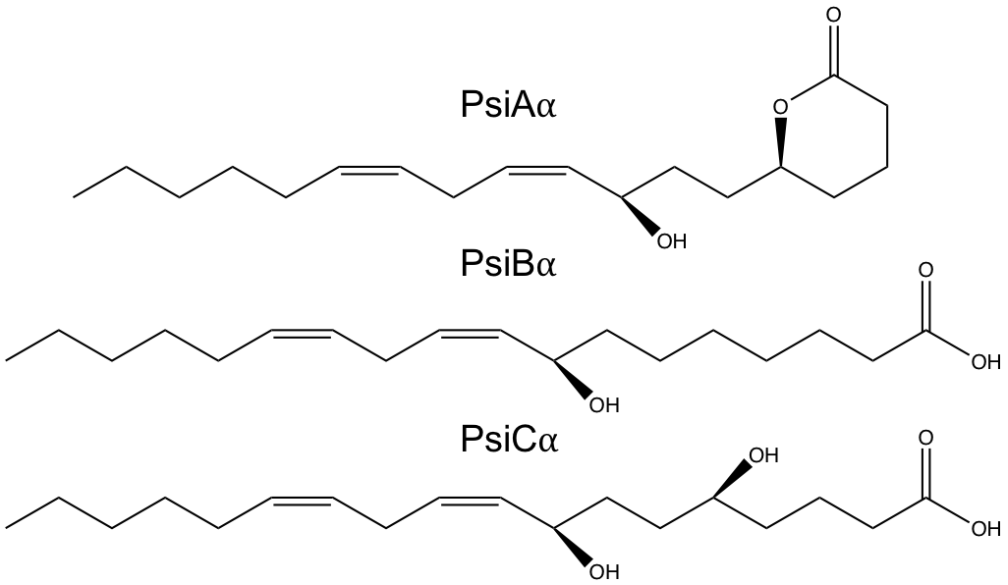


Figure 4.7 *– Chemical structure of hormones psiAα, psiBα and psiCα from* A. nidulans*, which have a key role in regulation of sexual development.*

Investigations have been undertaken to try and determine the genetic basis of psi factor production, leading to the discovery of PpoA, a dioxygenase protein. Deletion of *ppoA* decreases levels of psiBα, leading to increased asexual sporulation and reduced sexual spore development in *A. nidulans*. Overexpression of *ppoA*, however, resulted in a dramatic six-fold increase in sexual sporulation in comparison to asexual sporulation (Tsitsigiannis *et al.,* 2004b). Discovery of the function of PpoA in *A. nidulans* was followed by the identification of PpoC and its role as a dioxygenase contributing to the production of oleic acid derived psiBβ. By contrast to deletion studies of *ppoA*, the deletion of *ppoC* resulted in decreased levels of psiBβ and an increase in sexual spore development in comparison to asexual spore production (Tsitsigiannis *et al.*, 2004a). Interestingly, the discovery of yet another dioxygenase PpoB in *A. nidulans* further complicated the mechanisms controlling sexual spore development (Tsitsigiannis *et al.*, 2005). It was found that this dioxygenase also contributed to the production of psiBβ, similar to *ppoC*. However, deletion of *ppoB* led to a decrease in psiBβ levels and increase in asexual spore development in comparison to sexual

spore production. This was in contrast to the effect seen following *ppoC* deletion.

Phylogenetic analysis of the *ppo* genes has revealed their presence throughout the Ascomycota and Basidiomycota, indicating their likely important functions in many fungi (Tsitsigiannis *et al.*, 2005). Within the aspergilli*, ppo* genes have also been identified in *A. fumigatus* and *Aspergillus niger*, potentially consistent with sexual reproduction within these species (Garscha *et al.*, 2007; Wadman *et al.*, 2009).

4.1.4.3 SF morphogens (sex factor)

Another key example of a fungal sex hormone in the Ascomycota is sex factor (SF) from *P. brassicae* (Siddiq *et al.*, 1992). This species is known to cause light leaf spot on winter oilseed rape and production of apothecia sexual structures was formally described in 1978 (Rawlinson *et al.*, 1978). In 1984, two mating types were identified in *P. brassicae* leading to its confirmation as a heterothallic species (Ilott *et al.,* 1984). In 1986 the first sexual morphogen from this species, later to be termed SF, was extracted using ethanol (Ilott *et al.,* 1986). In this study, it was shown that addition of the ethanol extract to cultures containing a single mating type led to the production of sterile premature apothecia and suppressed asexual sporulation. By contrast, premature apothecia production could not be induced when extracts were applied from non-sexual (single mating type) cultures.

Further studies by Siddiq *et al.* (1992) agreed with previous findings, that application of SF led to a decrease in asexual sporulation of *P. brassicae* in a dose-dependent manner. Siddiq *et al.* (1992) further determined that applying SF to mating cultures not only increased the number of apothecia produced, but also decreased their maturation time. Interestingly, SF application was show to be most effective during a critical period of 3 to 6 days of growth post inoculation. These results indicate a certain time period in which factors involved with sexual development are being highly expressed.

Additionally, as discussed in Chapter 1 (section **1.1.5**) a putative MAT1-1-4 protein produced by *P. brassicae* contains a metallotheinein domain. It has been suggested that expression of this gene is initiated due to leaf senescence, conditions suitable for apothecia production (Singh *et al.,* 1999; McCartney and Lacey, 1990). However, this has yet to be tested. Identification of SF has also led to the identification of proteins involved in sexual development in *P. brassicae*. The protein 'sex factor induced 1' (SFI1) was found to be induced in both *MAT1-1* and *MAT1-2* cultures when SF was applied to the fungal cultures and is thought to have an important role within the developing apothecia (Ashby, 1998).

It could therefore be hypothesised that due to the ability of SF to inhibit asexual reproduction and induce sterile apothecia development in single mated cultures, agricultural application of hormones may have great potential for use in the control of fungal plant diseases (Dyer *et al.,* 1992). For example, by applying SF to plants infected with *P. brassicae,* it may be possible to prevent asexual growth on the plant material and thereby prevent dispersal of the pathogen by asexual conidia, growth instead being diverted to the production of sterile apothecia. Additionally, application of SF from *P. brassicae* may have potential to repress asexual sporulation in a number of other species i.e. use in plant disease control. This is supported by findings that application of SF altered sexual development in 30 other species of fungi (Chamberlain *et al.*, 1995).

4.1.5 Aims

Work in the present chapter aims to gain further knowledge about the involvement of sex hormones in fungal sexual development, and their possible exploitation.

Firstly, work was undertaken to identify and characterise fungal sex hormones from *N. fischeri*, with the hope that it might be possible to obtain sex hormones produced by this species that would have the ability to 'cross talk' and induce sexual sporulation or inhibit asexual sporulation in closely

related species i.e. be of applied use. Work focussed on organic-solvent extracts from sexually reproducing or control cultures, given that previous studies with sex morphogens have indicated these to be lipoidal in nature (Dyer *et al.,* 1993; Mazur *et al.*, 1991; Chamberlain *et al.*, 1995).

Secondly, work was undertaken to explore the function of Ppo proteins in *A. fumigatus.* Through a research collaboration with Nancy Keller and Greg Fischer (University of Wisconsin, USA) a series of mutant strains of *A. fumigatus* with either deletions or alterations in levels of *ppo* gene expression were obtained. It was hoped to use these to determine the role of Ppo proteins in sexual reproduction in this heterothallic species, to compare results with previous studies with the homothallic *A. nidulans.* In parallel, mutant strains of *A. fumigatus* with alterations in lipoxygenase (LOX) gene expression were also obtained from the same collaborators. LOX genes have been identified in a number of species such as *Penicillium roqueforti* (Perraud and Kermasha, 2000) and *Aspergillus flavus* (Horowitz Brown *et al.*, 2008). Deletion of Af*lox* was shown to result in increased numbers of sclerotia and decreased conidia production in *A. flavus* when growth plates were inoculated with a high density of conidia. This differs from the normal situation, where high spore density inoculations induce higher numbers of conidia (Horowitz Brown *et al.*, 2008). Given that sclerotia are a precursor to sex in *A. flavus* (Horn *et al.,* 2009a), it was therefore, of interest to evaluate the *ppo* and *lox* mutants from both oxygenase sub-categories for their role in sexual reproduction of *A. fumigatus*.

4.2 Methods

For media composition, refer to **Appendix**.

It should be noted for all experiments described in this section that Tween 80 (Polysorbate 80) is derived from polyethoxylated sorbitan and oleic acid, with possibly palmitic and linoleic acid also present. As discussed previously (**4.1**), these are precursor molecules of the psi factor hormones of *A. nidulans*. Therefore, care was taken to control for the levels of Tween 80 in our experimental setup described below.

4.2.1 Selection of model fungal species for hormone extraction

In order to attempt to identify and extract fungal sex hormones, it was first necessary to identify model fungal systems for studies. Various criteria were used in preliminary screening work. These included that, ideally, studies should include species forming a diverse range of sexual structures (e.g. perithecia, cleistothecia, apothecia); plentiful production of sexual structures *in vitro*, which would be presumed to be correlated with increased hormone production; the ability to consistently induce the sexual cycle under controlled conditions; easily identifiable sexual structures; and that species would exhibit a homothallic reproductive cycle, thereby reducing complications of mating procedures needed for heterothallic taxa. Initial work involved much consultation of the existing scientific literature to identify suitable species. Following this, a number of species were identified for possible use in hormone isolation experiments. These comprised: *Neosartorya fischeri* and *Eurotium repens* (cleistothecia producers) (Ashour, 2014); *Fusarium graminearum* (perithecia producers) (Hallen *et al.*, 2007); *Pyronema confluens* and *Sclerotinia sclerotiorium* (apothecia producers) (Nowrousian and Kück, 2006; Schwartz and Steadman, 1978). It was also a requirement that sexual reproduction could be reliably induced *in* vitro, and that a bioassay for hormone activity could be developed based on different modes of reproduction.

4.2.2 Culture storage, collection and revival

In order to use these species effectively for hormone extractions, highly sexually active strains were required, given the risk that levels of sexual fertility can often decrease significantly under laboratory sub culture (Dyer and Paoletti, 2005; Ashour, 2014). In order to identify these, where possible contact was made with external researchers working on the sexual reproductive cycle of the selected species. Furthermore, where work had already been performed at the University of Nottingham on a chosen species, strains were selected based on previous experience of high fertility (Ashour, 2014). In all other cases, cultures were ordered from the CBS-KNAW culture collection (Westerdijk Fungal Biodiversity Institute, Netherlands).

Upon receipt of strains, fungi were routinely subcultured onto malt extract agar (MEA) before aliquots were suspended in 20% glycerol (1:5 in sterile distilled water) and frozen under liquid nitrogen for long-term storage. For revival of fungal samples, stocks were taken from the liquid nitrogen collection and allowed to thaw before inoculation onto malt extract agar (MEA) 9 cm plates (Thermo Scientific, USA) and grown at 28 °C. The species and strains selected are shown below (**Table 4.1**).

Table 4.1 *– Table of sexual species identified as candidates for hormone extraction and appropriate strains of each species*

Species	**Strain**
Neosartorya fischeri	53-15, 53-16
Eurotium repens	51-4
Fusarium graminearum	PH-1
Pyronema confluens	64-2
Sclerotinia sclerotiorium	103-1

4.2.3 Conditions for induction of sexual reproduction

In order to verify that the fungal species chosen could reliably produce sexual structures in a controlled environment, each species was grown under conditions previously reported to promote sex. Conditions used to induce sex for each species are shown below:

- *N. fischeri* – Incubated for 10 days in darkness on MEA at 28 °C. Point inoculated at the centre of 5 cm diameter Petri dishes (SLS, UK) with 500 spores (5 µl of a 1×10^5 spores/ml of 0.05% Tween 80 spore [Sigma-Aldrich, USA] suspension), with plates sealed with Parafilm.
- *E. repens* – Incubated for 7 days in light on M40Y at 28 °C. Point inoculated at the centre of 5 cm diameter Petri dishes with 500 spores (5 µl of a 1×10^5 spores/ml of 0.05% Tween 80 spore suspension).
- *F. graminearum* – Incubated for 10 days on carrot agar in 5 cm diameter Petri dishes under bright fluorescent lights at 20 °C, centre inoculated with 500 spores (5 µl of a 1×10^5 spores/ml of 0.05% Tween 80 spore suspension). During incubation, once the mycelium reached the outer edge of the Petri dish, aerial mycelia were then removed with a sterile toothpick. Once removed, 1.0 ml of sterile 2.5% Tween 60 is distributed on the surface of the media with a sterile L-shaped spreader (STARLAB, Germany). After this, the fungal culture is returned to the lit incubator until perithecia have been produced.
- *P. confluens* – Incubated for 10 days in constant light on synthetic growth media at 25 °C, inoculated with a 0.7 cm agar plug of a previous fungal culture on a 5 cm diameter Petri dish.
- *S. sclerotiourium* – Incubated for 4 weeks on oatmeal agar (OA) at 20 °C to induce sclerotia production, centre inoculated with 500 spores (5 µl of a 1×10^5 spores/ml of 0.05% Tween 80 spore suspension) on a 5 cm diameter Petri dish.

After the appropriate incubation period, cultures were observed under a dissection microscope to identify the possible formation of sexual structures, which were then counted visually to determine viability for use in

hormone isolations. In addition to solid media, relevant liquid media was used for all species to test for any differences in sexual reproduction.

4.2.4 Control non-sexual culture conditions

In order for a bioassay for sex hormone activity to be developed, it was necessary to identify conditions under which growth but no sexual reproduction occurred in the study species. This would therefore allow the 'control' extractions to be made from such negative 'non-sexual' cultures as a control for comparison to extracts from sexually reproducing cultures. In order to act as an accurate control, culture conditions were identified which was as similar as possible to those used to induce sexual reproduction, but which lacked any sexual reproduction. The culture conditions for induction of solely asexual reproduction are shown below:

- *N. fischeri* – Incubated for 10 days in darkness on *Aspergillus* complete medium (ACM) at 28 °C, centre inoculated with 500 spores (5 µl of a 1×10^5 spores/ml of 0.05% Tween 80 spore suspension) on a 5 cm diameter Petri dish and sealed with Parafilm (Bemis, USA).
- *E. repens* – Incubated for 10 days in darkness on *Aspergillus* complete medium (ACM) at 28 °C, centre inoculated with 500 spores (5 µl of a 1×10^5 spores/ml of 0.05% Tween 80 spore suspension) on a 5 cm diameter Petri dish.
- *F. graminearum* – Incubated for 10 days on carrot agar in a 5 cm diameter Petri dish in darkness at 20 °C, centre inoculated with 500 spores (5 µl of a 1×10^5 spores/ml of 0.05% Tween 80 spore suspension).
- *P. confluens* – Incubated for 10 days in darkness on synthetic growth media at 25 °C, inoculated with a 0.7 cm agar plug of fungal culture on a 5 cm diameter Petri dish.
- *S. sclerotiourium* – Incubated for 4 weeks on ACM at 20 °C to induce sclerotia production, centre inoculated with 500 spores (5 µl of a

1×10^5 spores/ml of 0.05% Tween 80 spore suspension) on a 5 cm diameter Petri dish.

Post-sexual growth, cultures were observed under a dissection microscope to identify any sexual structures. In addition to solid media, relevant liquid media was used for all species to test for any differences in asexual reproduction.

4.2.5 Hormone extraction

Two different methods of hormone extraction were utilised:

- **Chloroform:methanol**

The chloroform:methanol hormone extraction was performed in accordance with that of Dyer *et al.* (1993) based on the methods of Christie (1982). For sexually reproducing cultures, crude lipid was extracted from both the supernatant and biomass of static liquid MEA cultures of *N. fischeri* grown in 5 cm Petri dishes (total of 40 dishes) for 10 days at 28 °C in darkness. The collected tissue was then flash frozen and lyophilised (ModulyoD, Thermo) for 18 hours to remove excess moisture before being homogenised in chloroform (Fisher Scientific, USA): methanol (Fisher Scientific, USA) (2:1 v/v) (30 ml solvent g^{-1} tissue) for 5 mins using an UltraTurrex at 8000 rpm. The homogenate was filtered using Whatman no. 3 filter paper (Sigma Aldrich, UK) to remove excess solids and subsequently filtered through Whatman no.1 filter paper (Sigma Aldrich, UK).

The filtrate was then evaporated to dryness in a rotary evaporator at 35 °C (Büchi Rotavapor R124) before being resuspended in 12 ml chloroform. The resuspended lipid extract was filtered through glass wool into four pre-weighed 4 ml glass vials. Chloroform was then evaporated using a SpeedVac (Savant SPD121P, Thermo Scientific) at 35 °C for approx. 2 hrs . From this point the lipid extract was resuspended at a concentration of 33 mg crude extract residue ml^{-1} chloroform, which was stored under nitrogen gas at -20 °C in a glass vial. The same method was used to extract crude lipids from control cultures of *N. fischeri* incubated in liquid ACM broth for 10 days at 28

°C in darkness. It is important to note that although primarily aimed at extraction of lipids, this method will yield extracts that also include some non-lipid molecules as well.

For use in hormone activity bioassay experiments, the crude extract residue was diluted to 0.33, 3.3, 33, 330 μg crude extract residue ml^{-1} chloroform and stored at -20 °C in glass vials.

- **Ethyl acetate:ethanol**

The ethyl acetate (Fisher Scientific, USA): ethanol (Fischer Scientific, USA) extraction method was performed in accordance to Lin *et al.* (2004). Similar to the chloroform:methanol extraction method, crude lipid was extracted from both supernatant and biomass of static liquid MEA cultures of *N. fischeri* grown in 5 cm Petri dishes (total of 40 dishes) for 10 days at 28 °C in darkness. The collected tissue was then flash frozen and lyophilised for 18 hours to remove excess moisture before being homogenised in ethyl acetate/ethanol (1.8:0.8) (30 ml solvent g^{-1} tissue) for 5 mins using an UltraTurrex at 8000 rpm. The homogenate was filtered using Whatman no.3 filter paper (Sigma Aldrich, UK) to remove excess solids and subsequently filtered again through Whatman no. 1 filter paper (Sigma Aldrich, UK).

The filtrate was then separated into organic and aqueous phases via the addition of distilled water containing potassium chloride (0.88 % KCl w/v). Post separation, the organic phase was removed with a glass pipette and evaporated to dryness in a rotary evaporator at 35 °C before being resuspended in 12 ml ethyl acetate. The resuspended lipid extract was then separated into four pre-weighed 4 ml glass vials. Ethyl acetate was then evaporated using a SpeedVac at 35 °C for approx. 2 hrs. From this point the lipid extract was resuspended at 33 mg crude extract residue ml^{-1} chloroform, which was stored under nitrogen gas at -20 °C in a glass vial. The same method was used for crude extractions from control cultures of *N. fischeri* incubated in liquid ACM broth for 10 days at 28 °C in darkness. It is important to note that although primarily aimed at extraction of lipids, this method will again yield extracts that also include some non-lipid molecules as well.

For use in hormone activity bioassay experiments, the lipid residue was diluted to 0.33, 3.3, 33, 330 μg crude extract residue ml^{-1} ethyl acetate and stored at -20 °C in glass vials.

4.2.6 Hormone bioassay

Two different methods were used to assay the bioactivity of the crude lipid extracts as described below.

- **Paper discs**

The first hormone bioassay used to test the activity of *N. fischeri* crude lipid extracts was based on a method described by Dyer *et al.* (1993). *N. fischeri* spore suspensions were prepared to a final concentration of $1x10^6$ spores ml^{-1} using a haemocytometer and 50 μl was spread plated onto 5 cm diameter Petri dishes containing solid ACM or MEA media. Crude extracts were diluted in chloroform to concentrations ranging from 0.33-330 μg crude extract residue ml^{-1} chloroform. 10 μl of each diluted crude extract (corresponding to 3.3-3,300 ng total extract) was added to an individual 10 mm diameter Whatman no. 1 filter paper disc (Sigma Aldrich, UK) and allowed to dry completely before being placed in the centre of the inoculated culture.

Inoculated *N. fischeri* cultures containing the hormone filter disc were sealed with one layer of Parafilm and incubated at 28 °C in darkness to simulate working conditions for sexual reproduction. During this incubation period, the cultures were scored for the ability to form sexual structures, cleistothecia (defined as approximately 100 μm in size and cream in colour) at 0, 2, 4, 6, 8 and 10 days. Asexual *N. fischeri* cultures were scored on the number of conidia within the treatment area (0.7 cm^2). In order to score conidia, a 1 cm^2 area containing the treatment area was extracted from the agar plate and conidia put into 0.05% Tween 80 suspension. A chloroform-only paper disc control was included in all treatments, in order to eliminate confounding factors.

- **Direct inoculation**

In order to minimise any effect the paper filter disc may have on sexual structure formation, an alternative direct inoculation method was used. Using the same methodology, 10 μl of crude extract ranging from 0.33-330 lipid extract residue ml^{-1} chloroform (corresponding to 3.3-3,300 ng total extract) was inoculated onto the centre of individual ACM and MEA agar plates. Once dried, 50 μl of a $1x10^6$ *N. fischeri* spore suspension was spread plated onto the agar surface. Inoculated *N. fischeri* cultures underwent the same method of assessing the activity of crude extract as above.

4.2.7 Hormonal 'cross talk'

To identify whether putative fungal hormone extracts exhibited any cross-species activity, the impact on growth of several other fungal species were tested using the 'direct application' methods described above. In the case of homothallic species, any possible increase (or decrease) in production of sexual structures was evaluated. The ability of the crude lipid extract to inhibit asexual reproduction in single mating-type cultures, via scoring of conidia numbers, was tested in heterothallic species. In order to score conidia, a 1 cm^2 area containing the treatment area was extracted from the agar plate and mixed thoroughly in 1 ml 0.05% Tween 80 suspension. The species used to test for possible hormonal activity were: *A. niger*; *A. fumigatus*; and *A. nidulans*. These were chosen due to their phylogenetic relatedness to *N. fischeri*, and/or previous use as models to determine sex hormone activity.

For each species, different growth conditions were used to induce asexual reproduction and sexual reproduction. Each culture was treated in the following ways. Either: 330 ng of crude lipid extract residue from a sexual culture was applied to the centre of the culture prior to inoculation; 330 ng of crude lipid extract residue from asexual culture was applied to the centre of the culture prior to inoculation; or there was no application to the culture.

- *A. niger* – grown on Czapek's yeast autolysate (CYA) for 10 days at 25 °C in darkness on 9 cm Petri dishes, in order to induce sclerotia production. Strain 8-161 was used.
- *A. fumigatus* – grown on oatmeal agar (OA) for 10 days at 30 °C in darkness on a 9 cm Petri dish to replicate growth conditions for sexual reproduction. Supermater strains 47-267 and 47-55 were used.
- *A. nidulans* – grown on ACM for 7 days at 37 °C in darkness on a 9 cm Petri dish to replicate growth conditions for sexual reproduction. Strain 2-231 was used.

For each hormone bioassay experiment, a negative control using chloroform with no extract residue present was used. After the appropriate incubation time, the presence of sexual structures and/or absence of asexual reproduction were evaluated using a dissection and light (40X) microscope. Asexual spore and sexual structure numbers were counted within the 1 cm^2 area where crude lipid extract was inoculated.

4.2.8 *Aspergillus fumigatus* oxygenase mutants - strains used and media supplementation

MAT1-2 parent strain Af293 and mutants derived from this strain, received from Nancy Keller and Greg Fischer (University of Wisconsin, USA), were studied in order to determine any impact on sexual development due to gene deletion or modification of the *ppo* and *lox* genes. To account for loss of activity from the *pyrG* and *argB* gene replacement in the Af293 derived mutants, where appropriate oatmeal agar (OA) was supplemented with either 0.2% (w/v) uracil and 20 mM uridine in mutants containing a *pyrG* gene replacement; 0.2% (w/v) arginine in mutants containing an *argB* gene replacement; and a combination of uracil/uridine supplementation and arginine in mutants containing both *pyrG* and *argB* gene replacements. The *A. fumigatus* strains used are listed below (**Table 4.2**).

Table 4.2 – A. fumigatus *strains tested for sexuality, listing the* MAT1-2 *wild type parent (Af293) and derived mutant strains.*

Strain	*MAT* Type	Genotype
47-267	*MAT1-1*	Wild type supermater (Sugui 2014)
47-55	*MAT1-2*	Wild type supermater (O'Gorman *et al.* 2009)
Af293	*MAT1-2*	Wild type
TJW55.2	*MAT1-2*	*pyrG1, A. parasiticus pyrG*
TJMP28.4	*MAT1-2*	*pyrG1, argB1, A. fumigatus argB and pyrG*
TDCW1.13	*MAT1-2*	*pyrG1, ΔppoA:: A. parasiticus pyrG*
TDWC2.4	*MAT1-2*	*pyrG1, ΔppoB:: A. parasiticus pyrG*
TDWC4.17	*MAT1-2*	*pyrG1, argB1, ΔppoC:: A. nidulans argB, A. parasiticus pyrG*
TJMP143.28	*MAT1-2*	*pyrG1, pyrG::gpda(p):ppoA*
TJW62.2	*MAT1-2*	*ΔppoABC* by RNAi with *A. parasiticus pyrG* replacement
TJMP38.2	*MAT1-2*	*pyrG1, argB1, ΔloxA:: A. parasiticus pyrG, A. fumigatus argB*
TJMP39.6	*MAT1-2*	*pyrG1, argB1, ΔloxB:: A. parasiticus pyrG, A. fumigatus argB*
TTRD12	*MAT1-2*	*pyrG1, argB1, ΔloxA:: A. parasiticus pyrG, ΔloxB:: A. fumigatus argB*
TGJF15.12	*MAT1-2*	*pyrG1; gdpa(p):loxA* in pJMP7, *A. parasiticus pyrG*
TGJF1.5	*MAT1-2*	*pyrG1; gdpa(p):loxB* in pJMP7, *A. parasiticus pyrG*
TGJF20.19	*MAT1-2*	*pyrG1, loxB(t):loxB:hbd(p):loxA:loxA(t):pyrG*, histone H3-H4 promoter from *A. nidulans, A parasiticus pyrG*

4.2.9 Sexual crosses

Each strain was sexually crossed with the sexually compatible *MAT1-1* supermater strain 47-267 (Sugui *et al.*, 2011). Strains were grown for 7 days on ACM. Spore suspensions were then made and adjusted to a final concentration of 5×10^5 conidia ml^{-1} in 0.05% Tween 80. Two aliquots of 1 μl from the *MAT1-1* isolate were point inoculated onto 9 cm diameter Petri dishes containing Odlum's Irish oatmeal agar (OA) approximately 4 cm apart

and perpendicular to aliquots of 1 μl of the *MAT1-2* isolate. The sexual crosses were then sealed with one layer of Parafilm and incubated for three months inverted at 30 °C in darkness as described by O'Gorman *et al.* (2009)(O'Gorman *et al.,* 2009). An additional *MAT1-2* strain 47-55 was also used as a control in crossing experiments to verify fertility of the *MAT1-1* supermater strain.

4.2.10 Scoring of *A. fumigatus* sexual crosses

After a three-month incubation period, each cross was examined for the production of cleistothecia. Typically, these would appear at the barrage zones between two complementary mating partners. To allow numbers of cleistothecia formed in sexual crosses to be counted accurately, it was first necessary to carefully remove superficial asexual spores from each crossing plates, because these often obscured or hid the presence of cleistothecia. A 'hoover' was therefore created which was attached to a suction airflow based on the methods of Sugui *et al.* 2013 (Sugui and Kwon-Chung *pers. Comm*; Ashton and Dyer, in press). This resulted in the superficial asexual spores being removed from the Petri dish and deposited in 60% isopropanol within the 'hoover' trap. A pipette tip was attached to the tubing of the 'hoover', cut to an appropriate size to allow the conidia to be removed but not remove the larger cleistothecia. Following suction of asexual spores from the barrage zones of each cross, total numbers of cleistothecia numbers formed on replicate Petri dishes were counted using a dissection microscope at 3X magnification (Nikon SMZ-2B, Japan).

4.2.11 Harvesting cleistothecia and single ascospore progeny

Sexual crosses were inspected using a dissection microscope within a laminar flow hood to determine the state of cleistothecia development. Where cleistothecia were found, they were isolated with a sterilised fine point needle. Sterile distilled water was then used to wash the cleistothecia of excess conidia, rolling the cleistothecia through the liquid. 100 μl 0.05%

Tween 80 was then aliquoted into sterile 2 ml Eppendorf tubes, and cleistothecia were then added to the 2 ml Eppendorf tube (one cleistothecia/Eppendorf tube) and crushed against the side. The volume of the solution was then adjusted to 2 ml by adding 1.9 ml 0.05% Tween 80. The ascospore suspension was vortexed for 1 min to ensure all the ascospores had been released and that asci had been disrupted. Using a heat block at 70 °C, the ascospore suspension was heat treated for 1 hour to kill any remaining conidia. Post heat treatment, 100 μl aliquots of the ascospore suspension were spread plated onto 2% w/v malt extract agar (MEA) plates and incubated 36 h at 28 °C. Following this incubation period, single germinating ascospores were identified via the use of a dissection microscope within a laminar flow hood. Upon identification, the germinating ascospores were isolated by carefully picking them up using a sterilised fine point needle or flattened platinum wire. Each isolated ascospore was transferred onto a 2% w/v MEA Petri plate and incubated at 28 °C until fully grown.

4.2.12 RAPD PCR

To ensure that recombination had occurred within the progeny of the sexual cross, random amplification of polymorphic DNA (RAPD)-PCR was performed, as described by Murtagh *et al.* (1999). A total reaction volume of 25 μl was used per PCR containing: 1x DyNAzyme II DNA polymerase reaction buffer; 500 μM of each dNTP; 50 μmol of a single 10-base pair primer; between 0.1-1 ng of DNA template; 1 U DyNAzyme II DNA polymerase; and adjusted to 25 μl using sterile distilled water. The primers used to assess recombination in *A. fumigatus* were previously identified as suitable to reveal polymorphisms in *A. fumigatus* (**Table 4.2**; data not shown). The PCR protocol used for RAPD analysis was as follows: Initial denaturation of 5 mins at 95 °C; 45 cycles of 30 secs at 93 °C at a ramp rate of 30 °C min^{-1}, 40 secs at 37 °C at a ramp rate of 30 °C min^{-1}, 1 min 20 secs at 72 °C at a ramp rate of 20 °C min^{-1}; followed by a final elongation stage for 5 mins at 72 °C (Dyer and Paoletti, 2005). The

products of the RAPD-PCR were resolved on 1.5% TBE agarose gels and visualised by ethidium bromide staining (Sigma-Aldritch, USA) and a UV transilluminator (Bio-Rad, USA). Resultant bands were then graded on size and intensity and scored accordingly. The RAPD profile-banding pattern for each putative progeny was compared to that of the crossing parents, and genetic similarity was determined.

4.2.13 Data analysis

All statistical analysis was performed using Graphpad Prism 7, using a one-way ANOVA statistical test with a Dunnet's multiple comparisons test and a Bonferoni post-test.

4.3 Results

4.3.1 Selection of sexual species for hormone extraction

For fungal species to be used as models for hormone extractions, it was important that the sexual and asexual life cycles were easily inducible *in vitro*. However, using the strains provided, it was found that only three species, *N. fischeri* strain 53-16, *E. repens* strain 51-4 and *F. graminearum* strain PH-1, could be induced to reproduce sexually *in vitro*. The easily identifiable sexual structures of *F. graminearum* and *N. fischeri* are shown below (**Figure 4.8**). The strains ordered from the CBS culture collection service failed to produce sexual structures, possibly due to their prolonged storage times and unknown fertility. Further inspection of the three species showed that *N. fischeri* produced significantly more cleistothecia in comparison to *E. repens* (data not shown). *N. fischeri* also had the additional benefit that by changing the growth media used (MEA or ACM) it was possible to induce either sexual or asexual reproduction, respectively, whereas in *E. repens* it was not possible to obtain solely asexual reproduction as some cleistothecia were formed under all conditions assayed. Meanwhile, although a sexual cycle could be induced in *F. graminearum*, the process was found to be unreliable for reasons unknown. Therefore, *N. fischeri* was chosen as a model species for hormone extraction experiments.

In addition, it was found that use of liquid cultures for growth in *N. fischeri* (MEA) permitted development of the sexual cycle. Therefore, to maximise total lipid extraction and minimise confounding effects solid agar may have, liquid cultures were used for further experiments.

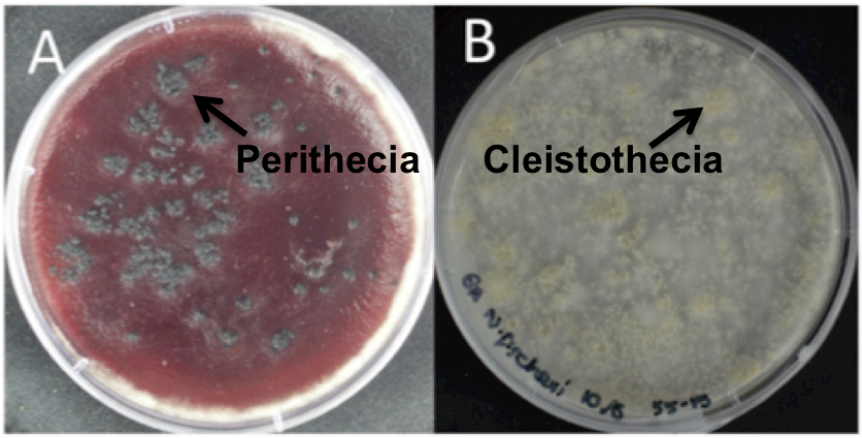


Figure 4.8 *– Sexual cultures of two fungal species: A)* Fusarium graminearum *and B)* Neosartorya fischeri*. Black arrows, showing black perithecia for* F. graminearum*, and cream-coloured cleistothecia for* N. fischeri*, indicate the sexual structures of each species*.

4.3.2 Hormone bioassay

4.3.2.1 Chloroform:methanol extractions

The chloroform:methanol extraction method was successfully used to extract crude lipids from cultures undergoing sexual reproduction, or control cultures exhibiting solely asexual reproduction. Extracts were then bioassayed for activity as follows.

- **Paper discs**

Differing amounts of crude lipid extracts from sexual or control cultures were placed on a filter paper disc to examine if the crude lipid extraction from the sexual cultures contained a sex hormone produced by *N. fischeri* cultures. The discs were added to cultures that were incubated in conditions ideal for sexual or asexual reproduction (MEA and ACM growth medium, respectively). Under conditions suitable to induce sexual reproduction, it was found that cleistothecia were formed only after 10 days on the filter paper discs. No cleistothecia were formed under conditions suitable for asexual reproduction even after 10 days. The effect of addition of the sexual or control crude lipid extractions on cleistothecial production at 10 days is shown below (**Figure 4.9**).

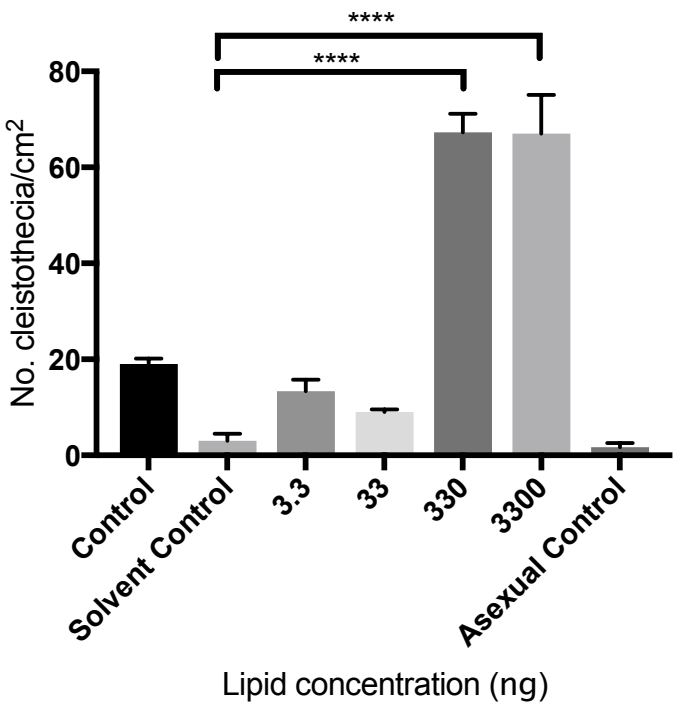


Figure 4.9 *– Analysis of the effect of methanol/chloroform extract dilutions on sexual reproduction of* N. fischeri. *Concentrations range 3.3 to 3300 ng were applied via sterile paper disc on MEA medium inoculated with* N. fischeri *strain 53.15. Statistical analysis was performed using one-way ANOVA with a Bonferoni post-test with p<0.0001 = ****. Error bars represent SEM from 3 technical replicates and 2 biological replicates.*

It was found that very few cleistothecia were formed on the solvent-only control disc, although there was abundant formation of cleistothecia elsewhere on the growth plate i.e. the presence of the filter paper disc seemed to inhibit cleistothecial formation. Production of cleistothecia was not significantly different from the paper-only control at the lowest levels of crude lipid addition (3.3 and 330 ng crude lipid residue) (**Figure 4.8**). However, the number of cleistothecia produced was increased significantly following addition of both 330 and 3300 ng crude lipid residue from sexual cultures compared to the solvent only control (One-way ANOVA; F=66.56; P<0.0001) (**Figures 4.9** and **4.10**). By contrast, addition of crude lipid extract from asexually reproducing cultures of *N. fischeri* did not show any increase in production of cleistothecia relative to solvent only paper-disc control (**Figure 4.9**).

It therefore appeared that a threshold of crude lipid extract from the sexual cultures was required in order to stimulate sexual development on the otherwise inhibitory surface of the filter paper disc.

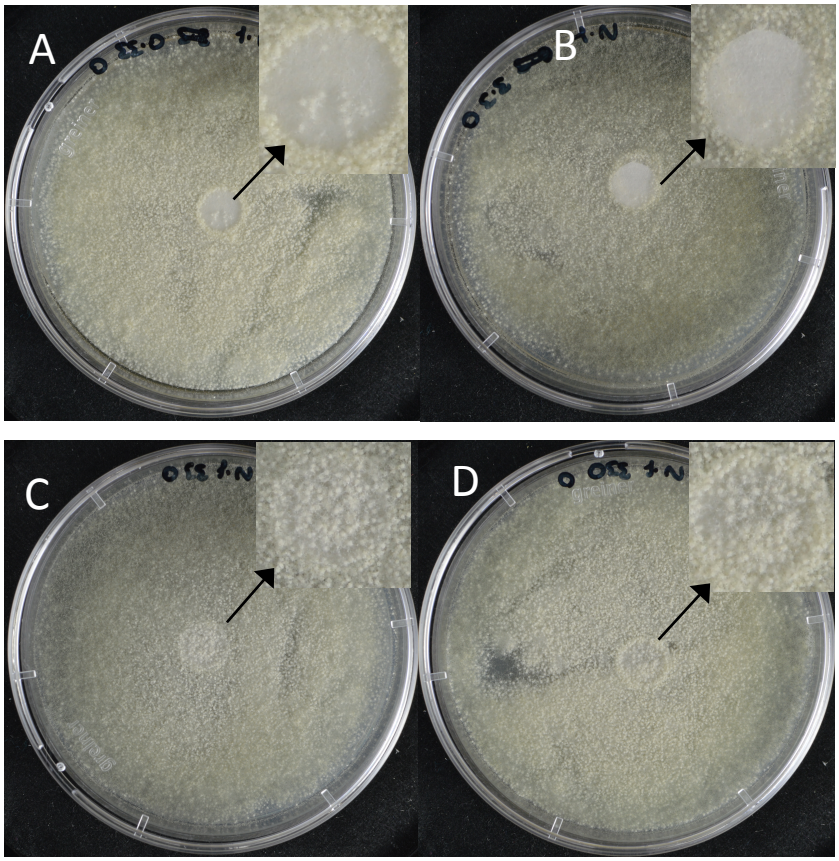


Figure 4.10 – N. fischeri *strain 53-15 cultures 10 days post treatment with crude lipid extraction from sexually active cultures. A) 3.3 ng crude lipid residue; B) 33 ng crude lipid residue; C) 330 ng crude lipid residue; and D) 3300 ng crude lipid residue. Labels on the Petri dish lid refer to ng crude lipid residue* ml^{-1}.

When scoring conidial production in asexual culture conditions for *N. fischeri*, the filter paper disc completely inhibited asexual production on its surface area. Therefore, no conidia were produced in any treatment condition.

- **Direct application**

In order to investigate if the sterile filter paper disc was having an inhibitory effect on cleistothecial production and impacting on the lipid bioassays, the activity of the extracted crude lipids was tested using an alternative 'direct application technique. Here, the crude lipid extracts from sexually

reproducing and asexual control cultures were applied onto Petri dishes containing either MEA or ACM growth medium (appropriate for sexual and asexual reproduction, respectively).

The effect on production of cleistothecia by addition of the various crude lipid extracts at 10 days post inoculation on MEA is shown below (**Figure 4.11**). No visible sexual structures were formed before this time point. No cleistothecia were produced on ACM following addition of the various crude lipid extracts under conditions suitable for asexual reproduction even after 10 days.

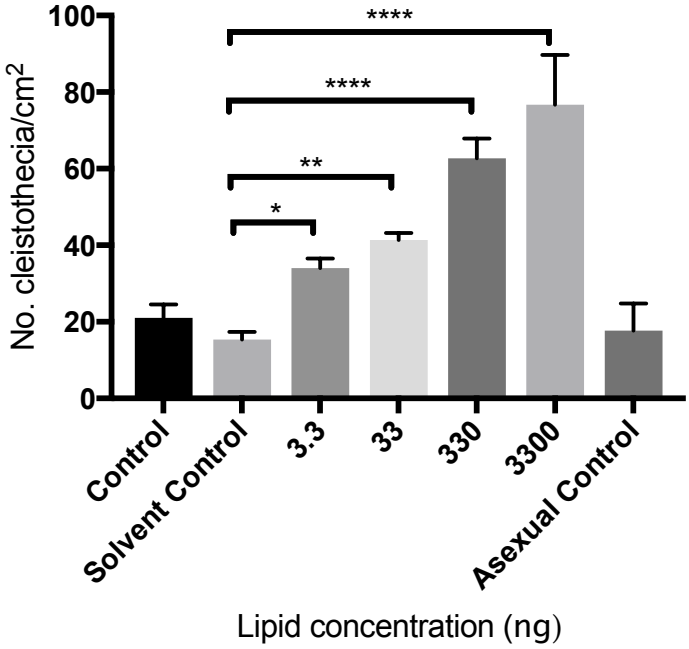


Figure 4.11 *- Analysis of the effect of methanol/chloroform extract dilutions on sexual reproduction of* N. fischeri. *Concentrations ranging from 3.3 to 3300 ng were directly applied to the center of a 4 cm Petri dish of MEA medium prior to inoculation with* N. fischeri *strain 53.15. Statistical analysis was performed using one-way ANOVA with a Bonferoni post-test with: p<0.05 = *; p<0.01 = **; p<0.001 = ***; p<0.0001 = ****. Error bars represent SEM from 3 technical replicates and 2 biological replicates.*

From **Figure 4.11**, it can be seen clearly there is a positive relationship between the addition of crude lipid extract from sexual culture and the number of cleistothecia produced, with the effect more marked than that

seen for the previous paper-disc assay. For example, while there was no difference in production of cleistothecia between the solvent control and the lowest amounts of crude lipid extract applied when using a filter paper disc (3.3 and 33 ng crude lipid residue), in this case when the crude lipid was directly applied, a significant 2-fold increase in cleistothecia production was seen (One-way ANOVA; $F=31.27$; $P<0.0001$). This data indicates that *N. fischeri* appears more sensitive to the technique using direct application of crude lipid extracts to the growth media rather than filter paper disc application, possibly due to the crude lipid extract being more easily obtainable to the fungal cultures in the former. Meanwhile, production of cleistothecia showed an approximate 3- and 4-fold increase in comparison to the solvent control when 330 and 3300 ng crude lipid residue from sexual cultures was applied, respectively i.e. there was a concentration dependent effect on sexual reproduction in *N. fischeri* according to the level of crude lipid extract applied. This was similar to the increase in number of cleistothecia produced when using the same hormone concentration with the filter paper disc assay (**Figure 4.9**). It was important to note that addition of crude lipid extracts from the control asexual *N. fischeri* cultures did not lead to any significant increase in cleistothecial production relative to the solvent control (**Figure 4.11**).

- **Direct application – conidia production in asexual conditions**

Conidial production was examined in *N. fischeri* cultures under growth conditions favouring asexual reproduction, with a range of crude lipid extract concentrations being applied in comparison to a control with no treatment (representing 3.3 ng, 33 ng, 330 ng; and 3300 ng crude lipid residue).

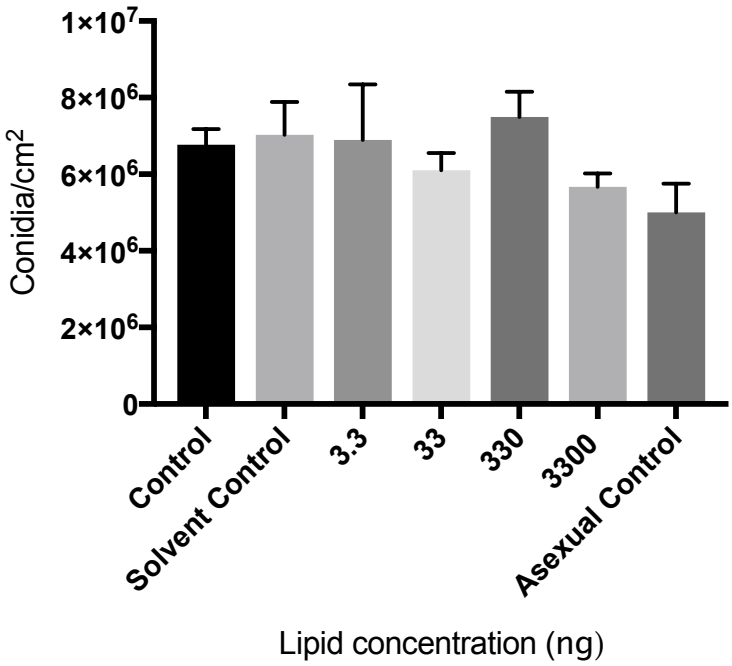


Figure 4.12 *– Analysis of the effect of methanol/chloroform extract dilutions on sexual reproduction of* N. fischeri. *Concentrations ranging from 3.3 to 3300 ng were directly applied to the center of a 4 cm Petri dish of ACM medium prior to inoculation with* N. fischeri *strain 53.15 and grown under asexually stimulating conditions. Statistical analysis was performed using one-way ANOVA with a Bonferoni post-test. Error bars represent SEM from 3 technical replicates and 2 biological replicates.*

The effect of asexual production when crude lipid extract has been applied is shown in **Figure 4.12**. There was no significant difference in the number of conidia produced between treated cultures and the untreated control. If the crude lipid extract contained an appropriate hormone, some repression of asexual reproduction might have been expected (One-way ANOVA; F=1.386, P=0.2966).

4.3.2.2 Ethyl acetate:ethanol

- **Direct application**

It was found above that filter paper discs appeared to have an inhibitory effect of on the growth of *N. fischeri*. Therefore, when testing the activity of crude lipids obtained via extraction with ethyl acetate:ethanol, a direct

application method was used for the bioassay. Similar to the method used for the chloroform:methanol extracts, crude lipid extract was applied onto Petri dishes containing either MEA or ACM growth medium (appropriate for sexual and asexual reproduction, respectively). The effect on production of cleistothecia on MEA growth media by addition of the various crude lipid extracts at 10 days post inoculation is shown below (**Figure 4.13**). All other time points produced no visible sexual structures on media promoting sexual reproduction. No cleistothecia were produced following addition of the various crude lipid extracts to ACM under conditions suitable for asexual reproduction even after 10 days.

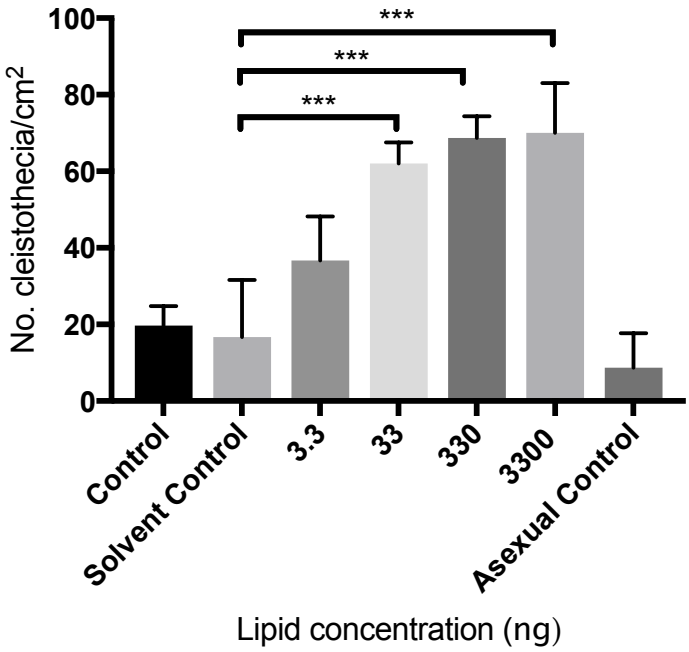


Figure 4.13 *– Analysis of the effect of ethyl acetate:ethanol extract dilutions on sexual reproduction of* N. fischeri. *Concentrations ranging from 3.3 to 3300 ng were directly applied to the center of a 4 cm Petri dish of MEA medium prior to inoculation with* N. fischeri *strain 53.15. Statistical analysis was performed using one-way ANOVA with a Bonferoni post-test with p<0.001 = ***. Error bars represent SEM from 3 technical replicates and 2 biological replicates.*

A similar trend in the effect on production of cleistothecia was seen as was observed with direct application of crude lipid extractions from the

chloroform:methanol methodology. A significant 3-fold or greater increase in cleistothecia was seen relative to the solvent only control when applying crude lipid extracts from sexual cultures of *N. fischeri* cultures at levels of 33 ng crude lipid residue and above (One-way ANOVA; F=19.61, P<0.0001). Although not statistically significant, addition of 3.3 ng of crude lipid extract also appeared to have a positive effect with regards to cleistothecia production. It should also be noted that addition of total crude lipid extract from sexually active cultures above the level of 33 ng extract residue showed very little increase in the number of cleistothecia produced (**Figure 4.13**). This potentially shows that a maximum threshold of cleistothecia production per cm^2 by *N. fischeri* had been reached under these conditions. .

Similar to results of the 'direct inoculation' with the chloroform:methanol extractions, the crude lipid treatment derived from an asexual control culture using ethyl acetate:ethanol extractions had no significant effect on cleistothecial production compared to the solvent-only control (**Figure 4.13**).

4.3.3 Hormone 'cross talk'

Once biologically active crude lipid extracts had been identified from *N. fischeri* (using two separate lipid extraction methods) it was of interest to identify if the extracts showed bioactivity in other related species. To test this, either inhibition of asexual reproduction or stimulation of sexual reproduction was assessed in *A. niger, A. fumigatus* and *A. nidulans* following addition of the crude lipid extracts. In the heterothallic species *A. niger* and *A. fumigatus* neither inhibition of asexual sporulation nor the formation of sexual structures occurred when crude lipid extracts were added to single mating-type cultures (**Figure 4.14** [one-way ANOVA; F=0.5505, P=0.6032] and **Figure 4.15** [one-way ANOVA; F=0.2243, P=0.8055], respectively). Similarly, in the homothallic species *A. nidulans,* cleistothecial production was not significantly different from the solvent only control cultures in conditions inducing sexual reproduction (**Figure 4.16**) [one-way ANOVA; F=1.149,

P=0.3868]. However, it is noted that the failure to observe an increase in production in *A. nidulans* might have been due to the already large number of cleistothecia being formed – possibly representing a maximum number of sexual structures produced under these conditions. These results indicate the crude lipid compound extracted from sexually reproducing cultures of *N. fischeri* may be species specific in their activity, unlike the SF obtained from *P. brassicae* (Chamberlain *et al.*, 1995) but similar to psi factor from *A. nidulans* (Champe *et al.*, 1987).

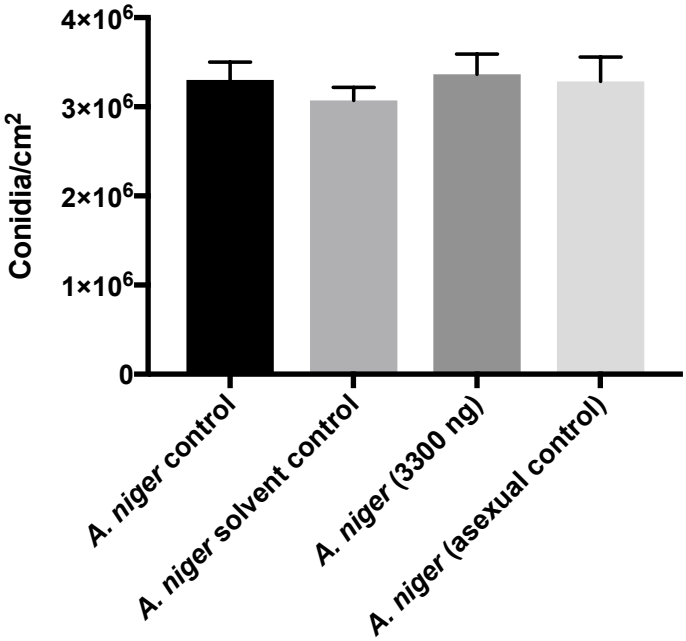


Figure 4.14 *– Analysis of the effect of ethyl acetate:ethanol extract from N. fischeri on asexual reproduction of* A. niger. *3300 ng was directly applied to the center of a 4 cm Petri dish of CYA medium prior to inoculation with* A. niger *strain 8-161. Statistical analysis was performed using one-way ANOVA with a Bonferoni post-test. Error bars represent SEM from 3 technical replicates and 2 biological replicates.*

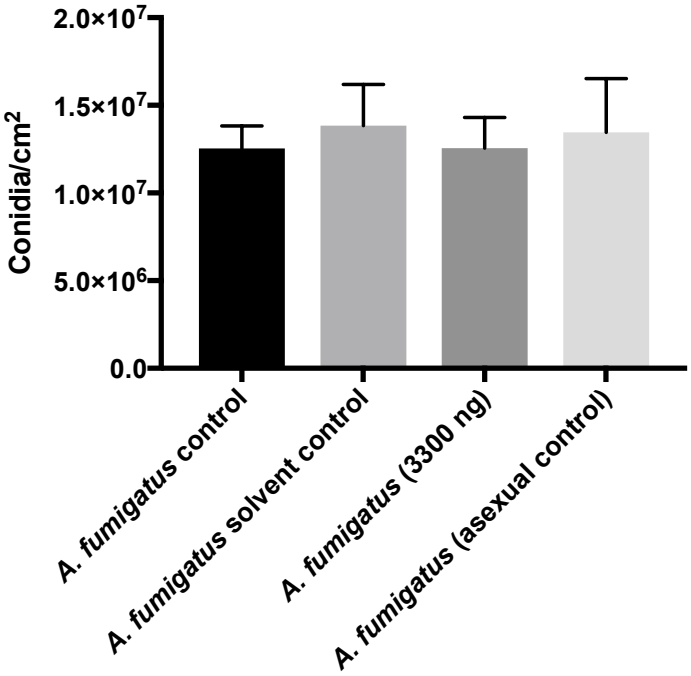


Figure 4.15 *– Analysis of the effect of ethyl acetate:ethanol extract from N. fischeri on asexual reproduction of* A. fumigatus. *3300 ng was directly applied to the center of a 4 cm Petri dish of OA medium prior to inoculation with* A. fumigatus *strain 47-55. Statistical analysis was performed using one-way ANOVA with a Bonferoni post-test. Error bars represent SEM from 3 technical replicates and 2 biological replicates.*

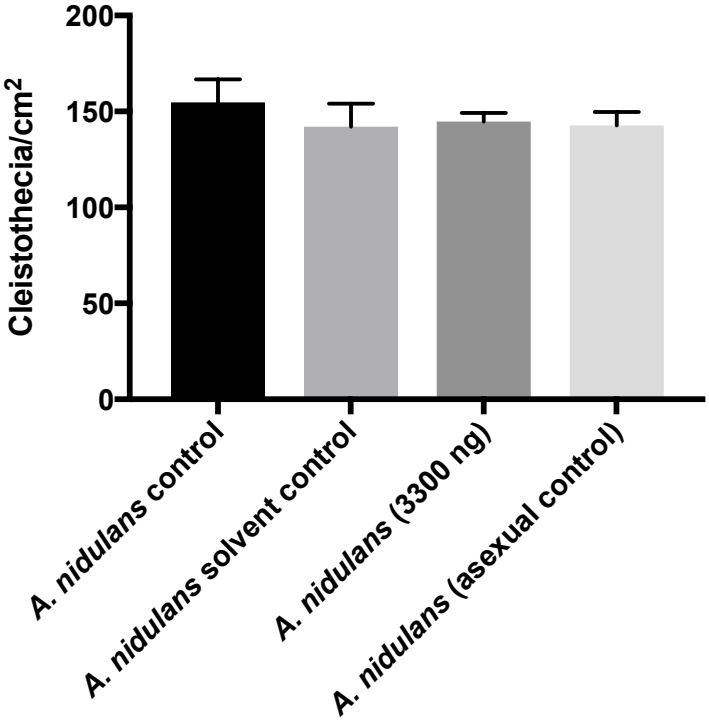


Figure 4.16 *– Analysis of the effect of ethyl acetate:ethanol extract from N. fischeri on asexual reproduction of* A. nidulans. *3300 ng was directly applied to the center of*

a 4 cm Petri dish of ACM medium prior to inoculation with A. nidulans *strain 2-231. Statistical analysis was performed using one-way ANOVA with a Bonferoni post-test. Error bars represent SEM from 3 technical replicates and 2 biological replicates.*

4.3.4 Production of cleistothecia in oxygenase mutants of *A. fumigatus*

In order to assess the role of *A. fumigatus* lipoxygenase genes, sexual crosses were set up between various oxygenase genetic mutants of *A. fumigatus* and known fertile mating partners in order to determine if the genetic background of the mutants impacted on the fertility of crosses – as assessed by counting the number of cleistothecia produced in sexual crosses. The results are shown below (**Figure 4.17**).

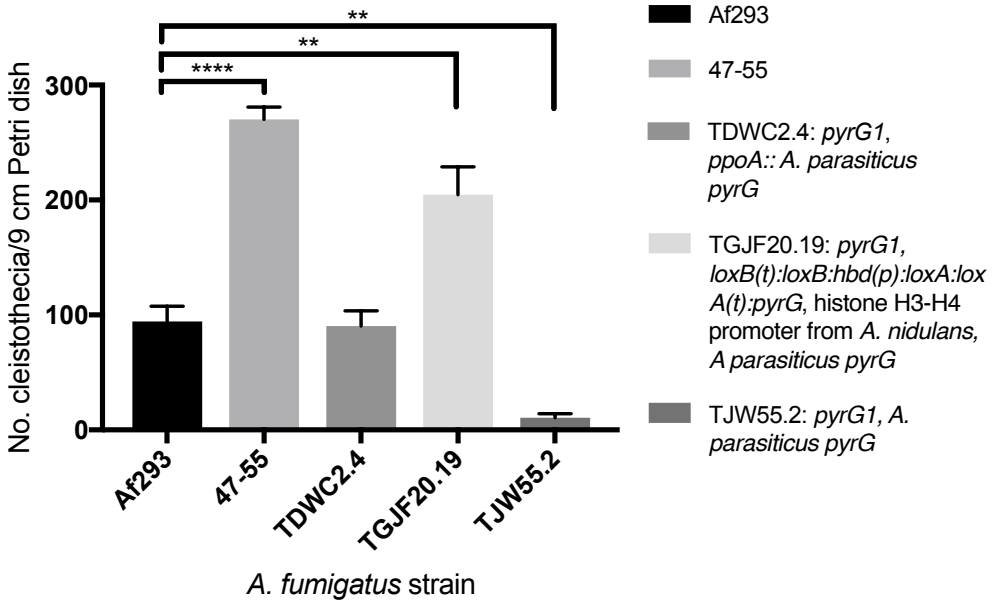


Figure 4.17 *– A graph representing the number of cleistothecia produced in a 9 cm diameter Petri dish by wild-type parent Af293, supermater 47-55 and Af293 derived mutants (TDWC2.4, TGJF20.19 and TJW55.2) when crossed with supermater 47-267. ** = p<0.01; **** = p<0.0001 ± SEM. Technical replicates per condition = 3; biological replicates = 2.*

Of the oxygenase mutants derived from the wild type parental Af293 strain, only isolates TDWC2.4 (Δ*ppoB*), TGJF20.19 (OE loxAB) and TJW55.2 (*pyrG* replacement control) were able to produce cleistothecia. Crosses of the other

mutant strains failed to form cleistothecia, even after incubation for an additional three months. Therefore, deletion of *ppoA, ppoC, ppoABC, loxA, loxB, loxAB* as well as over-expression of *loxA or ppoA* resulted in a loss of fertility. Of the mutant strains of Af293 that were able to cross, no significant difference in cleistothecial production was found between the wild type and TDW2.4, the Δ*ppoB* deletion strain. However, the overexpression *loxAB* mutant showed a significant 2-fold increase in the number of cleistothecia produced in comparison to the parental wild type.

Perhaps surprisingly, the transformation 'control' strain TJW55.2 produced a significantly lower number of cleistothecia compared to the parental wild-type strain (a 9-fold decrease in numbers) (one-way ANOVA; $F=49.38$, $P<0.0001$).

The genetic difference between these strains is the replacement of the endogenous *pyrG* gene in Af293 with the *pyrG* from *A. parasiticus*. This indicates a fertility decrease due to this heterologous gene replacement. Therefore, it was arguably more pertinent to examine the differences in fertility between the transformation 'control' TJW55.2 and the other mutant strains (as shown in **Figure 4.18**) than it is to observe the differences to the wild type Af293 strain. In which case, the Δ*ppoB* deletion strain TDWC2.4 produced approximately a 10-fold increase in cleistothecia numbers relative to the control, indicating an increase in fertility. Similarly, the *loxAB* overexpression strain TGJF20.19 produced a 20-fold increase in the number of cleistothecia in comparison to the transformation control strain, indicating a very large impact on sexual fertility from these genes (one-way ANOVA; $F=49.38$, $P<0.0001$) (**Figure 4.18**).

It is also noted that the supermater strain 47-55 produced significantly higher numbers of cleistothecia than the wild type Af293 in crossing work, this was to be expected due to the increased fertility of the supermater strain (Sugui *et al.*, 2011).

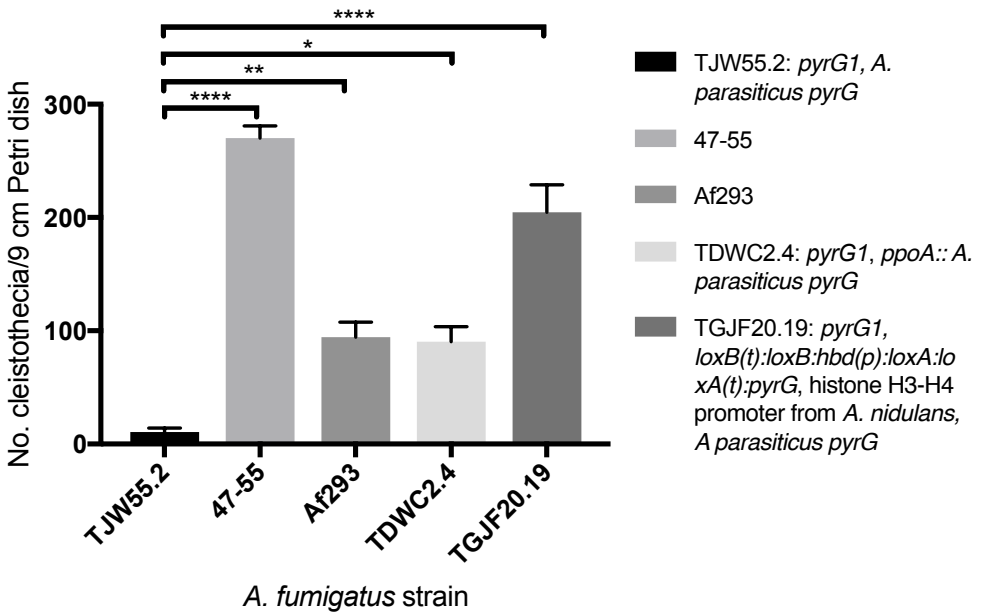


Figure 4.18 *– A graph representing the number of cleistothecia produced by parental wild type Af293, supermater 47-55 and Af293 derived mutants (TDWC2.4, TGJF20.19 and TJW55.2) when crossed with supermater 47-267. * = p<0.05; ** = p<0.01; **** = p<0.0001 ± SEM. Technical replicates per condition = 3; biological replicates = 2.*

In order to try to restore the fertility of the *pyrG* transformation control to wild-type levels, and therefore more accurately test relative fertility of these oxygenase mutants, media supplementation to the OMA crossing media was performed: in *pyrG* replacement mutants, uracil/uridine was added; in *argB* replacement mutants, arginine was added; and for *pyrG* and *argB* double replacements, uracil/uridine and arginine were added. However, in these circumstances it was found that the sexual cycle of *A. fumigatus* was completely inhibited - even in the supermater crosses - resulting in the production of no cleistothecia. This highlights the fastidious nature of *A. fumigatus* when initiating a sexual cycle (O'Gorman *et al.,* 2009). Indeed, this observation is of extreme pertinence to understanding induction of the sexual cycle – showing how minor changes to the media composition (in this case providing additional nutritional content for *A. fumigatus*) can negate the ability to form cleistothecia. This effect was even, unexpectedly, seen in the

supermater control sexual cross where no nutritional replacements were required.

4.3.5 RAPD PCR recombination

Following the successful production of cleistothecia in crosses between Af293, TJW55.2, TDWC2.4, TGJF20.19 and the positive control strain 47-55 with supermater 47-267, RAPD analysis was performed. RAPD analysis would confirm if sexual recombination had occurred between the sexually compatible strains. Using RAPD primers, A-05, J-01, W-10, W-19 and X-05, recombination could be confirmed in isolated progeny from all sexual crosses that produced cleistothecia. Representative RAPD PCRs can be shown below for progeny from TJW55.2 and TGJF20.19 using primers UBC20 and UBC90, respectively (**Figure 4.19**). Distinctive DNA bands highlighting recombination are represented by white arrows. In addition, there are several cases where additional bands are present in the progeny that are absent in both parents. It should be noted this phenomenon can occur due to novel combinations of suitable binding sites for the RAPD primers in recombined progeny, as described elsewhere (Dyer *et al.,* 1996).

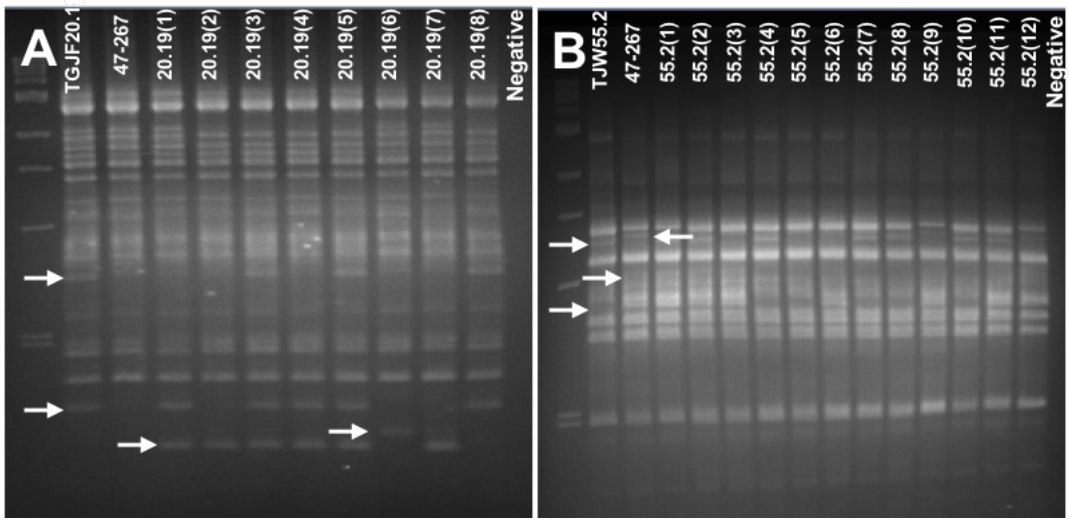


Figure 4.19 *– Representative RAPD using primer UBC20 with progeny from 'TJW55.2x47-267'(A) and UBC90 with progeny from 'TGJF20.19x47-267' (B) to identify sexual recombination. Sexual recombination was identified by presence of*

unique DNA bands from both parents in the progeny or absence of bands present in both parents. The lanes are as follows:

A) – *1) 1 kb DNA ladder; 2) TGJF20.19 parental isolate; 3) 47-267 parental isolate; 4) progeny 20.19(1); 5) progeny 20.19(2); 6) progeny 20.19(3); 7) progeny 20.19(4); 8) progeny 20.19(5); 9) progeny 20.19(6); 10) progeny 20.19(7); 11) progeny 20.19(8); 12) negative control.*

B) – *1) 1 kb DNA ladder; 2) TJW55.2 parental isolate; 3) 47-267 parental isolate; 4) progeny 55.2(1); 5) progeny 55.2(2); 6) progeny 55.2(3); 7) progeny 55.2(4); 8) progeny 55.2(5); 9) progeny 55.2(6); 10) progeny 55.2(7); 11) progeny 55.2(8); 12) progeny 55.2(9); 13) progeny 55.2(10); 14) progeny 55.2(11); 15) progeny 55.2(12); and 12) negative control.*

4.4 Discussion

4.4.1 Fungal sex hormones

Research has been conducted on fungal sex hormones for many years. However, there is still relatively little known about the extent of activity of sex hormones in fungi compared to better understood animal and plant systems (Janeczko and Skoczowski, 2005; Erdal, 2012; Santner and Estelle, 2009; Vilain and McCabe, 1998). Therefore, studies were undertaken with *N. fischeri* with the aim of extracting crude lipids from sexually reproducing cultures in order to see if these exhibited the ability to effect sexual and/or asexual reproduction both in the host and other fungal species.

The lipid class of chemicals was chosen because of the lipid nature of previously identified sex hormones (psi factor and SF factor) from other members of the Ascomycota (Siddiq *et al.*, 1992; Champe and El-Zayat, 1989). *N. fischeri* was chosen from an initial wider screen of fungal species. It was selected both for the ability to rapidly and reliably undergo sexual reproduction *in vitro*, as well as the fact that it undergoes exclusively asexual reproduction in alternative conditions. Furthermore, *N. fischeri* is a member of the aspergilli and therefore phylogenetically related to *A. nidulans*, a known producer of the psi sex hormone complex (Samson *et al.* 2014). A further consideration was to ensure that the extraction method used would be suitable for recovery of the desired types of lipid. Therefore, ethyl acetate:ethanol and chloroform:methanol extraction methods (Lin *et al.*, 2004; Dyer *et al.*, 1993) were chosen to ensure extraction of lipids in general, including the extraction of sterol lipids (Harkewicz and Dennis, 2011) and prenol lipids.

It indeed proved possible to obtain crude lipid extracts from sexually reproducing cultures of *N. fischeri*, which were able to significantly increase the production of cleistothecia compared to crude lipid extracts from control cultures undergoing asexual reproduction. Indeed, both bioassay techniques used were shown to stimulate production of cleistothecia in *N. fischeri.* This was despite the filter paper disc method, used to apply the crude lipid

extract, appearing to have an inhibitory effect on the growth of *N. fischeri* possibly due to the surface chemistry of the filter paper disc. By contrast, no such inhibitory effect was seen in when paper discs were used in studies of perithecial formation in *Nectria haematoccoca* by Dyer *et al.* (1993) and apothecial formation in *P. brassicae* (Siddiq *et al.*, 1992).

Previous work has suggested that fungal sex hormones might have wide-ranging bioactivity against other fungal species, with potential uses in biocontrol (Chamberlain *et al.*, 1995; Dyer *et al.*, 1992). It was therefore of great interest to test the bioactivity of the crude lipid extracted from *N. fischeri* against a range of species. If successful, this might provide a platform to induce sexual reproduction in species lacking a known sexual cycle or improve sexual yields in known sexual species.

In the present study, such possible hormone activity was assessed by the stimulation of sexual reproduction or the repression of asexual reproduction in the treatment zone of test species. In the closely related *A. fumigatus* and *A. niger,* asexual growth was not significantly inhibited when isolates were exposed to the crude lipid extract, nor were either sexually stimulated. Therefore, it can be concluded that the *N. fischeri* crude lipid extract has no effect on closely related heterothallic aspergilli. However, these are heterothallic species so there might be specific hormone or gated requirements in these systems. As seen in other heterothallic mating systems in fungi, such as *S. cerevisiae*, sexual reproduction is initiated by the sensing of pheromones from the opposite mating type (Haber, 2012). This specificity may be a requirement for inducing sexual reproduction or inhibiting asexual reproduction in heterothallic species.

The *N. fischeri* crude lipid extract was therefore also tested for activity on the homothallic species *A. nidulans*. No significant differences in cleistothecia production or decrease in asexual reproduction were observed. This was unexpected and in contrast to results of hormone studies in *P. brassicae* where SF was found to have a bioactivity in other species (Chamberlain *et al.*, 1995). However, this result was in accordance with findings that the application of psi factor from *A. nidulans* had no effect on reproduction in

other closely related species (Champe *et al.*, 1987). At this point it is not possible to conclude our crude lipid extract from *N. fischeri* definitely has no effect on cleistothecia production in *A. nidulans*. This is due to the confounding factor of psi hormone being produced in control *A. nidulans* cultures, regardless of the application of our crude extract. Therefore, there will be inherent cleistothecia production, with the possibility that maximal production of cleistothecia had already been achieved. To counter this, use of a psi factor deletion mutant would allow accurate analysis of our crude extract in a homothallic species. Additionally, to increase our understanding of cross species sexual activation, use of psi factor to assess cleistothecia production in *N. fischeri* would provide critical evidence towards a similar hormone structure and function between the species. Indeed, previous work on psi factor may inform us towards parameters for structural analysis. For example, initial enrichment of the psi extract was required until it was 5% of the original mass (20 times enrichment) for use in TLC and purification. This led to purified psiA yields of 500 $\mu g\ L^{-1}$ and psiB yields of 20 $\mu g\ L^{-1}$ (Champe and El-Zayat, 1989). Curiously, it is interesting to note that our crude lipid extract from *N. fischeri* had an effect on cleistothecia production at as little as 3.3 ng and a significant effect at 33 ng upwards. This is in comparison to initial reports that psiA was active at 200 ng and psiB at 25 ng (Champe and El-Zayat, 1989). Unfortunately psi factor is not commercially available to allow such investigations, and the original expert in this field (Sewell Champe) has passed away.

Another factor to discuss at this point is the difficulty determining whether the crude lipid extracts obtained from sexually reproducing cultures of *N. fischeri* contained a 'true' sex morphogen(s), with hormonal activity, or whether the extracts simply contained higher levels of certain growth substances promoting sexual reproduction (which were absent from the asexual control crude lipid extracts). Therefore purification and structural analysis is needed to provide critical insights into this possibility. In parallel work, Dyer *et al.* (1993) failed to identify a sex hormone from lipid extracts of mated cultures of *N. haematococca,* but were able to identify sexual growth

substances (particularly linoleic acid) that were absent in the same quantities from asexually reproducing control cultures.

Time constraints prevented further work on the crude lipid extracts from *N. fischeri.* Future work will therefore be needed to identify the active compound(s) in the crude lipid extract of *N. fischeri*. Such work might be achieved using high-performance liquid chromatography (HPLC) (Nissen and Kreysel, 1990; Frisvad, 1989). Typically, flame ionization detectors and light scattering detectors have been employed to analyze lipid extracts. However, the most commonly used method now involves the use of a charged aerosol detector (CAD), which acts to evaporate the solvent and charge the aerosol particles by nebulizing the column effluent (Moreau, 2006). This would hopefully allow the identification of the active compound(s) from the whole lipid extract, allowing more accurate examination of hormonal activity and reduction of confounding factors within the extract. Such work would require the separation of components according to retention time frames over several rounds of HPLC. Matrix assisted laser desorption/ionization (MALDI) coupled with mass spectrometry (MS) methods could then be used for structural analysis (Berry *et al.*, 2011). Common MS workflows utilize ultra-high mass resolution and accuracy (UHRAM) measurements and tandem MS (MS/MS). This allows for the identification of lipid class, number of carbons and carbon double bonds. However, *cis/trans* arrangements and double bond positions cannot be determined without use of other techniques (Siegel *et al.*, 2019).

As discussed previously, hormone isolations from various fungal groups and the oomycetes have shown such chemicals to be active at very low concentrations (Austin *et al.,* 1969; Raper and Haagen-Smit, 1942). Once the active compound(s) from *N. fischeri* has been isolated, structural determination, in tandem with bioassays to determine the concentrations at which the fraction showed activity compared to known fungal hormone compounds, could then be performed (Siddiq *et al.*, 1992; Mazur *et al.*, 1991). If the active compound extracted from *N. fischeri* is similar to an already identified hormone, chemical properties of this hormone can be

determined. It is noted that the extract from *N. fischeri* is unlikely to be identical to psi factor due to the fact it can neither repress asexual sporulation nor induce sexual sporulation in *A. nidulans*. However, as discussed, a psi factor deletion mutant strain of *A. nidulans* will be needed to fully assess this effect.

It will also be of great interest to determine the genetic pathway(s) controlling the production of the bioactive products(s) from *N. fischeri*. Given that the species is closely related phylogenetically to *A. nidulans*, it is possible that *ppo* or *ppo*-like genes may be necessary for the production of sexual factors in *N. fischeri* (Tsitsigiannis *et al.*, 2005). Therefore, generating *ppo* deletion strains of *N. fischeri* would provide further insights into the physiology of sexual reproduction in this species. The genome sequence of *N. fischeri* was reported in 2008, which provides a valuable tool to enable to such studies (NCBI).

To conclude, the crude lipid extract from sexually reproducing cultures of *N. fischeri* contains active compounds that stimulate sexual structure production in this species. However, the compound appears to be species-specific and unable to 'cross-talk' to other species, be they homothallic or heterothallic. Further studies should focus on the identification of the active compound(s) and genetic factors controlling its production.

4.4.2 *Aspergillus fumigatus* oxygenase mutants

As discussed in the introduction to this chapter (section **4.1.4.2**), the *ppo* gene family has been studied extensively in the homothallic *A. nidulans,* resulting in the identification of three genes (*ppoA*-*C*). The identified *ppo* genes in *A. nidulans* have found to both stimulate and repress sexual reproduction. Deletion of *ppoB* resulted in increased sexual reproduction in *A. nidulans* whereas deletion of *ppoA* and *ppoC* caused an inhibition of sexual reproduction, reduction of ascospore production and increased asexual sporulation (Tsitsigiannis *et al.,* 2004a; Tsitsigiannis *et al.*, 2005).

Furthermore, deletion of *ppoB* was linked with downregulation of *ppoA* and upregulation of *ppoC*, indicating a regulatory loop between the three genes for sexual cycle regulation (Tsitsigiannis *et al.*, 2005). In addition, *lox* genes have also been studied with regards to their effect on the sexual cycle. Previous studies within *A. flavus* showed that deletion of Af*lox* was linked with increased sclerotia development and lower levels of asexual reproduction at high cell densities, in contrast to the normal situation (Brown *et al.*, 2008). Outside of the sexual cycle, *lox* genes from *Gaeumannomyces graminis* produce a secreted enzyme potentially involved in plant tissue invasion (Oliw, 2002). Therefore, in order to assess any possible role of the oxygenase sub-categories, Ppo and LOX, in sexual reproduction of the heterothallic *A. fumigatus*, sexual crosses were set up between the highly fertile strain 47-267 and the parental and mutants of strain Af293 in which the *ppo* and *lox* genes had been subjected to gene manipulation.

Only three of the mutants derived from the parental wild-type strain Af293 (TJW55.2, the *pyrG* replacement control; TDWC2.4, Δ*ppoB*; and TGJF20.19, OE *loxAB*) were able to produce cleistothecia when crossed with 47-267.

When comparing the fertility of successful crosses with the *pyrG* replacement control, cleistothecia production was significantly increased in strains TDWC2.4 and TGJF20.19. With regards to the *ppo* gene family the *ppoB* deletion mutant exhibited increased cleistothecia production in comparison to the control strain TJW55.2. This indicates that *ppoB* has a negative regulatory effect on sexual reproduction in *A. fumigatus*. This contrasts with results in *A. nidulans* where deletion of *ppoB* resulted in decreased sexual reproduction (Tsitsigiannis *et al.*, 2005). Furthermore, *ppoA* and *ppoC* deletion mutant strains were unable to undergo a complete sexual cycle in *A. fumigatus*. This is in contrast with experimental evidence from *A. nidulans* where deletion of either gene results in increased sexual reproduction (Tsitsigiannis *et al.*, 2004a; Tsitsigiannis *et al.*, 2004b). However, the *ppoA* overexpression mutant TJMP143.28 was also sterile in the present study. Finally, strain TJW62.2 where *ppoABC* was downregulated by RNAi was

unable to complete a sexual cycle. This contrasts with *A. nidulans* where a triple Δ*ppoA*Δ*ppoB*Δ*ppoC* showed increased sexual reproduction (Tsitsigiannis *et al.*, 2005).

Regarding the *lox* gene family, deletion of *loxA* in strain TJMP38.2, deletion of *loxB* in strain TJMP39.6 and deletion of both genes in the double mutant *loxAB* strain TTRD12 all resulted in a total loss of sexual fertility. This indicates that the *lox* gene family is required for sexual reproduction in *A. fumigatus*. Consistent with this hypothesis, it was found that overexpression of *loxAB* in strain TGJF20.19 resulted in an increased production of cleistothecia compared to the control TJW55.2.

Thus, results overall indicate a role for oxylipin signaling in sexual reproduction of *A. fumigatus*. However, there is a major concern that use of the *pyrG* marker from *A. parasiticus* and *argB* marker, from either *A. fumigatus* or *A. nidulans*, as selection markers resulted in a significant decrease in fertility compared to the parental wild-type strain Af293. This indicates that the heterologous genes affected fertility, and were most likely unable to synthesize metabolites as effectively as the *A. fumigatus* host genes. Another possibility could be that integration of the *pyrG* or *argB* markers in the control strains had occurred at sites in the genome needed for normal sexual development. Indeed, a similar effect of *pyrG* replacement has been seen on the fertility of *A. nidulans* where the use of a *pyrG* marker resulted in reduced sexual fertility (Robellet *et al.*, 2010). Furthermore, arginine auxotroph strains were found to be sexually sterile even when exogenous arginine was supplemented (Eckert *et al.*, 1999). Therefore, the effect seen in the mutant strains where no sexual reproduction occurred may have been mainly due to the *pyrG* or *argB* gene replacement rather than any *ppo* or *lox* gene manipulation. To try and circumvent this problem, the OMA crossing media for *A. fumigatus* was supplemented with uracil/uridine and/or arginine (as appropriate for specific gene replacement mutants) in an attempt to restore fertility to the parental wild-type levels. However, when supplementation was done, fertility of all strains (including the supermater control 47-55) was completely reduced. This could be due to the sensitivity of

A. fumigatus to media conditions in order to undergo a sexual cycle. Therefore, enriching the chemical makeup of the media for sexual reproduction in the present work proved to be detrimental to the induction of sex in *A. fumigatus*. This is a significant finding because even in the supermater control cross (47-55 × 47-267) fertility was completely lost. It would therefore be interesting to see the effect arginine or uridine supplementation have on gene expression in comparison to standard crossing procedures. This might be assessed via use of a microarray system with genes involved in the sexual pathway in *A. fumigatus*. It is interesting to note that in contrast to the effect on sexual reproduction, there was no apparent change in formation of conidia or growth rate as a result of nutrient supplementation. This may confirm that *A. fumigatus* is unable to mate under nutrient rich conditions (i.e. a preference for relative nutrient limitation), or the addition of arginine or uridine interferes with already existing nutrients within the oatmeal agar.

Despite these reservations, the increase in fertility seen from *ppoB* deletion and *loxAB* overexpression appeared to be a genuine effect despite the confounding factor of use of the *A. parasiticus pyrG* marker. It should also be noted that Af293, the parental wild-type strain, has been found to be a strain of relatively low fertility strain from previous research experience in the Nottingham group (Swilaiman 2013). More research is therefore required into the roles of oxygenase genes in the aspergilli. Future studies with oxygenase mutants from *A. fumigatus* could be better conducted in mutants that have no *pyrG* or *argB* replacement background. This would eliminate the issue of reduced fertility in *A. fumigatus.* Additionally, if the *ppo* and *lox* family mutants could be generated outside of the Af293 background and within a high fertility strain, the effects of each gene deletion or overexpression could be better characterized. Indeed, current work is underway at the University of Manchester to generate gene knockouts of all genes of *A. fumigatus* in a CEA10 genetic background using hygromycin as a selective marker (Bromley, unpublished). These would provide a very good alternative source of strains, provided such strains are sexually fertile.

Furthermore, it will be of interest to examine novel gene combinations. For example, when comparing the fertility of TDWC2.4 and TGJF20.19 to control mutant TJW55.2, it was observed that both a *ppoB* deletion and *loxAB* overexpression increase fertility. Therefore, a combination of these mutations would be interesting to examine to gauge if there is an additive or synergistic effect involved.

4.4.3 Concluding remarks

To conclude, the genetic manipulation of various oxygenase genes in *A. fumigatus* was shown to have a clear effect on fertility. However, due to the *pyrG* and *argB* selection markers used in construction of the strains (including the effect of nutrient supplementation to counteract this), a full understanding of the role of specific genes is difficult to determine. From the data obtained to date, it appears that *loxAB* overexpression and deletion of *ppoB* has positive effects on cleistothecia production. This may indicate *loxA* and *loxB* as positive regulators of the sexual cycle in *A. fumigatus*, whereas *ppoB* acts as a negative regulator of the sexual reproduction. Although the effects in *A. fumigatus* contrast with those seen in *A. nidulans*, they further demonstrate the vital role of oxygenase genes in sexual reproduction in the aspergilli.

5. General Discussion

5.1 Exploitation of Fungal Sex for Biotechnology and Disease Control

The overall uniting aim of work in the present thesis was to gain knowledge about aspects of the fungal sexual cycle that would allow the practical exploitation of fungal sexual reproduction for use in biotechnology and disease control. It was also hoped that knowledge gained would provide fundamental insights into fungal sexual processes, of relevance to a broad range of fungi. Considering the thesis as a whole, it can be argued that progress was certainly made with regard to these aims. Some breakthroughs were made, but in many cases, this has led onto further work that needs to be conducted before definitive insights can be made. A common theme of interest that was identified between all aspects of this thesis was the specific, or narrow, conditions required by many of the study fungal species for induction of the sexual cycle.

Our first set of objectives aimed to identify whether it might be possible to induce a functional sexual cycle in certain members of the black aspergilli. These were the citric acid producer *A. niger* and a separate species *A. sclerotiicarbonarius*.

In *A. niger*, a potential sexual cycle was induced for the first time. It was possible to induce the production of structures distinct from conidia, which resembled ascospores. Furthermore, we observed a novel red pigmentation within the sexual structures in these reproducing cultures that had not been seen in this species previously. This provides a piece of the sexual puzzle in *A. niger.*

Identification of a potential sexual cycle in *A. niger* is significant for several reasons. Firstly, using the sexual cycle desirable traits could be selected for in the sexual progeny in a much more direct fashion, whilst acknowledging that the parasexual cycle of *A. niger* (Niu *et al.*, 2015) may also provide an alternative framework for strain improvement in this species.

Indeed, the current incubation times necessary for a potential sexual cycle to occur in *A. niger* is six months. This is in comparison to the much faster parasexual cycle, taking 1 week to complete. An exciting possibility in either case is that a 'transgressive segregation' effect may be observed, increasing the desirable yields further than those of either parent (Rieseberg *et al.*, 1999).

However, it was not possible to isolate and germinate the putative ascospores of *A. niger* and confirm recombination, despite numerous methods being trialled. Therefore, future work is suggested to involve the use of flow cytometry to isolate ascospores from others and surrounding conidia. This may lead to the isolation of single ascospores from *A. niger,* which can then undergo DNA extraction allowing recombination to be determined using the technique used in *A. sclerotiicarbonarius* (Darbyshir, 2014).

Indeed, for *A. sclerotiicarbonarius*, recombination between parental isolates in the sexual progeny was shown in the present work. Identification of sexual recombination in such a way, using SNP identifiers, provides a novel and effective framework to identify sex in other members of the fungi. Such methods of inducing a sexual cycle can now be applied to other 'asexual' members of the black aspergilli. Furthermore, crude lipid extraction of sexually active *A. sclerotiicarbonarius* mating partners at key time points may yield an active sexual hormone using methods similar to **Chapter 4**. Due to the similarity between the black aspergilli, an active sex hormone from this species may aid in the induction of a sexual cycle in species lacking a known sexual cycle, such as *A. niger*, or reduce total incubation times required.

The use of genes transcribed exclusively during meiosis, an indicator of true sexual reproduction, could also be used to verify a sexual cycle in *A. niger.* Such genes include *spo11, hop1* or *mnd1* that act specifically during meiosis (Schurko and Logsdon, 2008). By testing for the presence of transcribed mRNA at set time points during the sexual cycle of *A. niger,* meiosis might be confirmed to occur, confirming the presence of sex. This technique, if proven viable, would allow us to assess the transcription of

meiosis genes in other fungi where the precursor of a sexual cycle is identified.

Once a sexual cycle has been determined, it can be exploited for a number of reasons. The second objective of my thesis was to use the sexual cycle of *A. fumigatus* to be able to identify novel azole resistance genes using bulk-segregant analysis (BSA) in combination with next generation sequencing. This work was of medical importance, given that a number of isolates have been identified with unknown resistance mechanisms and that without efficient treatment mortality rates of above 50% can occur from invasive aspergillosis (Dagenais and Keller, 2009).

The BSA technique was implemented in various ways in the present study, using different numbers of rounds of backcrossing together with the use of different numbers of progeny in the BSA pools. We have shown that we could reduce the number of genes highlighted from the whole genome sequencing by increasing the number of backcrosses or number of progeny sequenced. However, it is cautioned that there may be a maximum number of backcrosses that can be performed in order to continue decreasing the genome region with the gene of interest – given the limited distribution of crossover hotspots in the genome.

Unfortunately, it was belatedly discovered that work in the present thesis used an *A. fumigatus* strain containing a previously known *cyp51A* resistance mutation (M220K). The next stage of this method development will involve the use of an *A. fumigatus* strain(s) with a novel unknown resistance mutation, using the research presented in this thesis as a framework for experimental work. For future work, improvements in BSA identified elsewhere (e.g. Niu *et al.*, 2015; Kershaw *et al.*, 2019; Hu *et al.,* 2015) could be incorporated into the workflow.

Overall, it is argued that use of this technique will provide an effective method of functional gene identification in the medically important species *A. fumigatus*. Results are anticipated to aid in drug development in the

future, with the aim of improving treatment of invasive Aspergillosis in the face of rapidly evolving resistance strategies. Additionally, use of this technique has the potential to be applied to any number of sexually reproducing fungal species where gene identification is necessary.

Looking towards the future use of BSA, prospects of this technique will be enhanced upon the discovery of a sexual cycle in *A. niger*. This could circumvent the use of a parasexual cycle, as used previously, and allow us to use the methodology described in **Chapter 3** to identify unknown genes for desirable phenotypes, such as for citric acid production.

To enhance our understanding of the sexual cycle, the third part of my thesis aimed to identify bioactive fungal sex hormones from model Pezizomycotina species and then to see if such hormones exhibited cross-species activity. Furthermore, the role of oxygenase genes in the sexual cycle of the heterothallic species *A. fumigatus* was examined using a range of mutant strains, building on earlier published work on *ppo* gene function in the homothallic *A. nidulans* (Tsitsigiannis *et al.,* 2004b; Tsitsigiannis *et al.*, 2005).

Neosartorya fischeri was chosen as a model homothallic species, and total lipid extractions and their bioactivity tested in this species and closely related species. It was found that the crude lipid extracts from sexually reproducing cultures of *N. fischeri* had a strong positive effect on the induction of cleistothecia under conditions favourable for sex. However, as discussed, this induction of sexual structure alone does not provide sufficient evidence that a fungal sex hormone had been detected. This effect could be due to the addition of specific lipids to the fungal culture providing extra metabolic energy for the sexual cycle. Further evidence for this possibility is suggested from the lack of any repression of asexual conidiation in non-sexual cultures, whereas this effect has been observed in other fungal sex hormone studies (Champe and El-Zayat, 1989; Siddiq *et al.,* 1992). By testing it on other a species, we observed no effect on asexual or sexual reproduction. We confirmed that either the crude lipid extract of *N. fischeri*

may not contain a sexually active hormone, due its lack of cross talk among fungal species, or if there is an active sex hormone present, it is not similar in activity to the psi factors of *A. nidulans*. However, it is currently not possible to conclude that our lipid extract has no effect on sexual reproduction in *A. nidulans* due to the presence of background psi factor promoting the sexual cycle naturally. However, to obtain a suitable psi negative strain of *A. nidulans* may require the action of multiple mutations within the *ppo* oxygenase family to fully eliminate the action of psi factor (Tsitsigiannis *et al.*, 2005).

In future work it will be of interest to identify the potential active compound(s) present in the lipid extracts from *N. fischeri.* This might be achieved through the use of high-performance liquid chromatography (HPLC) and/or gas-liquid chromatography (GC) in tandem with mass spec analysis (-MS). This future work will provide greater knowledge of the sexual cycle in *N. fischeri* and also provide a framework for isolating sexual hormones from other species. In addition, if an active compound can be identified by *N. fischeri,* it would be of interest to assess its ability on sexually active cultures of *A. fumigatus*. So far, only asexual cultures have been tested, but if the sexual cycle of this species can be enhanced further by the application of a sexual hormone this would provide a vital benefit in the use of BSA. Simultaneously, the same method could be used if a sexual cycle is identified in *A. niger,* potentially decreasing incubation times and providing a more viable method for strain improvement.

A further future direction of this work would be to isolate sexual hormones from known plant pathogens, with the hope of applying them to the host species in the field. This might result in the repression of asexual reproduction as observed for *P. brassicae* (Siddiq *et al.,* 1992) and inhibition of disease progression.

With regards to the study of oxygenase genes, evidence has been presented for a role of both *ppo* and *lox* gene families in sexual reproduction of the heterothallic species *A. fumigatus*. Results from *ppoB* deletion mutants agreed with those seen in *A. nidulans* (Tsitsigiannis *et al*., 2005). Whereas,

Δ*ppoA* and Δ*ppoC* mutants in *A. fumigatus* contradicted observations seen in *A. nidulans* (Tsitsigiannis *et al.*, 2005; Tsitsigiannis *et al.*, 2004a; Tsitsigiannis *et al.*, 2004b). Therefore, there is an indication of a role of oxylipins in sexual reproduction in *A. fumigatus*, distinct from that of *A. nidulans*. However, this effect cannot be confirmed until future work has been undertaken using a different parental genetic background with increased base fertility. This will be needed to reveal the true nature of these genes in sexual reproduction, due to the relative infertility of the parental strain Af293 used in this study (Dyer *et al.*, unpublished). Furthermore, as discussed previously, the use of *pyrG* and *argB* auxotrophs for mutant production has led to decreased sexual fertility in *A. nidulans* in other studies (Robellet *et al.*, 2010; Eckert *et al.*, 1999). Indeed, supplementation of sexual crosses using uracil or arginine (in the case of *pyrG* or *argB*, respectively) led to complete sterility of the sexual partners, even in the supermater mating partners – a significant insight into the impact of nutrition with regards to sex in *A. fumigatus*. Thereby, further work using different selectable markers may provide a more accurate analysis of these genes. This further analysis will enhance our knowledge of the sexual cycle in this medically important species, which might act as a model for oxygenase gene function in heterothallic species in general. Furthermore, the effect of nutrient supplementation with regards to sexual reproduction should be studied in more detail, categorising which supplements have the greatest impact in this species. This has the potential impact of enhancing sexual reproduction in *A. fumigatus*, which can be exploited in BSA for reduced incubation times.

5.2 Concluding Remarks

In conclusion, this thesis has contributed to the knowledge of fungal sex in a number of different areas: induction of sexual cycles in the black aspergilli; use of the sexual cycle of *A. fumigatus* for gene identification; fungal sex hormones in *N. fischeri*; and oxygenase gene function in *A. fumigatus*.

Through the exploitation of the fungal sexual cycle, it is possible to expand on the classical genetic techniques currently available, using BSA to identify genes of unknown functions. Indeed, as more sexual cycles are discovered in supposed ‘asexual’ species, powerful research techniques can be implemented to study gene function, phenotypic analysis and secondary metabolite production. Finally, investment in research into fungal sex hormone (currently a poorly researched area of biology) could have far reaching effects on fungal disease control. In summary, work presented in this thesis explores how the sexual cycle of fungi is an exciting topic of research and how it is anticipated that future work in this area will yield important findings of industrial, medical and academic importance.

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Appendix – Growth Media

1 Growth media (per litre)

1.1 *Aspergillus* complete medium (ACM):

10 g of glucose (Fisher Scientific, USA); 1 g of yeast extract (Oxoid, UK); 2 g of peptone (Oxoid, UK); 1 g of casamino acids (BD, USA); 0.075 g of adenine (Sigma-Aldrich, USA); 10 ml of *Aspergillus* vitamin solution; 20 ml of *Aspergillus* salt solution; 20 g of agar (Sigma-Aldrich, USA); and adjusted to pH to 6.5 with NaOH (Sigma-Aldrich, USA) in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 117 °C for 30 minutes.

1.2 *Aspergillus* salt solution:

26 g of potassium chloride (KCl) (Sigma-Aldrich, USA); 26 g of magnesium sulfate ($MgSO_4.7H_2O$) (Sigma-Aldrich, USA); 76 g of potassium dihydrogen phosphate (KH_2PO_4) (Fisher Scientific, USA); and 10 ml *Aspergillus* trace element solution in in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 117 °C for 30 minutes.

1.3 *Aspergillus* trace element solution:

40 mg of sodium tetraborate decahydrate ($Na_2B_4O_7.10H_2O$) (Sigma-Aldrich, USA); 800 mg of copper sulfate pentahydrate ($CuSO_4.5H_2O$) (Sigma-Aldrich, USA); 800 mg of ferric orthophosphate monohydrate ($FePO_4.H_2O$) (Sigma-Aldrich, USA); 800 mg of sodium molybdate dihydrate ($NaMoO_4.2H_2O$) (Sigma-Aldrich, USA) in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 117 °C for 30 minutes.

1.4 *Aspergillus* vitamin solution:

400 mg of p-aminobenzoic acid (Sigma-Aldrich, USA); 50 mg of thiamine HCl (Sigma-Aldrich, USA); 2 mg of d-Biotin (Sigma-Aldrich, USA); 100 mg of nicotinic acid (Sigma-Aldrich, USA); 250 mg of pyridoxine hydrochloride (Sigma-Aldrich, USA); 1.4 g of choline chloride (Sigma-Aldrich, USA); and 100 mg of riboflavin (Sigma-Aldrich, USA) in 800 ml distilled water. The volume is

then readjusted to 1 L with distilled water and autoclaved at 117 °C for 30 minutes.

1.5 Carrot agar:

400 g of fresh carrots (washed, diced and autoclaved at 121 °C for 30 minutes in 400 ml distilled H_2O) and then pureed; 20 g of agar (Sigma-Aldrich, USA) in an additional 500 ml of distilled H_2O and autoclaved at 121 °C for 30 minutes.

1.6 Cornmeal agar:

17 g of cornmeal agar (Sigma-Aldrich, USA); 1 g of yeast extract (Oxoid, UK); and 20 g of agar (Sigma-Aldrich, USA) in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 121 °C for 30 minutes.

1.7 Czapek's yeast autolysate:

30 g of sucrose; 5 g of yeast extract (Oxoid, UK); 3 g of sodium nitrate ($NaNO_3$) (Honeywell, USA); 1 g of potassium phosphate dibasic (K_2HPO_4) (Fisher Scientific, USA); 0.5 g of magnesium sulfate ($MgSO_4.7H_2O$) (Sigma-Aldrich, USA); 0.01 g of iron sulfate heptahydrate ($FeSO_4.7H_2O$) (Acros organics, USA); 0.5 g of potassium chloride (KCl) (Sigma-Aldrich, USA); and 15 g of agar (Sigma-Aldrich, USA) in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 117 °C for 30 minutes.

1.8 M40Y:

20 g of malt extract (Sigma-Aldrich, USA); 5 g of yeast extract (Oxoid, UK); 400 g of sucrose (Fisher Scientific, USA); 20 g of agar (Sigma-Aldrich, USA) and adjust the pH to 6.5 in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 117 °C for 30 minutes.

1.9 Malt extract agar (MEA):

20 g of malt extract; 6 g of peptone (Oxoid, UK); and 16 g of agar (Sigma-Aldrich, USA) in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 121 °C for 30 minutes.

1.10 Oatmeal agar (OA):

40 g of Odlums Irish pinhead oatmeal (Odlums, Ireland); 20 g of agar (Sigma-Aldrich, USA) added in 1 L of tap water.

Instructions to make OA:

1. Add oatmeal to water in a 5 L flask and bring to the boil using a hotplate. Stir with a magnetic flea and ensure oatmeal is constantly stirred. Cover the top with foil to prevent water loss.
2. After the water boils, lower the heat so the mixture is gently bubbling and cook for 45 minutes.
3. Once cooked, filter the solution through two layers of cheesecloth to remove any solids.
4. Restore the volume to 1 litre using tap water.

Autoclave OA at 121 °C for 30 minutes, followed by a slow pressure release to prevent boiling over.

1.11 RPMI 1640:

1. Make to 2X concentration: 20.8 g of RPMI 1640 (Sigma-Aldrich, USA); 40 g of glucose (Fisher Scientific, USA); and adjust the pH to 7.0 with morpholinepropanesulphonic acid (MOPS) buffer (Sigma-Aldrich, USA) in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and filtrated using two 0.22 μm pore size 500 ml bottle top filters.
2. Make to 2X concentration: 30 g of agar (Sigma-Aldrich, USA) in 800 ml distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 121 °C for 30 minutes.

For use, add a 1:1 ratio of 1. and 2.

1.12 Synthetic growth medium:

1 g of potassium nitrate (KNO_3) (Fisons Scientific, UK); 1 g of potassium dihydrogen phosphate (KH_2PO_4) (Fisher Scientific, UK); 0.5 g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Sigma-Aldrich, USA); 1 g of calcium chloride ($CaCl_2$) (Sigma-Aldrich, USA); 0.1 ml of trace element solution; 2 g of glucose (Fisher Scientific, USA) and 15 g agar (Sigma-Aldrich, USA) in 800 ml

distilled water. The volume is then readjusted to 1 L with distilled water and autoclaved at 121 °C for 30 minutes.

1.13 Synthetic growth medium trace elements:

5 g of citric acid; 5 g of zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) (Sigma-Aldrich, USA); 1 g of ammonium iron (II) sulfate hexahydrate ($[NH_4]_2Fe[SO_4]_2.6H_2O$) (Sigma-Aldrich, USA); 0.25 g of copper sulfate pentahydrate ($CuSO_4.5H_2O$) (Sigma-Aldrich, USA); 0.05 g of manganese sulfate monohydrate ($MnSO_4.1H_2O$) (Sigma-Aldrich, USA); and 0.05 g of boric acid (H_3BO_3) (Sigma-Aldrich, USA) in 100 ml H_2O and filtrated using a 0.22 μm pore size 500 ml bottle top filter (Corning, USA).

1.14 Water agar

40 g of agar (Sigma-Aldrich, USA) was added to 800 ml distilled water. The volume was then readjusted to 1 L and autoclaved at 121 °C for 30 minutes.