Evolution and Control of Sexual Reproduction in *Aspergillus* species

Nadhira Shaban Salih

BSc, MSc

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

April 2016

Abstract

Having an understanding of the reproductive mode of an organism is of great importance for gaining insights into the potential for the evolution of a given species, whilst the sexual cycle also provides a valuable tool for strain improvement of species used in industrial applications. About one third of *Aspergillus* species are reported to be capable of sexual reproduction, but the majority of species are only known to reproduce asexually. However, sexual cycles have recently been discovered in a number of *Aspergillus* species that were previously thought to be strictly asexual. This has provoked increased research interest both in the possible genetic basis of asexuality and the molecular genetic control of sexual development. The aim of the present study was to investigate the evolution and control of sexual reproduction in *Aspergillus* species.

To achieve this goal, studies were first undertaken to identify novel genes required for sexual development in *Aspergillus* species. A group of 33 genes was selected, most of which were of unknown function, that had previously been shown to be differentially expressed greater than 10-fold according to whether a *MAT1-1* or *MAT1-2* gene was resident at the *MAT* locus of *A. oryzae*. Homologous genes were identified in the homothallic model species *A. nidulans* where possible, and then a systematic deletion of these genes were performed in *A. nidulans* using *pyrG* marker deletion cassettes. Where transformant strains were obtained these were tested for sexual fertility. A range of phenotypes was observed, from no obvious impact (AN3239 and AN5993), to moderate loss or gain of fertility (AN1356, AN1958, AN4686, AN5791, AN6881, AN7818, AN8184 and AN11253), to complete loss of sexuality (two genes, AN3562 and AN8656). Sexual crossing was then used to restore the deleted genes of interest to confirm by complementation that observed major changes in sexuality were due to the deletion of the target gene.

Secondly, the possible roles of high-mobility group domain (HMG) proteins in sexual development of *Aspergillus* species was investigated. Nine putative high mobility group transcription factor genes were identitfied in *A. nidulans* by BLAST searching. Three of these had previously been characterized as having prominent roles in sexual development. Systematic deletion of the remaining putative HMG genes revealed that the majority of these also were involved in sexual development. Deletion of one gene (AN3549) resulted in a complete loss of fertility, while deletion of six putative HMG (AN1267, AN1962, AN2755, AN2885, AN4734 and AN5073) resulted in a significant decrease of fertility i.e. they acted as inducers for sex. Deletion of three HMG genes (AN0879, AN3580 and AN10103) had no obvious effect.

Finally, investigations were made to determine the potential of sex in some supposedly 'asexual' *Aspergillus* species, some of them being of economic and biotechnological importance. The presence and functionality of mating-type genes and elements of the pheromone signalling pathway were explored. All of the asexual species were found to contain one mating-type gene, consistent with a heterothallic breeding system, and also contained *ppgA* homologues encoding a pheromone precursor, and *preA* and *preB* homologues encoding pheromone receptors. Both the mating-type and pheromone signalling genes were shown to be expressed to mRNA level under conditions favourable for sexual reproduction. These results are of importance as they provide evidence of cryptic sexuality in these species, and the possibility of inducing a sexual cycle in these supposedly asexual species if the correct environmental conditions can be identified.

Overall, the identification of several new genes required for sexual reproduction provides significant further insights into the biological control of sexual reproduction in ascomcycete fungi. Indeed, mutation in any of these genes might be the reason for asexuality in several asexual strains; future gene manipulation may lead to induction of sexuality in such asexual species.

Acknowledgements

All praise and glory be to Allah, who is the most merciful and grateful for helping me in finishing my research.

I would like to express my special gratitude to my supervisor Profesor Paul Dyer for his continued support and guidance, also for giving me an opportunity to undertake this project.

My particular thanks go to the members of Archer/ Dyer lab for their technical support, especially Dr. Michaela Novodvorska, Dr. Nathalie Narraidoo, Dr. Paul Daly and Dr. Roxane Raule. Also, thanks go to Lee Shunburne, Matthew Kokolski, Marylin Wagner and Elaine for assistance and providing the lab materials and equipments.

Special thanks goes for both Dr. Ozgur Bayram and Dr. Carol Ringelberg for providing some deletion cassettes that used in HMG deletion work.

I would like to thanks my sponsor human capacity development programme of Kurdistan regional government of Iraq for financial support of this project throughout my study.

My special gratitude also goes for my parents, brothers and sisters and also for my husband and son for being patient and continues support that helped and encouraged me to continue my work.

Last but not the least, my thanks to all the friends who helped me in completing this research.

Abbreviations

ACM	*Aspergillus* complete media
AMM	*Aspergillus* minimum media
ANOVA	Analysis of variance
AspGD	*Aspergillus* Genome Database
BLAST	Basic Local Aligment Search Tool
bp	Base pair
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DW	Distilled water
EDTA	Ethylene diamine tetra-acetic acid
FGSC	Fungal Genetics Stock Center
MAT	Mating-type gene
mRNA	Messenger ribonucleic acid
HMG	High Mobility Group
PABA	Para amino benzoic acid
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
Psi	Precocious sexual inducer
pyro	Pyridoxine HCL
qRT-PCR	Quantitative real time polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
VeA	Velvet A

Table of Contents

Chapter 1 Introduction to Reproduction in Fungi

1.1 General background

The process of reproduction is one of the most variable features of the living world (Billiard *et al.*, 2012). Organisms may reproduce either sexually or asexually, and in the former case either by selfing or out crossing (Dyer *et al.*, 1992).

In asexual reproduction only one parent participates to produce progeny by mitosis; in such cases the progeny inherit the same complete copy of the genome as is present in the parent (Varga *et al.*, 2014). By contrast, the sexual mode of reproduction involves bringing together two nuclei to form a diploid zygote followed by meiosis. This often involves mating and recombination, with two parents involved in the reproductive process (Taylor *et al.*, 1999). Given that each of them has a different genome and evolutionary history, novel genotypes would be expected in the progeny as they receive half a set of genes from each parent by meiosis (Ni *et al.*, 2011). However, sexual reproduction can also involve selfing, with fusion of identical nuclei, which leads to clonality as seen in asexual reproduction.

In contrast to the majority of animal species, sex is facultative in a number of fungi. The majority of fungal species can produce their offspring either sexually or asexually (Dyer *et al.*, 1992), but approximately 20% of species have no known sexual reproduction (termed 'imperfect' fungi) while a few species are restricted to sexual reproduction (Sun and Heitman, 2011; Dyer and O'Gorman, 2012). Environmental conditions play an essential role in the life cycle of fungi, especially on the mode of reproduction. Certain environmental conditions can favour asexual or sexual reproduction depending on the fungal species in question, and one mode of reproduction can inhibit the occurrence of the other mode (Dyer *et al.*, 1992; Cary *et al.*, 2012; Dyer and O'Gorman, 2012).

Where sexual reproduction occurs, fungi exhibit either homothallic (self fertile) or heterothallic (self sterile) breeding systems (Pal *et al.*, 2007); the latter requires the presence of compatible mating partners for sexual reproduction to occur (Paoletti *et al.*, 2007; Czaja *et al.*, 2011).

Work in the present thesis will focus on an investigation of the genetic basis of sexual reproduction in ascomycete fungi. *Aspergillus nidulans* will be used as a model organism to identify new genes that were not previously known to be involved with the control of sexual reproduction. In addition studies will be undertaken to assess the potential of sex in a number of supposedly 'asexual' *Aspergillus* species.

1.2 Introduction to ascomycete fungi

The Ascomycotina are considered to be the largest and most diverse group of fungi (Scazzocchio, 2006; Schoch *et al.*, 2009). Members of the Ascomycotina are found in the natural environment either as saprophytes or parasitic or mutualistic symbionts on plants and animals, and the group contains species of considerable industrial and economic importance (Dyer *et al*., 1992, Schoch *et al.*, 2009; Debuchy *et al.*, 2010).

Ascomycete fungi are divided into two morphological groups: either multicellular (filamentous or mycelial) or unicellular (yeast) forms (Pöggeler *et al.*, 2006; Billiard *et al*., 2012). Filamentous ascomycetes are commonly characterized by formation of septate hypha with a Woronin body, a particular cell wall composition (Pöggeler *et al.*, 2006) and the formation of a special fruiting body called an ascocarp or ascomata, which contains the asci and ascospores, when they reproduce sexually; this features distinguishes the Ascomycotina from the rest of the fungal kingdom (Pöggeler and Kück, 2001).

The life cycle of most ascomycete fungi consists of two phases (**Figure 1-1**): a vegetative phase, in which a branched haploid filamentous hypha gives rise to mycelium; and a sexual phase, in which the mycelium differentiates to form male

and female sex organs, namley unicellular antheridia and multicellular coiled ascogonia, respectively (Bistis, 1998). Most fungal species in the Ascomycotina can propagate by both sexual and asexual means (Lee *et al.*, 2010a), although the precise morphology and nature of reproductive systems shows much variation amongst filamentous ascomycetes (Nauta and Hoekstra, 1996).

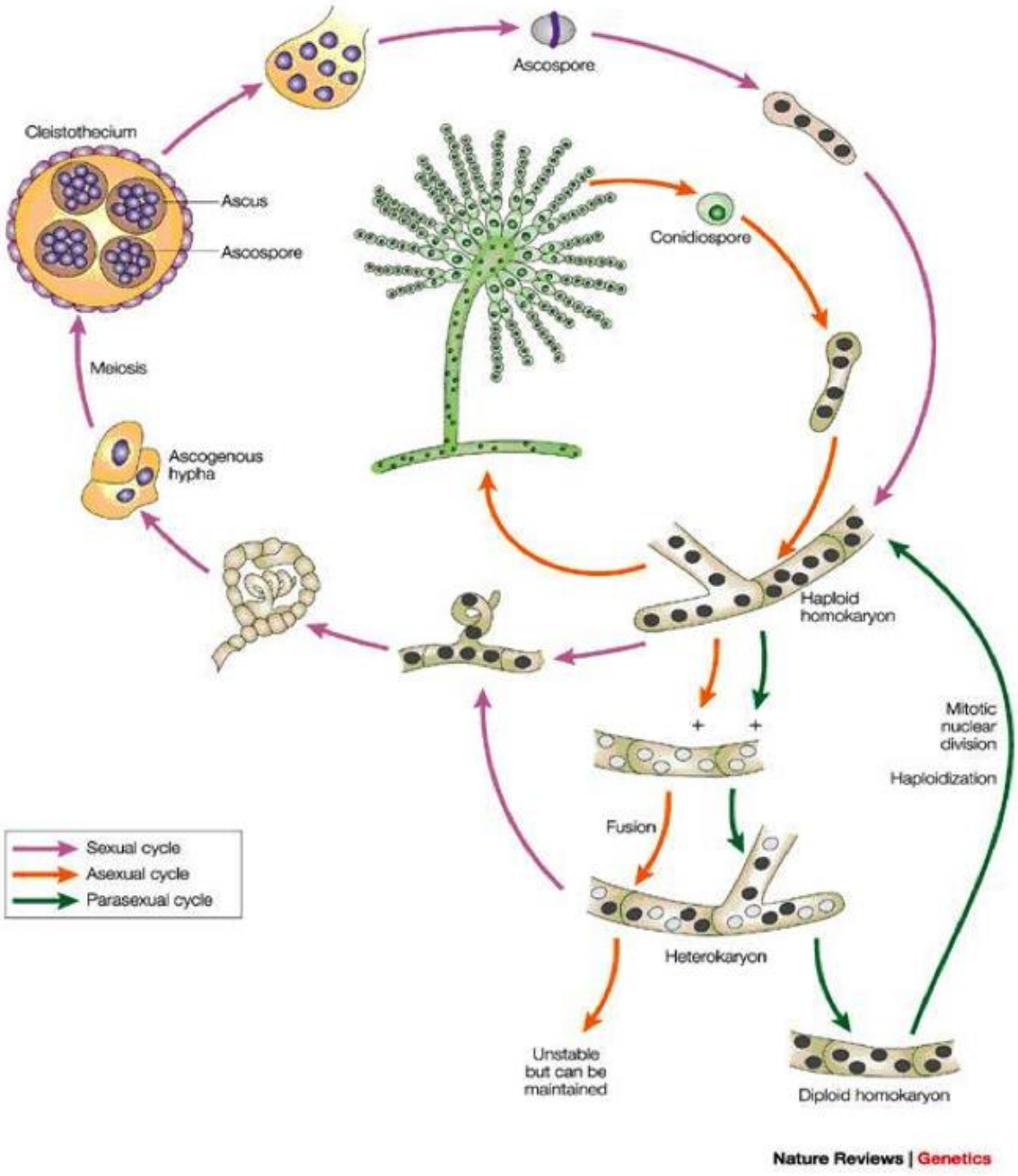


Figure 1-1 Life cycle of *Aspergillus* species showing the asexual, sexual and parasexual cycles. During asexual reproduction mitospores are produced which are called conidia; while during sexual propagation two nuclei fuse and undergo meiosis to produce meiospores called ascospores. The parasexual cycle involves fusion of compatible vegetative hyphae (heterokaryon formation) followed by karyogamy and mitotic division (Casselton and Zolan, 2002).

1.2.1 Asexual reproduction (including effect of environment)

Asexual reproduction is a predominant mode of propagation seen in higher fungi. In the Ascomycotina it involves the production of mitosporic spores called conidia (Adams, 1998; Kwon *et al.*, 2010a). The main advantages of asexual reproduction are that it maintains beneficial genotypes and allows production of high numbers of propagules at relatively low metabolic cost for dispersion of species, while the disadvantages include the risk of accumulation of deleterious mutations and the fact that this method is less efficient in generating genetic diversity to allow adaptation to environmental change than sexual reproduction (Sun and Heitman, 2011; Heitman *et al.*, 2013).

The process of conidiation can be divided into two different phases: the vegetative and developmental phases (Ni *et al.*, 2010). The vegetative stage includes spore germination and formation of hyphae and a mycelium (Adam *et al.*, 1998). Under certain environmental conditions, such as exposure to air and light, some of the hyphae may then undergo the developmental phase (Yu *et al.*, 2006; Garzia *et al.*, 2010). Other environmental factors that can also have a profound influence on the shift between growth forms include changes in the growth medium for example depletion of nutrients (Harris, 2009; Arratia-Quijada *et al.*, 2012); accumulation of secondary metabolites and osmotic change (Etxebeste *et al.*, 2010a). Among the environmental factors that affect development, light plays a crucial role in balancing asexual and sexual development. A study of light-dependent gene expression in *A. nidulans* by Ruger-Herroros and co-workers, identified changes in the transcript level of more than 500 genes under light induction; more than 400 genes were upregulated, including genes necessary for conidiation whilst expression of more than 100 genes decreased (Ruger-Herreros *et al.*, 2011).

In *Aspergillus* species the asexual reproductive structure consists of a foot cell, a tubular structure called a conidiophore, which expands at the apex to form a vesicle that bears metulae, phialides and chains of conidia (Adam *et al.*, 1998; Etxebeste *et al.*, 2010a) (Figure **1-2a**).

Asexual sporulation is regulated and controlled by expression of certain genes; notably bristle (*brl*A), abacus (*aba*A) and wetwhite conidia (*wet*A) have a major role in this process (Ward *et al.*, 2005; Kwon *et al.*, 2010a; Etxebeste *et al.*, 2010a).

Activation of *brlA* is required for commencement of conidiophore formation. *BrlA* encodes a transcription factor (C_2H_2 zinc finger), which controls the expression of other genes necessary for asexual sporulation (Adam *et al.*, 1998; Harris, 2009; Yu *et al.*, 2006). Deletion of *brlA* resulted in aberrant conidiophore development and failure in the formation of vesicle and phialide (Park and Yu, 2012).Whereas *abaA* has an essential function in the mid stage of asexual development, being involved in phialide formation; *abaA* is induced by *brlA*. The deletion of *abaA* resulted in a failure to produce phialides and conidia (Sewall, 1990; Yu, 2010).

BrlA activation is controlled by upstream genes, which form a complex network known as the 'upstream developmental activators' (UDAs) (Garzia *et al*., 2009; Harris, 2009) (**Figure 1-2b**). *Aspergillus* upstream developmental genes include a series of 'fluffy' *flu* genes (*fluG, flbA, flbB, flbC, flbD* and *flbE*), which act as positive regulators of asexual reproduction (Garzia *et al.*, 2009; Yu, 2010; Arratia-Quijada *et al.*, 2012). *FluG* is a key gene in this network. It encodes an extracellular protein, which initiates transition of vegetative growth to reproduction through *flbA* activation (Park and Yu, 2012), which suppresses vegetative growth via its effect on *fadA*; in addition *fluG* overcomes the negative effect of the *sfg* gene on developmental growth (Garzia *et al*., 2009).

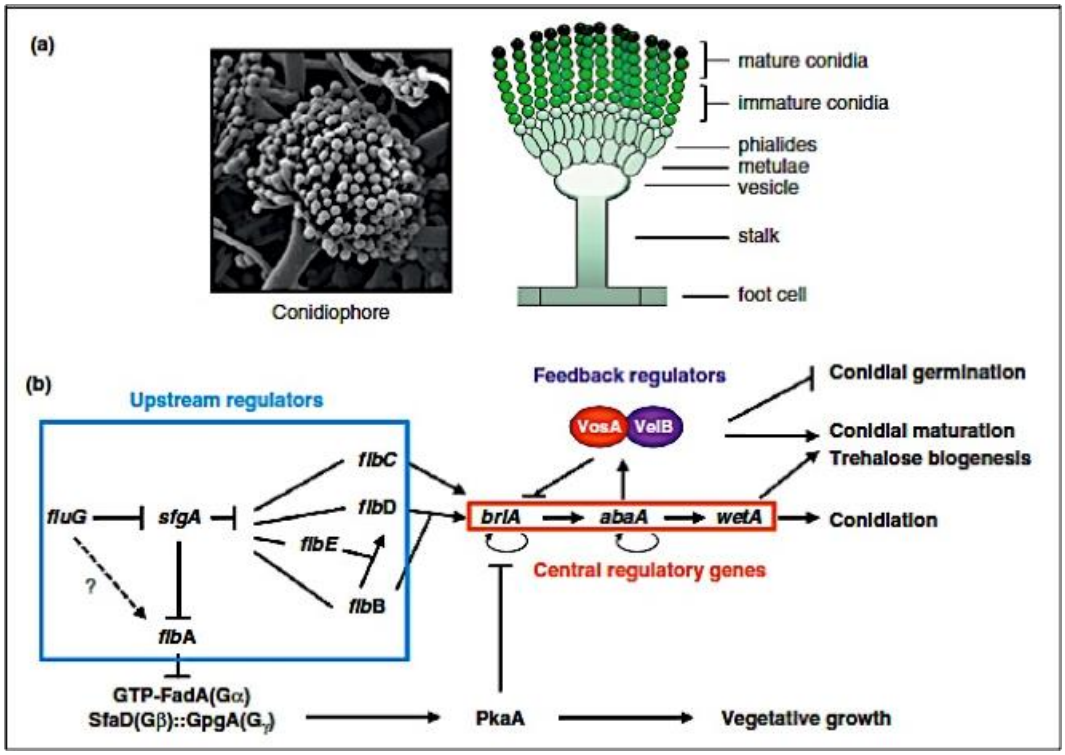


Figure 1-2 Asexual reproduction in *Aspergillus nidulans*: (a) asexual reproductive structure. (b) Upstream regulation pathway for asexual sporulation. FluG I is a central activator of asexual reproduction which inhibits vegetative growth through *flbA* activation, and suppresses G- protein signaling (RGS) balancing vegetative growth and differentiation. In addition FluG represses *sfgA*, then activates a set of genes (*flbB, flbC, flbD* and *flbE*) essential for activation of *brlA* (adapted from Park and Yu, 2012)**.**

1.2.2 Sexual reproduction (including effect of environment and different types of fruit body development).

Sexual reproduction has an essential role in the life cycles of most eukaryotic organisms, having a crucial effect on the evolutionary biology of species by allowing adaptation to environmental change and elimination of deleterious mutations (Dyer and Paoletti, 2005; Kück and Pöggeler, 2009; Lee *et al.*, 2010a; Ni *et al.*, 2011; Billiard *et al.*, 2012; Ene and Bennett, 2014). In the specific case of ascomycete fungi sex can in addition lead to the formation of resistant thick-walled sexual spores, many of which are resistant to harsh conditions, and

production of fruiting bodies which can serve as overwintering structures (Sun and Heitman, 2011; Dyer and O'Gorman, 2012). Sexual reproduction also offers a valuable tool for strain improvement and genetic analysis (Sun and Heitman, 2011; Dyer and O'Gorman, 2012).

Despite the advantages of sexual reproduction, there are some disadvantages. Sexual reproduction is relatively expensive in terms of metabolic costs, requiring nutrient input during the developmental stage of fruiting body formation; in the case of outbreeding sex two compatible parents are required, and in addition, sex may result in the loss of beneficial combinations of traits due to recombination (Dyer *et al.*, 1992; Ene and Bennett, 2014). Also sexual reproduction requires a longer period of time to occur than asexual spore formation (Dyer and O'Gorman, 2012). For example, *Aspergillus fumigatus* requires very specific conditions for sex and can take a very long period; sexual reproduction was observed only after six months of incubation at 30^{o}C in the dark on Parafilm-sealed oatmeal agar medium for many isolates (O'Gorman *et al.*, 2009).

Sexual compatibility/identity and development in fungi are unlike that seen in most animals where morphological differences are evident between the sexes. By contrast, mating identity in fungi is determined by genes found at the mating-type '*MAT*' locus, and the mating partners are morphologically indistinguishable although they can differentiate to form male and female elements (Harley, 2003; Kashimada and Koopman, 2010).

In the Ascomycotina, sexual reproduction is initiated by the interaction of two complementary cell types. Depending on the species, these cells may be morphologically differentiated into female (ascogonium) and male (antheridium) cells, or may remain undifferentiated (Pöggeler *et al.*, 2006). Fertilization does not occur until a trichogyne (an apical extension of ascogonium) fuses to an antheridium (Bistis, 1998).

The process of cell fusion is determined by diffusible pheromones and pheromone receptors in the plasma membrane (Kim and Borkovich, 2004; Kim and Borkovich, 2006; Ni *et al.*, 2011). Pheromones are involved with signalling and

recognition between receptive cell types, such as microconidia and trichogynes, of opposite mating type (Bölker and Kahmann, 1993; Kim and Borkovich, 2006). In *Neurospora crassa*, the growth orientation of female trichogynes is determined by the presence of pheromones produced by male cells of the opposite mating type (Kronstad and Staben, 1997). The donor male cell (the antheridium) might be uninucleate spermatia, conidia or hyphae (Glass and Kuldau, 1992; Coppin *et al.*, 1997). The union of the male and female cells results in fertilization (plasmogamy). However, nuclear fusion (karyogamy) does not follow immediately (Casselton, 2002); instead nuclei propagate to produce numerous nuclei in a syncytium (Nelson, 1996; Shiu and Glass, 2000; DeBuchy and Turgeon 2006). After that a pair of nuclei [one from each parent of opposite mating type in the case of heterothallic species (see below)] are transfered to ascogenous hypha from which crozier cells are formed (Coppin *et al.*, 1997; Ni *et al.*, 2011).

Karyogamy takes place in a binucleate crozier cell, which immediately undergoes meiosis to form four ascospores, or in some species a mitotic division also occurs to produce eight ascospores. These ascospores are located in a sac like structure called an ascus, which is embedded within a fruiting body called an ascocarp (Glass and Kuldau, 1992; Pöggeler *et al.*, 2006).

Depending on the morphology and structure of ascocarps (fruiting body; ascomata), ascomycete fungi have traditionally been classified into those forming perithecia (pyrenomycetes), apothecia (discomycetes), cleistothecia (plectomycetes), or pseudothecia (loculoascomycetes) (Pöggeler, 2001 and Pöggeler *et al.*, 2006) (**Figure 1-3**). This classification has been supported partly by molecular phylogenetic studies, except for the loculoascomycete grouping (Coppin *et al.*, 1997).

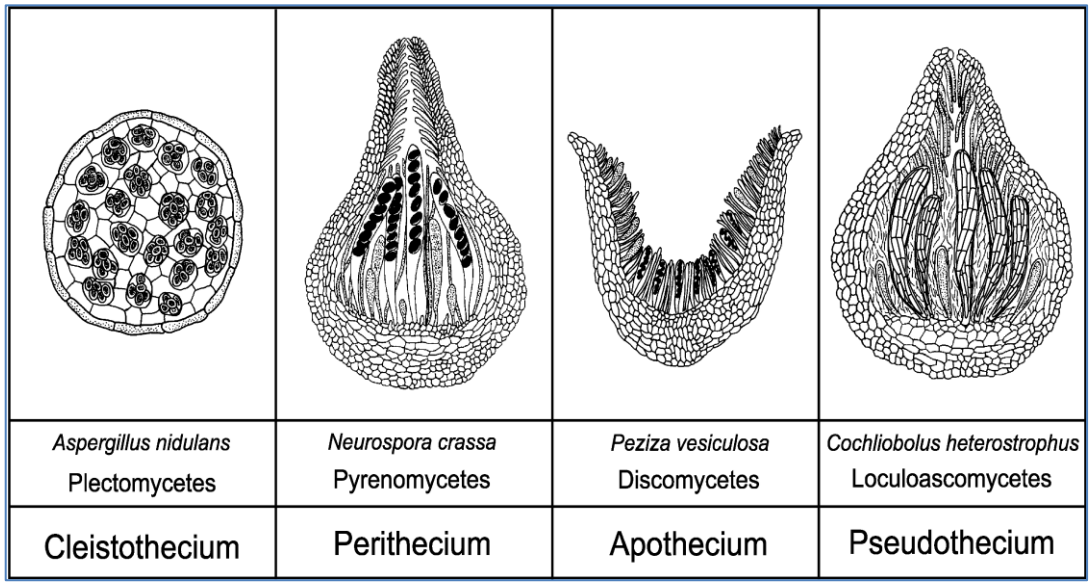


Figure 1-3 Types of fruiting body in the Ascomycotina (adapted from Pöggeler et al., 2006).

Environmental conditions influence sexual reproduction, some factors inducing sex whilst others repress it. There has been extensive research into this topic and a comprehensive review is not appropriate for this introduction; but some examples will now be provided. The formation of ascogonia is often triggered when nutrient supply becomes limited (Glass and Kuldau, 1992). A carbon source is essential for both sexual and asexual reproduction. For example, in *A. nidulans* the concentration of glucose has a major affect; a range of 0.5% - 6% glucose is required for sexual reproduction; whereas a glucose concentration lower than 0.5% and above 6% led to decrease in cleistothecial formation (Han *et al*., 2003). Similarly, in *Ophiobolus graminis* a glucose range of (0.75% - 10%) with an optimum of 1.5% is require for perithecia formation (Moore-Landecker, 1992); any decrease and increase in this range significantly affects the fertility. Also in *Saccharomyces cerevisiae* low nutrient levels can induce sexual reproduction, and vegetative growth is restored after transferring to a rich media, even when a cell is still at meiosis I (Debuchy *et al.*, 2010). Oxygen concentration also affects the developmental processes; limitation of oxygen induces sexual and inhibits asexual reproduction in *A. nidulans* (Han *et al*., 2003). Light is another factor; although

the influence of light on sexual reproduction is highly variable; is can either act as an inducer or inhibiter (Moore-Landecker, 1992; Pöggeler *et al.*, 2006; Debuchy *et al*., 2010). In *A. nidulans*, light of particular wavelengths suppresses sex and induces asexual reproduction (Butler, 2010), whereas ascocarp formation in *Pyronema domesticum* is completely reliant on light, with a loss of fertility in the dark (Pöggeler *et al.*, 2006).

Whereas most fungi are capable of vegetative growth over a wide range of temperatures, the formation of fruiting body and sexual spores usually occurs over a much narrower range of temperatures. The optimum temperature for fruiting body formation in a considerable number of ascomycete species is between 20-24^{o}C, an increase or decrease in the temperature adversely affects the sexual cycle (Moore-Landecker, 1992).

1.2.3 Parasexual reproduction - vegetative incompatibility

Parasexual reproduction is an unusual form of reproduction seen in certain ascomcycete fungi. The process involves fusion of compatible vegetative hyphae (heterokaryon formation) followed at a later stage by karyogamy and mitotic division. The process can result in genetic exchange through crossing over and recombination, albeit at a very low rate (Pontecorvo, 1956; Pál *et al.*, 2007; Schoustra, 2007; Varga *et al.*, 2014) (**Figure 1-4**). The parasexual cycle provides an alternative method to sexual reproduction to produce a recombinant strain through mitosis (Ward *et al.*, 2005). However, as with sexual reproduction compatible partners are required for the parasexual cycle (Pál *et al.*, 2007).

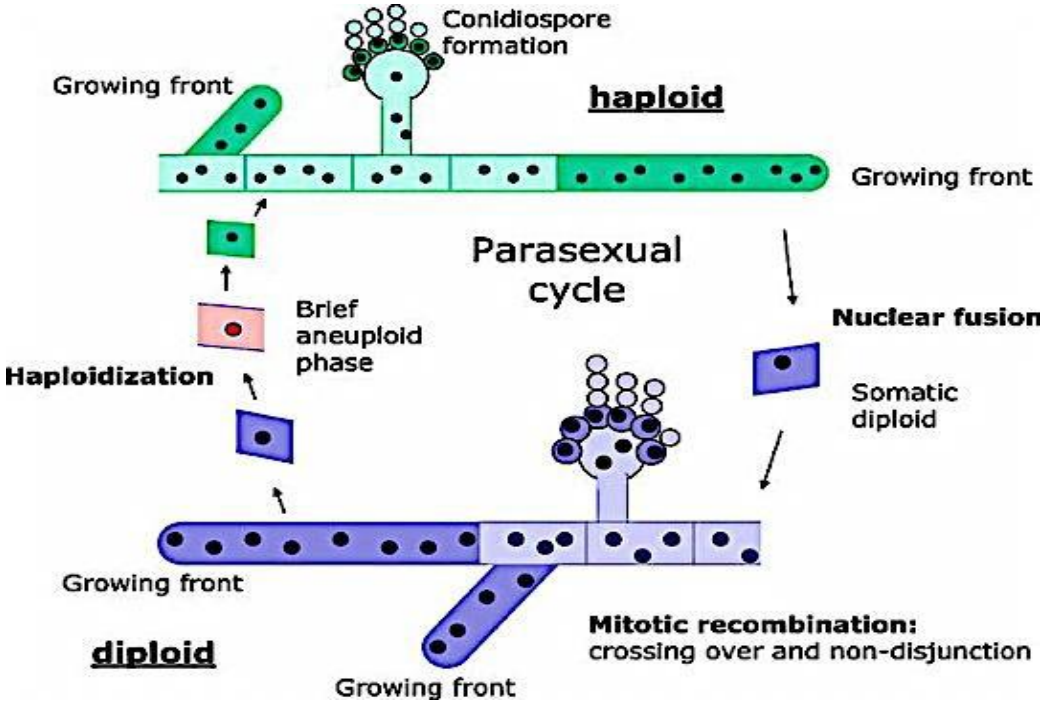


Figure 1-4 The parasexual cycle in *Aspergillus nidulans* showing the main events of the process (alteration between diploidization and haploidization in vegetative growth) (Schoustra *et al.*, 2007).

An important and initial step in parasexual reproduction is the formation of a heterokaryon, a process which is governed by vegetative incompatibility genes located at '*het*' loci (Leslie, 1993). Vegetative incompatibility or heterokaryon incompatibility describes all the mechanisms which prohibit the interaction between genetically incompatible strains thereby preventing the formation of heterokaryons between those nonallelic strains (Nauta and Hoekstra, 1996; Shui and Glass, 2000).

Vegetative incompatibility occurs between two strains when dissimilar alleles are present in at least one *het* locus, and leads to autolysis and cell death (Glass and Kuldau, 1992; Glass *et al.*, 2000; Saupe *et al.*, 2000; Xiang and Glass, 2004). The main events leading to cell death during vegetative incompatibility are similar to

programmed cell death in higher eukaryotes, including formation of septa to separate the heterokaryon hyphae from other hyphae, degradation of organelles, formation of vacuoles and finally shrinkage of cells (Glass *et al.*, 2000; Xiang and Glass, 2004). Genetic studies in model ascomycete fungi indicate the existence of variable numbers of *het* loci. There are at least 8 in *A. nidulans* (Anwar *et al.*, 1993; Loubradou and Turcq, 2000), 11 in *N. crassa* (Saupe, 2000; Smith *et al.*, 2000), and 9 in *Podospora anserina* (Saupe *et al.*, 2000). The genetic system of heterokaryon incompatibility is normally allelic but some nonallelic forms have been found in *P. anserina* and *Heterobasidion annosum* (Loubradou and Turcq, 2000).

1.3 Sexual reproduction strategies in ascomycete fungi

1.3.1 Breeding systems

Three different types of breeding systems are found in filamentous ascomycete fungi: homothallism, heterothallism and psuedohomothallism (Metzenberg and Glass, 1990; Poggeler, 2001; Lu *et al.*, 2011; Nygren *et al.*, 2011). The behaviour of species and presence of different organisations of mating-type (*MAT*) genes (see following **Section 1.3.2**) is a key determinant of, and element used for distinguishing, these breeding systems (Galagan *et al.*, 2005).

Homothallic fungi are by definition self-fertile (i.e. undergo sexual reproduction without the need of a compatible partner), and in most cases contain both HMG- and alpha-mating-types genes (see below) in the same genome, either in close proximity (Kück and Pöggeler, 2009) or on different chromosomes as seen in *A. nidulans* (Dyer *et al.*, 2003; Galagan *et al.*, 2005; Scazzocchio, 2006). Mycelium that arises from germination of sexual ascospores or asexual conidiospores is capable of completing the sexual cycle without the need for a compatible partner (Dyer *et al.*, 1992; Pöggeler *et al.*, 1997; Pöggeler, 2001). Importantly, most homothallic species retain the ability to outcross so are not limited to self-fertility (Varga *et al.*, 2014).

Pseudohomothallic or ‘secondary homothallic’ fungi are similar to homothallic fungi in that they are self fertile, but mating occurs between two genetically different nuclei, each of opposite mating type (Nelson, 1996; Coppin *et al.*, 1997; Poggeler, 2001). *Neurospora tetrasperma* is an example of a pseudohomothallic ascomycete; it produces four ascospores per ascus; each ascospore contains two nuclei of opposite mating type (Kronstad and Staben, 1997; Debuchy and Turgeon, 2006; Corcoran *et al.*, 2012).

By contrast, only one mating-type gene is present in heterothallic fungi; therefore these fungi are self-sterile and require a partner with a compatible mating-type gene for sexual reproduction to occur (Pöggeler *et al.*, 1997; Ni *et al.*, 2011; Dyer and O'Gorman, 2012; Wilken *et al.*, 2012). Heterothallism was described for the first time from the Zygomycotina, when Blakeslee in 1904 noted that some strains of Mucorinae were able to undergo sexual reproduction only when two compatible, different spores anastomosed to form zygotes (Coppin *et al.*, 1997). There is a long-standing debate as to whether heterothallism or homothallism is the ancestral mode of reproduction in ascomycete fungi. There is evidence from the structural organization of the *MAT* locus that homothallism descended from heterothallism in *Cochliobolus* spp. (Yun *et al.*, 1999; Geiser, 2009; Ni *et al.*, 2011). The structural organization of the *MAT* locus was found to be similar between all heterothallic species examined (*C. heterostrophus, C. carbonum, C. victoriae, C. ellisii, and C. intermedius*). By contrast, it was very variable in homothallic species with each homothallic species having a *MAT* locus organization unique to the species, indicating divergent evolution from heterothallic ancestors (Yun *et al.*, 1999). Similarly, the organization of the *MAT* locus was conserved between heterothallic *Neurospora* species, whereas four homothallic *Neurospora* species were each found to have different *MAT* arrangements (Gioti *et al.*, 2012). It is believed that homothallic *Fusarium* and *Pencillium* species are also evolved from heterothallic ancestors (Billiard *et al.*, 2012). In contrast it has been argued that for some *Aspergillus* species homothallism may be the ancestral state and that heterothallism has been derived by gene deletion (Billiard *et al.*, 2012), with the predominant presence of homothallic species within genus *Aspergillus* supporting this theory (Dyer, 2007).

1.3.2 Mating-type genes in the Ascomycotina

It is necessary for partners of different 'mating-type' to be present to allow sexual reproduction to occur in heterothallic species, comprising the processes of heterokaryon formation, diploidization and finally formation of haploid spores via meiosis (Bölker and Kahmann, 1993). Mating-type identity in fungi differs from most of the best-studied eukaryotes in that it is determined in the haploid rather than diploid stage (Billiard *et al.*, 2011). Gene(s) residing at the mating-type '*MAT*' locus are responsible for the determination of sexual identity by encoding for the production of transcription factors, which regulate the expression of pheromones and pheromones receptors necessary for cell recognition (Pöggeler *et al.*, 2006b; Coelho *et al.*, 2011). Mating-type genes are considered as master regulatory loci controlling sexual reproduction (Pöggeler *et al.*, 1997).

Almost all heterothallic ascomycete species exhibit bipolar mating systems, in which two alternative mating-type loci control sexual identity (Bennett, 2010). In heterothallic filamentous ascomycetes there is a single *MAT* locus, which contains dissimilar DNA sequences according to the mating identity of an isolate. These dissimilar regions have been termed idiomorphs, rather than alleles, to emphasise their divergence (Metzenberg and Glass, 1990; Arnaise *et al.*, 1993; Turgeon and Yoder, 2000; Scazzocchio, 2006). By definition *MAT1-1* isolates contain an idiomorph with a *MAT1-1* (sometimes abbreviated to *MAT1*) gene encoding an alpha box domain protein, whereas *MAT1-2* isolates contain an idiomorph with a *MAT1-2* (sometimes abbreviated to *MAT2*) gene encoding a high mobility group domain (HMG) protein (**Figure 1.5**). *MAT* loci may contain additional genes according to specific taxonomic grouping (Kück, and Pöggeler 2009). By contrast, homothallic fungi normally contain both *MAT1-1* alpha and *MAT1-2* HMG mating-type genes in the same genome (Dyer *et al.*, 2003; Dyer and O'Gorman, 2011) (**Figure 1-5**).

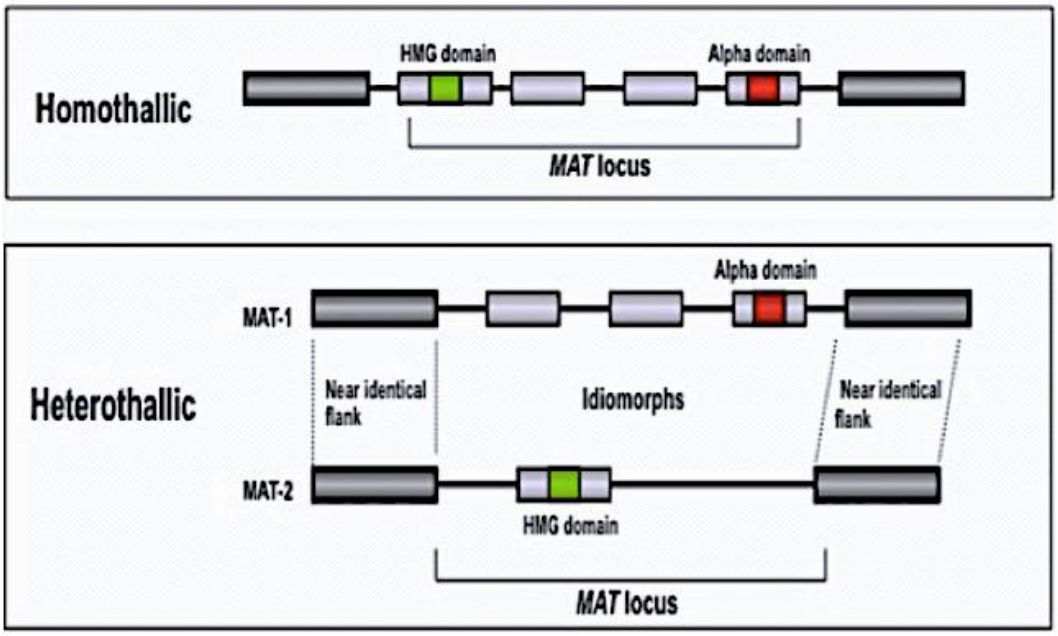


Figure 1-5 Mating-type locus in ascomycete fungi: homothallic strains contain both alpha and HMG-domain mating-type genes in the same genome, whereas heterothallic strains have only one mating-type gene (either alpha or HMG-domain) present (Dyer, 2007).

Neurospora crassa is an example of a heterothallic filamentous ascomycete, strains containing either a *mat A* or *mat a* idiomoph, encoding an alpha or HMG protein amongst other proteins (Metzenberg and Glass, 1990). The *mat A* idiomorph of *N. crassa* contains three genes: *mat A-1*, *mat A-2* and *mat A-3* (Staben, 1996; Kronstad and Staben, 1997; Xiang and Glass, 2004). The *mat A-1* gene is essential for sexual development and *mat* A identity (Glass *et al.*, 1990; Bobrowicz *et al.*, 2002); while deletion of either *mat A-2* or *mat A-3* gene has no phenotypic effect, but deletion of both genes together drastically decreased ascospore formation (Ferreira *et al.*, 1998). Both *mat A-2* and *mat A-3* are likely to be active after fertilization and are not required for mating-type compatibility (Silva *et al.*, 2009). In contrast *mat a* contains only a single gene *mat a-1* gene, which is required for *mat a* identity and sexual development (Staben and Yanofsky, 1990; Philley and Staben, 1994). Interesting the mating-type genes in *N. crassa* have dual function, in addition to their roles in cell recognition and

sexual reproduction, they also have roles in vegetative incompatibility (Glass and Kuldau, 1992 and Saupe *et al.*, 2000 and Xiang and Glass, 2004). Similarly in *Pencillium chrysogenum*, mating-type genes have functions beyond their role in cell identity. In this case *MAT* genes have been shown to influence penicillin production, with deletion of both *MAT* genes leading to a reduction of penicillin production; *MAT* genes were also shown to influence hyphal morphogenesis and conidiation (Böhm *et al.*, 2013).

1.3.3 Evolution and divergence of mating-type genes

Mating-type genes show high sequence divergence in the fungi, even between closely related genera, compared to housekeeping genes (Kronstad and Staben, 1997). In ascomycete fungi, genes at the *MAT* locus differ in size (between 1-6 kilobases) and also in number (ranging from 1-3 genes), and a more complex mating system still is found in the Basidiomycotina where there may be twenty or more mating-type genes and *MAT* locus regions of more than 100 kilobases. However, one feature is shared in the filamentous ascomycetes, being that the most conserved *MAT* genes encode either alpha-box domain or high-mobility group domain (HMG) transcription factors (Dyer, 2008).

HMG domain genes are generally the most conserved and are considered as ancestral sex genes within the fungal kingdom; the presence of HMG domain *MAT* genes in the Zygomycotina, which represents a basal grouping in the evolutionary tree of fungi, supports this theory (Idnurm *et al.*, 2008). *Phycomyces blakesleenus*, a member of the Zygomycotina, has a unique *MAT* locus or 'sex-determining region' in which only a gene encoding an HMG transcription factor is found (**Figure 1-6**), and no alpha-box domain gene is present. The corresponding regions of chromosomes of both plus and minus strains were found to encode HMG proteins, although this region is occupied by dissimilar DNA in both strains (Idnurm *et al.*, 2008). Among ascomycete fungi, the *MAT* locus in dothidiomycetes is relatively simple, encoding either an HMG domain or alpha box domain protein i.e. there is only one type of *MAT1* gene, the other *MAT1-2* and *MAT1-3* genes seen elsewhere in the Ascomycota are missing (Casselton,

2008). The presence of similar HMG domains in the *MAT1-2* and downstream region of the *MAT1-1* genes of *Cochliobolus*, together with the evidence of having only one HMG domain in *Phycomyces* suggests that they both may have diverged from a common ancestor (Turgeon and Debuchy, 2007). Indeed, Martin and co-workers have provided evidence that alpha-box domain proteins evolved from ancestral MATA-HMG domain family proteins, as supported by sequence similarity between them and phylogenetic studies that showed both were evolutionarily related. The alpha domain clustered as a monophyletic group in the MATA-HMG domain grouping, showing closer relationship to the MATA-HMG domain more than either SOX or HMGB-lineages (Martin *et al.*, 2010).

Elsewhere, the mating-type genes of *Sacchromyces cerevisiae* consist of two genes termed *MATa* and *MAT∝*, located on chromosome three (Butler *et al.*, 2004; Butler, 2007). In addition to *MATa* and *MAT∝* there are additional 'silent cassettes' that occupy the *HML* and *HMR* loci, which are homologous to the *MATa* and *MAT∝* genes (Astell *et al.*, 1981; Lee *et al.*, 2010a). The mating-type locus of *Schizoccharomyces pombe* differs from that of *S. cerevisiae*. A homologue of the *a1* gene is not found; instead it is replaced by an HMG-domain transcription factor encoded by *mat1-Mc.* The latter resides at the *Mat1-M* locus resembling that of the *MATa* locus of *S. cerevisiae.* In addition to *mat1-Mc* gene, there is another gene *mat1-Mm* localized to the same locus although this encodes a protein of unknown function (Casselton, 2002). The mating system in the majority of the Basdiomycotina involves a tetrapolar arrangement of mating-type loci, consisting of two unlinked loci and unlike other fungi HMG and alpha box domain genes are not present. Instead, both pheromone and receptor genes are linked together at one mating-type locus, conferring mating identity, whilst the other locus encodes two homeodomain transcription factors *HD1* and *HD2*, which are homologues of ascomycete yeast ∝2 and a1 factors, respectively (**Figure 1-6**) (Casselton, 2002; Coelho *et al.*, 2011).

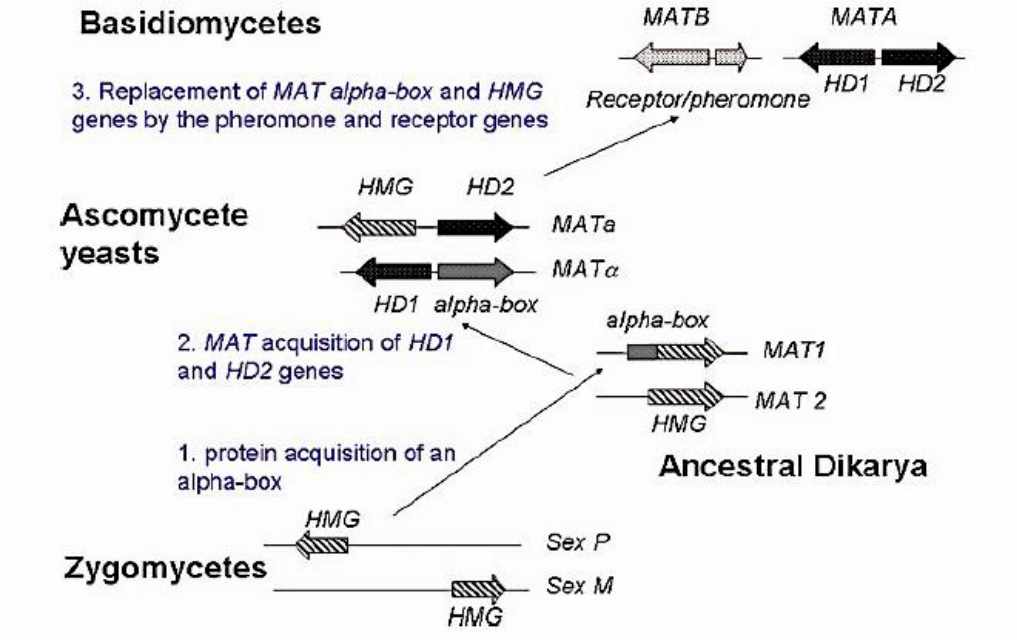


Figure 1-6 Hypothetical evolution of the mating-type locus in fungi. 1. In zygomycetes there is only HMG domain. 2. An alpha-box protein evolved in the filamentous ascomycete MAT locus. 3. Acquisition of *HD1* and *HD2* in yeast MAT loci. 4. Finally in basidiomycetes both alpha and HMG domain are replaced by pheromones and their receptors (Casselton, 2008).

1.4 Role of pheromone signaling in sexual reproduction

Pheromones are diffusible peptide mating factors that have a crucial role in specific cell recognition in many fungal species (Bölker and Kahmann, 1993; Schmoll *et al.*, 2010). Two classes of pheromones have been identified in ascomycete fungi (Bölker and Kahmann, 1993; Martin *et al.*, 2011). These have been most extensively studied in *S. cerevisiae*, which provides a model for understanding pheromone signalling in the fungal kingdom. In *S. cerevisiae*, initiation of mating between compatible partners is triggered by pheromone and their receptors (Naider and Becker, 2004); pheromone sensing results in polarized growth and formation of shmoos toward the compatible cell of opposite mating type (Bobrowicz *et al*., 2002; Casselton, 2002). Two pheromone and cognate receptor genes have been identified in *S. serevisiae*. MATa cells encode an a-

factor pheromone and a receptor for ∝-factor and vice versa i.e. MAT∝ cells secrete ∝-factor and have a receptor for the a-factor pheromone (Hirsch and Cross, 1992; Bardwell, 2004; Jones and Bennett, 2011). However, haploid cells of *S. cerevisiae* harbour genes for both types of pheromone and receptor but only one of these sets is expressed, which is controlled by mating-type genes in each cell (Pöggeler and Kück, 2001; Coppin *et al.*, 2005). The mature ∝-factor pheromone of *S. cerevisiae* is hydrophilic and consists of 13 amino acids and is produced by modification of two large putative pheromone precursor proteins 165 and 120 amino acids encoded by *MF∝ 1* and *MF∝ 2* genes respectively (Bölker and Kahmann, 1993; Naider and Becker, 2004). The ∝-factor pheromone is secreted by a classical secretory pathway and becomes bound to the Ste2 receptor on the surface of cells of opposite mating type (Jones and Bennett, 2011). Whereas the a-factor pheromone is hydrophobic and consists of 12 amino acids, being encoded by *MFa 1* and *MFa 2* genes, which encode 34 and 36 amino acids precursors respectively; the pheromone is secreted by a transporter and becomes bound to the Ste3 receptor on the surface of cells of opposite mating type (Bölker and Kahmann, 1993; Jones and Bennett, 2011). Both pheromone receptors belong to a large family of G-protein coupled receptors (GPCR) (Bölker and Kahmann, 1993; Pöggeler and Kück, 2001; Naider and Becker, 2004; Seo *et al.*, 2004; Jones and Bennett, 2011). The GPCR is composed of three sub-units: ∝, β and γ, forming a complex bound to the plasma membrane (Seo *et al.*, 2004; Seo *et al.*, 2005; Dyer, 2007; Jones and Bennett, 2011). Pheromone binding to its cognate receptor results in exchange of GDP to GTP followed by dissociation of the subunits of the GPCR, with G∝ cleaved from Gβγ (Dohlman and Thorner, 2001; Seo *et al.*, 2004; Deflorio *et al.*, 2013; Krijgsheld *et al.*, 2013). The released Gβγ complex then interacts with two other molecules: Ste20 and Ste5 (Maeder *et al.*, 2007). Activated Ste20 phosphorylates Ste11 resulting in the transmission of the signal to Fus3 through Ste7 as a phosphate signal; finally Fus3 become free and enters the nucleas to activate Ste12, which is a nuclear transcription factor required for initiation and control of sexual development (**Figure 1-7**) (Bayram *et al.*, 2012).

Related pheromone precursor and receptor genes have been identified in filamentous heterothallic ascomycete species (Imai and Yamamoto, 1994; Zhang *et al.*, 1998). Here, the direction of growth of sexual mycelia such as trichogynes towards the antheridium of an opposite mating type is also induced by diffusible pheromone signalling (Shui and Glass, 2000; Schmoll *et al.*, 2010). For example, in *N. crassa*, two pheromone precursor genes have been identified (*mfa-1* and *ccg-4*), which encode for a and α factor pheromones respectively; their expression seems to be regulated by *MAT* genes as a mating-type mutant strain failed to produced pheromones and trichogynes (Bobrowicz *et al.*, 2002). Pheromone signalling also appears to be important for sexual development in homothallic ascomycete fungi. Both pheromone precursor and receptor genes similar to *S. cerevisiae* are found in the ascomycetes *Sordaria macrospora* and *Aspergillus nidulans*, where they are transcribed during sexual development (Pöggeler, 2000; Mayrhofer *et al.*, 2006). Mayrhofer and co-workers suggested the requirement of at least one cognate pair of a pheromone and matching receptor for occurrence of sexual reproduction in homothallic *S. macrospora*, as deleting a single pheromone gene had no effect whereas a double deletion of both a- and α-factors impaired sexual development (Mayrhofer *et al.*, 2006).

Recent studies suggest that in addition to the role of pheromones in cell recognition and fertilization; they also play important role in post-fertilization events (Bölker and Kahmann, 1993; Kim and Borkovich, 2006; Schmoll *et al.*, 2010). In *Saccharomyces pombe* pheromone signalling is necessary for induction of *mat1-Pm* and *mat1-Mm* expression. These genes play important role in the initiation of meiosis through activation of the *mei3* gene (Willier *et al.*, 1995).

In contrast to the Ascomycotina, only a- factor pheromone precursor genes have been found in basidiomycete fungi (Bölker and Kahmann, 1993; Spellig, 1994; Chikashige *et al.*, 1997; Casselton, 2002).

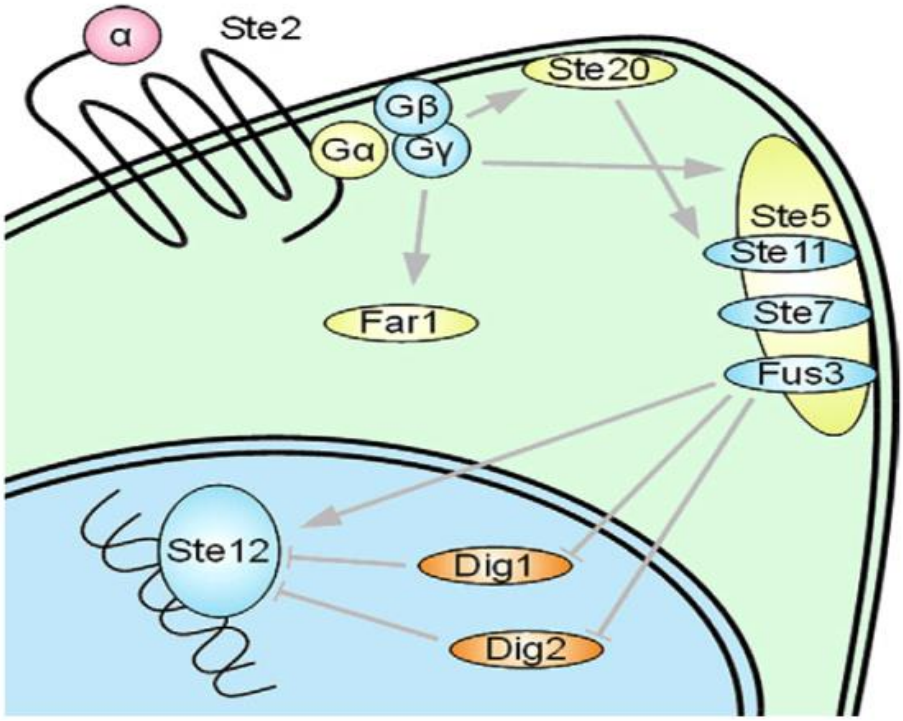


Figure 1-7 Mechanism of pheromone signalling in *Saccharomyces cerevisiae* showing pheromone binding to receptor and signal transmission through GPCR to activate Ste12 (Jones and Bennett, 2011).

1.5 Asexuality in fungi

Approximately 20% of fungal species are only known to reproduce by asexual means i.e. they lack sexual reproduction (Dyer and Paoletti, 2005; Hosid *et al.*, 2010). These fungi are thought to have evolved from sexual ancestors by, for example, a point mutation, nucleotide substitution, or frame shift mutation in a critical gene involved in sexual reproduction, resulting in a non-functional gene and therefore lack of sex (Dyer and Paoletti, 2005; Sun and Heitman, 2011). Indeed, recent genome sequencing studies of several imperfect fungi, both filamentous and yeast-like, have detected the presence of mating-type and other sex-related genes, providing a clue to the evolutionary origins of asexual species from sexual ancestors (Geiser *et al.*, 1996; Kück and Pöggeler, 2009). A lack of

isolates of compatible mating type might also provide an explanation for the failure to sexually reproduce (Dyer and Paoletti 2005; Sun and Heitman, 2011).

In the following subsections some of the possible factors that might lead to asexuality in the fungi will be described in more detail noting that these explanations are not mutually exclusive.

1.5.1 Mutation in a key sex-related gene(s)

Asexual species may arise from a sexual ancestor (Geiser *et al.*, 1996) as a result of mutation in a gene required for sexual reproduction (a 'sex-related' gene) or even complete loss of such a gene, resulting in a loss of sexual fertility (Dyer and Paoletti, 2005). Sexual reproduction is a complex process, involving a network of at least 200 – 400 genes (Dyer *et al.*, 1992). More than 200 mutations have been found in *N. crassa*, which affect fertility (Geiser *et al.*, 1996). Genetic analysis of *Aspergillus niger* revealed the presence of a complete set of genes known to be required for sex to occur, with a single mutation found only in a sex-related *Ppo* gene (Pel *et al.*, 2007; Dyer and O'Gorman, 2011). Genome screening of the supposedly asexual *A. fumigatus* and *A. oryzae* similarly revealed the presence of 215 genes with a role in sexual development, all seemingly encoding functional genes, suggesting that asexuality in these species may be due to mutation in other unidentified genes (Galagan *et al.*, 2005). Indeed, a sexual cycle was recently found in *A. fumigatus*, requiring a prolonged incubation time of 6 months under specific conditions (O'Gorman *et al.*, 2009).

3.3.1 Lack of compatible mating partner

As mentioned above, there are two breeding systems in ascomycete fungi: homothallism (self fertility) and heterothallism (self sterility). In heterothallic species, compatible partners of opposite mating are required for sex to occur, so the lack of availability of compatible mating partners in the environment may be a possible reason for absence of sex in some asexual species (Dyer, 2007; Geiser,

2009). For example, in the heterothallic basidiomycete *Cryptococcus neoformans* great variation has been found in the distribution of mating partners with predominance of the MATα type; only two MATa strains were found among 105 isolated strains from the natural environment (Kwon-Chung and Bennett, 1978). Intriguingly this might have lead to the phenomenon of unisexual mating (Heitman *et al.,* 2014). There is a similar possible argument that many asexual *Aspergillus* species might be heterothallic but are asexual due to the extinction of one mating type in populations (Dyer, 2007). For example, in *A. niger* there is imbalance in the distribution of mating type strains in nature, with a dominance of *MAT1-1* compared to *MAT1-2* strains (Pel *et al.,* 2007; Dyer and O'Gorman, 2011). This imbalanced distribution is also seen in the asexual *A. welwitschia*, (section Nigri) where here is a 6:1 ratio of *MAT1-1* to *MAT1-2* strains (Varga *et al.*, 2014).

1.5.2 Slow decline in sexual reproduction

The potential for sexual reproduction might simply decrease with time under certain conditions. For example, there is evidence that sexual fertility is decreased as a consequence of sub-culturing of vegetative mycelium in the laboratory (Dyer and Paoletti, 2005). Fruit body formation in *A. glaucus* was observed to decrease as a result of sub culturing of this species via the asexual form in the laboratory (Mather and Jinks, 1958). Recently a similar trend was found for *A. nidulans*, *Emericella repens* and *N. fischeri*, in which sexual fertility declined after several rounds of asexual sub culture (Ashour, 2014). Also in the plant pathogenic species *Magnaporthe grisea* only the anamorphic state has been found in the field, and field isolates showed generally only very low fertility when crossed with fertile laboratory tester strain (Notteghem and Silue, 1992). It was suggested that the reason for low fertility might be due to a decline in sexual fertility after extended periods of asexual reproduction in the field. Such a 'slow decline' in sexual fertility, rather than abrupt loss, might be due to the variation in the expression of genes required for sex, epigenetic changes, and/or internal factors that govern the compatibility between male and female in heterothallic species.

1.5.3 Failure to identify a teleomorph-anamorph relationship or oversight

Another possible reason for asexuality in fungi may be due to inability to identify an anamorph-teleomorph relationship. For example the plant pathogenic species *Pseudocercosporella herpotrichoides* causing eyespot disease in cereals was for a longtime thought to propagate only by asexual mean despite being well studied. It was only as result of careful field and laboratory studies that it was then realized that sexual fruiting bodies (apothecia) found in the field on straw stubble actually represented the sexual state *Tapesia* of the eyespot fungal species (Dyer and Paoletti, 2005).

1.6 The genus *Aspergillus*

Work in the current project will focus on members of the genus *Aspergillus*, which will now be described in more detail.

1.6.1 General background

The genus *Aspergillus* is amongst the largest and most diverse group of fungi known, comprising ~ 300- 350 species (Pitt and Taylor, 2014; Varga *et al.*, 2014). The majority is asexually reproducing fungi with no known sexual state (Dyer and O'Gorman, 2011). Species of aspergilli are saprophytic and cosmopolitan (Dyer, 2007), capable of degrading a variety of substances (Krijgsheld *et al.*, 2013), and producing a vast number of small air dispersible spores, these factors contribute to the frequent distribution of *Aspergillus* species in the indoor and outdoor environments (Klich, 2009).

The genus *Aspergillus* consists of species ranging from those harmful to those beneficial to mankind (Galagan *et al.*, 2005; Yu, 2010; Dyer and O'Gorman, 2012). Both *A. fumigatus* and *A. clavatus* are human pathogens that cause disease such as pulmonary respiratory disease and pneumonitis, respectively (Butler, 2010; Lee *et al.*, 2010a). In addition *A. flavus* and *A. parasiticus* are plant

pathogenic fungi and adversely affect human life because of production of toxins called aflatoxins, which are carcinogenic (Abe *et al.*, 2006; Yu *et al.*, 2006; Bennett, 2009). By contrast, *A. oryzae* and *A. niger* are important beneficial species, widely used to produce valuable compound and various enzymes (Archer and Dyer, 2004; Yu *et al.*, 2006; Yu, 2010). Also *A. nidulans* is an important experimental eukaryotic model organism used to study molecular and genetics processes (Archer and Dyer, 2004; Dyer, 2007).

About one third of *Aspergillus* species have the ability to reproduce both sexually and asexually (Pitt and Taylor, 2014). Where species reproduce sexually they can have either homothallic or heterothallic breeding systems. The existence of a broad range of reproductive modes means that the aspergilli provide a valuable model to study the genetics of fungal reproduction (Sohn and Yoon, 2002; Dyer, 2007; Dyer and O'Gorman, 2012).

1.6.2 Morphology of *Aspergillus*

All of the species of the aspergilli share a common morphological character, which is the formation of an asexual structure called an aspergillum (**Figure 1-8**), which is composed of a conidiophore, specialized hyphae that expand at the top to produced a vesicle, which bears a chain of metullae and phailides which then produce asexual spores called conidia (Bennet, 2009; Lee *et al.*, 2010a; Dyer and O'Gorman, 2012).

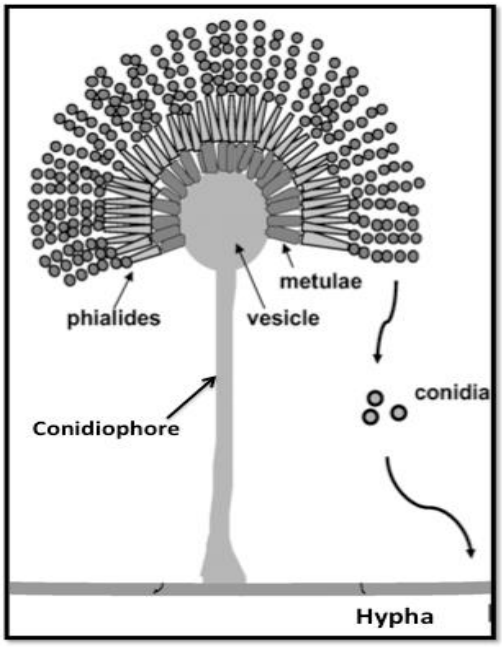


Figure 1-8 Morphology of the asexual structure of the genus *Aspergillus* (Tsitsigiannis *et al.*, 2004b).

1.6.3 Taxonomy of *Aspergillus*

The genus *Aspergillus* comprises more than 300 species; these are classified into subgenera and sections according to their phenotypic, physiological and molecular genetic features (Klich, 2009; Chang and Ehrlich, 2010). Phylogenetic analysis of mitochondrial DNA and the nuclear genome indicates that the majority of aspergilli have a common sexually reproducing ancestor (Geiser *et al.*, 1996). The Genus *Aspergillus* is classified within kingdom Fungi as follows (Hibbett *et al.*, 2007).

Kingdom: Dikarya

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Eurotiomycetidae

Order: Eurotiales

Family: Trichocomaceae

Genus: *Aspergillus*

1.6.4 Economic importance of the genus *Aspergillus*

The genus *Aspergillus* represents one of the most widely distributed fungal species in the natural environments. In part due to this ecological diversity, some *Aspergillus* species have evolved sets of physiological features that mean they can greatly affect human life.

For example, *A. oryzae* and *A. sojae* are important species that have been used for centuries for the production of Japanese fermented food, such as soy sauce and sake (Abe *et al.*, 2006; Scazzocchio, 2006; Bennett, 2009). *A. oryzae* is regarded as a generally safe organism, as it cannot produce toxins (Kobayashi *et al.*, 2007; Amaike and Keller, 2011). *A. oryzae* has the capability to produce and secrete various extracellular enzymes therefore it has been used as a host for heterologous protein production (Machida *et al.*, 2005; Abe *et al.*, 2006; Wada *et al.*, 2014). In contrast, the closely related *A. flavus* and *A. parasiticus* (all belonging to section Flavi with *A. oryzae*) produce toxic compounds that affect human life through contaminating crops; *A. flavus* also causes aspergillosis in human and animal (Horn *et al.*, 2009b; Bennett, 2010; Amaike and Keller, 2011). *A. niger* is a ubiquitous, opportunistic fungus that can cause disease in immunocompromised patients. Despite this, it is considered one of most useful filamentous fungi to mankind, being widely used in the biotechnology industry (Schuster *et al.*, 2002; Baker, 2006). Its major use is in the production of citric acid, a weak acid widely used in industry in the production of soft drinks (Schuster *et al.*, 2002; Bennett, 2009; Bennett, 2010). *A. niger* is also used as a host for industrial production of recombinant enzymes and proteins (Bennett, 2010). In addition, *A. niger* plays an important ecological role in the recycling of carbon; producing a variety of degrading and oxidizing enzymes, which break down plant lignocellulose material

(Baker, 2006). Meanwhile, *A. terreus* produces the medically important compound lovastatin, a drug used to lower cholesterol levels (Bennett, 2009). However, *A. fumigatus* is a predominant airborne opportunistic fungal pathogen being a causative agent of pulmonary aspergillosis, which can be a fatal disease in the immunosuppressed patient (Paoletti *et al.*, 2005; Kwon-Chung and Sugui, 2009; O'Gorman *et al.*, 2009; Chamilos *et al.*, 2010).

1.7 Overview of sexual development in the aspergilli

1.7.1 Breeding systems in *Aspergillus*

Approximately one-third of *Aspergillus* species are capable of reproducing both asexually and sexually (Geiser *et al.*, 1996; Geiser, 2009; Bennett, 2010). Sexually reproducing species exhibit either self fertile (homothallic) or outcrossing (heterothallic) breeding systems (Paoletti *et al.*, 2007; Große and Krappmann, 2008). As mentioned above (**Section 1.3.1**), the sexual identities of ascomycete fungi are determined by *MAT1-1* and *MAT1-2* mating-type genes (Dyer *et al.*, 1992; Turgeon and Yoder, 2000; Galagan *et al.*, 2005). In homothallic aspergilli, both mating-types genes are present, located either tightly linked in the same *MAT* locus as in *Petromyces alliaceus*, (Horn *et al.*, 2011) or in the same genome but unlinked on different chromosomes as seen in *Neosartorya fischeri* (Bennett, 2010) and *Aspergillus* (*Emericella*) *nidulans* (Dyer *et al.*, 2003; Galagan *et al.*, 2005; Paoletti *et al.*, 2007). *A. nidulans* is a homothallic *Aspergillus* species, but also has ability to out cross preferentially with other fertile strains of *A. nidulans* under certain conditions, so the term 'relative heterothallism' has been used to explain this phenomena (Czaja *et al.*, 2011 and Dyer and O'Gorman, 2012).

Among the species of *Aspergillus*, heterothallism was first reported in *A. heterothallicus* (Lee *et al.*, 2010a). In contrast to homothallic species, in the heterothallic fungi *A. flavus* (Horn *et al.*, 2009a), *A. fumigatus* (O'Gorman *et al.*, 2009), and *A. parasiticus* (Horn *et al.*, 2009b) each strain only contains a single

mating-type gene; so compatible strains of opposite mating type are required for the development of sexual structures (**Figure 1-9**).

1.7.2 Mating-type genes in *Aspergillus*

A *MAT1-2* HMG-domain family gene was the first mating-type gene to be discovered in the aspergilli, identified from the homothallic *A. nidulans* by degenerate PCR (Paoletti *et al.*, 2007). An α-domain gene was later found by BLAST searching of the newly available *A. nidulans* genome (Dyer *et al.*, 2003; Paoletti *et al.*, 2007). Unlike most other homothallic species, these mating-type genes were found not to be linked, but instead resided on different chromosomes (3 and 6, respectively) in the same genome (Dyer *et al.*, 2003 and Galagan *et al.*, 2005; Pál et al., 2007; Paoletti *et al.*, 2007). Deletion of either of the *MAT1* or *MAT2* genes resulted in the aberrant formation of cleistothecia that lacked ascospores, without any obvious effect on mycelial growth or the asexual cycle i.e. deletion of either type results in the loss of sexual fertility (Paoletti *et al.*, 2007; Czaja *et al.*, 2011; Krijigsheld *et al.*, 2013).

In *A. nidulans* expression of *MAT1* (synonym *matA*) is regulated by a silencer element (SE) located in the upstream region. The SE suppresses the expression of *MAT1* (*matA*) during vegetative growth; deletion of SE resulted in over expression of *MAT1* (*matA*) in vegetative growth and about three times upregulation in the developmental tissue. In addition there is a promoter and initiator (Inr) elements that induce the expression of *MAT1* (*matA*) during the sexual developmental phase. These promoters do not require TATA or CAAT boxes, which are other well-known promoters (Czaja *et al.*, 2011).

Two scenarios have been proposed to explain the evolution of mating-type genes in the genus *Aspergillus*, according to whether homothallism or heterothallism is the ancestral state. Some lines of evidence supports a homothallic ancestor, notably the prevalence of homothallic species within the genus *Aspergillus* together with existence of an HMG sequence fragment in the flanking region of the *A. fumigatus* alpha MAT1-1 gene. This has been used to propose the theory

that heterothallic species evolved from homothallic ancestors by segregation and then loss of one mating-type gene (Galagan *et al.*, 2005; Dyer, 2007; Lee *et al.*, 2010a). This would be consistent with the unusual organization of mating-type genes observed in *A. nidulans* and *Neosartorya fischeri*, where they seem to be derived by a translocating break and segmental duplication, respectively, from a homothallic ancestor (Figure 1-9) (Dyer, 2007; Lee et al. 2010a). The second model is the possibility of a heterothallic ancestor and derivation of homothallism. Comparing the structure of the mating-type locus between the aspergilli supports this hypothesis. All known heterothallic species share a similar organization of the *MAT* locus, with either an HMG or alpha-domain gene present, normally flanked by the presence of *APN1* and *SLA2* homologues (Dyer, 2007). By contrast, the organization of *MAT* loci among homothallic species is not consistent, with a few different arrangements found suggesting divergent evolution from heterothallic ancestral species (Dyer, 2007; Lee *et al.*, 2010a)

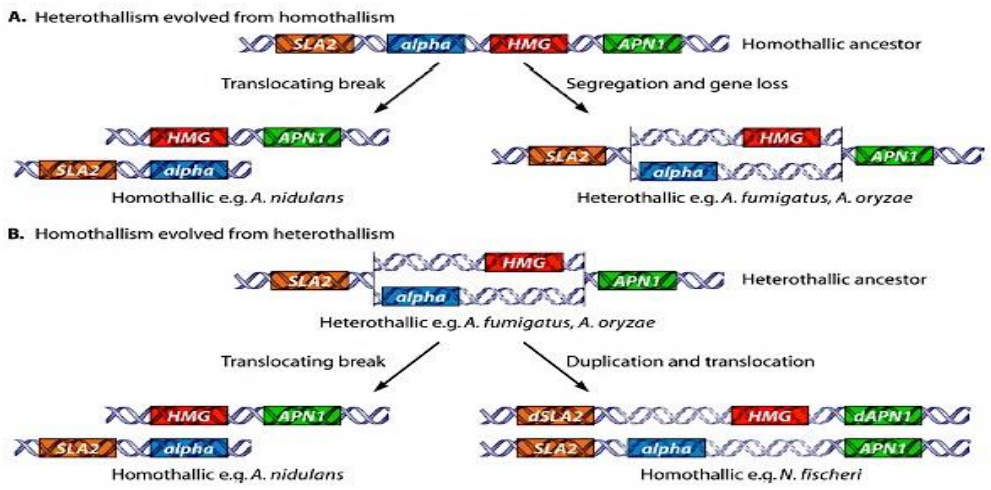


Figure 1-9 Evolution of mating-type loci in the genus *Aspergillus*. (A) Possibility of a homothallic ancestor and heterothallic species being derived by segregation and loss of either HMG or alpha box domain genes. (B) Possibility of a heterothallic ancestor and evolution of homothallic species by a translocating break as in *A. nidulans* or by chromosomal translocation and duplication as in the case of *N. fischeri*. Adapted from Galagan *et al.* (2005), Dyer (2007), and Lee *et al.* (2010a).

1.7.3 Morphology of sexual states in the aspergilli

The genus *Aspergillus* contains both sexual and asexual reproducing species, the majority are asexual; only 70 species are known to reproduce sexually (Dyer, 2007; Geiser *et al.*, 2007). The genus *Aspergillus* produces enclosed globose fruiting body structures called cleistothecia, the meiospores (ascospores) are produced in a sac like structure called an ascus which is scattered within the ascomata (Dyer, 2007; Geiser, 2009). Each cleistotheicium may contain approximately 100,000 asci, each enclosing (with very rare exceptions) eight ascospores (Pontecorvo *et al.*, 1953; Braus *et al.*, 2002).

The sexually reproducing species fall into 12 genera; four of them (*Eurotium*, *Emericella*, *Neosartorya* and *Petromyces*) being the most common genera found in the environment according to number of species (Geiser *et al.*, 2007; Dyer and O'Gorman, 2012). Each of these genera produces fruiting bodies (cleistothecia) that are morphologically different from other genera (**Figure 1-10**). The principal difference used to distinguish them is variation of the colour and composition of the cleistothecial wall (termed the 'peridium') (Benjamin, 1955; Geiser *et al.*, 2007; Pitt and Taylor, 2014). The cleistothecial wall in *Eurotium* is characterized by one layer of yellow large broad cells (**Figure 1-10a**) (Geiser, 2009); in contrast in *Emericella* it is composed of two layers of flattened cells with purple-like colour, in *A. nidulans* these cells are connected together with an unknown electron-dense substance found in the intercellular space (**Figure 1-10b**) (Sohn and Yoon, 2002). The peridial wall of *Neosatorya* (**Figure 1-10c**) consists of a multilayer network of white to light yellow flattened cells that are interconnected together forming a network of hyphae. In *Petromyces*, cleistothecia are enclosed in another structure called stromata (sclerotium), which is a hard, dark, thick-wall resting body, that enables fungi to withstand the extreme environmental conditions (Geiser, 2007; Dyer and O'Gorman, 2012).

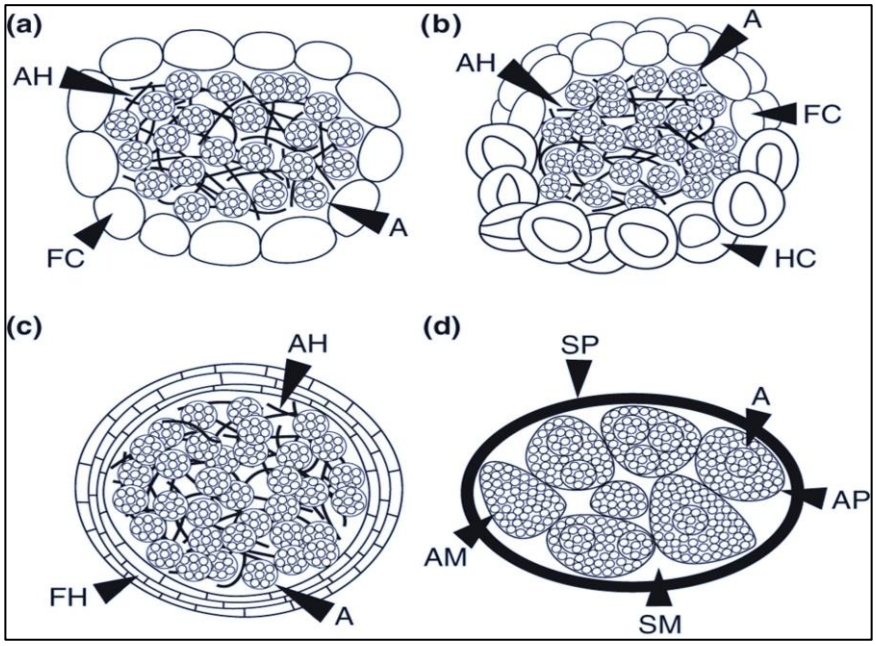


Figure 1-10 Schematic representation of four different cleistothecial forms of the *Aspergillus* teleomorphic genera showing differences in the cell wall composition: (a) *Eurotium*, (b) *Emericella*, (c) *Neosartorya* and (d) *Petromyces*. (Dyer and O'Gorman, 2012).

1.8 Genes involved with sexual development in the aspergilli

More than 75 genes have been identified in the genus *Aspergillus* which are necessary for sexual reproduction; these play essential roles in the control of mating, sensing of environmental signals and the production of cleistothecia and ascospores (**Fig, 1-11**) (**Table 1-1**) (Dyer and O'Gorman, 2011). These will now be described in more detail.

Table 1-1 Summary of known genes involved with sexual reproduction in the aspergilli (adapted from Dyer and O'Gorman 2012).

Gene	Protein function (domain)	Sex effect[a]	Locus ID[b]	Reference
Perception of environmental signals				
fphA	Red-light sensing phytochrome (P2, GAF, PHY, HKD, RRD)	Repressor	AN9008	Blumenstein *et al.* (2005)
lreA	Blue-light sensing white collar (LOV, PAS, zinc-finger)	Activator	AN3435	Purschwitz *et al.* (2008)
lreB	Blue-light sensing white collar	Activator	AN3607	Purschwitz *et al.* (2008) (PAS, zinc-finger)
cryA	Blue-light and UVA sensing cryptochrome (PHR)	Repressor	AN0387	Bayram *et al.* (2008a)
veA	Light/dark response (velvet complex)	Activator	AN1052	Kim *et al.* (2002)
velB	Light/dark response (velvet complex)	Activator	AN0363	Bayram *et al.* (2008b)
velC	Light/dark response	Activator	AN2059	Park & Yu (pers. comm.)
laeA	Light/dark response (methyl transferase)		Bimodal[c]	AN0807 Sarikaya Bayram *et al.* (2010)
imeB	Light response, low glucose sensing (?) (TXY MAP kinase)		Bimodal	AN6243 Bayram *et al.* (2009)
silA	Light response (zinc-finger)	Repressor	AN1893	Han *et al.* (2008)
silG	Light response (zinc-finger)	Repressor	AN0709	Han *et al.* (2008)
cpcA	Amino acid sensing (bZIP)	Repressor	AN3675	Hoffman *et al.* (2000)
cpcB	Amino acid sensing (WD repeat)	Activator	AN4163	Hoffman *et al.* (2000)
lsdA	High salt sensing	Repressor	AN2330	Lee *et al.* (2001)

phoA	Low phosphorous sensing (cyclin-dependent kinase)	Repressor	AN8261	Bussink & Osmani (1998)
An-pho80	Low phosphorous sensing (cyclin)	Activator	AN5156	Wu *et al.* (2004)
esdC	Early sexual development (glycogen binding domain)	Activator	AN9121	Han *et al.* (2008b)
fhbA (*fhbB*)	NO response (flavohemoglobin)	Repressor	AN7169 (N/A [d])	Baidya *et al.* (2011)

Mating processes and signal transduction

MAT1	Mating type (alpha)	Activator	AN2755	Paoletti *et al.* (2007)
MAT2	Mating type (high mobility group)	Activator	AN4734	Paoletti *et al.* (2007)
ppgA	Pheromone precursor (α-type)	Activator[e]	AN5791	Paoletti *et al.* (2007)
preA/gprB	**a**-type pheromone receptor (GPCR seven transmembrane)	Activator	AN7743	Dyer *et al.* (2003) Seo *et al.* (2004)
preB/gprA	α-type pheromone receptor (GPCR seven transmembrane)	Activator	AN2520	Dyer *et al.* (2003) Seo *et al.* (2004)
fadA	G protein α subunit	Activator	AN0651	Rosén *et al.* (1999)
sfaD	G protein β subunit	Activator	AN0081	Rosén *et al.* (1999)
gpgA	G protein γ subunit	Activator	AN2742	Seo *et al.* (2005)
flbA	Regulator of G protein signaling	Activator	AN5893	Han *et al.* (2001)
phnA	Phosducin-like chaperone	Activator	AN0082	Seo & Yu (2006)
STE20	MAP kinase, kinase, kinase, kinase	N/A	AN5674	Dyer *et al.* (2003)
steC/*steB*	MAP kinase, kinase, kinase	Activator	AN2269	Wei *et al.* (2003)
STE7	MAP kinase, kinase	Activator[e]	AN3422	Paoletti *et al.* (2007)
mpkB	MAP kinase	Activator	AN3719	Paoletti *et al.* (2007)
steA	Transcription factor (homeodomain and zinc finger)	Activator	AN2290	Vallim *et al.* (2000)
ste50	Kinase cascade regulator	Activator[e]	AN7252	Paoletti *et al.* (2007)

rasA	Small G-protein (GTPase)	Repressor	AN0182	Hoffmann *et al.* (2000)
gprD	G protein coupled receptor (seven transmembrane)	Repressor	AN3387	Han *et al.* (2004)
gprK	G protein coupled receptor (seven transmembrane)	Activator	AN7795	Yu (pers. comm.)
gibB	G protein (β subunit-like)	Activator	N/A	Kong & Yu (pers. comm.)
ricA	GDP/GTP nucleotide exchange factor	Activator	N/A	Kwon & Yu (pers. comm.)
sakA/hogA	Osmotic and oxidative stress response (MAP kinase)	Repressor	AN1017	Kawasaki *et al.* (2002)
atfA	Stress response (bZIP domain)	Repressor	AN2911	Lara-Rojas *et al.* (2011)

Transcription factors and other regulatory proteins

stuA	Transcription factor (bHLH, APSES)	Activator	AN5836	Wu & Miller (1997)
medA	Transcription factor	Activator	AN6230	Busby *et al.* (1996)
devR	Transcription factor (bHLH)	Activator	AN7553	Tüncher *et al.* (2004)
dopA	Transcription factor (leucine-zipper)	Activator	AN2094	Pascon & Miller (2000)
nsdC	Transcription factor (zinc-finger)	Activator	AN4263	KIm *et al.* (2002a)
nsdD	Transcription factor (zinc-finger)	Activator	AN3152	Han *et al.* (2001)
nosA	Transcription factor (zinc-finger)	Activator	AN1848	Vienken & Fischer (2006)
rosA	Transcription factor (zinc-finger)	Repressor	AN5170	Vienken *et al.* (2005)
flbC	Transcription factor (zinc-finger)	Repressor	AN2421	Kwon *et al.* (2010a)
flbE	Transcription factor (putative)	Repressor	AN0721	Kwon *et al.* (2010b)
fhpA	Transcription factor (forkhead)	Activator	AN4521	Lee *et al.* (2005)
nrdA/msnA	Transcription factor (zinc-finger)	Repressor	AN1652	Jeon *et al.* (2009)
rcoA	Developmental regulation (WD repeat)		Activator	AN6505 Todd *et al.* (2006)
csnA	COP9 signalosome subunit (PCI)	Activator	AN1491	Busch *et al.* (2007)

csnB	COP9 signalosome subunit (PCI)	Activator	AN4783	Busch *et al.* (2007)
csnD	COP9 signalosome subunit (PCI)	Bimodal	AN1539	Busch *et al.* (2003, 2007)
csnE	COP9 signalosome subunit (MPN+ with JAMM deneddylase)	Bimodal	AN2129	Busch *et al.* (2007) Nahlik *et al.* (2010)
csnG/acoB	COP9 signalosome subunit (PCI)	Activator	AN3623	Lewis & Champe (1995) Busche *et al.* (2007)
candA-C	Protein neddylation	Activator	AN2458	Helmstaedt *et al.* (2011)
candA-N	Protein neddylation	Activator	AN10306	Helmstaedt *et al.* (2011)
Endogenous physiological processes				
ppoA	Oxylipin biosynthesis (dioxygenase)	Activator	AN1967	Tsitsigiannis *et al.* (2004)
ppoB	Oxylipin biosynthesis	Activator	AN6320	Tsitsigiannis *et al.* (2005)
ppoC	Oxylipin biosynthesis	Repressor	AN5028	Tsitsigiannis *et al.* (2005)
noxA	Generation of reactive oxygen species (NADPH oxidase)	Activator	AN5457	Lara-Ortiz *et al.* (2003)
sidC	Intracellular siderophore synthesis (non-ribosomal peptide synthetase)	Activator	AN0607	Eisendle *et al.* (2006)
trxA	Regulation of cellular redox state (thioredoxin system)	Activator	AN0170	Thön *et al.* (2007)
mutA	α-1,3 glucanase/mutanase	Activator[e]	AN7349	Wei *et al.* (2001)
hxtA	High affinity hexose transporter	Activator[e]	AN6923	Wei *et al.* (2004)
Ascospore production and maturation				
grrA	Protein ubiquitinylation (F-box)	Activator	AN10516	Krappmann *et al.* (2006)
samB	Cell polarity, nuclear positioning	Activator	AN0078	(Kuger & Fischer 1998)

	(zinc-finger)			
strA	Striatin scaffolding and Ca signaling	Activator	AN8071	Wang *et al.* (2010)
tubB	Microtubule assembly (alpha tubulin)	Activator	AN0316	Kirk & Morris (1991)
vosA	Trehalose production	Activator	AN1959	Ni & Yu (2007)

[a]Genes listed as 'Activators' are required for sexual reproduction, and gene overexpression may enhance sexual fertility. Expression of genes listed as 'Repressors' may reduce sexual fertility in general or operate only under particular environmental conditions. See main text for full details.

[b]Comprehensive details of gene and protein sequence are as available from the AspGD website: http://www.aspergillusgenome.org/. Also see listing of *Aspergillus* gene names at: http://www.fgsc.net/Aspergillus/gene_list/loci.html.

[c]Acts as a repressor or activator under different environmental conditions and/or at different stages of the sexual cycle. Effects might only be seen under forced gene expression.

[d]Data not yet available.

[e]Based on marked upregulation during sexual morphogenesis.

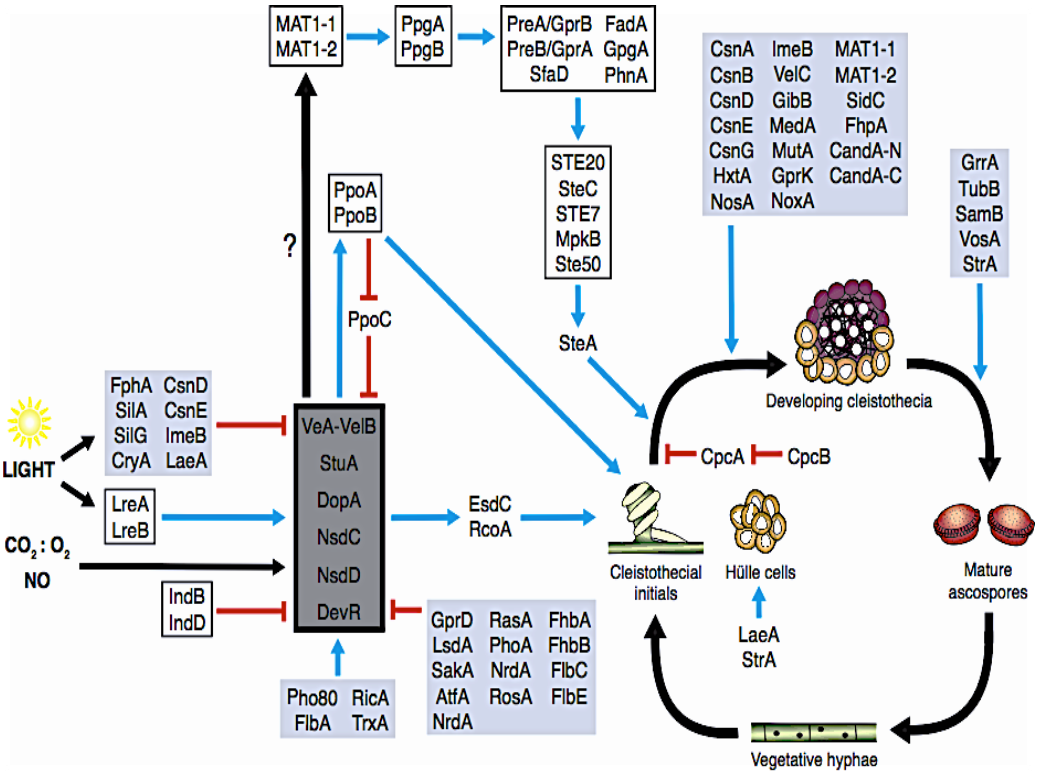


Figure 1-11 Genetics of sexual reproduction in *Aspergillus*, showing known sex-related proteins and proposed interactions between them. Proteins found in the same box are proposed to act at the same stage of development. Blue lines represent an activation effect on the gene while the red line is used to indicate repression and the black line for connecting the stage of picture. Adapted from Dyer and O'Gorman (2012).

1.8.1 Perception of environmental signals - light

A variety of proteins are involved with light detection in *A. nidulans* (**Figure 1-12**). Sexual development in *A. nidulans* is repressed by red light, mediated by the *veA* (velvet A) gene, which is considered as a major regulator of differentiation (Hatakeyama *et al.*, 2007; Stinnett *et al.*, 2007; Calvo, 2008; Araujo-Bazan *et al.*, 2009; Etxebeste, *et al.*, 2010b; Sarikaya Bayram *et al.*, 2010). The *veA* gene encodes the VeA protein that belongs to the velvet superfamily, playing an

essential role in the regulation of reproduction and secondary metabolism (Araújo-Bazán *et al.*, 2009; Butler, 2010). Localization of VeA is light dependent, in the dark it is localized in the nucleus and the levels decrease when exposed to light, suggesting that light might stimulate degradation of the VeA protein (Stinnett *et al.*, 2007; Calvo, 2008; Kim *et al.*, 2009a). In the absence of light, VeA binds to the other proteins VelB and LaeA to produce a heterotrimeric complex VelB-VeA-LaeA (termed the 'velvet complex'), which induces the initiation of sexual reproduction (Bayram *et al.*, 2008b; Sarikaya Bayram *et al.*, 2010). The velvet complex seems to have the same role in *A. flavus*, given that mutation of *veA* and *laeA* results in the failure in formation of aflatoxins and sclerotia (Kale *et al.*, 2008; Amaike and Keller, 2009). Deletion of *veA* led to a complete loss of sexual reproduction while overexpression resulted in an increase in fruiting body formation and production of sexual structures under conditions not favourable for sexual sporulation such as in liquid culture (Kim *et al.*, 2002; Dyer, 2007; Krijgsheld *et al.*, 2013). In addition to VeA, VelB is another velvet family protein, which also plays an important role in sexual development (Sarikaya Bayram *et al.*, 2010; Bayram and Braus, 2012). The heterodimer complexes VelB-VosA act as a suppressor for asexual development and both VelB and VosA also play essential roles in the maturation of asexual and sexual spores (Sarikaya Bayram *et al.*, 2010 and Bayram and Braus, 2012). The *laeA* gene is important for balancing sexual and asexual reproduction. In contrast to *veA*, deletion of *laeA* repressed conidia formation and stimulated fruit body formation. LaeA also has an essential role in light-dependent regulation of VelB and VosA protein levels (Bayram and Braus, 2012; Krijgsheld *et al.*, 2013). In addition LaeA regulates the VeA protein by impairing post translation modification of VeA (Sarikaya Bayram *et al.*, 2010).

VosA and VelC are also members of velvet family; together with VeA and VelB they share a common conserved velvet domain consisting of 150 amino acids (Bayram and Braus, 2012). VelC acts as an inducer of sexual development, given that deletion of *velC* led to a reduction in formation of cleistothecia and increased asexual conidiation, whereas overexpression had the opposite effect (Dyer and O'Gorman, 2012).

In addition to the genes of velvet family, there are other genes (*fphA, lreA, lreB* and *cryA*) found in fungi that have essential roles in sensing red, blue and UV light, respectively (Bayram *et al*, 2008a; Dyer and O'Gorman, 2012; Krijgsheld *et al.*, 2013). FphA (fungal phytochrome A) is red light photosensor in *A. nidulans* that represses sexual reproduction in the red light when it binds to a biliverdin, with deletion of *fphA* leading to formation of cleistothecia even under red light (Blumenstein *et al.*, 2005; Bayram *et al.*, 2008a). This photosensor has also been found in *A. fumigatus, Gibberella moniliformis,* and the basidiomycetes *Ustilago maydis* and *Cryptococcus neoformans* (Blumenstein *et al.*, 2005). FphA interacts with the protein kinase ImeB (inducer of meiosis), deletion of the *imeB* gene resulting in ascospore formation in *A. nidulans* even in the presence of light; deletion of both *fphA* and *imeB* resulted in a complete loss of light sensing (Bayram *et al*, 2009; Bayram and Braus, 2012; Dyer and O'Gorman, 2012).

Both LreA and LreB are blue light white-collar sensors, homologues of the *N. crassa* white-collar proteins 1 and 2, which stimulate sexual reproduction and supress asexual conidiation (Purschwitz *et al.*, 2008; Krijgsheld *et al.*, 2013). Deletion of *lreA* and *lreB* caused an increase in conidial formation and decrease in cleistothecial formation by 70% and 30%, respectively, in the dark compared to wild-type (Purschwitz *et al.*, 2008). CryA is the only cryptochrome/photolyase gene identified in *A. nidulans*, which supresses sexual reproduction under UV light, deletion of this gene resulting in the formation of fruiting bodies under conditions not normally favourable for sex (Bayram *et al*, 2008a; Bayram and Braus, 2012).

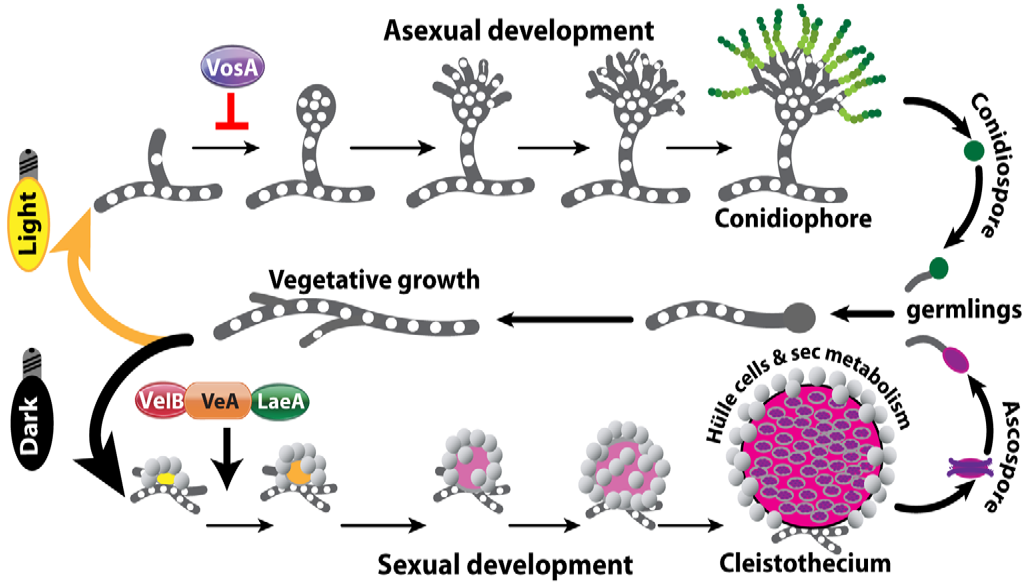


Figure 1-12 Effect of light on *Aspergillus* development and the genes that are involved. In the presence of light asexual conidia are produced, whereas in the dark conidiation is suppressed and sexual development is initiated. Red line indicates repression, and black line activation, effect on genes (Sarikaya Bayram *et al*., 2010).

1.8.2 Perception of environmental signals - nutrient and stress level

Sexual reproduction is a complex process. The differentiation of mycelium to form specialized fruiting structures requires the formation of a variety of proteins, which necessitates many different amino acids. Limitation of amino acid supply results in the activation of a set of genes called 'cross pathway' control genes, which are able to produce amino acids through various biosynthetic pathways (Hoffmann *et al.*, 2000; Bayram and Braus, 2012). *cpc*A and *cpc*B are two types of cross pathway control genes that sense amino acid limitation in the surrounding environment. CpcA represses sexual reproduction at low amino acid concentrations, only small cleistothecia being formed which fail to produce mature ascospores. In contrast when amino acids are available, CpcB induces sexual reproduction through inactivation of *cpc*A (Hoffmann *et al.*, 2000;

Pöggeler *et al.*, 2006; Dyer, 2007). The blockage of sexual reproduction by amino acid limitation can be restored by addition of amino acids to the media (Hoffmann *et al.*, 2000).

Salt concentration is another factor that can have a potential effect on sex. *isd*A is a gene involved with sensing high concentration of salts and which represses the sexual cycle. Impairment of this gene led to fruiting body formation in a condition normally unfavourable for sex (Lee *et al.*, 2001). The concentration of phosphorus ions can also affect sexual reproduction. The *pho*A gene encodes a cyclin-dependent kinase, which inhibits sex at low phosphorus concentrations (Bussink and Osmani, 1998; Wu *et al.*, 2004). In contrast to *pho*A, An-*pho*80 is another cyclin gene that induces sex in low phosphorus concentration; the effect of both genes on sexual reproduction are pH dependent (Wu *et al.*, 2004; Pöggeler *et al.*, 2006; Dyer and O' Gorman, 2012).

1.8.3 Signal transduction pathways

Changes in environmental conditions are sensed by signal transduction pathways, which control and regulate the expression of specific genes that are necessary for proper cell development and differentiation (Pöggeler *et al.*, 2006b; Futagami *et al.*, 2011). Mitogen activated protein kinases (MAPK) play an essential role in transmitting these signals to the nucleus. The MAP kinase system consists of three kinases: MAPK, MAP2K and MAP3K, the MAP kinases being highly conserved from yeast to human (Pöggeler *et al.*, 2006b; Jun *et al.*, 2011; Bayram *et al.*, 2012). Two MAP kinase genes, *mapk*B and *mapk*C, have been studied in *A. nidulans*. Deletion of the *mapk*C kinase had no effect on sexual reproduction *A. nidulans* (Jun *et al*., 2011). In contrast, *mapk*B gene deletion resulted in the complete loss of cleistothecia formation and retardation of vegetative growth (Paoletti *et al.*, 2007). The possible reason behind the blockage of sexual development in the null *mapkB* might due to impairment of pheromone signaling (see below). It has also been suggested that *mapkB* might have a role in hyphal anastomosis (heterokaryotic formation) and post karyogamy functions, as indicated by failure of heterokaryotic hypha produced by fusion of two mutant

strains of *mapkB* to complete the sexual cycle regardless of the formation of dikaryotic cells (Jun *et al.*, 2011).

Pheromone signaling requires transmission via pheromone receptors, which are bound to heterotrimeric G protein. As mentioned above (**Section 1.4**) the latter is composed of three subunits (alpha, beta and gamma) encoded by *fadA*, *sfaD* and *gpgA* respectively. Impairment in the function of any of these genes by mutation or deletion results in blocked fruit body formation (Dyer and O'Gorman, 2012; Krijigsheld *et al.*, 2013).

The *steA* gene was first isolated in *A. nidulans* by Vallim et al. (2000). This gene is a homologue to *ste*12 of yeasts and other filamentous fungi, which is found at the base of the MAPK signaling cascade. This gene is required for sexual reproduction, whereas deletion had no obvious effect on vegetative growth and conidial production. The deletion of *steA* resulted in the formation of sterile hyphae under conditions favorable for sexual reproduction (Vallim *et al.*, 2000; Dyer, 2007; Krijgsheld *et al.*, 2013). Similarly, *A. nidulans ste*C is homologue to *ste*11 of yeast identified by Wei and coworker (2003), which also affect sexual reproduction. Deletion of *ste*C in *A. nidulans* has the potential to affect both vegetative and sexual reproduction. Mutation of *steC* resulted in a slowdown of the vegetative growth rate and production of modified hyphae that have more branches, and production of larger conidia from secondary conidiophore protruding from the vesicle. It was thought that *steC* deletion impaired fruiting boy formation through inhibition of hyphal fusion (Wei, 2003).

The *sakA* gene of *A. nidulans* is a homologue of the *hog1* gene of *S. cerevisiae* first identified by Kawasaki and coworkers (2002). It is a member of the mapk stress family. *SakA* responds to osmotic and oxidative stress, mutation of this gene increased levels of sexual spore formation and decreased the resistance of conidia to heat shock and oxidative stress compare to the wild-type parent (Kawasaki *et al.*, 2002).

1.8.4 Genes encoding transcription factors and other regulatory proteins

Transcriptional initiation is a critical process for the regulation of gene expression. Substantial research has been performed to find proteins that act as regulatory proteins, which act either as transcription activators or repressors on specific gene promoters (Caddick *et al.*, 2006).

As mentioned above (**Section 1.8.1**), VeA is considered as a major regulatory protein that regulates substantial numbers of genes that are required for and involved with vegetative growth, asexual and sexual reproduction. VeA also regulates various genes necessary for secondary metabolism (Stinnett *et al.*, 2007; Bayram *et al.*, 2008a; Calvo, 2008; Han, 2009; Bayram and Braus, 2012). Other genes encoding the transcription factors include *rosA,* which encodes a putative Zn(II)2Cys6 transcription factor. This is a homologue of *pro1* from *Sordaria macrospora,* which suppresses sexual reproduction (Vienken *et al.*, 2005), deletion of this gene resulted in the formation of fruiting bodies under conditions unfavorable for sex such as high salt and low glucose concentrations (Vienken *et al.*, 2005; Dyer, 2007; Calvo, 2008; Han, 2009; Dyer and O'Gorman, 2012). Also this gene seems to regulate the expression of *veA* as indicated by up-regulation of *veA* transcripts level in null *rosA* strains (Vienken *et al.*, 2005; Calvo, 2008; Han, 2009; Krijgsheld *et al.*, 2013). *NosA* (number of sexual spore) encodes a second putative Zn(II)2Cys6 transcription factor in *A. nidulans*, and a second homologue of *pro1* from *S. macrospora*. This gene is expressed at relatively low levels throughout the entire life cycle, the expression being increased during later stages of conidiation and under carbon limitation. In contrast to RosA, NosA acts as an inducer of sexual development, being necessary for maturation of primordia, with only very few and small cleistothecia containing reduced number of ascospores being produced in a *nosA* mutant strain (Vienken and Fischer, 2006).

StuA is another regulatory protein; together with *medA* (medusa) this regulates both *brl*A and *aba*A genes, which have key roles in asexual development (Wu and Miller, 1997). In addition StuA affects sexual development as indicated by a *stu*A

mutation strain, which exhibited complete loss of sexual reproduction, even failure to produce Hülle cells (Wu and Miller, 1997 and Dyer, 2007). StuA appears to affect sexual development through regulating the expression of several genes involved in the sexual reproduction. For example, *cpe*A produces a Hülle cell specific catalase-peroxidase, and *stuA* acts as an activator for the expression of *cpe*A (Scherer *et al.*, 2002). Similarly *med*A mutation resulted in the blockage of sexual development in the early stage beyond the Hülle cells formation, with only abnormal Hülle cells being produced (Dyer, 2007).

NsdD (never in sexual development) D is a transcription factor, which has a GATA-type zinc finger motif, which also affects sexual development. Overexpression of *nsdD* resulted in the formation of Hülle cells and cleistothecia in submerged culture conditions normally unfavorable for sex. Deletion of *nsdD* resulted in complete abortion of sexual development (Han *et al.*, 2001). Similarly NsdC is another type of transcription factor that regulates sexual development independently of *nsdD* and *veA*. Deletion of *nsdC* affected both asexual and sexual reproduction; leading to a slowdown in growth rate, negative control of conidiation, and complete loss of sexual reproduction in conditions normally inducing sex. In contrast overexpression of this gene induced fruiting body formation under stressful conditions (Kim *et al.*, 2009b). Also Cary and coworkers (2012) demonstrated that both *nsdC* and *nsdD* homologues of *A. nidulans* are found in *A. flavus*, both of which are essential for conidiation, aflatoxin production and formation of sclerotia linked to cleistothecia development (Cary *et al.*, 2012). The mutant strain failed to produce sclerotia in both light and dark conditions (Cary *et al.*, 2012). Meanwhile FlbC is a transcription factor that contains two zinc finger domains consisting of 354 amino acids, which is conserved within the genus *Aspergillus*. This protein is required for normal vegetative growth and is also essential for sexual development and conidiation, a mutant strain producing more cleistothecia with decreased and delayed conidiation (Kwon *et al.*, 2010).

The *dop*A gene encodes a relatively large protein, homologous to Dop1p of *S. cerevisiae*, required for both sexual and asexual development and cellular

morphogenesis. Deletion of this gene resulted in complete loss of sex and production of conidiophores with abnormal morphology (Pascon and Miller, 2000). Similarly RcoA is crucial for both asexual and sexual development. A null mutant strain was unable to undergo sexual reproduction, whereas overexpression of *RcoA* rescued the suppression affect of the *veA1* phenotype, leading to an increase in cleistothecial formation similar to the *veA* background (Todd *et al.*, 2006).

Finally, SclR (sclerotium regulator) is a transcription factor belonging to a basic helix-loop-helix (bHLH) protein family, which has an essential role in controlling vegetative growth and development. Mutation of *sclR* in *A. oryzae* induced asexual spore formation and the formation of only few and scattered sclerotia in a strain normally producing sclerotia. In contrast, overexpression of *sclR* led to an increase in sclerotial formation with a decrease and retardation in conidiation (Jin *et al.*, 2011).

1.8.5 Genes linked to endogenous physiological process

Some endogenous physiological process controls the transition from asexual to sexual reproduction. In *A. nidulans*, oxylipins called psi factors (precocious sexual induction) are hormone-like signaling compounds that have an essential role in controlling the balance between asexual and sexual reproductive cycles (Champer *et al.*, 1987; Champer and El- Zayat, 1989; Krijgsheld *et al.*, 2013). Three types of fatty acid oxygenases (PpoA, PpoB and PpoC) are found in *A. nidulans* that are proposed to produce the psi factors (Tsitsigiannis *et al.*, 2005; Brodhun *et al.*, 2010; Dyer and O'Gorman, 2012; Krijgsheld *et al.*, 2013). PpoA is a fatty acid dioxygenase that has a role in the biosynthesis of linoleic acid derived oxylipin psiB∝, which balances asexual and sexual reproduction (Tsitsigiannis *et al.*, 2004a). Asexual spore formation is increased compared to sexual sporulation as a result of the deletion of *ppoA*. On the other hand, when *ppoA* was overexpressed the ratio of asexual to sexual spores decreased drastically (Tsitsigiannis *et al*., 2004a; Dyer, 2007). Similarly, *ppoB* is involved in the

production of oleic acid derived oxylipin psiBβ, the deletion of this fatty acid oxygenase resulting in a reduction in the ratio of asexual to sexual spore production (Tsitsigiannis *et al*., 2005). In contrast, *ppoC* is a putative dioxylipin, deletion of *ppoC* resulting in an increase in the ratio of asexual to sexual spore formation (Tsitsigiannis *et al.*, 2004b).

The deletion of all three fatty acid oxygenases (*ppoA*, *ppoB* and *ppoC*) resulted in an unexpected result, being an increase in the activation of sexual reproduction. In addition the strain with these mutations was capable of producing sexual spores and cleistothecia even in liquid media, whereas the wild-type does not sexually reproduce under such conditions (Tsitsigiannis *et al*., 2005; Dyer, 2007). The protein OdeA catalyzes the conversion of oleic acid to linoleic acid, the deletion of the *odeA* gene resulting in impairment in the conversion of oleic acid to linoleic acid. This then caused a reduction in the concentration of linoleic acid, which is a component of psi factor, which then finally affected asexual and sexual reproduction. Decreased formation of asexual spores was observed and a delay in sexual reproduction, although a final increase in the number of ascospores compared to the wild-type when strains were incubated in the dark (Calvo *et al.*, 2001).

The *nox*A gene is another gene found in *A. nidulans* and other fungal species that produce ascomata. This gene encodes an NADPH oxygenase enzyme, which produces reactive oxygen species (ROS) that have a direct effect on sexual development (Malagnac *et al.*, 2004; Pöggeler *et al.*, 2006b; Dyer, 2007). The inactivation of *nox*A results in ROS reduction that causes a blockage in early stages of cleistothecia develoment, with only Hülle cells and cleistothecia primordia being produced, whereas vegetative growth and conidia formation are unaffected (Aguirre *et al.*, 2005; Dyer, 2007; Bayram and Braus, 2012). Finally, the *basA* gene of *A. nidulans* is a homolog of the *sur2* gene from *S. cerevisiae*, which has role in the biosynthesis of phytosphingosine. Deletion or mutation in this gene results in a shift from asexual to sexual reproduction by increasing the expression of both *ppoA* and *steA*, which have regulatory effects on development (Li *et al.*, 2007).

1.8.6 Genes involved in ascospore production

Ascospores are produced in a sac like structure called an ascus. Several asci are embedded and scattered in the cavity of the ascomata (ascocarps). In the case of the aspergilli the specific ascomata is a closed, globose fruiting body termed a cleistothecium (Geiser, 2009).

In *A. nidulans* the alpha tubulin protein is encoded by both *tub*A and *tub*B genes. TubA has an important role in mitotic and nuclear migration (Kirk and Morris, 1991). By contrast, TubB is essential for late stages of sexual reproduction (ascosporogenesis), such as karyogamy or meiosis I, because *A. nidulans* null *tub*B strains produced fruiting body and asci but no viable ascospores, whereas these could be produced when out-crossed with the wild-type strain (Kirk and Morris, 1991; Dyer, 2007; Dyer and O'Gorman, 2012). Similarly GrrA is a substrate adaptor protein, orthologue to *Grr1* of *S. cerevisiae*, identified in *A. nidulans,* which has a role in the formation of ascospores. Deletion of *grr*A resulted in a block in the formation of ascospores; this gene having no effect on vegetative growth and asexual cycle, or on the early stage of sexual development. Thus it appears to only be involved with ascospore development through an effect on meiosis (Krappmann *et al.*, 2006). The study of Jun and coworkers (2011) suggested that *mapk*B that encoded for MAPKB protein might have essential role in post-karyogamy (Jun *et al.*, 2011).

A final gene that has a role in the formation of ascospore is *strA* (striatin), which encodes a putative scaffolding protein with four domains as first described by Wang and coworkers (2010). Deletion of *strA* impaired sexual development, resulting in the production of only a few small cleistothecia with reduced numbers of ascospores; 11% of these ascospores were abnormal in shape, and some of them were fused together i.e. they failed to separate completely. The effect of *strA* mutation was even more pronounced in a *veA1* strain, in which more than half of cleistothecia failed to produce ascospores. In contrast, overexpression of *strA* resulted in a three-fold increase in cleistothecial formation in a high salt media compared to a wild-type control, and the formation of Hülle cell in shaking liquid

media (Wang *et al.*, 2010). These results provide evidence of the positive role of StrA in sexual development.

1.9 *Aspergillus nidulans*

A. nidulans is a homothallic *Aspergillus* species, reproducing both asexually (producing conidia) and sexually (forming ascospores) (Han *et al.*, 2004; Seo *et al.*, 2004; Pöggeler *et al.*, 2006b; Todd *et al.*, 2007; Harris *et al.*, 2009). The ability to propagate the species by both modes of reproduction under laboratory conditions, alongside the availability of a whole genome database, together with vast amounts of published research has made *A. nidulans* an excellent and popular model organism to study genetics and molecular biology (Adams *et al.*, 1998; Galagan *et al.*, 2005; Pöggeler *et al.*, 2006; Todd *et al.*, 2007; Ni *et al.*, 2011).

All of these factors have allowed studies to be made in *A. nidulans* of various metabolic and physiological processes such as chromatin and mitochondrial DNA structure, cytoskeleton functioning, gene regulation and mechanisms of transcriptional control, DNA repair and the genetic basis of disease in humans (Caddick, *et al.*, 2006; Ni *et al.*, 2011). Also other characteristic features such as the pigmentation of conidia allow different strains of *A. nidulans* to be easily observed by microscopic examination (Adam *et al.*, 1998).

A. nidulans is homothallic fungi which undergoes sexual reproduction without the need of compatible partner, but it also has the ability to outcross (Dyer, 2007; Czaja *et al.*, 2011; Krijgsheld *et al.*, 2013). During sexual development *A. nidulans* produces globose, enclosed, fruiting body structures normally between 120-200 µm in diameter called cleistothecia (Seo *et al.*, 2004). The initial step of sexual reproduction is the formation and appearance of Hülle cells (Czaja *et al.*, 2011), which are golden yellow, thick-wall multinucleated cell serve as a nursing cell for developing cleistothecia (Pöggeler *et al.*, 2006; Krijigsheld *et al.*, 2013). Then the hyphae fuse to form dikaryotic hypha, although no clearly differentiated female and or male cells have yet been identified (the genus *Fennelia* is an exception) except for possible proto-ascogonia (Benjamin, 1955; Gieser, 2009).

Fertilization is not followed directly by karyogamy, instead both nuclei undergo several mitotic divisions to form dikaryotic cells (Czaja *et al.*, 2011), then karyogamy take place in an ascus mother cell, followed by meiosis to produce four haploid ascospores. Each ascospore undergoes one mitotic division, resulting in the formation of eight binucleated red-coloured ascospores within each ascus (Pöggeler *et al.*, 2006; Varga et al., 2014).

1.10 Aims of the study

The main purpose of the present study is to investigate the genetic and biochemical factors which are involved in sexual reproduction of *Aspergillus* species. Studies are focused on the genus *Aspergillus*, because it contains various species of economic and medical importance, and considerable bioinformatic and experimental resources are available to support research efforts. Most work will be focussed on the model fungus *A. nidulans*. Specific aims and the rationale for the study are as follows.

1. To identify novel genes involved with control of sexual reproduction

Sexual reproduction is a complex process requiring the involvement of numerous genes. As described above, over 70 genes have so far been identified that are involved in sexual development in ascomycete fungi. However, it has been suggested that between 200-400 genes may involved in this process (Dyer *et al.* 1992). Genomic analysis of *A. nidulans* indicated that approximately 50% of *A. nidulans* genes have as yet no known function. Therefore in this study we aim to identify novel genes related to sexual development. The identification of such novel genes would provide fundamental insights into the biology of sex. At the same time this might have practical applications. For example, it might be possible to show that mutations in such genes are present in asexual species, which would prevent the occurrence of the sexual cycle. Gene manipulation might then be used to restore sexuality to any such industrially useful species to allow

strain improvement and genetic analysis via the sexual cycle. Alternatively, over-expression of such genes might be used to manipulate and enhance the sexual cycle in otherwise recalcitrant species, again of practical benefit.

2. To investigate the role of high mobility group (HMG) domain transcriptional factors in sexual reproduction of *Aspergillus nidulans*

High-mobility group (HMG) domain proteins act as transcriptional factors regulating a wide diversity of cellular activities and developmental processes in a variety of eukaryotic organisms, especially relating to sexual development. So far very few HMG-domain proteins have been identified and characterised from ascomycete fungi. We hypothesized that there might be so-far unidentified HMG genes required for sexual reproduction in *Aspergillus* and that manipulation of such genes might be used to enhance sexual development, with potential useful downstream applications.

3. To investigate the potential for sexual reproduction in supposedly 'asexual' *Aspergillus* species of economic importance

Recently it has been reported that sexual reproduction can be induced in a number of previously considered 'asexual' *Aspergillus* species, such as *A. flavus* and *A. fumigatus*. Linked into an international genome consortium research project, in this study we aim to investigate whether key genes required for sexual reproduction are present within the genome of a series of supposedly 'asexual' *Aspergillus* species. A further objective is to check whether these genes, if present, are expressed or not. Given that many of the species under study are used in industry, the discovery of sexual potential in these species would provide an important indication of the possible use of the sexual cycle as a tool for strain improvement and classical genetic analysis by industry.

Chapter 2 Materials and Methods

The materials and methods described here are relevant to multiple chapters in the thesis. Materials and methods specific to certain chapters will be presented in the relevant chapter. All media components were purchased from Sigma (U.K) unless specified otherwise.

2.1 Materials

2.1.1 Media for culture growth

Ampicillin Sodium Salt (Antibiotic)

A final concentration of 100 µg/ml of ampicillin (sodium Salt) was prepared by 1 g of ampicillin (sodium Salt) in 10 ml distilled water and then filter sterilised through a 0.22 µm filter, which was previously pre-washed with 50 ml of sterile dH_2O. The ampicillin stock solution was dispensed into aliquots and stored at -20 ^oC for up to a year or at 4 ^oC for a month. 1 ml/L was then added to LB media as required.

***Aspergillus* Complete Media**

ACM was prepared according to the method described by Paoletti *et al.* (2005), by dissolving the following components in 900 ml of distilled water (Bardwell): 10 g D-glucose powder, 1 g yeast extract powder (Oxoid, U.K.), 2 g Peptone (Oxoid, U.K.), 1 g casamino acids, 0.075 g adenine, 10 ml *Aspergillus* vitamin solution and 20 ml *Aspergillus* salt solution and 20 g agar (Oxoid, U.K.). The pH was adjusted to 6.5 and then the volume made up to 1L with DW before final autoclaving at 117 ^oC for 30 min.

Aspergillus salt solution was prepared by dissolving 26 g potassium chloride (VWR International, U.K), 26 g magnesium sulphate (Fisher, U.K.), 76 g

potassium dihydrogen phosphate (Fisher, U.K) in an appropriate volume of distilled water and adding 10 ml *Aspergillus* trace elements solution, then the volume was finally made up to 1L with distilled water.

The *Aspergillus* trace elements solution consisted of the following: 40 mg sodium tetraborate decahydrate (VWR International, U.K), 800 mg copper sulphate pentahydrate (Fisher, U.K), 800 mg ferric orthophosphate monohydrate (Sigma, U.K), 800 mg manganese sulphate tetrahydrate (Fisher, U.K), 800 mg sodium molybdate dihydrate (Fisher, U.K) and 8 g zinc sulphate (Fisher, U.K), made up to 1L with distilled water.

The *Aspergillus* vitamin solution consisted of the following: 400 mg p-aminobenzoic acid, 50 mg thiamine HCl, 2 mg d-biotin, 100 mg nicotinic acid, 250 mg pyridoxine hydrochloride, 1.4 g choline chloride and 100 mg riboflavin made up to 1L with distilled water.

***Aspergillus* Minimum Media**

12.5 g D-glucose was dissolved in 800 ml of distilled water; 40 ml 25X MN salts (described below) and 1 ml 1000X trace solution (described below) were also added and adjusted to pH 6.5 with conc. NaOH, then 15 g agar was added, and made up to 980 ml with distilled water and autoclaved at 117°C for 30 min. After autoclaving, 20 ml of 50X $MgSO_4$ was added aseptically. Finally, after cooling down, PABA and/ or pyrodixine HCL were also added as required.

LB (Luria Bertani) Broth:

10 g tryptone, 5 g yeast extract and 10 g NaCl were added to 800 ml distilled water, adjusted to pH 7 with NaOH, then the volume made up to 1L before autoclaving at 121°C for 15 minutes. NB for LB agar media the same components were added with the addition of 15g agar per litre prior to autoclaving.

Para-Aminobenzoic acid stock solution (PABA)

PABA was prepared by dissolving 1 g of para-aminobenzoic acid (1% w/v) in 100 ml of distilled water, which was then filter sterilised (0.22 µm) and stored at 4 ^{o}C. 1.0 ml/L of this solution was added to ACM where required.

Pyridoxine HCl stock solution

0.1 g/100 ml of pyridoxine was dissolved in distilled water, filter sterilised (0.22 µm) and stored at 4 ^{o}C. 50 µl/L of this solution was then added to ACM or AMM as necessary.

Sorbitol Minimum Media

40 ml 25X MN salts and 1 ml 1000X trace elements solution were mixed with a suitable volume of distilled water (Bardwell) then 10 g D-glucose and 218.64 g D-sorbitol (final concentration of 1.2 M) were dissolved in this mixture. The volume was made up to 900 ml with DW and adjusted to pH 6.5 with concentrated NaOH. Next, 15 g agar was added and the volume made up to 980 ml with DW, before autoclaving at 117 ^{o}C for 30 min. Finally, 20 ml 50X $MgSO_4$ was added aseptically before pouring.

Uridine and Uracil stock solution

5.6 g uracil and 6.1 g uridine were dissolved in 100 ml distilled water. The resulting solution was autoclaved and 20 ml/L of this stock was added to ACM and AMM as necessary.

4% (W/V) Water Agar

40 g of agar (Oxoid, U.K.) was dissolved in a suitable volume of distilled water then the final volume made up to 1 L, before autoclaving at 121 ^{o}C for 15 min.

Yeast Extract and Glucose (YEG)

YEG was prepared according to Dyer *et al.* (1993), consisting of 40 g/L of D-glucose powder and 8 g/L yeast extract dissolved in 1 L distilled water. The mixture was sterilized by autoclaving at 117 ^{o}C for 30 min.

2.1.2 Solutions and Buffers

Colony DNA extraction buffer

This buffer was consisted of 10 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 M NaOH; made up in appropriate volume of distilled water.

Tris-HCl pH 7.5 was prepared by adding 80.6 ml 2M HCl to 100 ml 2M Tris solution, stirred thoroughly and allowed to cool to room temperature. Then the pH was adjusted to 7.5 with 2 M HCl, and the solution made up to a final volume of 200 ml with distilled water prior to autoclaving at 121 ^{o}C for 15 min.

Diethyl-Pyrocarbonate treated distilled water (DEPC-treated water)

0.1 ml of diethyl-pyrocarbonate (0.1% v/v) was added to 100 ml distilled water, incubated overnight at room temperature then autoclaved at 121 ^{o}C for 15 min.

DNA extraction buffer

For 100 ml of DNA extraction buffer, 25 ml of 1 M Tris-HCl pH 8.0, 25 ml of 1 M NaCl, 10 ml of 250 mM EDTA pH 8.0 and 5 ml of 10% SDS (pH 7.2) were mixed then made up to final volume 100 ml with dH_2O prior to autoclaving at 121 ^{o}C for 15 min.

DNA gel loading dye

2.4 g sucrose, 15 mg xylene cyanol and 15 mg bromophenol blue were dissolved in distilled water and made up to final volume of 6 ml with DW.

1.1 M KOH solution

KOH solution was prepared according to Szewczyk *et al.* (2007). Deionized distilled water was boiled in a 500 ml beaker, then a smaller beaker and a 100 ml cylinder were rinsed with this boiled deionised distilled water (bddw). After that 6.17 g KOH was dissolved in approximately 70 ml of bddw in the smaller beaker. The solution was transferred to the 100 ml cylinder and made up to a final volume of 100 ml with bddw. This was covered with Parafilm and mixed well by inversion. The solution was always prepared fresh before use because it reacts with atmospheric CO_2 (Szewczyk *et al.*, 2007).

KCl - Citric acid solution

A final concentration of 1.1M KCl, 0.1M citric acid monohydrate was prepared by dissolving 8.2 g KCl and 2.1 g citric acid monohydrate in 50 ml of deionised distilled water, adjusted to pH 5.8 with 1.1 M KOH and made up to a final volume of 100 ml with deionised distilled water, before being sealed with Parafilm and stored at 4 ^{o}C for a maximum of two weeks before use.

Lysis solution

Lysis solution was used for lysing bacterial cells, denaturing protein and genomic DNA, and for plasmid preparations. The solution was prepared by adding 2 ml of 10 M NaOH and 10 ml of 10% (w/v) SDS (sodium dodecyl sulphate), respectively, to 80 ml of distilled water before being made up to 100 ml final volume. The solution was always made fresh.

Mycelium wash

A (0.6 M) $MgSO_4.7H_2O$ solution was prepared in distilled water and autoclaved at 121 ^{o}C for 15 min then stored at 4 ^{o}C.

25X MN salt

37.5 g $NaNO_3$, 3.25 g KCl and 9.5 g KH_2PO_4 were dissolved in an appropriate amount of distilled water and then made up to a final volume of 250 ml prior to autoclaving at 121 ^oC for15 min.

50X $MgSO_4$

50X $MgSO_4$ was prepared by dissolving 6.5 g $MgSO_4.7H_2O$ in 250 ml of distilled water prior to autoclaving at 121 ^oC for 15 min and storage at 4 ^oC.

Neutralization solution

Composed of 3 M potassium acetate adjusted to pH 4.8 by adding glacial acetic acid, kept at 4 ^oC.

NM buffer

A final concentration of 1 M NaCl and 20 mM MES were prepared by dissolving od 11.7 g NaCl and 0.8 g MES in 150 ml of distilled water, adjusted to pH 5.8 then made up to a final volume of 200 ml with DW prior to autoclaving at 121 ^oC for 15 min.

PEG solution

0.12 g Tris base (final concentration 10 mM), 0.74 g $CaCl_2.2H_2O$ (final concentration 50 mM) were dissolved first in 20 ml of distilled water, then 60 g (60% W/V) polyethylene gycol (PEG) 6000 gradually added to the mixture step by step with microwaving for a few seconds each time to dissolve the added PEG. The resulting mixture was allowed to cool down before being adjusted to pH 7.5 using concentrated HCl and then being made up to a final volume of 100 ml with distilled water and autoclaving at 117 ^oC for 15 min.

Potassium Phosphate Buffer

1 M of K_2HPO_4 was prepared by dissolving 14.03 g of K_2HPO_4 in 61.5 ml distilled water. 6.81 g KH_2PO_4 was dissolved separately into 50 ml of DW to

prepared 1 M KH_2PO_4 then 38.5 ml from the first solution (1 M KH_2PO_4) was added to the 61.5 ml of the second solution (1 M K_2HPO_4). Further 1 M KH_2PO_4 was then added drop wise until a final value of pH 7.0 was reached. The solution was then made up to the final volume of 1 L with DW prior to autoclaving at 121 ^oC for 15 min.

2X protoplasting solution (128 mg/ml)

1.28 g of Vinoflow FCE (gift of Novo Nordisk, Switzerland) was dissolved in 10 ml of KCl-citric acid solution and filter sterilised using a low protein binding Miller GV filter (0.22 µm). Vinoflow FCE powder is commercially available for winemaking and is considered as an excellent protoplasting enzyme, with pectinase and beta 1,3-1,6 glucanase activity; the enzyme is more efficient on hyphae than germinating spores (Szewczyk *et al.*, 2007).

Sodium acetate (3 M, pH5.2)

40.0 g of $NaOAC.3H_2O$ was dissolved in an appropriate volume of distilled water, adjusted to pH at 5.2 using glacial acetic acid, then made up to a final volume of 100 ml with DW.

STC solution

A final concentration of 1.2 M D-sorbitol, 10 mM Tris base and 50 mM $CaCl_2.H_2O$ were prepared by dissolving 65.6 g D-sorbitol, 0.36 g Tris base and 2.2 g $CaCl_2.H_2O$ in approximately 250 ml of distilled water, adjusted to pH7.5 using concentrated HCl then made up to final volume 300 ml with DW prior to autoclaving at 117 ^oC for 30 min and storage at 4 ^oC.

Re-suspension buffer

This buffer was used for preparation of plasmid stocks. It had a final composition of 50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0. 1 mg/ml of RNase was added to this buffer.

Restriction enzymes

Unless specified in the relevant chapter, restriction enzymes used in the current study were obtained from New England Biolabs (U.K.) and were used following the manufacturer's instructions.

RNA gel loading dye

2X RNA loading dye consisted of 47.5% formamide, 0.01% SDS, 0.01% bromophenol blue, 0.005% xylene cyanol and 0.5mM EDTA pH 8.0. The dye was stored at -20 ^{o}C for up to 2 years.

1000X Trace element solution

100 ml of this solution was prepared by dissolving 2.2 g $ZnSO_4.7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2.4H_2O$, 0.5 g $FeSO_4.7H_2O$, 0.17 g $CoCl_2.6H_2O$, 0.16 g $CuSO_4.5H_2O$, 0.15 g $NaMoO_4.2H_2O$ and 5.0 g $EDTA.Na_2$ in 80 ml of distilled water in this order (ignoring any colour change and precipitation) prior to boiling and cooling down to about 60 ^{o}C. The solution was then adjusted to pH 6.5 with concentrated KOH before cooling to room temperature and being made up to a final volume of 100 ml with DW.

TE (Tris-EDTA) Buffer

TE buffer was prepared by mixing 10 ml of 1M Tris-HCl pH 8.0 solution and 2 ml 0.5 M EDTA, which was then made up to a final volume of 1 L with distilled water prior to autoclaving at 121 ^{o}C for 15 min.

1M Tris-HCl pH 8.0 prepared by adding 100 ml 2M Tris solution to 58.4 ml 2M HCl. The mixture was stirred thoroughly and allowed to cool to room temperature then further 2 M HCl was added, with stirring by a magnetic bar, to adjust to a final pH 8.0 (the mixture allowed to cool if necessary) before being made up to a final volume of 200 ml with DW.

0.5 M EDTA (Disodium Ethylene Diamine Tetraaceticacid) was prepared by dissolving 37.22 g of EDTA and 4 g NaOH in about 160 ml of distilled water,

then the pH was adjusted to 8.0 with 2 M NaOH. The solution was made up to a final volume of 200 ml with DW prior to autoclaving at 121 ºC for 15 min.

TAE (Tris Acetate EDTA) buffer

50X TAE buffer solution was prepared by dissolving 242 g Tris, 57.1 ml glacial acetic acid and 100 ml of 0.5 M stock solution EDTA in an appropriate volume of distilled water, which was then made up to a final volume of 1 L with DW prior to autoclaving at 121 ºC for 15 min.

TBE (Tris Borate EDTA) buffer

10X TBE solution was prepared by dissolving 108 g tris, 55 g boric acid and 9.3 g (or 20 mls 0.5 M solution) EDTA in an appropriate volume of distilled water, which was then made up to a final volume of 1 L with DW prior to autoclaving at 121 ºC for 15 min.

2.2 Methods

2.2.1 Fungal strains and culture maintenance

Two strains of *A. nidulans* were selected for study to allow gene deletion and eventual gene complementation. These were 2-258 (*pyrG89-*, *pyro A4-*, *nkuA::argB, veA+*; gift of O. Bayram, original code number AGB551), which was grown on ACM (**Section 2.1.1**) supplemented with uridine and uracil and pyridoxine (**Section 2.1.1**), and 2-259 [*paba A1-*, *yA2* (yellow spore), *veA+*, *pyro+*; gift of O. Bayram], which was grown on ACM supplemented with para-amino benzoic acid (paba) (**Section 2.1.1**). These were carefully selected to be *veA+* genotype, to avoid any confounding factors of the *veA1* mutation found in most lab strains of *A. nidulans*, and the strain 2-258 for transformation experiments was a *KuA* knockout strain to permit efficient gene targeting.

For short-term storage an ACM agar slope supplemented with strain-specific nutrient auxotrophic requirement was prepared by pouring approximately 12-15

ml of agar media into a universal tube (Sterilin) and left to set on an angle. The slope was inoculated with the selected strain and incubated at 28 ºC in the light for one week prior to storage at 4ºc.

For long-term storage, the conidia from above cultures were suspended in 1.5 ml of 10% glycerol solution in a sterile cryovial and vortexed rapidly, and then held at 4 ºC for 30 min before being placed in a freezer at -20 ºC for 30-40 min (or until the contents become frozen). The frozen cryovial was then transferred to a -80 ºC freezer and left for 30 min, prior to being transferred into liquid nitrogen in a cryovessel.

2.2.2 Spore suspension

Spore suspensions were prepared by agitating conidiating slopes with 0.01% Tween 80 using a sterile swab. The arising suspension was filtered through a sterile funnel with Miracloth (Merck Chemical Ltd., UK) to remove hyphal debris. The spore concentration was quantified by using an improved Neubauer haemocytometer (depth 0.1mm); spores were counted either in small (central) or medium (corner) squares until a count of approximately 200 spores was obtained. Then the average number of spores per square was multiplied by either 4 $x10^6$ or 2.5 x 10^5 in small (central) or medium squares, respectively, to obtain the number of spores/ml. The spore suspension was then diluted to a final desired concentration with 0.01% Tween 80.

2.2.3 DNA extraction

Colony DNA extraction

A fungal culture was prepared by inoculating ACM agar (supplemented with pyridoxine and/or uridine and uracil if required) with fungal inoculum which was then incubated at 28 ºC for 16-20 hr. Newly grown mycelium was then scraped off the surface and mixed with 100 µl of colony DNA extraction buffer (section 2.1.2). The mixture was boiled at 100ºC for 10 min in a Techne TC-

512FTC1FH2D thermal cycler (Bibby Scientific Ltd., Stone, Staffordshire). 1µl of this mixture was used as a DNA template in 25 µl PCR tests.

Phenol-Chloroform DNA extraction

Fungal cultures were grown in YEG media (supplemented with pyridoxine and/or uridine and uracil if required) for 2-3 days at 28 ºC with shaking before being filtered though sterile Miracloth and washed with Potassium phosphate buffer pH 8.0 (**Section 2.1.2**). The mycelia were then freeze dried overnight (AB4, HemLab, England). Next, mycelia were ground under liquid Nitrogen using a pestle and motor and then approximately 500mg was added to 500 µl of DNA extraction buffer (section 2.12) in a 1.5 ml Eppendorf tube which was inverted several times before being left for 30 min at 65 ºC. Tubes were then spun down, and 500 µl of the supernatant removed and mixed with and equal amount of Phenol/Chloroform in a 2.0 ml phase lock tube, which was inverted several times before being centrifuged at 13,000 x **g** for 10 min. The supernatant was removed and another 500 µl of phenol/chloroform was added and mixed by inversion, before being centrifuged again at 13,000 x **g** for 10 min. The aqueous layer was removed and mixed with 0.7 volumes of Isopropanol, inverted several times and incubated at -20 ºC for 30 min. After that, tubes were spun down again at 13,000 x **g** for 10 min and pellet was washed with 70% of Ethanol, before being centrifuged again at the same speed and duration. The supernatant was removed and the pellet left to dry in laminar airflow for 15 min before being re-suspended in 100 µl of TE buffer (**Section 2.1.2**). The DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Labtech International).

Nucleospin® Plant II kit DNA extraction

Fungal strains were grown, freeze-dried and ground under liquid nitrogen as described above for DNA extraction by the phenol-chloroform method. DNA was then extracted using a Nucleospin® plant II kit (Macherey-Nagel GmbH & Co. KG, Germany) according to manufacturer's instruction. Briefly, 20 mg of ground powder was mixed with 400 µl buffer PLI and 10 µl RNase A stock solution and

then incubated at 65ºC for 10 minutes. The crude lysate was filtered through a Nucleospin® filter (violet ring) placed in a 2 ml collection tube, which was centrifuged at 11,000 x **g** for 2 min. The filtered solution was transferred into a new tube, and DNA precipitated by mixing with 450 µl Buffer PC. The suspension was then filtered through a Nucleospin® filter (green ring) placed in a new 2 ml collection tube which was centrifuged again at 11,000 x **g** for 1 min. The filter (containing bound DNA) was washed three times: firstly 400 µl of PW1 was added and centrifuged at 11,000 x **g** for 1 min and the flow- through discarded; for the second wash 700 µl of PW2 was added and centrifuged at 11,000 x **g** for 1 min, and the flow- through discarded; finally 200 µl of PW2 was added, centrifuged at the same conditions for 2 min and the flow- through discarded. Finally the DNA was eluted by placing the Nucleospin® plant II column into a new 1.5 ml Eppendorf tube and adding 50 µl Buffer PE, incubating at 65 ºC for 5 min and then centrifuging for 1 min. DNA concentration was finally determined by using a Nanodrop ND-1000 spectrophotometer (Labtech International).

2.2.4 Agarose gel analysis

Agarose gels were prepared by dissolving 0.7-2.0% (w/v) of agarose (Lonza, Rockland, Maine, USA) in TAE or TBE buffer, and ethidium bromide added to a final concentration of 0.2 µg/ml of for visualising DNA and RNA, respectively. The DNA or RNA was mixed with DNA or RNA loading dye (**Section 2.1.2**), respectively, and then loaded into the wells and electrophoresis run at 100 -120 V to separate products. A Bio-Rad chemidoc XRS+ was used to capture gel images, using Quantity One 4.6.6 software (BioRad, Hemel Hempstead, Hertfordshire, UK).

2.2.5 Primer design

Unless specified in the relevant chapters, all primers used in this study were designed using the primer design (Primer3) function of MacVector version 14.0.4 (MacVector, Inc., North Carolina, USA).

2.2.6 Deletion cassettes and transformation protocol

All gene deletion cassettes (except for those used for deletion of the AN2755 and AN3447 genes) used in the following subsequent chapters for *A. nidulans* deletion work were obtained from the Fungal Genetics Stock Centre, USA (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm).

The deletion cassette provided by Fungal Genetic Stock Centre (produced at Dartmouth Medical School and sent to FGSC; K McCluskey pers. comm.), contained a *pyrG* selective marker gene from *Aspergillus fumigatus,* flanked by approximately 1 kb of gene specific upstream and downstream flanking region (**Figure 2-1**) (http://www.fgsc.net/Aspergillus/ko_cassettes/Strategy.htm), to facilitate homologous integration. The deletion cassettes were stored at 4 ^{o}C immediately upon receipt.

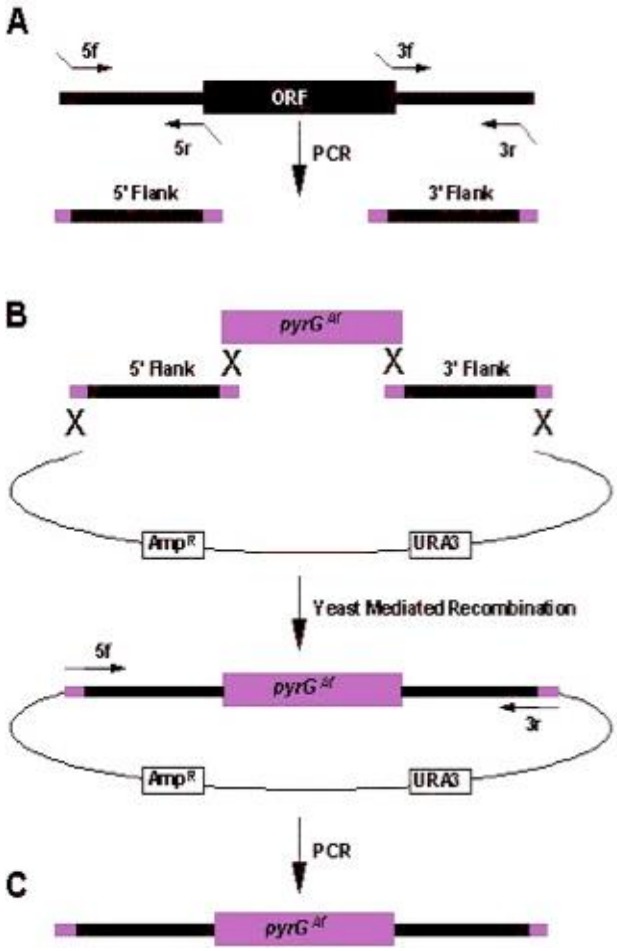


Figure 2-1 Strategy for generation of deletion constructs (http://www.fgsc.net/Aspergillus/ko_cassettes/Strategy.htm).

2.2.7 Amplification of deletion cassettes

Deletion cassettes from the USA required an initial amplification step, and were sent out with specific primer sets for this purpose. Each of the *A. nidulans* deletion cassettes was therefore amplified using the specific primers supplied with the cassette (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm). According to instructions provided, each deletion cassette template was diluted between 20 - 100 fold with TE buffer. For 50 µl PCR reaction, 1 µl of a 100-fold dilution of the cassette template with 1 µl of both primers and a final concentration of 400

μM of each deoxynucleoside triphosphate (dNTP) were used with Phusion® High-Fidelity DNA Polymerase (New Biolab England). The cassettes were amplified in a Techne TC-512FTC1FH2D thermal cycler (Bibby Scientific Ltd., Stone, Staffordshire) using a PCR cycling programme of 1 min 94ºC; then 35 cycles of 30 sec 94 ºC, 30 sec 63 ºC, 5 min 72 ºC; and a final 10 min 72 ºC.

The deletion cassette PCR products were analysed on 1.2% (w/v) of agarose gel (**Section 2.2.8**) and purified following instructions for use of the Nucleospin® gel and PCR clean up kit (not shown) (Macherey-Nagel GmbH & co. KG, Germany). The purified DNA was concentrated by adding 0.1 volumes 3M sodium acetate pH 5.2 (section 2.1.2) and 2 volumes of cold ethanol. The mixture incubated for 30 min at -20 ºC, then centrifuged and the pellet washed two times by adding 70% of ethanol; the pellet was then left to air dry and re-suspended in 7 μl of TE buffer (**Section 2.1.2**) then stored at 4 ºC and used for transformation.

2.2.8 Protoplasting

A. nidulans strain 2-258 was used for protoplast formation and transformation tests following the method of Szewczyk *et al*. (2007). A volume of 20.0 ml of *Aspergillus* complete media (ACM) broth (**Section 2.1.1**) supplemented with pyridoxine, uridine and uracil (**Section 2.1.2**) was inoculated with $1x10^8$ of spore (**Section 2.2.2**) then incubated with shaking for 13-14 hours at 30ºC. The culture was filtered through a sterile funnel with Miracloth (Merck chemical Ltd., UK) and washed with sterile cold mycelium wash solution (**Section 2.1.2**). The mycelium growth was collected and re-suspended in 8.0 ml of previous media and 8.0 ml of 2X protoplasting solution (**Section 2.1.2**), incubated at 28.0ºC for 2.0 hours with shaking at 100 rpm. Protoplast formation was monitored microscopically at 30 min intervals after two hour of incubation, until sufficient protoplasts were formed. Then purification of protoplast was performed by filtration through sterile polyallomer wool (Supa Aquatic Supplies Ltd, U.K.) in two purification columns. The latter consisted of two sterile 10.0 ml syringe barrels containing sterile polyallomer wool. The protoplast culture was poured

through the column; a small amount of cold NM buffer (**Section 2.1.2**) was added to wash the column. The filtrates were collected in a sterile 50 ml tubes (15ml per tube). Two volumes of NM buffer were added to each tube containing the protoplastic filtrate, which was mixed gently by inversion before being centrifuged at 2,500 x **g** at 4.0^{o}C for 10 min. The supernatant was discarded and the white upper region of pellet was scraped with a pipette tip and re-suspended in 1.0 ml of cold STC solution (**Section 2.1.2**). The suspended protoplasts were transferred to a new tube 50ml then made up to 50 ml with cold STC, mixed well by inversion and centrifuged at 2000 x **g** at 4.0^{o}C for 10 min. The supernatant was discarded and the pellet re-suspended in 1.0 ml of cold STC. Protoplasts were quantified using an improved Neubauer haemocytometer, diluted to 5 x 10^{7}/ml with STC and stored in ice before being used in transformation.

2.2.9 Transformation

Transformation was performed by PEG mediated transformation (Paoletti *et al.*, 2007) mixing 3-5 μg of the selected deletion cassette with 100 μl of a 5 x 10^{7}/ml protoplast suspension (**Section 2.2.6**) in a 15ml centrifuge tube, which were then mixed gently and incubated at room temperature for 25 min. A minus-DNA control was also prepared, which consisted of the protoplasts only without addition of cassette. The tubes were mixed again and 200 μl of PEG solution (**Section 2.1.2**) was added to each tube drop by drop with mixing, then another 200 μl of PEG and finally a further 850 μl of PEG were added to each tube gradually with gentle mixing before mixing by inversion and incubation for 20 min at room temperature. Each tube was then filled with cold STC (**Section 2.1.2**), which was gently added until the PEG had dissolved completely and spun at 2,000 x **g** for 10 min at 4.0 ºC. The supernatant was discarded and the pellet re-suspended in 600 μl of cold STC then 100 μl of this mixture was transferred to sorbitol minimal agar (**Section 2.1.1**) supplemented with pyridoxine (**Section 2.1.1**); the minus-DNA control was inoculated on sorbitol minimal agar supplemented with uridine and uracil (**Section 2.1.1**) and pyridoxine as a positive control. All of these plates were incubated at 28 ºC for 3-5 days.

Putative transformant colonies of *A. nidulans* were transferred to *Aspergillus* minimal agar (**Section 2.1.1**) slopes for further study.

2.2.10 Screening of transformants

The transformants together with parental strain and protoplast control were screened by PCR. DNA was extracted by the colony DNA extraction method (**Section 2.2.3**). Two primers were designed (**Section 2.2.5**) within the target gene (TG1 and TG2) for each *A. nidulans* target genes. If the target gene was disrupted (as shown by failure to amplify using TG1 and TG2, compared to control templates), correct homologous integration of the *pyrG* marker gene was confirmed by positional PCR as follows. Primers were designed slightly outside the flanking region of target gene and within the *pyrG* marker gene, and then the upstream (FP1 and TP3) and downstream (TP4 and FP2) regions were amplified (**Figure 2-2**). The DNA was extracted again from each *A. nidulans* target gene deleted strain, protoplast control and parental strain, using either the phenol-chloroform or Nucleospin plant II DNA extraction methods (**Section 2.2.3**).

For both PCRs the following PCR reaction mixture and cycling program was used. 1μl DNA template for 25 μl PCR reaction, 0.25 μl of phusion polymerase HF, 0.5 μl of dNTP, and 1.25 μl of 10mM each gene specific primer and a PCR cycling program of initial temperature 94 ^{o}C for 1 min; 25 cycles, 94 ^{o}C for 30 sec, 60-63 ^{o}C for 30 sec, 72 ^{o}C 30 sec; then a final extension at 72 ^{o}C for 3 min and final hold at 4 ^{o}C). Then PCR products were analyzed by gel electrophoresis (**Section 2.2.4**).

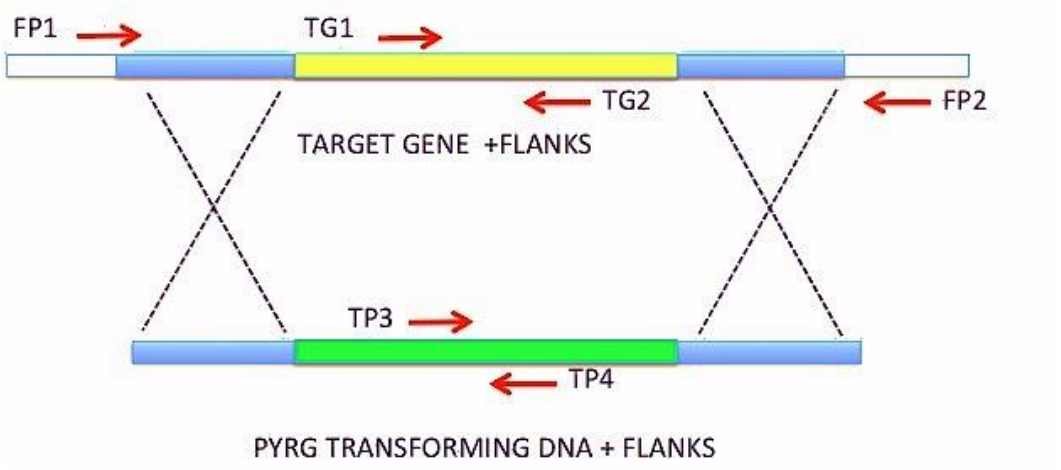


Figure 2-2 Schematic diagram to explain homologous transformation and to show the location of primers within the target gene (yellow), flanking regions (blue) and the *pyrG* marker gene (green).

2.2.11 Sexual fertility test

Sexual fertility tests were performed for all confirmed *A. nidulans* strains, which were the result of gene deletion and homologous integration. Fertility of protoplast and parental control strains were also determined for comparison to gene deletion strains.

A standard *A. nidulans* sexual fertility test was applied (Paoletti *et al.*, 2007). For each strain, six replica plates (5 cm Petri dish) were prepared; each containing 10 ml of ACM agar supplemented with uridine and uracil and pyridoxine (**Section 2.1.1**) (Robellet *et al.*, 2010). A spore suspension (50 µl of a 1 x 10^5 spore/ml suspension) (**Section 2.2.2**) was spread over the surface of each plate and incubated at 32 ^{o} C in the dark in a single layer (i.e. plates were not stacked). After 15-18 h of incubation, the plates were sealed with two layers of Parafilm to reduce the oxygen level and then incubated further for four weeks again in a single layer. Sexual fertility was assessed depending on the formation, density and diameter of cleistothecia and also on the formation and viability of ascospores compared to parental wild-type strains.

2.2.12 Transformation complementation

All mutant strains exhibiting a significant change in fertility were crossed with another *A. nidulans* strain 2-259 [*paba A1-, yA2* (yellow spore), *veA+, pyro+*] to re-insert (complement) the gene back, to confirm that the observed phenotype was due to loss of the target gene rather than other artefact.

Complementation by sexual crossing was done following techniques described by Todd *et al.* (2007). Both deletion strains derived from the green spore parent 2-258 (*pyrG89+, pyro A4-, nkuA::argB, veA+*) and *A. nidulans* 2-259 [*paba A1-, yA2* (yellow spore), *veA+, pyro+*] were stab inoculated by a sterile wire twist onto ACM media supplemented with Pyrodoxin and PABA (**Section 2.1.1**), 5 mm apart from each other, to allow hyphal anastomosis. Cultures were then incubated at 28 ºC for 2-3 days (**Figure 2-3**). A fragment of non-conidiating hypha was cut with a sterile wire at the edge of the colonies where both strains met; this was then transferred to a 5 cm Petri-dish containing *Aspergillus* minimal media agar (**Section 2.1.1**) without any nutrient supplementation, then incubated in the dark at 32 ºC for 2 days; the plate was sealed with two layer of Parafilm and further incubated for two weeks resulting in the formation of mature cleistothecia. A total of 4-5 large cleistothecia were selected for screening and cleaned under a dissecting microscope by rolling over the surface of a 4% water agar plate (**Section 2.1.1**) using sterile wire needle to remove Hülle cells and any hypha or conidia that might be fused to the wall of the cleistothecia. The clean cleistothecium was transferred into a sterile Eppendorf tube and ruptured on the side of the tube by squashing it before being suspended in 500 µl of sterile distilled water. To confirm that the cleistothecium was formed from heterokaryotic hyphae as a result of hybridisation, 5 µl of ascospore suspension were spread on the surface of a 9 cm Petri dish containing ACM and incubated at 28 ºC for 2-3 days. A hybrid cleistothecium would be expected to contain progeny from both parental strains (i.e. green and yellow conidia), while a cleistothecium produced by only one parental strain would be expected to contain either green or yellow conidia. A master plate of progeny was produced from hybrid cleistothecia and transferred onto three different plates: one contained only *Aspergillus*

minimal media agar (AMM), the second plate contained AMM + pyridoxine and the last one contained AMM + PABA; these were all incubated again at 28 ºC for 2 days. The green colonies which grown only on AMM + pyridoxine were chosen for further screening by PCR to ensure the re-insertion of target gene. After confirmation by PCR, a sexual fertility test was performed as described previously (**Section 2.2.11**) to check for phenotypic restoration to wild-type.

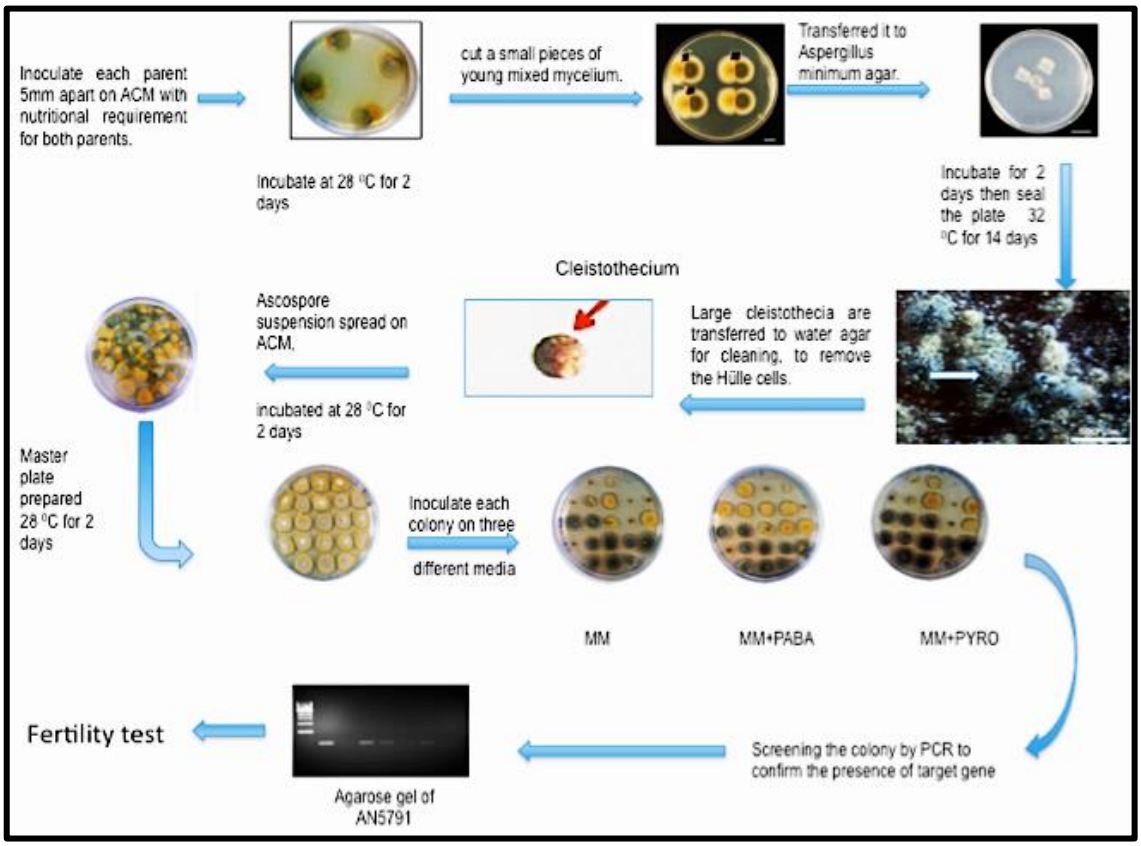


Figure 2-3 Schematic diagram explaining technique used for gene restoration (complementation) testing. Yellow colour colony corresponds to *A. nidulans* strain 2-259 and green colony to *A. nidulans* 2-258.

2.2.13 RNA extraction

RNA was extracted from solid culture media. Selected strains were grown on ACM supplemented with pyridoxine and uridine and uracil. A 1x 10^5 spore suspension was prepared and 50 µl of these suspension spread over the surface of 5 cm Petri dish (**Section 2.2.11**) previously covered with sterile Millipore nylon

filter paper (11µm pore size) (Merk Millipore Ltd., Ireland), incubated in dark at 32 ^{o}C for 15-18h then the plates were sealed with two layers of Parafilm and incubated for a further 6 days. Mycelia over the surface of nylon filter paper was then harvested by scraping with a scalpel with a Swann Morton number blade 10 (Swann, Morton, UK) and transferred to a 2 ml sterile microfuge tube, snap frozen in liquid nitrogen and immediately used for RNA extraction.

RNA extraction by TRIzol method

The frozen mycelia were ground under the liquid Nitrogen using a pestle and mortar, and transferred to a 2 ml Eppendorf tube containing 1ml of Trizol (Invitrogen Life Technologies) and mixed by inversion several times. Samples were then left at room temperature for 10 min before 200 µl of chloroform was added and mixed by vortexing and left for 2-3 min; suspensions were vortexed again and spun at 13,000 x **g** for 10 min. Approximately 750 µl of the aqueous layer was transferred to a fresh 1.5 ml tube and mixed with equal amount of Isopropanol, inverted several times and incubated at -20 ^{o}C for 20 min. Samples were then centrifuged at 13,000 x **g** for 10 min and the supernatant discarded; a gel-like pellet on the bottom and side of the tube was washed with 700 µl of 70% of ethanol in DEPC water then centrifuged again at 13,000 x **g** for 10 min. The ethanol was removed and pellets dried for 5-10 min in a laminar airflow cabinet before final resuspendion in 100 µl DEPC water. RNA was either stored at -80 ^{o}C or preceded to the following clean up steps to remove possible contaminating DNA.

For RNA purification a NucleoSpin® RNA kit (Macherey- Nagel GmbH & Co. KG, Germany) was used according to manufacturer's protocol. Briefly, 600 µl of buffer RA1- ethanol-premix (300 µl of RA1 mixed with 300 µl of ethanol 96-100% previously) was added to 100 µl of the RNA sample, mixed by pipetting and 700 µl were loaded to NucleoSpin® RNA clean up column (light blue ring) placed in a 2 ml collection tube and spun at 11,000 x **g** for 30 sec. The flow through was discarded and 350 µl of MDB was added to the membrane to desalt the silica membrane. Next followed a DNase treatment step. 10 µl of DNase

(provided in the kit) was premixed with 90 µl of rDNase buffer and 95 µl of this mixture was added to the membrane, left at room temperature for 1h. The membrane was then washed in three steps: 200 µl of RAW2 and 600 µl of RA3 were added respectively for first and second steps, each time spun at 11,000 x **g** for 30 sec. and discarded the flow off. Finally 250 µl of RA3 was added and centrifuged for 2 min at 11,000 x **g** to wash and dry the membrane. The column was transferred to a 1.5 ml tube provided with the kit; 60 µl of Elution buffer was added to the membrane and centrifuged for 1 min, with the flow through containing the RNA stored in ice.

The extracted and purified RNA was quantified by a Nanodrop ND-1000 (Labtech International); the purity and integrity of RNA was checked by PCR and gel electrophoresis, respectively. To confirm the RNA purity and absence of DNA, standard PCR was performed using 1 µl of RNA sample as a template (and genomic DNA as a control), trying to amplify the housekeeping gene actin. To check RNA integrity, 3 µl of RNA sample were mixed with an equal amount of RNA loading dye (**Section 2.1.2**) and resolved on 1.2% TBE agarose gels at 120 V for 30 min before final analysis under UV light using a Bio-Rad chemidoc XRS+, with Quantity one 4.6.6 program (BioRad, Hemel, Hempstead, Hertforshire, UK). Lanes were checked for appearance of both RNA bands (40S and 60S ribosomal sub units). RNA samples were then stored at -80 ^{o}C.

2.2.14 cDNA synthesis

RNA was reverse transcribed to cDNA by using a SuperScript III kit (Invitrogen). For a standard 20 µl reaction, 1 µl of oligo $(dT)_{20}$ (50 µM), 0.5 µg of total RNA and 1 µl of 10 mM dNTP mixture were added to a nuclease free-PCR tube and made up to 13 µl with sterile distilled water; heated for 5 min at 65 ^{o}C and stored on ice for at least 1 min. The contents were collected by centrifugation and 4 µl of 5X First-strand buffer, 1 µl of 1 M DTT and 1 µl of SuperScript III RT (200 units/ µl) were added to the tube, then mixed by pipetting and incubated at 50 ^{o}C for 60 min and finally the reaction was inactivated by incubating at 70 ^{o}C for 15 min.

The cDNA were stored at -20 ^{o}C then used as a template for either RT-PCR or qRT-PCR.

2.2.15 qRT-PCR

To determine any change in gene expression in particular *Aspergillus nidulans* mutant strains (where a single gene had been deleted by homologous transformation) the transcript level of the gene of interest was measured and compared to values obtained from the parental control strain.

Vegetative cultures were grown on solid media and RNA extracted and cDNA synthesised as described above (**Sections 2.2.13 and 2.2.14**). qRT-PCR amplification was performed using an Applied Biosystems 7500 Fast Real-Time PCR system. A standard curve method was used for quantification against known concentrations of genomic DNA. Three biological replicates were used for each measurement using either duplicate or triplicate technical repeats. 10 µl of PCR reaction mixtures were used, which contained 0.5 µl of cDNA, 0.4 µl of gene specific primers (175 nM final concentration), 5 µl of FAST SYBR-Green Master Mix (Applied Biosystems) and 4.1 µl of ultra purified water. The PCR programing parameters were denaturation at 95°C for 15 sec, up to 40 cycles of hybridization at 67°C for 30 sec., and a final extension at 60°C for 60 sec.

2.2.16 Cloning

It was necessary to clone certain genes of interest to allow scale up via plasmid extraction. The pAN52.1 cloning vector plasmid was used (**Figure 2-4**), which contains the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*A) promoter (a constitutive promoter), an ampicillin resistance gene as a selective marker for *Escherichia coli* selection on LB contained 10 µg/ml Ampicillin antibiotic (**Section 2.1.2**) after transformation, and a fungal *trpC* terminator region.

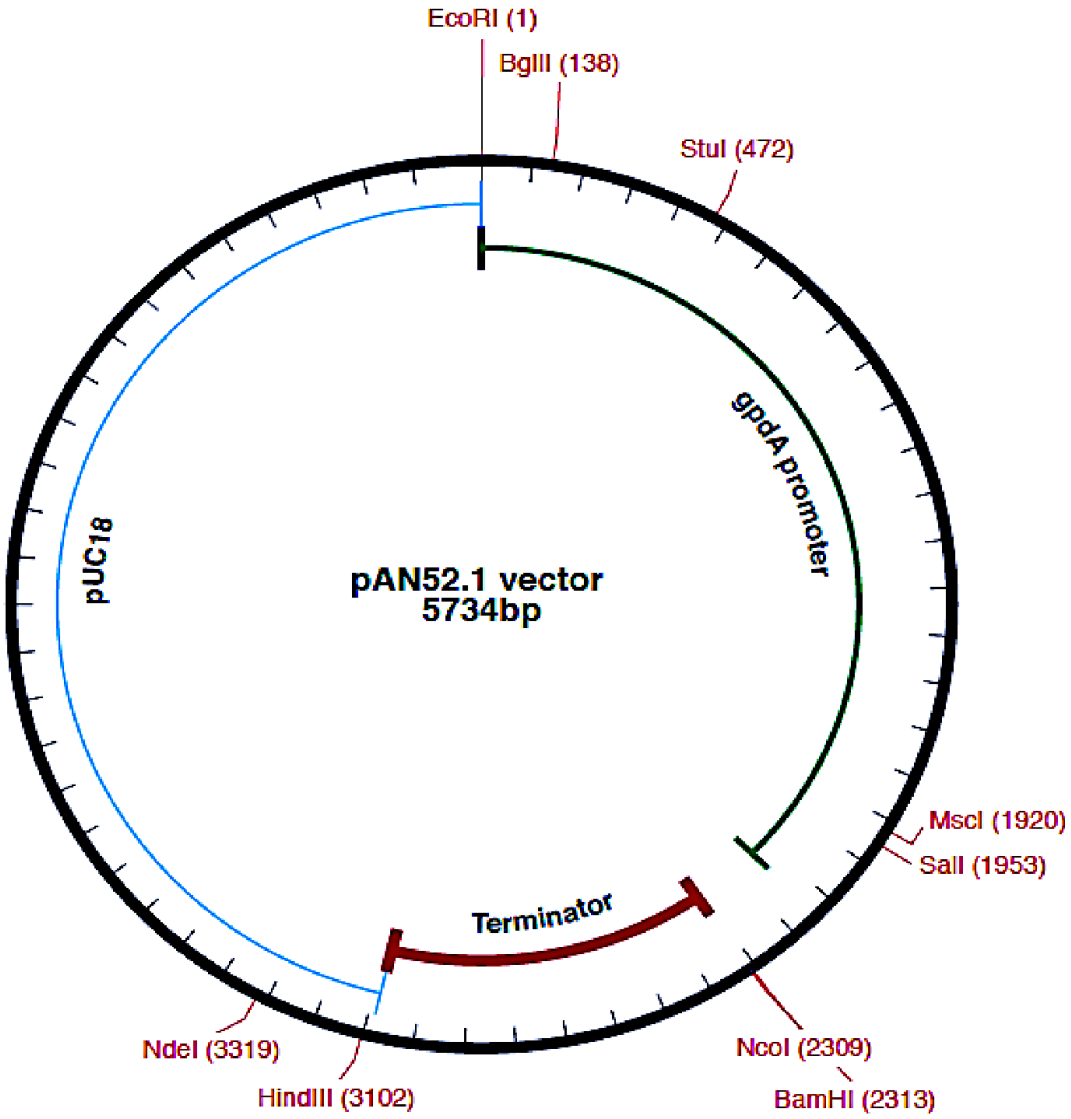


Figure 2-4 Map of the pAN52.1 vector.

Cloning vectors were designed using the NEBuilder assembling tool (http://nebuilder.neb.com), with the restriction digestion option selected to linearize vectors as appropriate. Primers used for amplification of fragments to be assembled into a new cloning vector were designed using the NEBuilder™ web tool (http://nebuilder.neb.com). Each primer contained overlapping sequences for the adjacent DNA fragments, allowing them to be joined into the new vector.

To construct overexpression vectors, three separate cloning steps were performed. Firstly, both 1 kb of the 3' flank of the target gene and the *pyrG* selective marker gene (from *A. fumigatus*, used for selection of *A. nidulans* strain after transformation) were assembled into pAN52.1 vector and transformed into *E.coli*. In the second step the target gene of interest was inserted into the subsequent vector. Finally 1 kb of the 5' flank of the target gene was inserted into the vector formed at the second step.

All of the fragments (3' flank, *pyrG*, target gene and 5' flank) were cloned into the vector by using the Gibson Assembly® Cloning Kit E5510 (New England Biolabs, inc. Uk), following manufacturer's instruction as follows. 10 µl of the Gibson assembling master mix, 0.1 µg of cloning vector, and a 3-fold excess of the fragments to be inserted were mixed and made up to 20 µl reaction with sterile distilled water. The mixture was incubated at 50 ^{o}C for 15 min (in a thermocycler) and then stored on ice or -20 ^{o}C for subsequent transformation.

For transformation NEB 5-alpha competent *E. coli* cells (provided with the kit) was used as a recipient cell for the assembled vector. 2 µl of the assembling reaction was added to 50 µl *E. coli* chemical competent cell, mixed by pipetting or flicking the tube, incubated on ice for 30 min. The tube was then transferred to 42 ^{o}C for 30 sec. and immediately placed the tube on ice for 2 min. 950 µl of SOC outgrowth media was added to the tube, which was incubated at 37 ^{o}C with shaking for 60 min. 100 µl from this culture was spread over the surface of LB agar contained penicillin (the plate was previously incubated at 37 ^{o}C) then incubated at 37 ^{o}C overnight. Some colonies were selected for screening and checking for the insertion of fragment by digesting the plasmid using an appropriate endonuclease enzyme and were then analysed by agarose gel electrophoresis.

2.2.17 Plasmid preparation

Cultures of *E. coli*, which contained the desired plasmid, were grown in LB broth supplemented with 10 µg/ml ampicillin. 5-10 ml of LB media was placed in a 30

ml universal tube and inoculated by using a sterile yellow tip then incubated overnight at 37 ºC with shaking. The bacterial cells were harvested from the culture media by placing 1.5 ml of culture in an Eppendorf tube and centrifuged at 13,000 x **g** for 15 sec, then the supernatant decanted. This step was repeated depending on the amount of plasmid required for subsequent tests. The plasmid was then purified by using one of the following methods.

Isolation of plasmid by alkaline lysis methods

The harvested bacterial cells (**Section 2.2.17**) were re-suspended in 100 µl of ice cold re-suspension buffer (**Section 2.1.2**) by either vortexing or pipetting and left on ice for 5 min. The cells were lysed by adding 200 µl of lysis solution (**Section 2.1.2**), mixed by inverting tube 4-6 times and incubated at room temperature for 3-5 min; then 150 µl of cold neutralization solution was added, mixed by inversion and incubated for 5 min. Tubes were then spun for 10 min at 13,000 x **g** in 4 ºC (Fisher Scientific accuspin ™ Micro R centrifuge, rotor 75003243). Approximately 450 µl of the supernatant was transferred into a 1.5 ml tube and mixed with equal amount of isopropanol, and centrifuged again at 13,000 x **g** for 30 min at room temperature. The supernatant was discarded and the pellet washed with 500 µl of 70% ethanol, which was then spun for 10 min at 13,000 x **g**; the supernatant was discarded and the pellet was dried, and then dissolved in 25 µl of either sterile distilled water or TE buffer pH 8.0, stored at -20 ºC until required.

PureYield™ Plasmid Miniprep System

Bacterial cultures was grown as described above (**Section 2.2.17**). The plasmid was purified by using a PureYield™ Plasmid Miniprep System kit (Promega, USA) following manufacturer's instruction. 600 µl of bacterial culture was transferred to a 1.5 ml Eppendorf tube and 100 µl of cell lysis buffer was added to it and mixed by inverting the tube 6 times. Within 2 min, 350 µl of cold (4-8 ºC) neutralization solution was added and mixed thoroughly by inversion, then the tube centrifuged at 13,000 x **g** for 3 min. Approximately 900 µl of supernatant was transferred to a PureYield™ minicolume placed into a PureYield™ collection tube, before centrifugation at 13,000 x **g** for 15 sec. The flow through

was discarded and 200 µl of Endotoxin solution added to the column and spun at 13,000 x **g** for 15 sec, then 400 µl of column wash was added and centrifuged at the same speed for 30 sec. The minicolunm was transferred into a 1.5 ml tube and 30 µl of Elution buffer added, and then the minicolunm was incubated at room temperature for 1 min and spun at 13,000 x **g** for 15 sec. The flow through was stored either at 4 or -20 ^{o}C.

2.2.18 Statistical analysis

The Statistical Package for Social Science (SPSS, version 22) for Macintosh (Mac OS X version 10.7.5) was used to perform statistical analysis whenever required. Specific details about the tests used for analysis will be given in the relevant chapters.

Chapter 3 Identification of Novel Genes Regulating Sexual Development through Differential *MAT* Expression Screen.

3.1 Introduction

Sexual reproduction is a complex process requiring the expression and interaction of a network of genes. More than eighty genes with defined genetic loci have been identified so far in *A. nidulans*, which are necessary for normal sexual development (Wada *et al.*, 2012). However, Dyer *et al.* (1992) suggested that several additional genes might be required for sexual development. Thus, fruiting body formation is under polygenic control and a series of gene participate in this process. Therefore, it is likely that many genes required for sexual reproduction in *Aspergillus* species remain to be identified. As previously explained (**Section 1.10**) it is of interest to identify such genes both from fundamental and applied perspectives.

Indeed, mutation studies by Han *et al.* (2003) identified a series of sexual developmental mutants of *A. nidulans.* Depending on the effect on sexual fertility of the resulting mutants, these genes were classified into three groups. Some of the genes were recognized as 'NSD' (never in sexual development), mutations that led to total sterility; some were defined as 'BSD' (block in sexual development) where sexual development began but was blocked at a particular stage; and finally some were termed 'ASD' (abnormal in sexual development), where mature fruiting bodies were formed but which were abnormal in the number and/or morphology of the sexual structures (Han *et al.*, 2003). At the time none of these mutations were linked to a specific genetic locus, although ongoing work has now identified the genetic location of some of these mutations such as *nsdC* and *nsdD* (Han *et al.*, 2001; Kim *et al.*, 2009b).

In ascomycete fungi sexual identity and reproduction are governed by mating-type genes (**Section 1.3.2**), which regulate the expression of genes involved in these processes. In addition *MAT* genes may regulate the expression of additional genes with roles in other cellular process which may only indirectly be involved in sexual development (Hornok *et al.*, 2007). For example, a comparative study of differential gene expression between a wild-type *Gibberella zeae* and a *MAT1-2* deletant strain revealed that 171 genes were down regulated in the mutant strain; when cultures were grown on carrot agar media for 10 days at 25 ^{0}C, then mycelim suspended in 1 ml of tween 60 (25% v/v%) before transfer to another plate for induction of peritherial formation (12 hours photoperiod using a mixture of fluorescent cool white and black light) for a further 10 days. The majority of these genes annotated 98 out of 171 genes; from these 98 genes 34 genes having a role in metabolism, the significance of which was unclear (Lee *et al.*, 2006). Similarly, Bohm et al. (2013) found that 2421 genes were differentially expressed between a *Penicillium chrysogenum* wild-type strain and a *MAT1-2* gene deletant strain (Böhm *et al.*, 2013). Many of these genes again had no obvious direct role in sexual reproduction, providing further evidence of the wide-ranging roles of *MAT* genes in cellular processes.

Similar to *MAT* genes, there is accumulating evidence that other genes identified through their role in sexual development can have wider roles in cellular processes. For example, a correlation is evident between the regulation of development and biosynthesis of certain secondary metabolites in fungi (Calvo *et al.*, 2002; Yu and Keller, 2005). The *veA* encodes transcription factor, which balances asexual and sexual reproduction in response to light conditions, also has an important role in sterigmatocystin biosynthesis by controlling the transcription of *aflR* genes necessary for the activation of the sterigmatocystin biosynthetic gene clusters (Kato *et al.*, 2003; Bayram *et al.*, 2008b). Deletion of *veA* resulted in loss of aflatoxin production as well as blockage in sclerotial formation in both *A. flavus* and *A. parasiticus* (Calvo *et al.*, 2004; Duran *et al.*, 2007). Similarly, *veA* homologues in other fungal species have roles in the biosynthesis of secondary metabolites such as penicillin and other β-lactam antibiotics in *P. chrysogenum* and *Acrymonium chrysogenum*, respectively (Bayram and Braus, 2012).

The work in the current chapter follows on from a study performed by Wada and coworkers (2012). They worked on *Aspergillus oryzae* (section *Flavi*), an economically important species used for centuries in the production of certain Asian foodstuffs. *A. oryzae* is regarded as a 'generally recognized as safe' (Stros *et al.,* 2007) species (Kobayashi *et al.,* 2007), due to its lack of production of aflatoxin, and long period of safe use in the fermentation industries. It is a close relative of the aflatoxin producer *A. flavus* (Chang and Ehrlich, 2010). Whereas *A. flavus* reproduces both sexually and asexually (Horn *et al.*, 2009b) *A. oryzae* is only known to propagate asexually i.e. it lacks a sexual life cycle (Kobayashi *et al.*, 2007). In initial studies only one mating-type gene (*MAT1-1*) was identified in *A. oryzae* (Galagan *et al.*, 2005). However, by screening a much larger sample set of over 150 isolates, Wada et al. (2012) were able to detect the presence of *MAT1-2* isolates with a *MAT1-2* gene in the mating-type locus by using a PCR diagnostic assay. They also they found that both isolates of both mating type were found in in a 1.1 ratio. Therefore *A. oryzae* appeared to have the *MAT* organization of a heterothallic *Apergillus* species, with strains containing either a *MAT1-1* or *MAT1-2* mating-type gene. To assess whether *MAT* genes might be functional in *A. oryzae,* Wada et al. (2012) then constructed isogenic strains of *A. oryzae* which contained either a *MAT1-1* or *MAT1-2* mating-type gene at the *MAT* locus. They then used microarray technology to determine whether there was evidence of differential gene expression in the arising strains. These microarray experiments involved growth under conditions suitable for expression of mating type gene [1 x 10^6 of spore suspension of both strains (*MAT1-1* (NSPlD1) and *MAT1-2* (NSPlD1-M2) of *A. oryzae* were grown on DPY media supplemented with uridine and uracil for 24 hr, then 200 mg of mycelium was transferred to PDY agar covered with cellulose acetate membrane, incubated for 48 hr]. This resulted in the identification of over 30 genes that showed over 10-fold changes in expression according to which mating-type gene was resident at the *MAT* locus (**Table 3-1**). Significantly, most of these genes were only listed as putative proteins without a known function.

Table 3-1. Differentially expressed genes in *A. oryzae* depending on which mating-type gene (*MAT1-1* or *MAT1-2*) is resident at the *MAT* locus, from Wada *et al.* (2012).

TABLE 2 Genes upregulated in the MAT1-1 mating type strain based on DNA microarray analysis

Gene ID	Fold change (MAT1-1/MAT1-2)	Protein[a]
AO080503000328	55.7	Acyl-CoA dehydrogenases
AO080508000390	55.7	Predicted protein
AO080503000008	39.4	AoPpgA, putative α-pheromone precursor
AO080508000389	27.9	Predicted protein
AO080541000465	26.0	Predicted protein
AO080523000564	24.3	Predicted protein
AO080511000446	21.1	Probable taurine catabolism dioxygenase
AO080521000309	19.7	Predicted protein
AO080502000077	18.4	Predicted protein
AO080521000174	18.4	NptB, neutral protease NPII
AO080564000028	18.4	NADP FAD-dependent oxidoreductase
AO080523000782	16.0	Predicted protein
AoEST3991	16.0	Predicted protein
AO080501000145	14.9	Predicted protein
AO080513000200	14.9	Predicted protein
AO080541000466	14.9	Predicted protein
AO080531000014	13.9	Cytochrome P450
AoEST3853	13.9	Predicted protein
AO080508000337	13.0	Predicted protein
AO080523000711	13.0	Cytochrome P450
AO080541000488	13.0	Predicted protein
AO080521000051	12.1	Predicted protein
AO080523000026	11.3	Chitosanase
AO070333000246	10.6	Predicted protein

[a] Acyl-CoA, acyl coenzyme A; FAD, flavin adenine dinucleotide.

TABLE 3 Genes upregulated in the MAT1-2 mating type strain based on DNA microarray analysis

Gene ID	Fold change (MAT1-2/MAT1-1)	Protein
AO080505000070	84.4	Predicted protein
AoEST5207	64.0	Predicted protein
AO080515000084	18.4	Predicted protein
AO080532000139	18.4	Vesicle coat complex COPII, subunit Sec31
AoEST6582	17.1	Predicted protein
AO080523000595	14.9	Predicted protein
AO080508000372	14.9	Predicted protein
AO080511000543	11.3	Predicted protein
AO080553000049	11.3	Predicted protein

3.2 Aims of the work

The objective of the work in the current chapter is to identify new genes involved in the sexual development of *Aspergillus* species. This work follows on from a study by Wada and co-workers (2012) who identified 33 genes in *A. oryzae* which were differentially expressed greater than 10-fold according to whether a *MAT1-1* or *MAT1-2* gene was resident at the *MAT* locus (**Table 3.1**). Given that these genes were regulated in a mating-type dependent manner we therefore reasoned that some of these genes might therefore be involved with sexual reproduction. Very significantly, 24 of these genes were of unknown function. To further

investigate the role of these genes, first the homologous genes from the model species *A. nidulans* will be identified by BLAST analysis where possible. Secondly, systematic gene deletion will be performed to functionally characterise these genes by disrupting the homologous target gene in *A. nidulans* by using deletion cassettes available from the fungal genetic stock centre (USA). This involved targeted replacement of the resident gene with a *pyrG* marker gene (from A. fumigatus) (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm). Finally, the effect of gene deletion in *A. nidulans* on sexual development would be monitored. *A. nidulans* provides an ideal model system for assay of sexual genes because it has a well established homothallic sexual cycle that can be induced easily in vitro, but which also requires the action of genes required for heterothallic outbreeding (Paoletti *et al*., 2007). Should any sex-related genes be identified by the strategies described above then these will be studied further by means of gene complementation and overexpression.

3.3 Materials and methods

General materials and methods not specific to the current chapter have already been described in **Sections 2.1.1** and **2.1.2.**

3.3.1 BLAST searching and deletion cassettes

Candidate genes from *A. oryzae* were selected according to the work done by Wada et al. (2012) (**Table 3-1**). The BLAST (Basic Local Alignment Search Tool) algorithm available from the AspGD web site (http://www.aspergillusgenome.org/cgi-bin/compute/blasti) was used to try and find any homologous genes in *A. nidulans* with regions of similarity. Full sequences of any homologous genes identified were then downloaded from the *Aspergillus* genome database (ASPGD) (http://www.aspergillusgenome.org/). Deletion cassettes were then ordered from the Fungal Genetics Stock Centre (USA) for any homologous genes identified in *A. nidulans* where the homologous gene was the same (higher matching) according to both TBLASTX and BLASTX

searches. A nominal cut of *e* value less than 1 x 10^{-10} was set for homology. These deletion cassettes featured approximately 1 kb flanking regions from both the upstream and downstream regions of the target gene of interest, with a *pyrG* selective marker inserted between these for selection purposes (**Section 2.2.6**).

3.3.2 Amplification of *A. nidulans* deletion cassettes

The deletion cassettes were amplified according to methods described in **Section 2.2.7** according to recommendations from the Fungal Genetics Stock Centre (USA). A 1 µl volume of a 100-fold dilution of the deletion cassette was normally used as a DNA template for amplification of all deletion cassettes except for gene (AN11253), which required 200-fold dilution.

3.3.3 Transformation

The deletion cassettes of homologous gene of *A. nidulans* were transformed by PEG-mediated transformation, with protoplasts prepared as described in **Section 2.2.8** and transformations performed according to previously described methods (**Section 2.2.9**).

3.3.4 Screening transformants by PCR

DNA was extracted from transformant colonies (**Section 2.2.3)** and screened to confirm deletion of the target gene using primer sets designed to amplify internal regions of the target gene (**Appendix 1**), with DNA obtained from the wild-type parent and protoplast controls used as positive controls. The correct integration of the *pyrG* gene was also checked by positional PCR using primers (**Appendix 2**) designed to anneal to flanking regions of the target gene and internal to the *pyrG* gene as as described in **Section 2.2.10.**

3.3.5 Sexual fertility test for mutant strains

All transformant strains with confirmed deletion of the target gene and correct integration of *pyrG* marker gene were tested to functionally characterise the result of gene deletion on sexual reproduction by determining the sexual fertility (**Section 2.2.11**) compared to parent and protoplast control strains. Three parameters were examined, namely the number and size of cleistothecia formed, and whether ascospores were produced. The number of cleistothecia were determined by counting the total number of cleistothecia (over 80 µm in diameter) in three viewing areas per plate from a total of four plates (representative of the 6 replica plates included) i.e. a total of 12 viewing areas were used for counting the cleistothecia for each strain under test. The average size of cleistothecia was determined by measuring the diameter of 100 cleistothecia per strain under x 45 objective of a dissecting microscope. Fertility test for all transformant and control strains were performed at the same time to allow direct comparison of results independent of any possible run-to-run variation in fertility. Photographic images were taken using an image-pro insight (Media Cybernetics) imaging system. Data regarding sexual fertility was statistically analysed by either one-way ANOVA, or if the Levene's test for homogeneity of variance was not satisfied then data was transformed prior to one-way ANOVA. The least significant difference test (LSD) was used in post hoc testing of the one-way ANOVA data to compare each mutant strain to the parental control.

3.3.6 Complementation test

Gene deletion mutant strains (green spore) which showed a significant change in sexual fertility were crossed to *A. nidulans* strain 2-259 [*paba A1-, yA2* (yellow spore), *veA+, pyro+*] to generate sexual progeny which included those in which the target gene was restored back in the original genetic background, to confirm that the observed phenotype was due to target gene deletion rather than any other factor(s) such as unexpected ectopic integration. Restoration of a deleted gene was obtained through sexual crossing and then monitoring of the segregation of

either the wild-type or mutant phenotype amongst the offspring; if a correlation between restoration of wild-type function and presence of the wild-type gene in the offspring was observed then this was taken as evidence of gene complementation. Complementation was performed as described in **Section 2.2.12**; the strains with restored target gene were tested for sexual fertility (**Section 2.2.11**) and checked for restoration of phenotype compared to the wild-type parent.

3.3.7 Determination of transcript level of some sex key genes

qRT-PCR was used to quantify the transcribed levels of some key sex-related genes (*MAT1*, *MAT2*, *ppgA*, *preA*, *preB*, and *steA*) in each of the ΔAN3562-1, ΔAN5791-1, ΔAN8184-1, and ΔAN8656-1 strains compared to the *A. nidulans* parental strain 2-258, using primer pairs listed in **Appendix 3**. These strains were grown on ACM agar supplemented with uridine and uracil and pyridoxine, and covered with a Nylon filter membrane (pore size 11 µm) as described in **Section 2.2.13**. RNA was extracted by a TRIzol method (**Section 2.213**). The purity of RNA was checked for DNA contamination by performing PCR of a suitable house keeping gene(s) on RNA samples compared to the genomic DNA. The cDNA were synthesised as previously described (**Section 2.2.13**) and qRT-PCR was performed using three independent biological and technical repeats as described (**Section 2.2.14**).

3.3.8 Overexpression of *A. nidulans* genes

Cloning vectors were constructed for genes AN5791, AN8184 and AN3562 (http://www.aspergillusgenome.org/) using either the NEBuilder Gibson or NEBuilder HiFi cloning kits (**Section 2.2.16**) to incorporate the *gpd* promoter in front of the gene of interest. At each step the insertion of desired fragments was confirmed by digesting the plasmid with specific endonuclease, the digested product was separated and analysed by agarose gel electrophoresis. For example, to construct the cloning vector for the AN8184 gene, in the first step the pAN52.1

plasmid was linearized using *HindIII* endonuclease enzyme, and both the *pyrG* marker gene and the 3' flank of AN8184 were amplified and purified by using a Nucleospin PCR clean up kit. The forward primer of *pyrG* (acctgcaggcatgcaGATTTCAGTAACGTTAAGTGG) was designed to contain extra nucleotides complementary to the 5' flank of pAN52.1 plasmid (shown in red), which opened with *Hind*III and the reverse primer (catcagaaaGACAGAAGATGATATTGAAGG) contained complementary nucleotides to the 5' flank of DNA fragment of the 3' flanking region of AN8184 (shown in green). Meanwhile, the primers used for amplification of the 3' flanking region of AN8184 also included complementary sequences; the forward primer (cttctgtcTTTCTGATGACATTA-CTCTGC) contained complementary nucleotides for the 3' flank of the *pyrG* gene and the reverse primer (cgacggccagtgccaACTGACTCGACCATCTATC) contained complementary nucleotides to the 3' flank of the pAn52.1 plasmid opened with *Hind*III. After assembling and transformation of this vector in *E. coli* (normally 2 µl of the assembling reaction was used for transformation, see results **Section 3.4.7** for further details), the insertion of these fragments (*pyrG* and the 3' flank of AN8184) was checked by digestion with the *Pst*I endonuclease. For the second step of cloning the constructed plasmid in the first step was opened by *Nco*I endonuclease, and AN8184 gene was amplified by using primer sets AN8184F (**cttgagcagacatcac**ATGTCCCCCATGGTCCTT) and AN8184R (**gtaacgttaagtg-gatcTCAGAGATTGACCAGGCG**), which contained extra complementary nucleotides for either sides of the linear plasmid, in this step the correct integration of AN8184 gene was checked by *SacI* endonuclease. Finally to insert the 5' flank of AN8184 gene, the plasmid in the second step was opened with *EcoRI* endonuclease; also the primers that were used for amplification of the 5' flank of AN8184 had extra nucleotides for the open plasmid by *Eco*RI, forward AN8184_5FF (ctatgaccatgattacgCTTGAAGGATAACGCAGG) and reverse AN8184_5FR (gtgtagagatacaagggCGTGAACAATCTCAAACC**)**. The final vector was checked using the *Stu*I endonuclease to confirm the insertion of the 5' flank of the AN8184 gene.

For AN5791 the cloning steps were slightly different in the order of insertion of the fragments because of complications arising from restriction sites present within the gene. Here, for the 3' flank 1 kb was selected after the first 150 bp of the starting the downstream of the gene (the first 150bp not used, because contained the restriction site for *EcoR*1, which used in the next step to open the vector and insert the 5 flank of the gene). In the first step of cloning the *ppgA* gene was inserted into the plasmid, and then both the 3' flank and *pyrG* marker gene and finally 5' flank of the gene were inserted. Similarly, the cloning vector for AN3562 was constructed by inserting the 5' flank of the gene, then the AN3562 gene (in this cloning step the plasmid was opened with *Bam*HI instead of *Nco*I endonuclease) before final insertion of both the 3' flank and the *pyrG* gene. Unfortunately, despite several attempts it was not possible to insert both of these final fragments into the vector. Co-transformation was therefore performed to transform the cloning vector and the *pyrG* marker gene into *A. nidulans* in the case of AN3562. The primers that were used for constructing the cloning vector of both AN5791 and AN3562 are presented **Appendix 4**.

Plasmid vectors were amplified in *E. coli* as described previously (**Section 2.2.16**). Resulting vectors (as circular plasmids) containing the overexpression cassettes were transformed into *A. nidulans* 2-258 (*pyrG-*, *pyro A4-*, *nkuA□::argB, veA+*) by PEG-mediated transformation as described in **Sections 2.2.8** and **2.2.9**. After transformation, representative colonies were selected and screened by PCR to confirm the integration of the vector within the genome of *A. nidulans*. For such screening, a forward primer was designed in the 5' flank of the gene and a reverse primer was designed internal within the gene. For example, to confirm the integration of the AN8184 vector within the genome, both AN8184_5FF and AN8184R were used (**Appendix 5**). Strains with the correct integration were then selected for further study. This included initially checking the expression levels of these genes by qRT-PCR (**Section 2.2.15**) in all suitable *A. nidulans* transformant strains (i.e. those which contained the desired cloning vector) compared to the parental strain. The expression level of AN81814 gene was quantified by using forward primer (CGTTCCACAGAGCGTTTGTG) and reverse primer (CATCGAAGGCATGCTTGAGC). Cultures for RNA extraction

were grown under conditions favourable for sexual reproduction (**Section 2.2.13**) and RNA was extracted by a TRIzol method **(Section 2.2.13)**. Finally, *A. nidulans* transformant and parental strains were tested for sexual fertility as previously described (**Section 2.2.11**) to assess whether gene overexpression resulted in any change in fertility such as alteration in the number and diameter of cleistothecia compared to the parental strain.

3.4 Results

3.4.1 BLAST search and deletion cassettes

Table 3-2 lists the 29 *A. oryzae* genes from the work of Wada *et al.* (2012) that were used in BLAST searches for homologous genes in *A. nidulans* [note that it was discovered that there was some duplication in the original list of 33 genes due to the same gene sometimes being listed as both a gene and an EST signal (**Table 3.2**)]. This resulted in the detection of 20 homologous genes in *A. nidulans* based on the best hits of the same gene in *A. nidulans* being detected by both TBLASTX and BLASTX searches, and using a nominal 1 x 10^{-10} cut-off E-value (**Figure 3.1**). Note that at this point if different genes were identified by TBLASTX and BLASTX searching then these deletion cassettes were not ordered due to time constraints. The deletion cassettes for most *A. nidulans* homologs were available from the FGSC except for cassettes AN6418 and AN0021 were reported as PCR failures at the website and not available, AN2806 also was not listed (not found), whilst the tube for AN3393 was reported as being empty. Meanwhile, for three of the *A. oryzae* genes two homologous genes from *A. nidulans* were identified with similar E-values e.g. for AO080531000014 (**Table 3-2**) and it was decided to investigate both such genes in this case. This resulted in a total of 19 possible target genes. Where homologous genes were found in *A. nidulans* and the matching deletion cassettes were available in Fungal Stock Centre, gene synteny (SYBIL) was checked using the Jaccard Orthologous cluster option at AspGD

website http://aspgd.broadinstitute.org. However, for most of the tested genes the syntenic position was not conserved (**Table 3-2**).

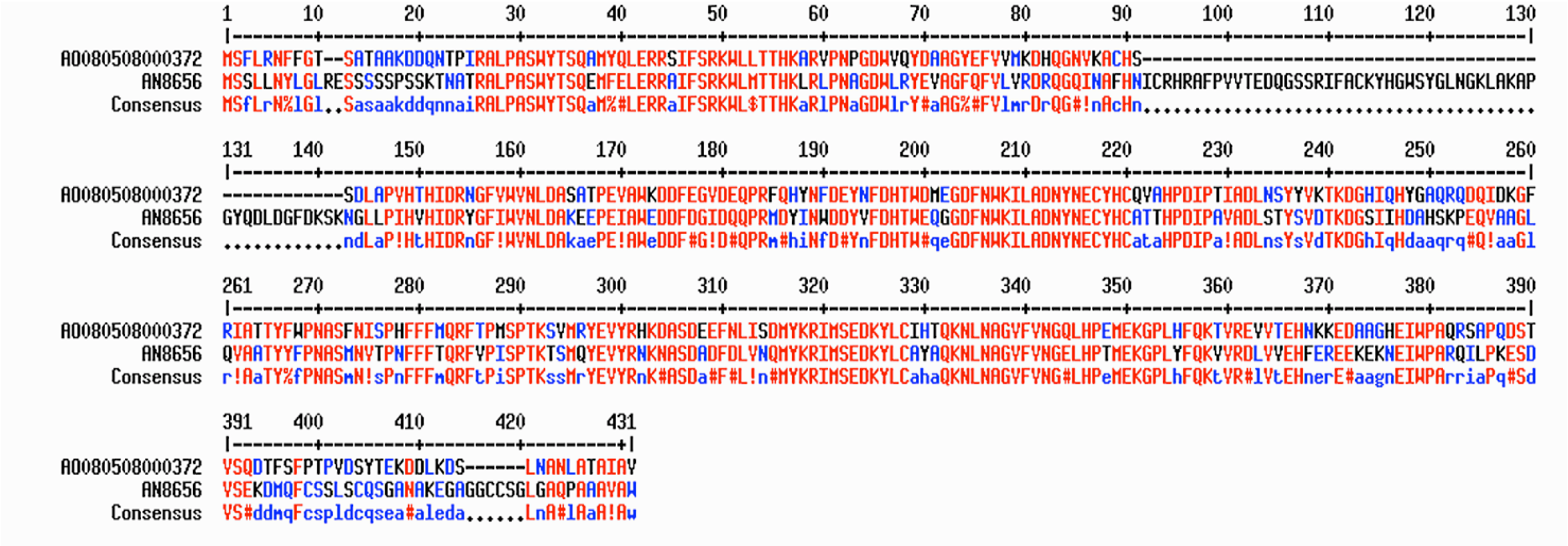


Figure 3.1 Representative example of alignment result between protein sequence of the *A. oryzae* AO080508000372 and the AN8656 sequence in *A. nidulans* using the MultAlin website (http://multalin.toulouse.inra.fr/multalin/) and the matrix BLOSUM 62. Red capital letters illustrate high consensus (the protein similar in both tested species) /whereas blue letters correspond to low consensus. E value = 3.0 e^{-123}.

Table 3-2 List of differentially regulated genes of *A. oryzae* identified by Wada et al (2012) and BLAST search results, together with synteny check and availability of deletion cassettes.

***A. oryzae* gene ID**	**Fold change in *MAT1* (M1) or *MAT2* (M2)**	**BLAST search to find homologous genes in *A. nidulans***				**Description of homologous gene in *Aspergillus nidulans***	**Synteny [a]**	**Availability of deletion cassette[b]**
		TBLASTX		**BLASTX**				
		Gene ID	**E value**	**Gene ID**	**E value**			
AO080503000328	55.7 (M1)	AN3239	1.0e-141	AN3239	3.0e-71	Has domain(s) with predicted acyl-CoA dehydrogenase activity, oxidoreductase activity, and acting on the CH-CH GROUP of donor activity.	**-**	**+**
AO080508000390	55.7 (M1)	AN8025	7.7e -02	AN8750	1.0e+0.0	Unknown (AN8025) Predicted oxidoreductase activity (AN8750)	**N**	**n/a**
AO080503000008	39.4 (M1)	AN5791	7.0e-14	AN5791	2.0e-56	*ppgA* pheromone precursor	**+**	**+**
AO080508000389	27.9 (M1)	AN6639	8.8e-02	AN3770	4.0e-01	Putative 2-methylcitrate dehydratase with a predicted role in lysine metabolism	**N**	**n/a**

						(AN6639). Unknown (AN3770)		
AO080541000465	26.0 (M1)	AN1168	3.0e-01	AN1285	3.4e+0.0	Putative voltage-gated calcium channel (AN1168) Unknown function (AN1285)	**N**	**n/a**
AO080523000564	24.3(M1)	AN8890	3.0e-62	AN8890	3.0e-57	Both have domain(s) with predicted carbohydrate binding, catalytic activity and role in carbohydrate catabolic process	**-**	**+**
		AN10820	4.e-30	AN10820	2.0e-36		**-**	**+**
AO080511000446	21.1 (M1)	AN2960	0.0e+0.0	AN2960	3.0e-174	Has domain(s) with predicted oxidoreductase activity and role in oxidation-reduction process	**+**	**+**
AO080521000309	19.7 (M1)	AN3562	1.0e-115	AN3562	9.0e-101	Unknown function	**-**	**+**
AO080502000077	18.4 (M1)	AN1540	6.0e-20	AN7406	8.0e-20	Unknown function	**N**	**n/a**
AO080521000174	18.4 (M1)	AN3393	3.0e-124	AN3393	5.0e-125	Protein with similarity to neutral metalloprotease II	**-**	**Empty tube**

AO080564000028	18.4 (M1)	AN10283	6.0e-08	AN6835	2.0e-10	Has domain(s) with predicted FMN binding, iron ion binding, oxidoreductase activity and role in oxidation-reduction process	**N**	**n/a**
AO080523000782	16.0 (M1)	AN5039	1.0e-61	AN5039	6.0e-40	Unknown function	**-**	**+**
AO080501000145	14.9 (M1)	AN6418	2.0e-72	AN6418	9.0e-39	Predicted role in transmembrane transport and integral component of membrane localization	**N**	**-**
AO080513000200	14.9 (M1)	AN0542	5.0e-12	AN9389	3.0e-12	Predicted role in cell wall macromolecule catabolic process	**N**	**n/a**
AO080541000466	14.9 (M1)	AN0056	3.5e-02	AN1700	1.4e+0.0	Arrestin-like protein; involved in UapA ubiquitination, endocytosis and vacuolar turnover, HulA-dependent ubiquitination at Lys-343 is critical for function (AN0056). Putative 26S proteasome regulatory subunit; transcript upregulated in response to camptothecin (An1700)	**N**	**n/a**

AO080531000014	13.9 (M1)	AN3275	2.0e-56	AN3275	9.0e-90	Cytochrome P450	**-**	**+**
		AN7818	2.0e-71	AN7818	1.0e-88	Putative sterigmatocystin biosynthesis P450 monooxygenase with a predicted role in sterigmatocystin/aflatoxin biosynthesis	**-**	**+**
AO080508000337	13.0 (M1)	AN3183	4.0e-02	AN1211	5.3e-01	Unknown function	**N**	**n/a**
AO080523000711	13.0 (M1)	AN1598	7.0e-77	AN1598	3.0e-104	Putative cytochrome P450; member of the PbcR-activated diterpene cluster	**-**	**+**
		AN8184	6.0e-71	AN8184	4.0e-107	Putative cytochrome P450	**-**	**+**
AO080541000488	13.0 (M1)	AN6458	8.0e-17	AN6458	9.0e-14	Unknown function	**-**	**+**
AO08052100005 (AoEST3991)	12.1 (M1)	AN0021	8.0e-9	AN0021	2.0e-12	Unknown function	**N**	**-**
AO080523000026	11.3 (M1)	AN4686	1.0e-08	AN4686	5.0e19	Putative chitosanase predicted glycosyl phosphotidyl linositol (GPI)-anchor	**-**	**+**
AO070333000246	10.6 (M1)	AN3551	9.0e-03	AN 2983	5.5e-02	Unknown function	**N**	**n/a**

AO080505000070 (AOEST5207)	84.4 (M2)	AN11253	2.0e-50	AN11253	2.0e-48	Has domain(s) with predicted transferase activity, transferring phosphorus-containing groups activity	+	+
AO080515000084	18.4 (M2)	AN6881	6.0e-48	AN6881	5.0e-41	Unknown function	-	+
AO080532000139	18.4 (M2)	AN6257	3.0e-75	AN6257	4.0e-68	Vesicle coat complex COPII, subunit 31	+	+
AO080523000059 (AOEST6582)	14.9 (M2)	AN5993	3.0e-51	AN5993	4.0e-40	Predicted role in calcium binding activity	+	+
AO080508000372	14.9 (M2)	AN8656	1.0e-145	AN8656	3.0e-124	Predicted 2 iron, 2 sulfur cluster binding, iron ion binding, oxidoreductase activity, oxidoreductase activity acting on paired donor	-	+
AO080511000543	11.3 (M2)	AN1356	6.0e-119	AN1356	1.0e-125	Unknown function	+	+
AO080553000049	11.3 (M2)	AN2806	3.0e-04	AN2806	1.0e-13	Unknown function	N	_

[a]**+** indicates conserved gene synteny (gene is localized on the same chromosome within species), **-** indicates no synteny, and **N** indicates that the synteny was not checked for this gene. [b]Availability of cassettes indicated by (**+**) available and (**–**) not available. n/a indicates not applicable as different genes identified by TBLASTX and BLASTX searching.

3.4.2 Amplification of deletion cassettes

The 19 deletion cassettes obtained from the FGSC were amplified using Phusion high fidelity enzyme and an annealing temperature of 63 ^{o}C. Most of them were successfully amplified using these conditions (**Figure 3-2**). However, for three cassettes (AN2960, AN5039 and AN6458) secondary products were produced and two cassettes (AN8890 and AN11253) only yielded faint products. For these latter cassettes it was necessary to perform gel purification and further rounds of PCR amplification.

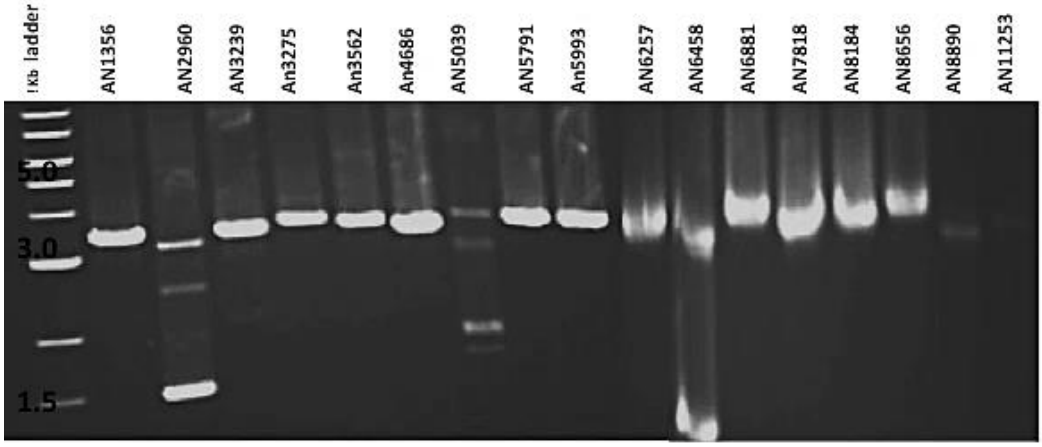


Figure 3-2 Agarose gel (1.2%) showing results of PCR amplification of *Aspergillus nidulans* deletion cassettes.

3.4.3 Transformation and screening of transformants by PCR

After amplification, clean up and concentration of deletion cassettes a total of 1-3 µg of DNA from each deletion cassette was used for transformation of *A. nidulans* strain 2-258 (*pyrG*$^{-}$, *pyro-*, *ve+* and *Ku*$^{-}$) via PEG mediated transformation. The *pyrG* marker gene was used as a selective marker. After transformation at least twelve colonies were selected for screening by PCR to assess possible deletion of the target gene; if the gene was not deleted in these twelve colonies then further

colonies were tested. Out of the 19 selected deletion cassettes of *A. nidulans*, transformation was successfully achieved for 13 genes in which the deletion of the target gene was confirmed by PCR (**Appendix 15)** (**Figure 3-3**). This required the screening of between 12-150 transformants per target gene.

Figure 3-3. Representative results of gene deletion screening illustrated by target gene AN8656. PCR amplification results (using a primer pair within the target gene) of the wild-type (parental) and control (- DNA protoplast control) compared to transformant colonies (1, 5 and 6) where the target gene had been deleted and failed to amplify any product. The –DNA control was included and yielded no product (data not shown).

After confirming deletion of the target gene, the correct integration (i.e. non-ectopic) of *pyrG* marker gene was checked and confirmed by positional PCR (**Appendix 16**). Representative results are shown in **Figure 3-4**, illustrating amplification products obtained where the *pyrG* gene was inserted to replace the AN8656 gene. Correct integration of the *pyrG* marker was confirmed for all 13 deletion cassettes.

For the remaining 6 target genes (AN2960, AN5039, AN6257, AN6458, AN8890 and AN10820), despite three different rounds of transformation, use of the *Ku*$^{-}$

marker and screening of numerous transformants (where these were obtained), it was not possible to obtain gene deletion mutants and no further work was undertaken with these. For the gene AN6257 approximately 150 colonies were screened but the gene was always found to be present, it was then discovered that its orthologue in *Saccharomyces cerevisiae* (*sec31*) is an essential gene, which indicated that the *A. nidulans* null mutant strain was not viable.

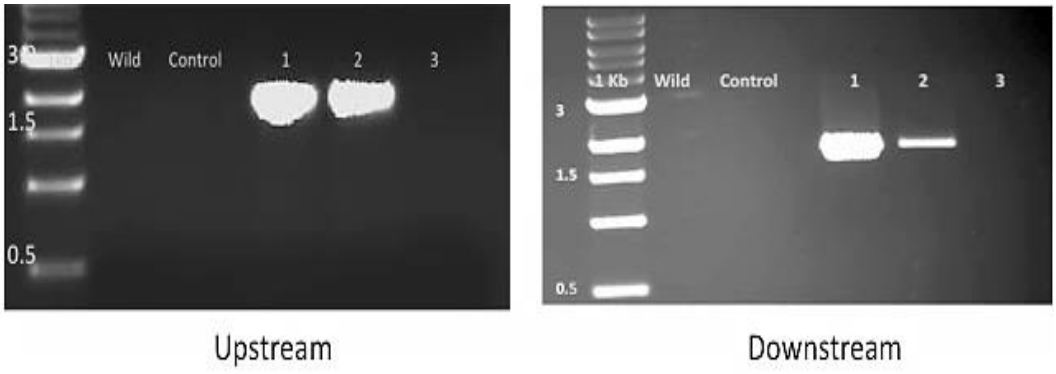


Figure 3-4 Agarose gel (1.2%) showing representative results of amplification products of *pyrG* positional PCR in the ΔAN8656 deletion strain (lanes 1 and 2) compared to wild-type and protoplast control strains. For amplification either upstream (FP1 and TP4) or downstream (TP3 and FP2) primers were used (**Section 2.2.10**).

3.4.4 Sexual fertility

All 13 deletant gene strains of *A. nidulans* together with the parent and –DNA protoplast control strains were assayed for sexual fertility as previously described (**Section 2.2.11**). Ideally, where available two independent gene deletion mutants of each target gene were included to ensure reproducibility of results. In all cases there was no significant difference between the parental and protoplast transformation control strains in the number and size of cleistothecia produced (data not shown). However, significant differences in sexual fertility were detected in some of the gene deletant strains as follows.

A: Number of cleistothecia formed

Results presented in **Figure 3-5** show the relative number of cleistothecia produced by the various transformant strains compared to the parental control. It was found that deletion of two genes (AN3562 and AN8656) resulted in a complete loss of fertility, with sexual development blocked at a very early stage with no evidence even for Hülle cell formation. By contrast, deletion of most (9 out of 11) of the remaining target genes had a more moderate effect of fertility, resulting in either a significant increase or decrease in the number of cleistothecia formed compared to parental control **(Appendix 6a)** (**Figs. 3-5**). Deletion of just two genes (AN3239 and AN5993) had no significant effect on fertility. All of gene deletion strains, which produced cleistothecia, also formed ascospores except for deletion strain ΔAN1356 in which the cleistothecia were sterile. One-Way ANOVA, Post HOC test (LSD) wee used to analyse the data (**Appendix 6a).**

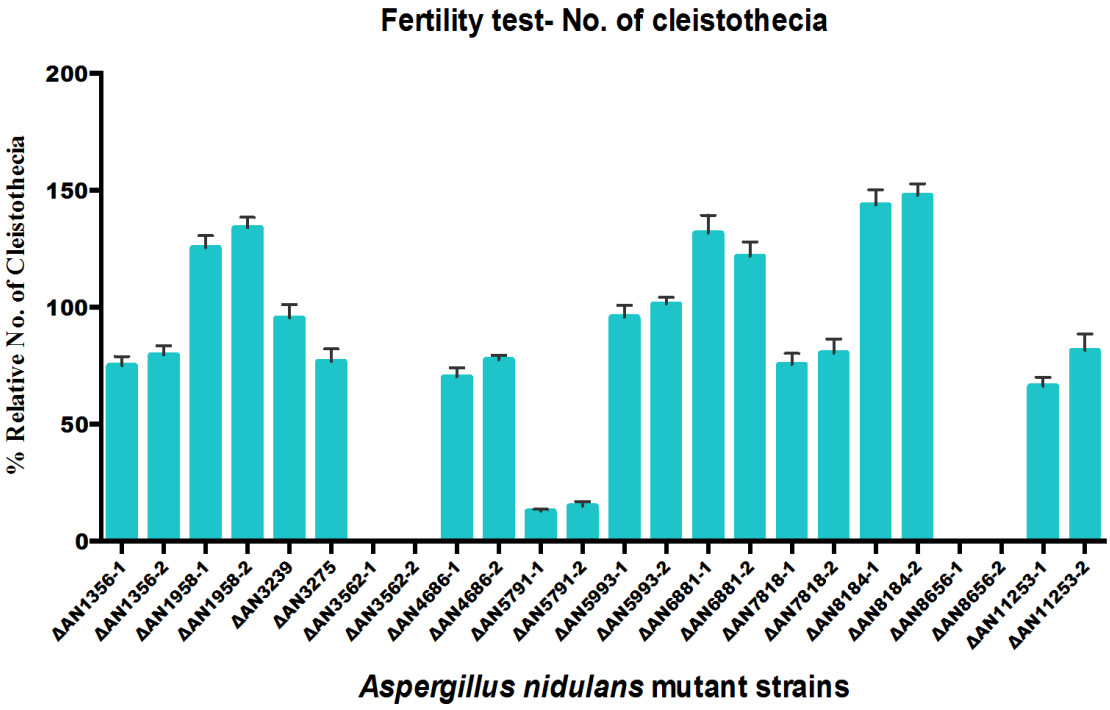


Figure 3-5 Effect on fertility in gene deletion strains in terms of numbers of cleistothecia produced. Data show the numbers of cleistothecia produced by various *A. nidulans* gene deletion strains relative to the parental control strain (as

a percentage). The number of cleistothecia were counted in four replicate plates and three viewing areas were taken for each plate from each tested strain (i.e a total of 12 viewing areas were counted). Note that for most deletion strains two independent strains (suffix -1 and -2) were assayed and that all were significantly different from the parental control except for (AN3239 and AN5993). Error bars represent the standard error of the mean. Between 1052.78-1341.63 cleistothecia were formed per cm^2 by the parental strain.

B: Size (diameter) of cleistothecia formed

Most gene deletant strains produced cleistothecia of a similar size to the parental control strain (**Figure 3.6**). The only exceptions were for the two gene deletants which failed to produce any cleistothecia (ΔAN3562 and ΔAN8656) and the ΔAN3275, ΔAN4686 and ΔAN5791 deletion strain that produced cleistothecia that were significantly larger than the wild-type strain (**Appendix 6b**) (**Figs. 3.6 and 3.7**) (a non-parametric Kruskal-Wallis test was used to analyse results because did not satisfy the Levene's test of homogeneity of variance) (**Appendix 6b**).

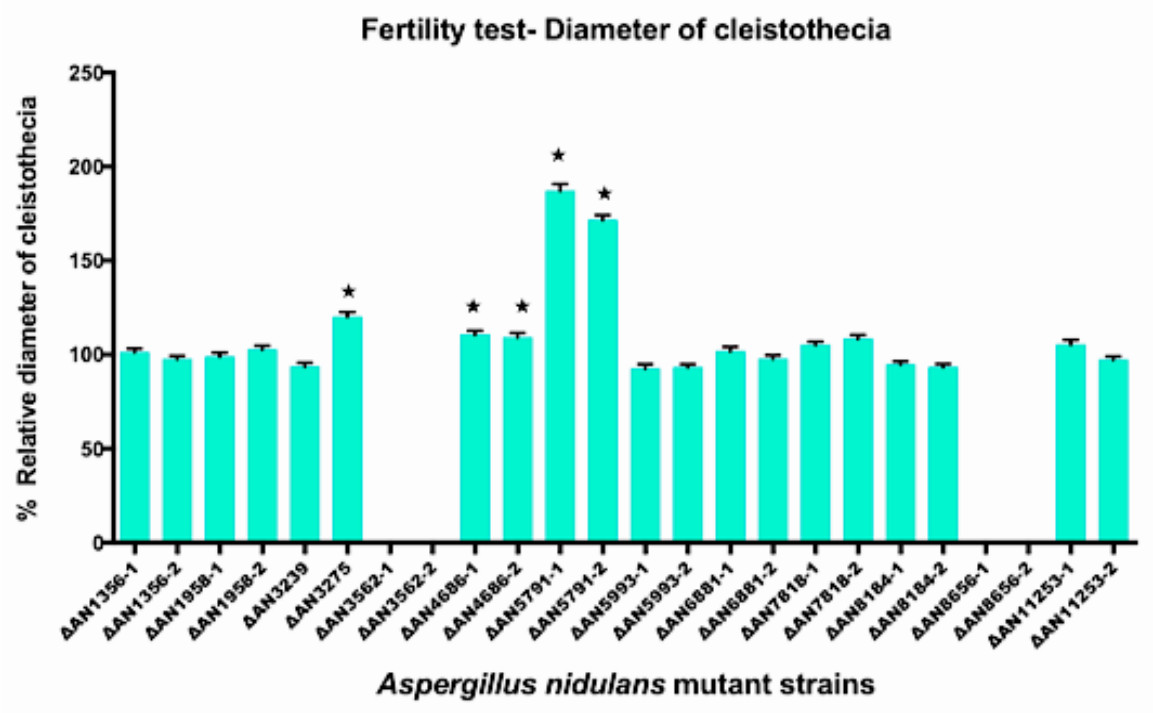


Figure 3-6 Effect on fertility in gene deletion strains in terms of size of cleistothecia produced. Data show the diameter of cleistothecia produced by various *A. nidulans* gene deletion strains relative to the parental control strain (as a percentage). Note that for most deletion strains two independent strains (suffix -1 and -2) were assayed. Presence of * above the results bar indicates a statistically significant difference from the parental control strain. Error bars represent SEM.

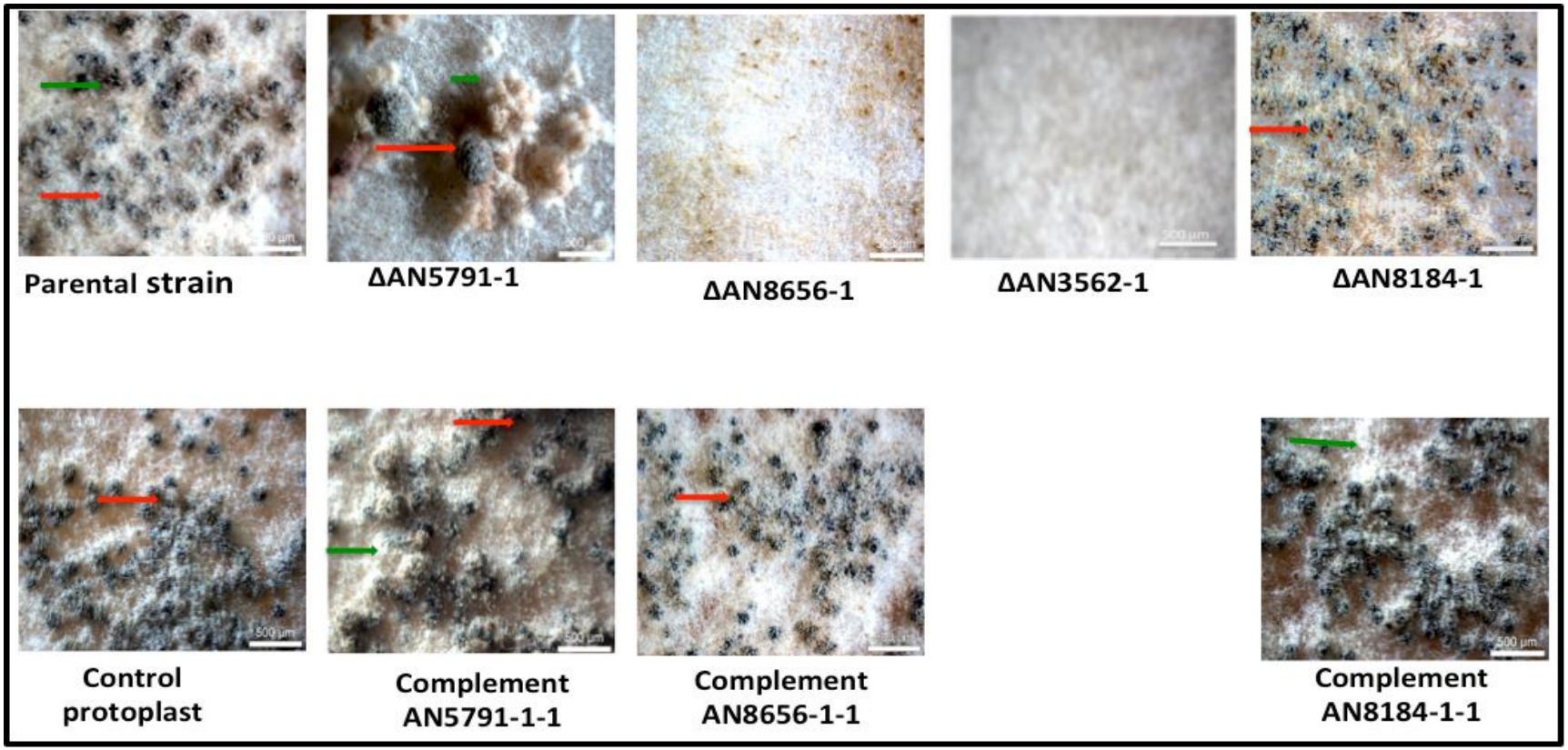


Figure 3-7 Images of representative gene deletion and parental and protoplast control strains to illustrate the effect of gene deletion on sexual fertility. Red arrows indicate cleistothecia produced by the various strains, whilst green arrows indicate the Hülle cells surrounding the cleistothecia. Scale bars represent 500 µm in all cases.

3.4.5 Complementation of transformants

All target gene deletion strains (*pyro-*, *pyrG*$^{+}$, Ku^{-}, green spore), which exhibited a significant change in sexual fertility were outcrossed with *A. nidulans* strain 2-259 [*paba A1-*, *yA2* (yellow spore), *veA+*, *pyro+*] as described by Todd *et al.*, (2007) (**Section 2.2.12**). Ascospores from hybrid cleistothecia were then evaluated for auxotrophic markers to assess restoration of the original genotype as evidence of complementation of the transformants. Green colonies that only grew on AMM supplemented with pyridoxine were selected and further tested by PCR to confirm the restoration of the target gene (**Appendix 17**). Representative results are presented in **Figure 3-8** for the gene AN8656. Here, four restored strains were screened for presence of the target gene by PCR (checks were also made in the wild-type and –DNA transformation control), with the deleted gene found to be restored in two of the complement strains (1 and 3) (**Figure 3-8**).

Figure 3-8. Agarose gel (1.2%) showing representative results of PCR amplification of the AN8656 target gene in four complement strains of AN8656 (labelled 1-4) together with amplification from parental wild-type and protoplast control strains.

The sexual fertility of all restored complement strains was assessed (**Section 2.2.11**), to confirm whether the observed phenotype resulting from the original gene deletion was indeed due to gene deletion rather than some other artefact. The number and diameter of cleistothecia in all complement strains was compared to the parental strain. Fortunately, all restored complement strains exhibited cleistothecia of similar phenotype to the parental type strain, with no significant difference in terms of number and size of cleistothecia formed (**Appendices 7a and 7b**) (**Figs, 3-9 and 3-10**).

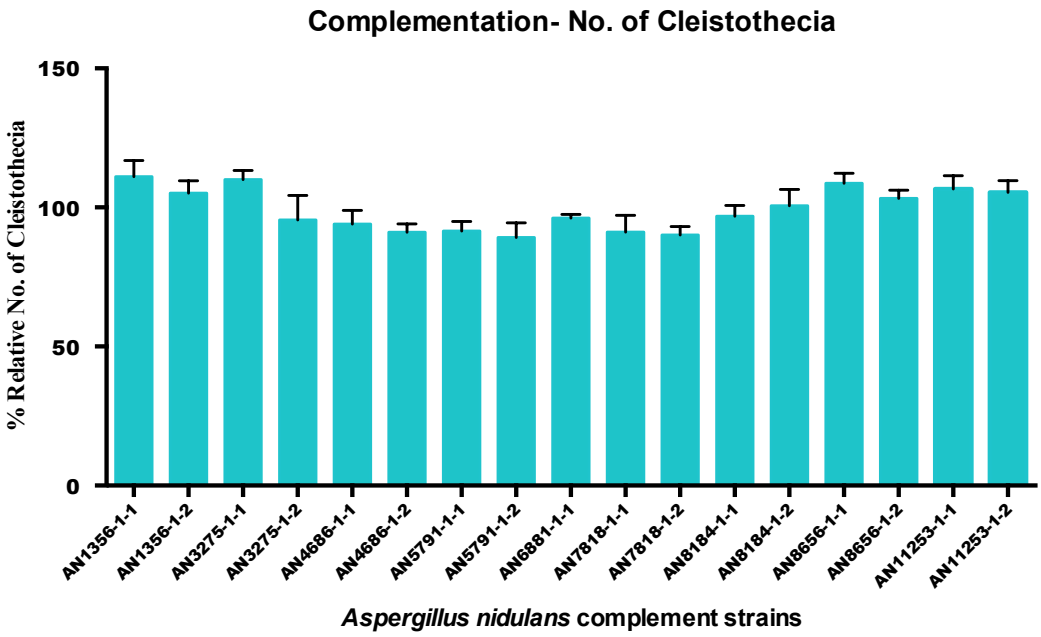


Figure 3-9. Relative number of cleistothecia produced by restored complement strains of *A. nidulans* compared to the parental strain (expressed as percentage relative to parental strain). There were no significant differences in the number of cleistothecia produced by restored complement strains compared to the parental control strain. Error bars represent SEM. Between 1052.78-1385.75 cleistothecia were formed per cm^2 by the parental strain.

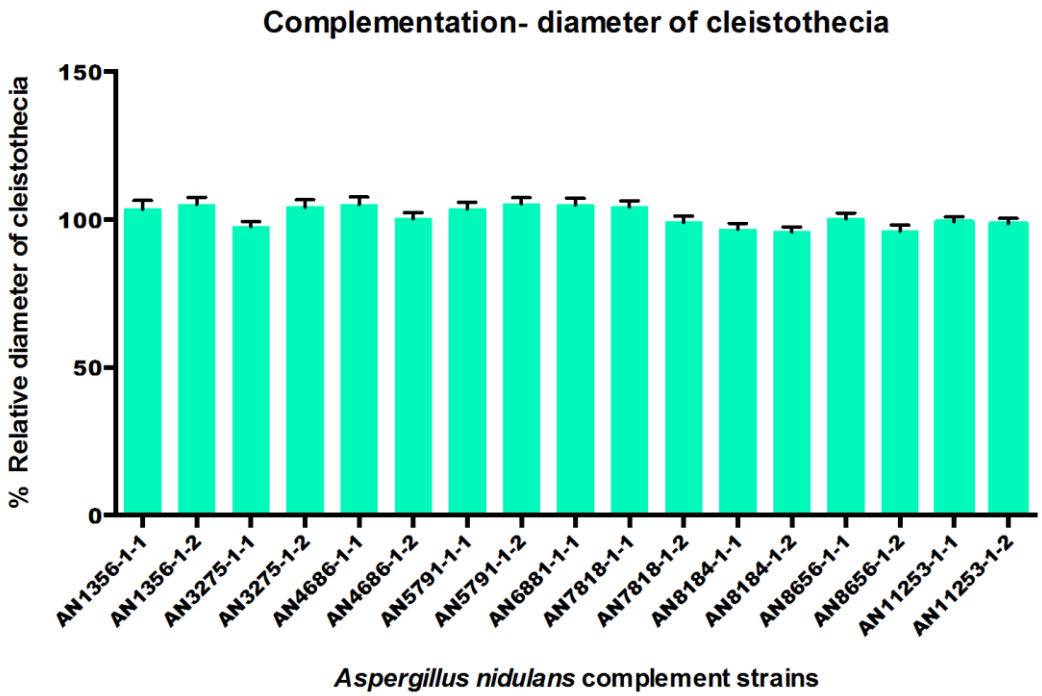


Figure 3-10. Relative diameter of cleistothecia produced by restored complement strains of *A. nidulans* compared to the parental strain (expressed as percentage relative to parental strain). There were no significant differences in the size of cleistothecia produced by restored complement strains compared to the parental control strain.

3.4.6 Determination of transcript level of some sex key genes

The transcript levels of some key genes for sexual reproduction were quantified by qRT-PCR in the parental, transformation control and also in a number of gene deletion strains [ΔAN3562-1 (sterile phenotype), ΔAN5791-1 (alpha pheromone deletant), ΔAN8184-1 (increased number of cleistothecia phenotype) and

ΔAN8656-1 (sterile phenotype)], which exhibited interesting phenotypes regarding sexual fertility compared to parental strain. RNA was extracted from 6 days old cultures grown under conditions favouring sexual development (incubation in the dark at 32 ^{0}C on solid media with sealed plate) and transcript levels of *MAT1*, *MAT2*, the alpha pheromone precursor (*ppgA*), both pheromone receptors (*preA* and *preB*) and s*teA* determined. Data were normalised as a percentage relative to the housekeeping *H2b* gene (**Figure 3-12**).

The transcript level of both *MAT1* and *MAT2* gene were drastically decreased in both ΔAN3562-1 and ΔAN8656-1 and also significantly decreased in ΔAN5791-1 compared to the parental strain **(Figure 3-12)**. In contrast expression of *preA* was not changed significantly in most of the strains compared to the parent except for strain ΔAN5791-1 **(Figure 3-12)** where there was a significant increase in the expression level [one way ANOVA using reciprocal transformed data; F (DF 5,12) = 3.178 and P-value < 0.047)].

Regarding the expression of s*teA*, there was a significant increase in *steA* transcript levels in both ΔAN5791-1 and ΔAN8656-1 strain compared to the parental strains [one way ANOVA post hoc test (LSD), F (5,12) = 8.72, P- value <0.001] but not in ΔAN3562-1 or ΔAN8184-1 **(Figure 3-12)**. Meanwhile, a drastic change in *ppgA* expression levels was observed in both the ΔAN3562-1 and ΔAN8656-1 strains compared to the parental strain and as expected there was no expression in ΔAN5791-1 because the gene was deleted in this strain. There was no significant change in the transcript level of *ppgA* gene in either the transformation control or ΔAN8184-1 strain (one way ANOVA post hoc test (LSD), F (5,12) = 478.50 and P- value <0.0001).

Finally, for the pheromone receptor *preA* showed a significant change in expression level in only the ΔAN5791-1 strain (0.0163 in ΔAN5791-1 strain compared to 0.0112 in the parental strain) [one way ANOVA of reciprocal transformed data; F (5,12)=53.178, P < 0.047; a post hoc test (LSD) was used to detect differences]. By contrast, the *preB* expression profile was changed in all of four deletion strains of *A. nidulans* compared to the parental strain (**Figure 3-13**).

A marked increase (7.13 fold) was especially apparent in the expression level of the ΔAN5791-1 strain. Significant increases were also apparent in the ΔAN8184-1 and ΔAN8656-1 strains, whilst a decrease was observed in the ΔAN3562-1 strain. However, the very low expression levels of *preB* relative to the *H2b* gene mean these latter results must be interpreted with caution [one way ANOVA of log transformed data; $F(5,12)=59.587$, $P < 0.0001$; a post hoc test (LSD) was used to detect differences].

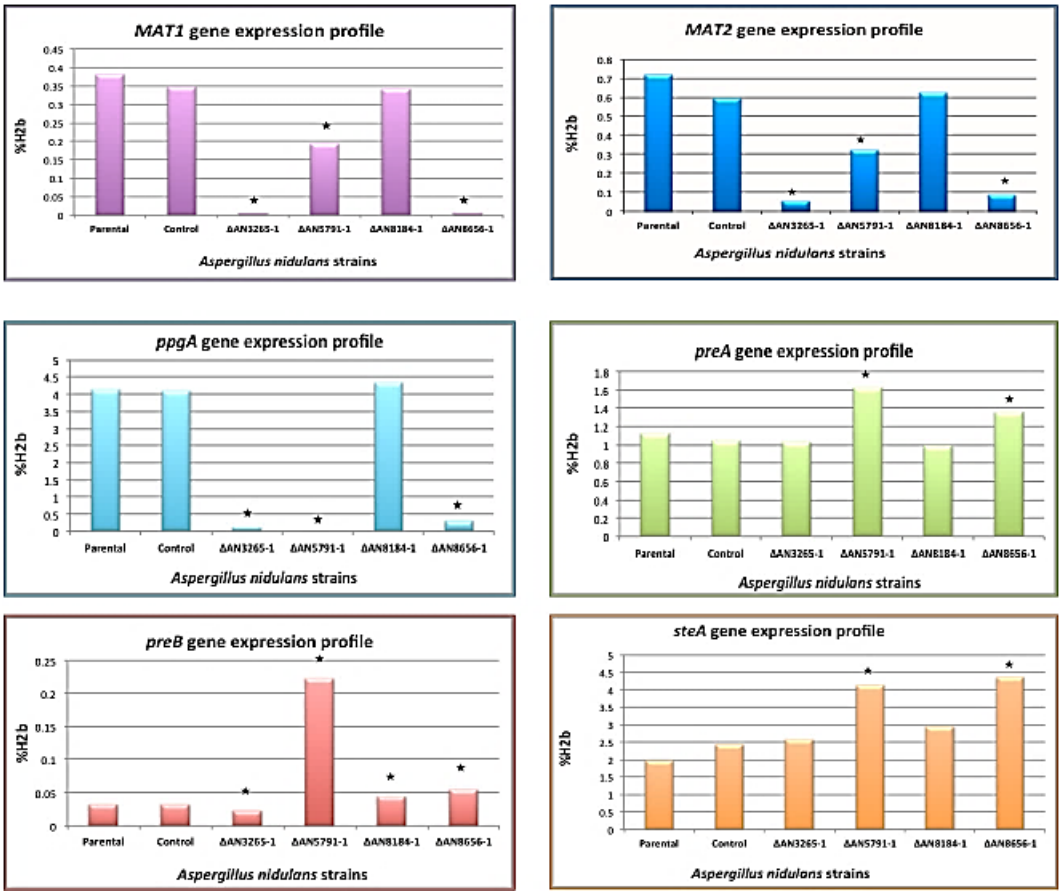


Figure 3-12 qRT-PCR analysis of gene transcript levels (relative to *H2b* housekeeping control gene) showing the expression profile of *MAT1*, *MAT2*, p*pgA*, *steA*, *preA* and p*reB* in the *A. nidulans* parental strain, transformation control and four different gene deletion strains (ΔAN3562-1, ΔAN5791-1, ΔAN8184-1 and ΔAN8656-1). ★ indicates conditions where gene expression was statistically significantly different compared to the parental strain (P value < 0.05).

3.4.7 Gene overexpression

3.4.7.1 Amplification of DNA fragments for cloning

Cloning was performed for each of AN3562, AN5791 and AN8184 in three different steps using either the Gibson or HiFi NEBulider cloning kits. For construction of cloning vectors, approximately 1kb of both flanking regions of the target gene, the target gene and the *pyrG* marker gene of *A. fumigutus* were amplified by PCR. The primer used for amplification of each of these fragments contained complementary nucleotides specific to the site of insertion in the pAN52.1 plasmid. **Figure 3-13** is a representative example showing amplification of fragments used for construction of the cloning vector for AN8184.

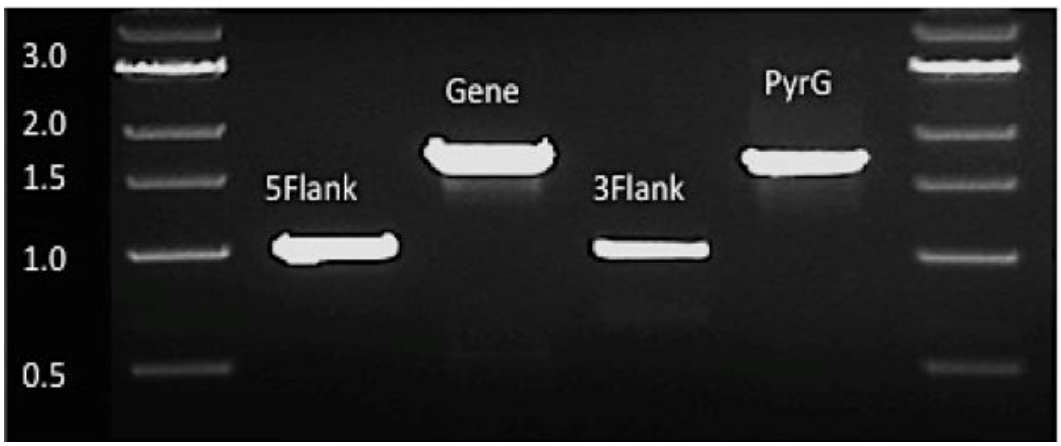


Figure 3-13. Gel electrophoresis of amplification products of DNA fragments used for cloning of AN8184 gene. The expected size for the PCR product of both upstream and downstream flanking region is 1000 bp, for the AN8184 gene is 1740 bp and for *pyrG* gene is 1732 bp.

3.4.7.2 Assembly of DNA fragments into vector

Amplified DNA fragments were assembled into the pAN52.1 vector containing the constitutive *A. nidulans gpdA* promoter and an *E. coli* penicillin resistance selective marker. The first cloning step involved insertion of both *pyrG* and the 3' flank of gene into pAN52.1 by linearizing with *Hin*dIII endonuclease and the NEBuilder Gibson cloning kit was used to assemble these two fragments as shown by the predicted map of the vector produced at this step (**Figure 3-14**).

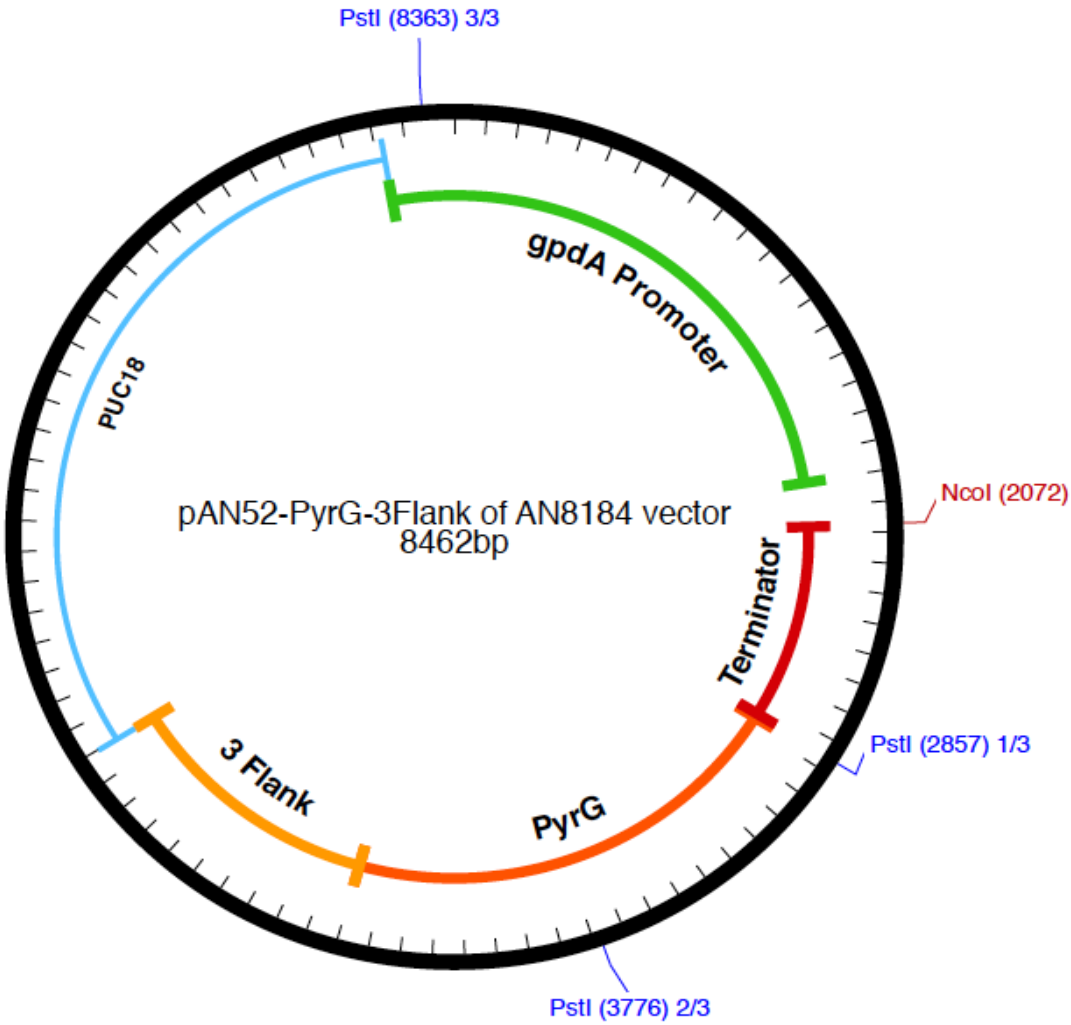


Figure 3-14. Predicted map of the pAN52.1 vector with the *pyrG* and the 3' flank of the AN8184 gene correctly inserted.

The assembling mixture was transformed into chemically competent *E. coli* (provided with the kit) by a heat shock method and cells cultured on LB media containing Ampicillin (sodium salt). Putative transformed colonies of *E. coli* were selected, plasmid extracted and then screened by digestion with *PstI* endonuclease to confirm the insertion of both fragments in the correct site within a vector (**Figure 3-15**).

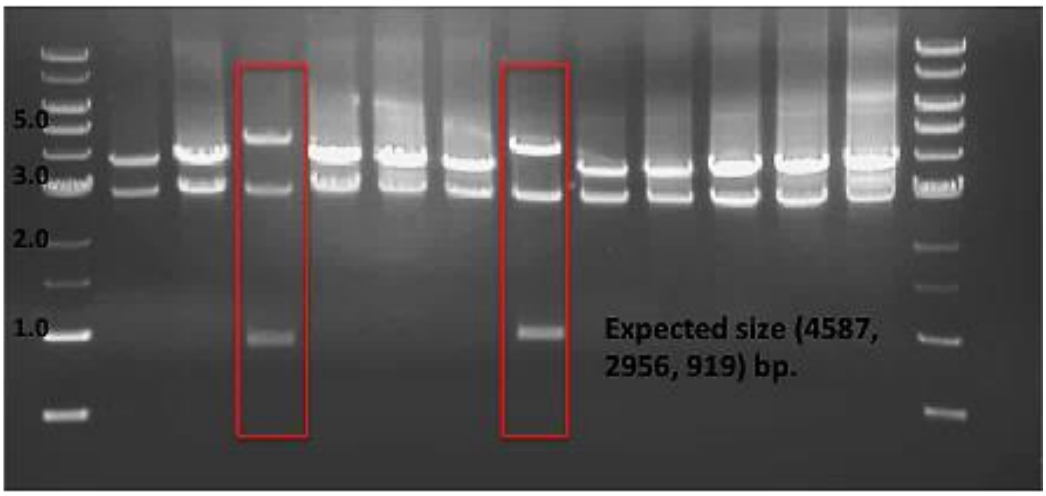


Figure 3-15. Screening of plasmid DNA digested with *Pst*I to test for insertion of both *pyrG* and 3' flank of the AN8184 gene in plasmid pAN52.1. Lanes 4 and 8 show correct predicted insertion pattern.

The pAN52.1-PyrG-3Flank plasmid constructed in the first step was then used in the second step of cloning. The plasmid was opened with the *Nco*I restriction enzyme then the AN8184 gene was assembled into the plasmid in the site between the *gpdA* promoter and terminator (see **Figure 3-16**) using the Gibson cloning kit.

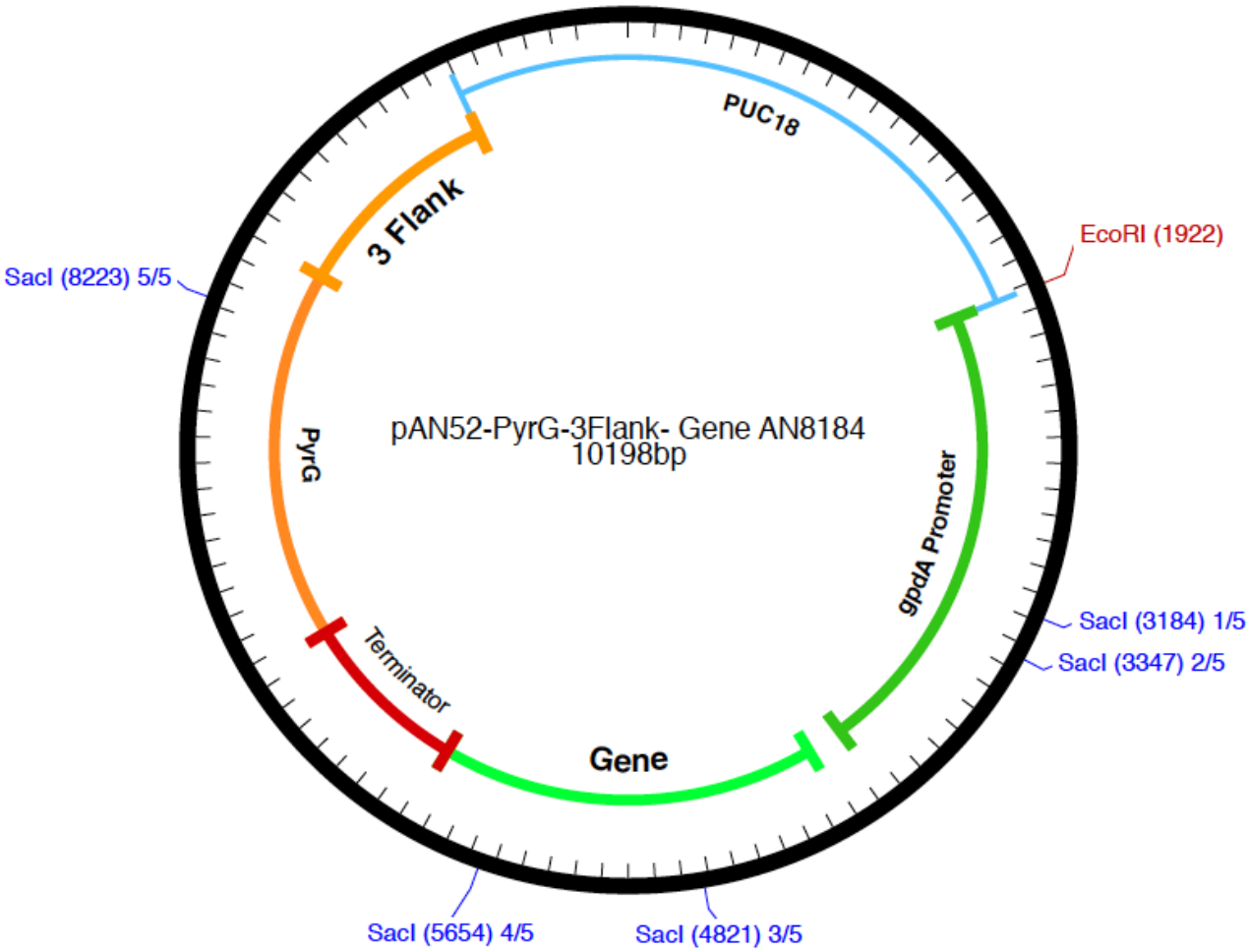


Figure 3-16 Predicted map of the pAN52-PyrG-3 Flank with AN8184 gene correctly inserted after the *gpdA* promoter.

The assembling mixture was transferred as in the first step to *E. coli* and representative colonies were selected and plasmid extracted. The plasmid was screened using the *Sac*I restriction enzyme to confirm correct insertion of the AN8184 gene into the pAN52-PyrG-3 Flank vector (**Figure 3-17**).

Figure 3-17 Screening of the pAN52-PyrG-3 Flank plasmid by digestion with *Sac*I to assess correct insertion of the AN8184 gene. Lanes 2, 3 and 12 show correct predicted insertion pattern.

The final step of vector assembly involved digestion of the pAN52-PyrG-3Flank-AN8184 product (from the second step of cloning) with *Eco*RI. The remaining 5' flank DNA fragment of AN8184 was inserted in the correct site of the vector to produce the final vector (**Figure 3-18**). After transformation, colonies of *E. coli* were selected for plasmid extraction and the correct integration confirmed by digestion of the plasmid with *Stu*I (**Figure 3-19**).

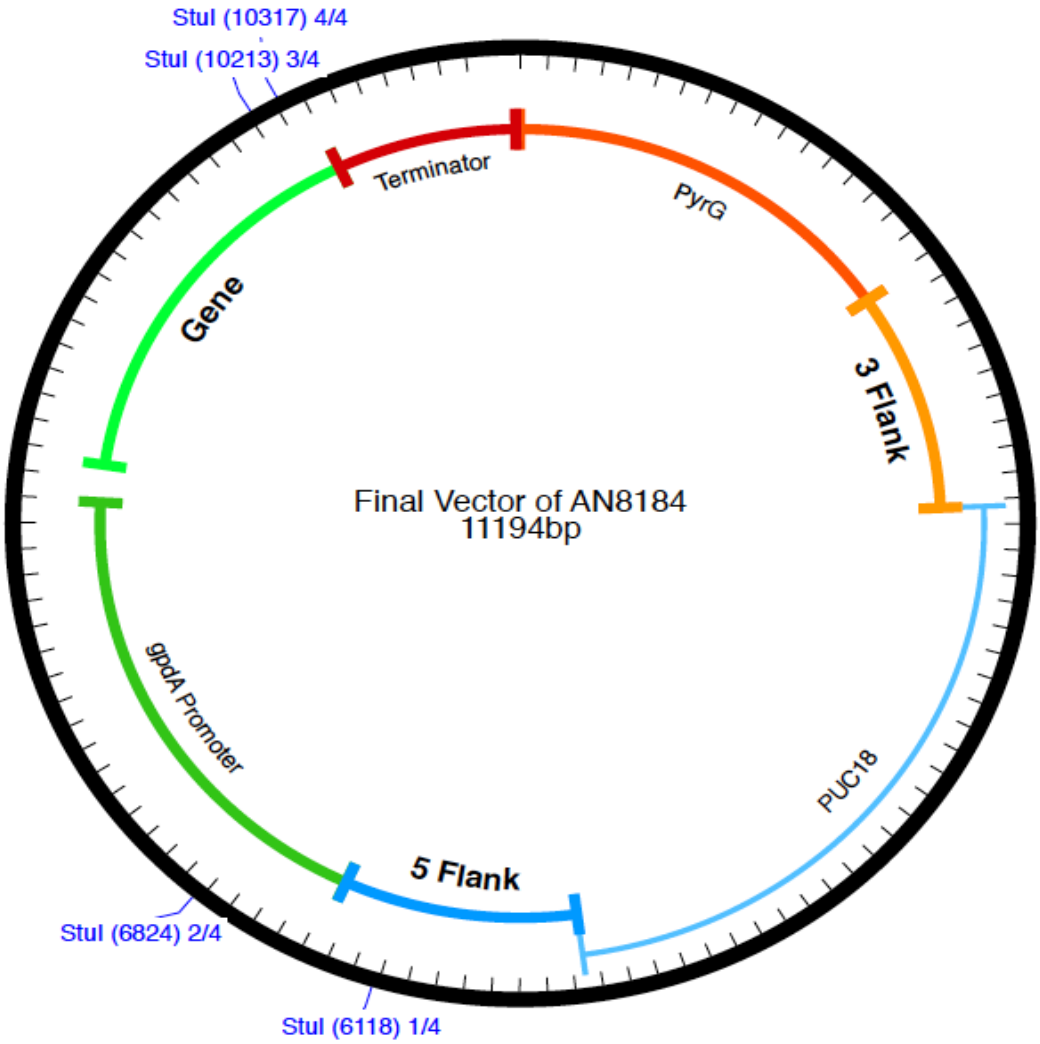


Figure 3-18. Predicted map of final vector for overexpression of gene AN8184.

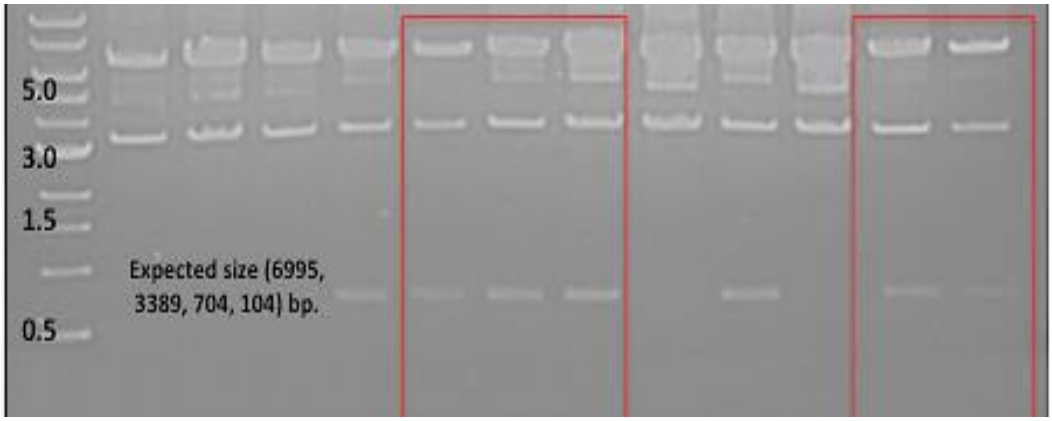


Figure 3-19 Screening the final overexpression vector of AN8184 by digestion with *Stu*I to assess correct insertion of the 5'flank from the AN8184 gene. Lanes 5, 6, 7, 8, 10, 12 and 13 show the correct predicted insertion pattern.

3.4.7.3 Transformation of *A. nidulans* and screening of transformants

Overexpression plasmids for genes AN8184, AN5791 and AN3562 were transferred to *A. nidulans* strain 2-258 (auxotrophic for uridine and uracil) by PEG-mediated transformation using the *pyrG* gene as a selective marker. After transformation, representative colonies that grew on sorbitol minimum media without uridine and uracil were selected and screened by PCR for correct integration of the overexpression vector using primer sets of a forward primer in the 5'flank of the target gene and a reverse primer within the target gene (**Figure 3-20**). If the plasmid had inserted in the correct site there was predicted to be only one amplicon, which should be larger in size than that in the parental strain because of insertion of the *A. nidulans gpdA* promoter between the 5'flank and the target gene.

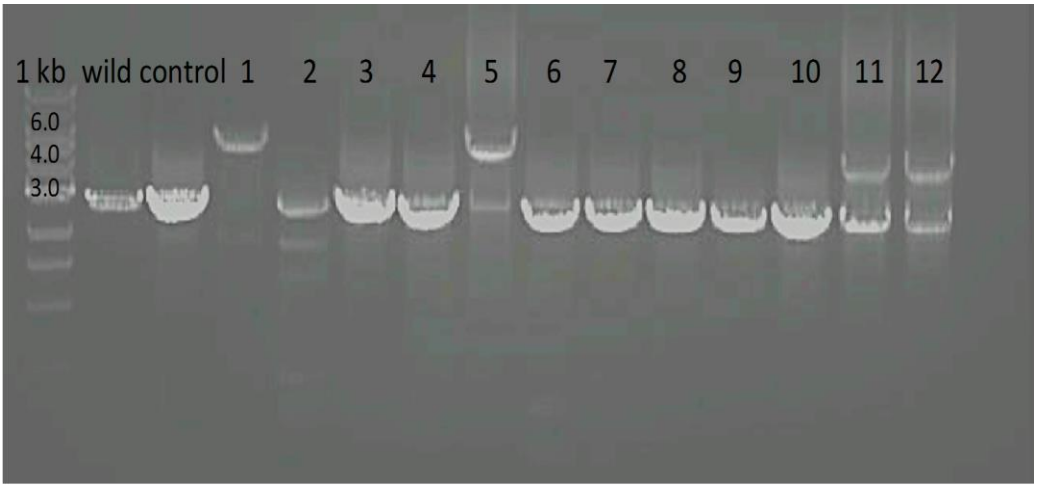


Figure 3-20. Screening by PCR of genomic DNA of transformant colonies for putative insertion of the AN8184 overexpression vector. The expected product size in the parental and protoplast controls is 2,804 bp but if the vector had inserted then a larger expected product size of 5035 bp would be predicted. Lane 1 shows an example of the correct insertion of the vector to replace the resident target gene, whereas transformant strains 5, 11 and 12 contained two copies of gene, one of them being the parental and other being an inserted copy from vector that had integrated somewhere else within the genome.

3.4.8 Determination of transcript level of overexpressed gene

The transcript level of AN8184 was quantified by qRT-PCR in the *A. nidulans* parental strain and in two selected transformant strains that contained the AN8184 cloning vector inserted in the correct place to replace the resident AN8184 gene (OvEAN8184-1), whilst for comparison expression levels were also determined for a strain (OvEAN8184-2) which contained two copies of the AN8184 gene - the parental one and the other arising from ectopic insertion of the cloning vector (**Figure 3-21**). One-way ANOVA-LSD revealed that there were significant differences in the expression levels of the AN8184 gene between the parental strain and the transformant strains, with the OvEAN8184-1 and OvEAN8184-2 strains showing a 154 and 274.4 fold increase over the parental strain, respectively [square root transformed data; $F(2,6) = 544.012$, $P < 0.0001$].

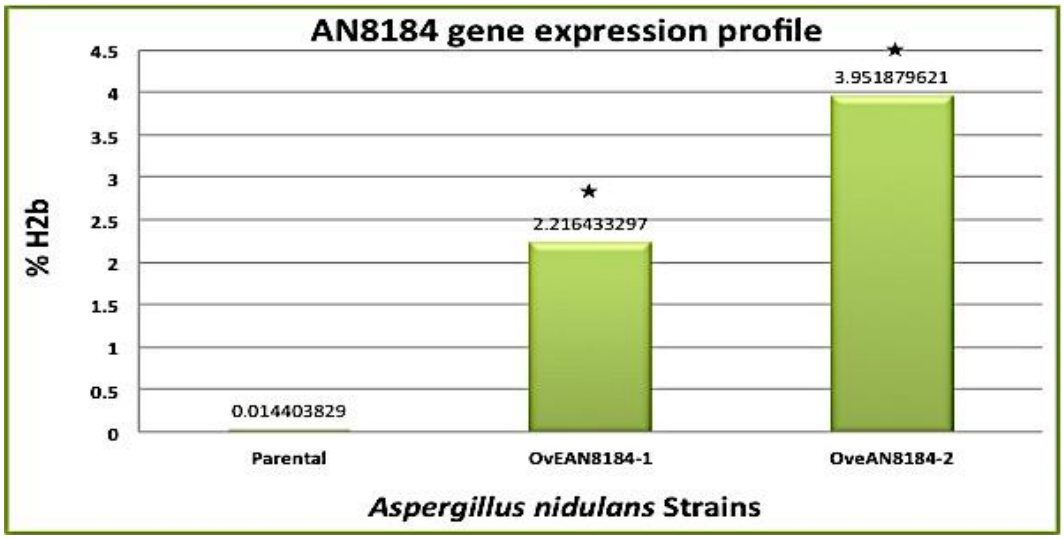


Figure 3-21 qRT-PCR analysis showing expression levels of the AN8184 gene in the parental strain and two transformant strains containing the AN8184 cloning vector. ★ indicates statistically significant differences in the expression of AN8184 gene between relevant strain and the parental strain.

3.4.9 Fertility test for overexpression strains

Transformant strains containing either the AN8184 overexpression cloning vector (OvEAN8184-1) or the cloning vector as well as the resident AN8184 gene (i.e. an extra ectopic copy; OvEAN8184-2 and OvEAN8184-3), and two strains of OvEAN5791 (both containing a single copy of the overexpressed AN5791 gene) were tested for fertility to determine the effect of overexpression of the target gene on fertility compared to the parental and protoplast transformant control strains. Perhaps surprisingly, there were no significant differences in fertility in terms of the number [one-way ANOVA-LSD; $F(4, 55) = 0.748$, $P > 0.05$] and size (diameter) of cleistothecia [$F(4, 495) = 2.171$, $P > 0.05$] produced by the various OvEAN8184, protoplast control and parental strains (**Figures 3-22, 3-23 and 3-24**). And even more surprising, overexpression of AN5791 totally suppressed the formation of cleistothecia (**Figures 3-22, 3-23 and 3-24**).

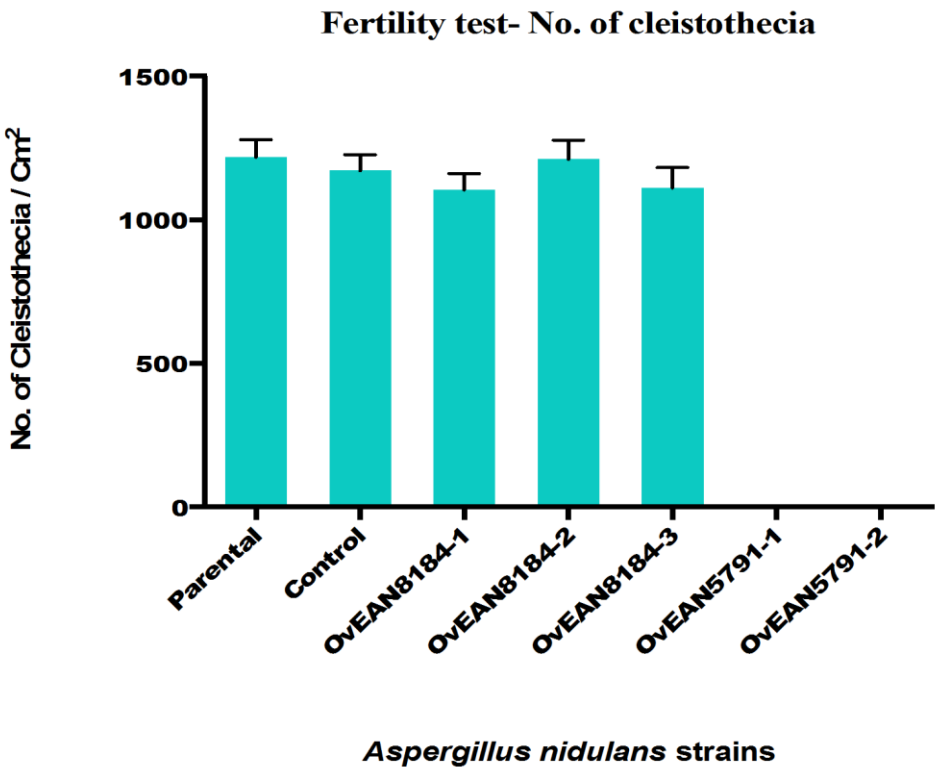


Figure 3-22 Fertility test showing the number of cleistothecia produced by parental, control and overexpressed strains of *A. nidulans*. The OvEAN8184-1 contains one overexpressed copy of the gene while both OvEAN8184-2 and OvEAN8184-3 had two copies of the AN8184 gene (one ectopic overexpressed, one resident), whereas both overexpression strains of AN5791 (OvEAN5791-1 and OvEAN5791-2) contained a single overexpressed copy of AN5791. Error bars represent SEM.

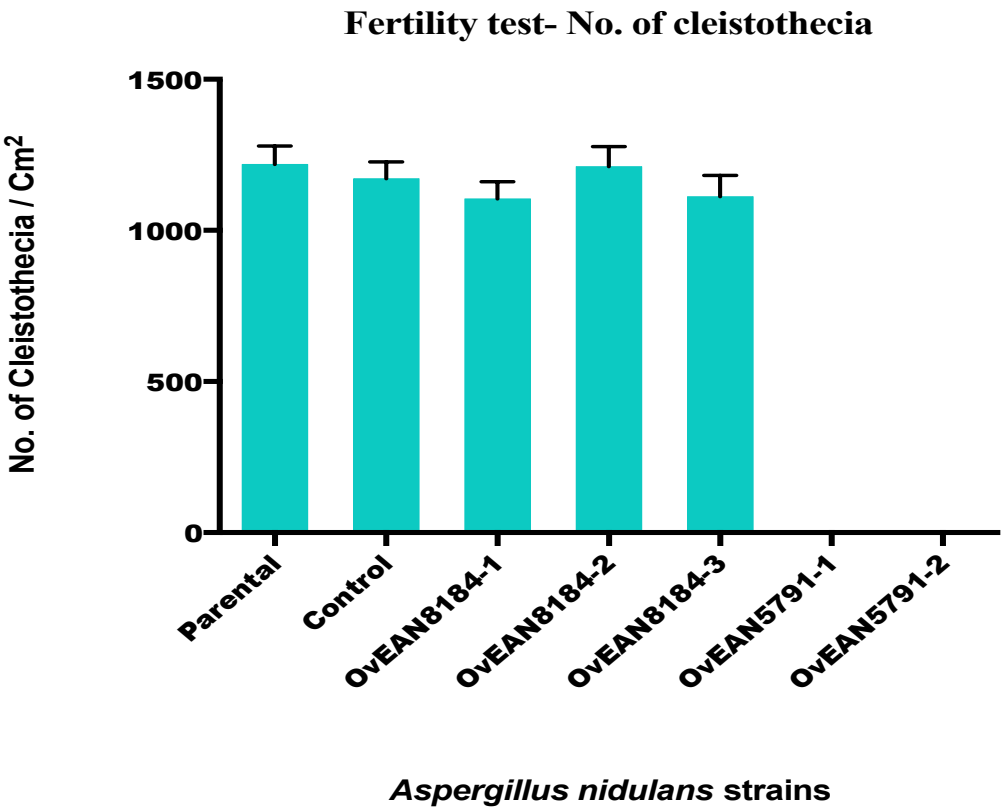


Figure 3-23 Fertility test showing the size (diameter) of cleistothecia produced by parental, control, three strains of OvEAN8184 and two strains of OvEAN5791 of *A. nidulans*. The OvEAN8184-1 contains one overexpressed copy of the gene while both OvEAN8184-2 and OvEAN8184-3 had two copies of the AN8184 gene (one ectopic overexpressed, one resident), whereas both overexpression strains of AN5791 (OvEAN5791-1 and OvEAN5791-2) contained a single overexpressed copy of AN5791. Error bars represent SEM.

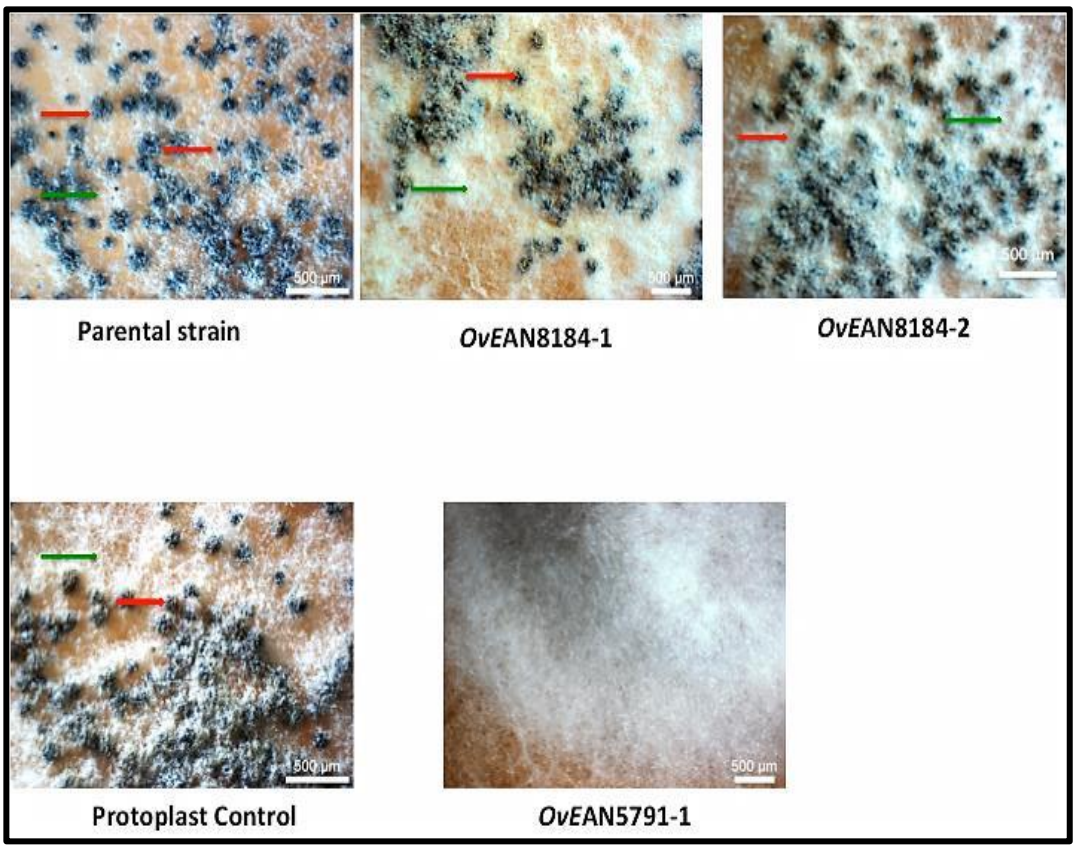


Figure 3-24. Images illustrating sexual fertility of various AN8184 and AN5791 overexpression strains as well as the protoplast and parental control. Red arrows indicate cleistothecia produced by the various strains, whilst green arrows indicate the Hülle cells surrounding the cleistothecia. Scale bars represent 500 µm in all cases.

3.5 Discussion

Fungal sexual reproduction is a complex processes requiring the involvement of a network of genes. Mating-type genes are considered as one of the master regulators of this process, regulating the expression of a variety of genes, which directly or indirectly participate in the development process. A key aim of the current work was to identify novel genes that might be involved in the control of sexual development in *Aspergillus* species.

3.5.1 Gene deletion studies

The work in the current chapter focused on 33 genes of *A. oryzae* which were reported by Wada *et al.* (2012) to be differentially expressed greater than 10-fold when the resident *MAT1-1* gene was substituted with a *MAT1-2* gene in *A. oryzae*. BLAST searches revealed that of these genes, only 20 homologous genes were present in *A. nidulans*. For the remaining 9 genes only low E-value matches were obtained, including unfortunately for the *A. oryzae* AO080508000390 gene that showed a very high fold change (55.7) in gene expression under the control of *MAT1-1* mating-type gene (**Table 3-2**). This result suggests that these latter genes are either highly divergent or that they may be absent in *A. nidulans* and therefore represent an extra suite of genes present *A. oryzae*. This might be due to the fact that the genome of *A. oryzae* is larger than *A. nidulans*, *A. fumigatus* and *A. flavus* and contains extra genes, which may relate to metabolic activity (Machida *et al.*, 2005; Khaldi, 2008) and/or some of these extra 9 genes might be required specifically for heterothallic mating and have been lost from the homothallic *A. nidulans*. Efforts were therefore made to study the possible role in sexual reproduction of the remaining 20 genes, which significantly included a number of genes listed as of 'unknown function'. The strategy used involved finding the homologous gene in the homothallic species *A. nidulans*, and then using this as a model system to determine whether gene deletion had any impact on sex in *A. nidulans* – thereby indicating whether or not the genes are indeed related to sexual reproduction. This strategy was facilitated by the availability of deletion cassettes

from an American project (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm). Unfortunately, deletion cassettes were not found for three of the *A. nidulans* homologous genes (AN0021, AN2806 and AN6418) and for one further gene the tube was empty, suggesting that these deletion cassettes had already been used up due to supply to other researchers (Kevin McClusky pers comm.). However, deletion cassettes for the remaining 16 genes were obtained and for three genes two homologous genes have been ordered (**Table 3-2**), so a total of 19 homologous genes were obtained and amplified. Again, unfortunately in three of them (AN2960, AN5039 and AN6458) secondary PCR product were produced. Several attempts were made to reduce the background PCR contamination e.g. by changing the annealing temperature and also using hot start PCR in an attempt to obtain a pure product, but sadly secondary products persist. Meanwhile, for the AN8890 cassette only a very faint band was obtained despite attempted amplification at a variety of annealing temperatures and using a range of cassette template concentrations of cassettes. Several attempts were made to perform transformations with gel-purified aliquots of these latter four cassettes, but no correct transformants were obtained – most likely due to the DNA concentration of the cassettes not being sufficient for successful transformation. Also transformation was not successful for two genes AN8890 and AN10820, which were homologues of the *A. oryzae* gene AO080523000564. Meanwhile, for the AN6257 cassette about 150 colonies were screened by PCR, unfortunately it was not possible to obtain a strain with the gene deleted, and it was later found that the homologous SEC31 gene in *S. cerevisiae* is an essential gene needed for viability. However, transformation was successfully achieved for the remaining 13 deletion cassettes, and for most of them (except for AN3275) two deletion strains were obtained for further characterisation studies.

3.5.2 Characterization of gene deletion mutants

The gene deletion strains were functionally characterized by testing them for fertility to determine the role of each gene in sexual reproduction. *A. nidulans* was used as a model organism because it is able to reproduce by both sexual and

asexual reproduction and due to being a homothallic species it does not require a compatible mating partner to undergo sexual reproduction. Although interestingly *A. nidulans* has retained the capability to outcross, this feature being used for restoration of deletion mutants as it allows reinsertion of deleted genes back into the genome.

The results of the sexual fertility tests are presented in **Figures 3-4, 3-5** and **3-6.** It was observed that four different groups of genes could be recognised depending on the effect of gene deletion on sexual fertility. Deletion of two of the genes (AN3562 and AN8656) resulted in the most extreme phenotype with failure to develop any cleistothecia and even Hülle cells were not produced. Thus, both genes seem to have a role in the early stages of sexual development. Hülle cells are thick-walled globose cells produced at the onset of sexual reproduction and are considered as nursing cells for developing cleistothecia (Hermann *et al*., 1983; Han, 2009). The AN3562 gene is located on chromosome II, consists of 1506 bp and encodes a 465 amino acid product annotated as of unknown function; while AN8656 is located on chromosome III, consists of 1610 bp and encodes a 431 amino acid product, also listed as of uncharacterized function but which is described as having a domain with predicted 2 iron, 2 sulfur cluster binding, with predicted oxidoreductase activity. The latter observation is of possible significance to the functioning of AN8656 given that the redox state of the cell is considered an essential factor during the early stages of sexual development (Dyer and O'Gorman, 2012). For example in *A. nidulans* deletion of *noxA*, which encodes an NADPH oxidase, resulted in a block in cleistothecial formation although Hülle cell formation was not effected (Lara-Ortíz *et al.*, 2003). Similarly, deletion of *trxA*, a component of the thioredoxin system, abolished both asexual and sexual sporulation; the adverse effect of the mutant phenotype could be rescued by addition of a small amount of reduced glutathione to the media (Thon *et al.*, 2007). These findings for AN3562 and AN8656 are similar to the effects of gene deletion of the *nsdD* gene, which encodes a putative GATA-type transcription factor, that results in a complete blockage of sexual reproduction under conditions favorable for sex (Han *et al.*, 2001). Similarly, *nsdC* acts as an activator of sexual reproduction and has a role in the early stages of sexual

development as confirmed by deletion of *nsdC*, which completely abolished sexual development including Hülle cell formation (Kim *et al.*, 2009b). In addition, deletion of the transcriptional regulator *stuA* also resulted in a complete loss of sexual reproduction including failure to produce Hülle cells (Wu and Miller, 1997).

Deletion of the alpha pheromone precursor gene AN5791 did not eliminate cleistothecial production, unlike AN3562 and AN8656. However, this resulted in a drastic decrease in the formation of cleistothecia and those cleistothecia, which were produced, were significantly larger than those of the parental strain. One other interesting consequence was an increase in the formation of Hülle cells, with massed aggregations of Hülle cells found scattered over growth plates (**Figure 3.5)**. A similar result was observed following mutation of the *ppg1* gene in the homothallic ascomycetes species *G. zeae,* where deletion of the alpha pheromone precursor gene led to a decrease in the perithecia production by 50 per cent and the formation of perithecia larger than those of the parental strain (Lee *et al.*, 2008). By contrast, in *S. macrospora* single deletion of each of the pheromone precursor genes (*ppg1* and *ppg2*) and their cognate receptor (*pre2* and *pre1*) had no any effect on vegetative and sexual reproduction whereas double deletion of both pheromone resulted in a drastic decrease in fruiting body formation (Mayrhofer *et al.*, 2006). It is speculated that the alpha pheromone precursor gene might be essential for sexual development in the genus *Aspergillus* as there might not be any a-factor pheromone present given that such extensive genome searching has failed to detect any such pheromone precursor gene in this genus (Dyer, 2003; Dyer and O'Gorman, 2012).

A third group of genes could be recognised where gene deletion only had a moderate effect on fertility – with some of these genes appearing to act as inducers and other as repressors of sexual development in terms of number of cleistothecia produced (although the size of cleistothecia was not affected). These genes represented a very interesting novel class of genes that may moderate sexual fertility rather than having a profound impact on development. Indeed, differences in the expression of such genes might partially explain differences

seen between the fertility of natural isolates of many fungal isolates. There have been many reports of variation in numbers of fruiting bodies produced in sexual crosses despite isolates being of compatible mating type, so mating-type identity alone does not fully explain sexual compatibility (Dyer *et al.* 1992; Swilaiman 2013). For example, deletion of five genes (AN1356, AN3275, AN4686, AN7818 and AN11253) led to decrease in sexual fertility as they produced significantly slightly fewer cleistothecia than the parental strain. Of these five genes:

(a) AN1356 is located on chromosome VIII and consists of a 1248 bp gene that encodes a putative 415 amino acid product of no known function. Interestingly, the ΔAN1356 mutants were sterile in that they were unable to produce ascospores i.e. there was developmental error in the last meiotic and ascosporogenesis steps of sexual development. This phenotype resembled that observed following deletion of the *MAT1* gene of *A. nidulans*, which also produced lower number of cleistothecia that were barren, without ascospore formation (Paoletti *et al.*, 2007). Similarly, deletion of both α*-tuB* (Kirk and Morris, 1991) and *grr*A (Krappmann *et al.*, 2006) resulted in a block in the formation of ascospores, deletion of both genes otherwise having no effect on vegetative growth, asexual reproduction, or the early stages of sexual development. Although fertility was decreased, deletion of the remaining four genes had no effect on ascospore development.

(b) AN3275 is located on chromosome VI and consists of a 2016 bp gene that encodes a putative 508 amino acid product annotated as a cytochrome P450 and homologue of *S. cerevisiae erg5* and *Schizosaccharomyces pombe ergll*. Both *erg5* and *erg11* are genes that have roles in the biosynthesis of ergosterol; impairment in the function of these genes blocked the formation of ergosterol. In *S. cerevisiae, erg5* catalyses the conversion of ergosta-5, 7, 24(28)-trienol to ergosta-5, 7, 22, 24(28)-tetraenol; mutation of erg5 led to accumulation of ergosta-5, 7, 24(28)-trienol (Dupont *et al.*, 2012). Impairment in the function of *erg5* resulted in a less severe phenotype than deletion of *erg6*, *erg2* and *erg3,* and did not require the addition of sterol to the media to maintain growth (Skaggs *et al.*, 1996). Jin *et al.* (2008) investigated the role of ergosterol in pheromone signalling and plasma membrane cell fusion in *S. cerevisiae*. Depletion of

ergosterol in this yeast was found to interfere with pheromone signalling, with mutation of erg6 leading to a decrease in polarized cell growth towards a pheromone signal (Jin *et al.*, 2008).

(c) AN4686 is located on chromosome III and consists of a 1014 bp gene that encodes a likely 327 amino acid product, annotated as a putative chitosanase with a predicted glycosyl phosphotidylelinositol (GPI)-anchor. Chitosanase is an enzyme that degrades chitosan, a component of the fungal cell wall. The main role of this enzyme may be degradation of chitosan molecules in the cell wall during spore germination or the enzyme might assist the hydrolysis of the hyphal wall during cell division (de Groot *et al.*, 2009). A correlation has been suggested between chitinase and sexual development as the cell wall is a dynamic structure continuously subjected to change during cell propagation and differentiation (Baker *et al.*, 2009). *In Cryptoccocus neoformans*, three endochitinases (*chi4*, *chi2* and *chi22*) and one exochitinase (*hex1*) gene have been identified. Triple deletion of all the endochitinase genes had no obvious effect on asexual and vegetative growth but resulted in impaired sexual reproduction (Baker *et al.*, 2009). The chitosanase enzyme of *A. nidulans* might have a similar role in cell wall hydrolysis and/or be involved with utilisation of chitin residues from the external environment for subsequent use as building blocks for the cell wall during fruit body development.

(d) AN7818 is located on chromosome IV and consists of a 1534 bp gene that encodes a likely 483 amino acid product, annotated as *stcF* with a putative sterigmatocystin biosynthesis P450 monooxygenase with a predicted role in sterigmatocystin and aflatoxin biosynthesis. Sterigmatocystin is a precursor of aflatoxin (Guzmán-de-Peńa *et al.*, 1998; Calvo and Cary, 2015). There is evidence of the coordinated regulation of sterigmatocystin biosynthesis and sporulation in *A. nidulans*, with conditions which induced sporulation also shown to increase sterigmatocystin production (Guzmán-de-Peńa *et al.*, 1998). Also the *veA* transcription factor affects both pathways (Kato *et al.*, 2003; Bayram and Braus, 2012). The gene *stcF* (AN7818) is a homologue of *avnA* in *A. parasiticus*, deletion of this gene resulting in loss of aflatoxin biosynthesis in the step of

converting averantin to averufin (Yu *et al.*, 1997). Trail et al. (1995) proposed that there is a possibility of interconnection between some aflatoxin biosynthetic genes and cellular differentiation and development, as mutation of the *A. parasiticus avnA* gene caused defect in both processes (Trail *et al.*, 1995). In the current work mutation of AN7818 resulted in a slight decrease in the number of cleistothecia, this finding is similar to mutation of a number of aflatoxin biosynthetic gene in *A. parasiticus* which resulted in the accumulation of intermediate precursors (averufin and versicolorin) of aflatoxin and decreased sclerotial formation (Skory *et al.*, 1992; Trail *et al.*, 1995).

(e) AN11253 is located on chromosome III and consists of a 1030 bp gene that encodes for a putative 323 amino acid product that has a predicted transferase activity, transferring phosphorus-containing group. Phosphorus limitation has a suppression effect on sexual development likely due to its involvement in ATP formation (Dyer and O'Gorman, 2012).

In contrast to the above genes, deletion of three remaining genes (AN1598, AN6881 and AN8184) led to a significant increase in the formation of cleistothecia (although the size of cleistothecia was not affected), indicating that these genes act as repressors for sexual fertility. Both AN1598 and AN8184 are putative cytochrome P450s, located on chromosomes VII and II (sizes 1706 bp and 1740 bp) and encode for 534 and 461 amino acid products, respectively. By contrast, AN6881 had no known function, is located on chromosome I (size 639 bp) and encodes for a 190 amino acid product. These findings are similar to the effect of deletion of the *flbC* gene in *A. nidulans*, which acts as a positive regulator for asexual reproduction, mutation of this gene was found to lead to an increase in cleistothecial formation (Kwon *et al.*, 2010a).

Finally, a group of genes was observed where gene deletion had no effect on sexual fertility. Although both *A. oryzae* homologues of AN3239 and AN5993 (AO080503000328 and AO080523000595, respectively) were up-regulated more than 14 fold in *A. oryzae* as a result of *MAT* gene switching (**Table 3.1**), deletion of these two genes in the *A. nidulans* had no any obvious effect on vegetative or

sexual development. AN3239 is located on the chromosome VI and consists of a 1246 bp gene that encodes for a putative 364 amino acid product with predicted acyl-CoA dehydrogenase activity. AN5993 is located on chromosome I and consists of a 351 bp gene that encodes a putative 116 amino acid product with predicted calcium binding activity. The function of these two genes may have inbuilt redundancies in *A. nidulans* or these genes might have a specific role in mating and outcrossing, so might not have any effect on selfing in *A. nidulans.* Another possible reason is that these genes may not be involved in sexual development, as mating-type genes are now known to have other roles than simply the regulation of sexual development as seen in *Penicillium chrysogenum* where *MAT* genes were shown to regulate genes involved in the production of the antibiotic penicillin (Böhm *et al*., 2013). In addition, a comparative study of *Fusarium verticillioides* gene expression between wild-type and Δ*MAT1-2* strain, revealed that 248 genes were down and up regulated in the Δ*MAT1-2* strain compared to wild-type strain; most of up regulted genes have roles in metabolism and protein synthesis (Keszthelyi *et al.*, 2007). Also in *S. cerevisiae* mating-type genes have a role in maintaining cell wall integrity (Verna and Ballester, 1999). The lack of effect of gene deletion of AN3239 was particularly surprising given that expression of the *A. oryzae* homologue AO080503000328 was increased by 55-fold in the *MAT1-1* background, the highest fold change of any gene (**Table 3.1**). By contrast expression of the AN5993 homologue AO080523000595 was increased by 14.6 fold in the *A. oryzae MAT1-2* background.

The deletion strains that exhibited significant phenotypic changes in sexual fertility as a result of target gene deletion were crossed with *A. nidulans* strain 2-259 for restoration of the target gene in the original genetic background. In all but one case the original phenotype was restored with a similar number and size of cleistothecia produced as seen in the parental strain (**Figures. 3-7, 3-8** and **3-9**), confirming that the phenotypes were due to gene deletion rather than any artefact. Unfortunately complementation was not successful for the deletion strain of AN3562 by applying this method. This strain was crossed several times but failed to produce hybrid cleistothecia, only small size cleistothecia were produced that originated from selfing of the other strain rather than from heterokaryotic

mycelium. This gene may be having a role in the early stage of sexual reproduction such as fusion of hyphae for formation of heterokaryotic mycelium.

3.5.3 Effect of Gene Deletion on sex gene expression

Deletion of four of the *A. nidulans* genes (AN3562, AN5791, AN8184 and AN8656) resulted in particularly interesting phenotypes. qRT-PCR was therefore used to analyse the expression of some key sex genes in these mutants. These comprised the mating-type (*MAT1* and *MAT2*), pheromone precursor (*ppgA*), both pheromone receptors (*preA* and *preB*), and finally *steA* genes. The expression of these genes was normalized relative to the housekeeping *H2B* gene, which encodes a histone protein used by a number of researchers as a housekeeping gene in *A. nidulans* (Futagami *et al.*, 2014; Grunbacher *et al.*, 2014). The expression of *MAT1*, *MAT2* and *ppgA* were decreased drastically in both of the totally sterile mutant strains ΔAN3562-1 and ΔAN8656-1 compared to the parental strain (**Figure 3.12**). These results indicated that ΔAN3562 and ΔAN8656 might have a role in signalling processes or sensing of environmental conditions before activation of the mating-type and pheromone pathway genes in sexual development. Deletion of the alpha pheromone precursor gene AN5791 also affected the transcript level of both mating-type genes, with a decrease in expression of both, indicating that pheromone signalling affects the expression of mating-type genes as has been reported elsewhere (Debuchy *et al.*, 2010). Surprisingly, the expression of both pheromone receptors (*preA* and *preB*) and also *steA* were highly increased in the α-pheromone deletion strain compared to the parental strain. The putative α-pheromone precursor gene is the only pheromone gene to have been found in the *Aspergillus* species, a precursor gene for an a-factor like pheromone not having yet been identified yet (Dyer *et al.*, 2003). Indeed it is not certain if an a-factor precursor exist within the genus. However, one may be present but it has not yet been detected due to BLAST searching difficulties with short DNA sequences, or it has unusual structure as seen with the a-factor pheromone of *Trichoderma reesei*, which has characteristics of both α- and a-factor pheromones (Schmoll *et al.*, 2010). One

possible explanation for the increase in the transcript levels of both pheromone precursors in the ΔAN5791-1 strains might be the existence of an a-factor pheromone that send signals to *preA*, or it might be that other factors such as environmental signals promoting sexual reproduction are responsible, both pheromones might have other functions in addition to sexual pheromone signalling, and/or both receptors are under the control of the same regulatory pathway. The increase in the transcript level of both pheromone receptors may have resulted in increased transcription levels of the *steA* gene, one downstream target of pheromone signalling. Both *preB* and *steA* were also highly expressed in the ΔAN8656-1 strain. Given that AN8656 has oxideoreductase activity, deletion of this gene might have resulted in a change in the redox state of the cell. Thus, cell stress signalling is likely to have been initiated to reprogramme the cell to overcome the stress (Moye-Rowley, 2003), which might have caused altered expression of the sex genes.

3.5.4 Effect of gene overexpression

Three genes (AN3562, AN5791 and AN8184) were selected from *A. nidulans* to study the effect of their overexpression given that deletion of these genes resulted in particularly interesting phenotypes. To examine the overexpression effect, plasmids were constructed to overexpress the genes under the strong constitutive *gpdA* (glyceraldehde-3 phosphate- dehydrogenase) promoter of *A. nidulans.* The *pyrG* marker gene from *Aspergillus fumigatus* was also assembled within the plasmid, which was used for selection of *A. nidulans* strain after transformation of cloning vector of these genes. The cloning vector was transformed into *A. nidulans* strain (*PyrG-*, *PyroA4-*, green spored colour and *paba+*, *veA+*).

Transformant colonies with the overexpressed genes for both AN5791 and AN8184 were tested for fertility (Figures 3.22, 3.23 and 3.24). Unexpected results were obtained for both of them. There was no impact of AN8184 overexpression on fertility (similar numbers of cleistothecia were produced as the parental strain) despite an increase in AN8184 gene expression for both tested strains (154 and

274.4 fold increase compared to parental strain). And surprisingly the overexpression AN5791 (the alpha pheromone precursor gene) strains produced an even more severe phenotype than the ΔAN5791 deletion strain; both OvEAN5791 strains failed to produce any fruiting bodies and also showed a reduction in vegetative growth under conditions favourable for sexual reproduction. This result was rather disappointing as it had been hoped that overexpression of OvEAN5791 would over do sex and get more fertility! Results indicate that a very limited range of the expression of AN5791 is required for correct sexual development.

Meanwhile it was necessary to perform co-transformations (with separate cloning vectors and *pyrG* gene) with AN3562 because several attempts to produce a single vector failed to assemble both the *pyrG* and 3'flank of the gene into the same vector. Unfortunately for AN3562 no colonies were obtained containing the cloned gene despite screening about 40 transformant colonies by PCR and lack of time precluded further screening attempts.

3.6 Summary and conclusions

1. Deletion of some *MAT*- regulated genes (e.g. AN3239 and AN5993) had no obvious phenotypic effect on sexual and asexual reproduction in *A. nidulans*.

2. However, deletion of two genes (AN3562 and AN8656) resulted in a complete loss of fertility without any apparent effect on vegetative growth and asexual reproduction. Both of these two genes appear to have a role in early stages of sexual reproduction.

3. Deletion of the *ppgA* α-pheromone precursor gene (AN5791) drastically decreased the number of cleistothecia formed. The mutant strain produced a few large cleistothecia with increased Hülle cell formation compared to controls, indicating that pheromone signalling is needed for normal mating and sexual development even in the homothallic *A. nidulans*.

4. Some of the *MAT*- regulated genes whilst not being essential for sex still have a significant effect on sexual fertility, acting either as repressors or inducers with their deletion resulting in a slight increase or decrease in sex. These represent a very interesting novel class of genes that may moderate sexual fertility and differences in their expression might partially explain differences seen between the fertility of natural isolates.

Chapter 4 Role of Genes encoding High-Mobility Group in Sexual Development of *Aspergillus nidulans*

4.1 Introduction

The high-mobility group (HMG) set of proteins is a large and diverse superfamily of proteins (Stros *et al.*, 2007). The name 'high-mobility group' is derived from original observations of the electrophoretic mobility behavior of these proteins after their extraction as a component of chromatin DNA as they migrated rapidly (i.e. exhibiting high mobility) within gels (Bustin, 2010; Malarkey and Churchill, 2012). The HMG group of proteins are transcriptional regulators and have been shown to have roles in a variety of cellular functions such as DNA repair, programmed cell death, DNA replication and genetic recombination (Bianchi and Agresti 2005). Based on the type of HMG box domain present, the HMG proteins are divided into three families: the HMG-AT-hook family (HMGA), the HMG-box family (HMGB), and the HMG-nucleosome binding family (HMGN) (Bustin, 2001; Agresti and Bianchi, 2003; Bianchi and Agresti, 2005; Stros *et al.*, 2007; Malarkey and Churchill, 2012).

HMGB proteins contain an HMG-box consisting of three α-helices, folded in an L-shaped structure (Stros *et al.*, 2007; Ueda and Yoshida, 2010). These proteins are characterised by their interaction with DNA structure instead of DNA sequence (Bianchi and Agresti 2005); they bind to the minor groove of DNA and this results in DNA bending (Sessa and Bianchi, 2007; Stros *et al.*, 2007). Members of the HMGB family contain an HMG-box consisting of a conserved motif of approximately 80 amino acids (Reeves, 2015).

HMGB proteins are divided into two main subfamilies: the MATA/TCF/SOX and the HMG/UBF families (Laudet *et al.*, 1993; Soullier, 1998). Members of the

MATA/TCF/SOX family are considered to be sequence-specific DNA binding proteins; most of them contain one HMG-box domain and have no acidic tail. On the other hand, the HMG/UBF family is characterized by the presence of at least two HMG-box domains with acidic tails; these proteins bind to DNA sequence non-specifically (Soullier, 1998; Stros *et al.*, 2007). The MATA/TCF/SOX subfamily is further sub-divided into two subgroups: the MATA-HMG subgroup, which is found in fungal species, and the TCF/SOX subgroup which is specific to animal species (Soullier, 1998).

Members of the SOX family play an essential role in the determination of gender in mammals. For example, the SRY protein, encoded by a single *sry* gene located on the Y chromosome, is responsible for male characteristics in mammals. This protein contains a single HMG-box, which plays a crucial role in the function of this protein (Stros *et al.*, 2007). Despite the early divergence of animals with fungi and their different modes of reproduction, there is evidence that both share common features regarding sex-determining systems (Fraser and Heitman, 2005). For example, the mating-type region of *Cryptococcus neoformans* occupies a large region >100 kb and contains more than 20 genes, some of them involved in differentiation and pathogenicity, unlike most other members of the Basidiomycota that have tetrapolar mating systems. Thus, this *mat* region in *C. neoformans* in some ways resembles the larger sex chromosomes of animals. Sexual identity in *C. neoformans* is determined by a particular gene that is only found in the *mat* locus of the α- mating type, which encodes for a homeodomain protein called Sxi1α factor, responsible for the determination of a or α cell type (Fraser and Heitman, 2003). Similarly, the *mat* locus of *Ustilago hordei* is unusual for basidiomycetes; here the tetrapolar *mat* loci are fused to form a large bipolar mating system which span a region >500 kb and this expansion of the sex determining region is unusual amongst fungi and again resembles that of the mammalian cell sex chromosomes (Fraser and Heitman, 2005).

In ascomycete fungi sexual identity is determined by genes located in a specific region of DNA termed the mating-type locus rather than there being sex chromosomes present such as seen in animals (Harley, 2003; Fraser and Heitman,

2004; Kashimada and Koopman, 2010). As mentioned in chapter one, in almost all filamentous ascomycete fungi two classes of mating-type genes have been found, *MAT1-1* and *MAT1-2*, which determine sexual identity (Arnaise *et al.*, 1993; Turgeon and Yoder, 2000; Scazzocchio, 2006; Dyer, 2007; Dyer and O'Gorman, 2012). *MAT1-2* belongs to the MATA-HMG subgroup (**Figure 4-1**) containing a single HMG- box, and has a prominent role in the sexual development of fungi, with deletion of this gene resulting in sterility (Paoletti *et al.*, 2007; Debuchy *et al.*, 2010; Lee *et al.*, 2010a). *MAT1-1* belongs to MATα_HMG subgroup (**Figure 4-1**), containing a single HMG- box (Martin *et al.*, 2010). There are significant similarities apparent between the core consensus sequence of α- domain and HMG domain; the core amino acid sequence is longer by two and four amino acids compared to Pleosporales and Pezizomycotina respectively (Martin *et al*.., 2010). *MAT1-1* also has a prominent role in the sexual development of fungi, deletion of this gene also resulting in sterility (Paoletti *et al.*, 2007; Debuchy *et al*.., 2010). *Saccharomycese cerevisiae* is an exception to this rule, the *MATa* mating type having lost the MATA-HMG gene during evolution and it now only contains the Matα1p which is evolutionary related and consider as a member of MATA-HMG gene (Martin *et al.*, 2010).

As well as *MAT1-1* and *MAT1-2*, ongoing bioinformatic investigations have revealed that most fungi also contain other types of HMG-type genes elsewhere in the genome. And where experimental work has been conducted this has suggested that such HMG genes found outside the mating region may also have regulatory roles involved in sexual development (Lee *et al.*, 2010a). For example, the Zygomycota, an early divergent branch of fungal species, also contain HMG genes. A blast search study of *Phycomyces blakesleeanus* revealed the presence of 10 HMG genes within the genome with only one of these HMG genes being responsible for sex identity that was present in (–) strains but not in (+) strains (Idnurm *et al.*, 2008). In *Podospora anserina* twelve HMG genes were identified, eleven of them were found to be essential for sexual reproduction following gene deletion experimental work. Further bioinformatics analyses identified similar numbers of HMG genes in a range of other filamentous ascomcycete species (**Figure 4.1**) (Benkhali *et al.*, 2013). Similarly, in *Schizosaccharomyces pombe*

the transcriptional regulator *ste11* belongs to the HMG-box superfamily and has essential roles in regulating mating-type genes and activation of sexual reproduction as a result of nitrogen depletion (Sugimoto *et al.*, 1991). Meanwhile, *S. cerevisiase* contains the ROX1 protein that also belongs to the HMG super family but which has a different role not involved with sexual reproduction, being a repressor of hypoxia (Deckert *et al.*, 1995). In *Aspergillus nidulans* preliminary bioinformatic studies identified eight putative HMG genes in the genome, and some of these were characterized [AN4734 (*MAT2*), AN2755 (*MAT1*) and AN3667) and were shown to have an essential role in sexual reproduction, (Paoletti *et al*., 2007; Ashour 2014). However, only AN3667 was studied in the Ve^{+} background.

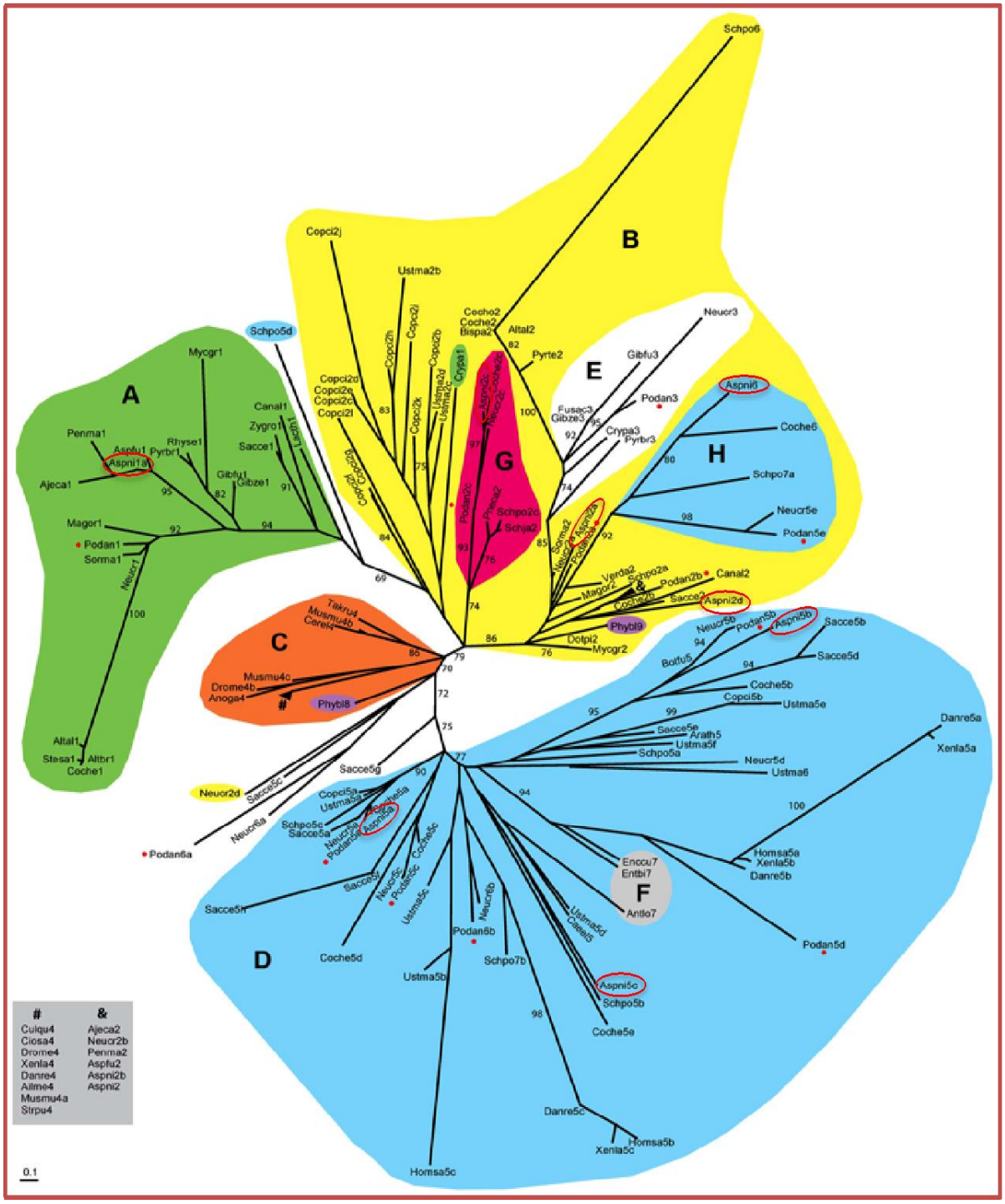


Figure 4-1 Phylogram of the HMG-box superfamily. The 154 amino acid HMG-box domains of some plant, animal and fungi were clustered into groups depending on core amino acid sequences. The HMG genes of *Aspergillus nidulans* are enclosed in red oval shape. Aspni1a (*MAT1*) belongs to MATα_HMG (clade A), both of Aspni2a (*MAT2*) and Aspni2d (AN3549) belong

to clade B (MATA_HMG), clade D (HMGB-UBF_HMG) contains Aspni5a (AN2885), Aspni5c (AN1267) and Aspni5b (AN10103), and each of Aspni2c (AN3667) and Aspni6 (AN3580) belong to clade G (MATA_HMG) and clade H (HMGB-UBF_HMG), respectively. Adapted from Benkhali *et al.* (2013).

4.2 Aims of the work

Work in the current chapter focuses on a group of genes encoding members of the large superfamily of High Mobility Group (HMG) domain transcription factor proteins. It was hypothesised that in addition to the previously characterised *MAT1* and *MAT2* HMG genes, other HMG genes present in the genome might also have roles in regulating sexual development in *A. nidulans.* Therefore work aimed to investigate the role of high mobility group (HMG) domain transcriptional factors in sexual reproduction of *A. nidulans* by: (a) identifying these genes using conserved amino acid sequences characteristic of HMG-box domains to BLAST search the *A. nidulans* genome, then (b) deleting the genes to functionally characterise their role in the sexual development of *A. nidulans.* In particular all work was conducted in the Ve^{+} genetic background, some previous analyses having been performed in the VeA1 background which might confound analyses (only AN3667 was previously deleted in a Ve^{+} strain) (Paoletti et al. 2007). Also the effect of each gene deletion on the expression of other putative HMG genes was determined by quantification of the transcript level of each of them in HMG gene deletion strains background compared to the parental strain by qRT-PCR. It was hoped that insights gained would provide a better understanding of sexual development as a whole, and that any genes identified might be used as tools to promote sexual development where required in asexual species.

4.3 Materials and methods

General materials and media used in this chapter are as previously described (**Sections 2.1.1 and 2.1.2**).

4.3.1 Identification of putative HMG genes

Putative HMG genes of *A. nidulans* were identified by using the conserved sequence of amino acids of the core HMG-boxes of both the *A. nidulans* MAT2 protein (KIPRPPNAFILYRQHHYPKVKEARPDLSNNEISVIIGKKWRAEPEE GKLHFKNLAEEFKKKHAEEXPDYQYTPRKPSEKKRR) and the *Podospora anserina* FMR1 protein (PRPPNAYILYRKDQQAALKAANPGIPNNDSVMTG GMWKKESPEVRAEYQRRASEIKAKLMSAHPHYRYVPRRSSEIRRR), using blastp searches against the *Aspergillus nidulans* FGSC A4 protein database (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html).

Psort was used to identify the putative subcellular localisation of all putative HMG genes (http://psort.hgc.jp/form2.html) (Nakai and Horton, 1999). The specific family of each HMG gene was determined via predicted structure analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

4.3.2 HMG gene deletion cassettes

Attempts were made to obtain deletion cassettes for the putative HMG genes of *A. nidulans* from the Fungal Genetic Stock center (USA). Deletion cassettes and primer sets for amplification were available for four of the putative HMG genes (AN0879, AN2885, AN3580 and AN5073). However, deletion cassettes for the remaining six putative HMG genes were no longer available. DNA template of four of these genes (AN1267, AN1962, AN3549 and AN10103) were obtained as a gift from Carol Ringelberg (Dartmouth College, USA) and primer sets for their amplification were synthesised according to the Fungal Genetic Stock center *A. nidulans* knockout cassettes datasheet (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm). Deletion cassettes for the remaining two genes (AN2755 and AN4734; the previously characterised mating-type genes) were constructed using fusion PCR (see below). All of the resulting deletion cassettes contained a *pyrG* selective marker gene from *A. fumigatus* flanked by approximately 1 kb of gene specific upstream and downstream nucleotides that enhanced homologous transformation (**Section 2.2.6**).

4.3.2.1 Amplification of HMG gene deletion cassettes

The first four deletion cassettes (AN0879, AN2885, AN3580 and AN5073) were amplified as described in **Section 2.2.7** using the primer mixture provided with the cassettes and an annealing temperature of 63 ^{o}C.

The second sets of deletion cassettes (AN1267, AN1962, AN3549 and AN10103) were amplified using primer sets listed in **Table 4-1**. 50 µl PCR volumes contained 2.5µl of each primer, 1 µl of deoxynucleotide, 10 µl of 5x phusion buffer, 32.5 µl of ultra-purified water, 0.5 µl phusion high fidelity DNA polymerase and 1 µl of deletion cassette template. The following PCR cycling parameters were used for amplification: initial denaturation 94 ^{o}C for 1 min, 35 cycles 94 ^{o}C for 30 sec, annealing temperature 63 ^{o}C for 30 sec, 72 ^{o}C for 5 min, maximum ramp rate, final extension 72 ^{o}C for 10 min and then hold at 4 ^{o}C.

PCR products were cleaned up using a NucleoSpin® Gel and PCR Clean-up kit according to manufacture protocol. If a contaminating secondary product was formed, bands of the desired size were gel extracted then purified using the NucleoSpin® kit. After clean up the products were precipitated in 0.1% of sodium acetate pH 5.8, washed in a 2-fold excess of 100% ethanol and then resuspended in buffer to get higher concentrations of DNA for use in transformations.

Table 4-1 List of primer sets used for amplification of HMG deletion cassettes

Gene ID	Forward primer	Reverse primer
AN 1267	GTAACGCCAGGGTTTTCCCAGTCACG ACGGCTCCGGTAATCTCTATCAC	GCGGATAACAATTTCACACAGGAAACA GCTTAGGTAGCTCTTCCCTCAC
AN 1962	GTAACGCCAGGGTTTTCCCAGTCACG ACGTACACGCTTCAGAGTCAGAG	GCGGATAACAATTTCACACAGGAAACA GCCCTGGGACTCATACACCTAT
AN 3549	GTAACGCCAGGGTTTTCCCAGTCACG ACGCATATCTCCTCCACATCAGG	GCGGATAACAATTTCACACAGGAAACA GCCTATCCTCGACTCAAGCTCT
AN 10103	GTAACGCCAGGGTTTTCCCAGTCACG ACGGACTGTATAGAGCTTGCCG	GCGGATAACAATTTCACACAGGAAACA GCTCTAGTAGCACAACGTACCG

4.3.2.2 Construction of deletion cassettes

Deletion cassettes for both mating-type genes [AN2755 (*MAT1*) and AN4734 (*MAT2*)] were constructed by fusion PCR (Yu *et al.*, 2004; Szewczyk *et al.*, 2007). To construct the deletion cassette, approximately 1 kb from both flanking regions of *MAT1* and *MAT2* gene were used and fused to either side of the *pyrG* gene from *Aspergillus fumigatus*, which was used as a selective marker for *A. nidulans* strains after transformation. Gene deletion cassettes were assembled in two steps. In the first step the upstream and downstream fragments of each gene and the *pyrG* marker gene were amplified and purified separately. The reverse primer used for synthesising the upstream flanking region had some extra-nucleotides complementary to the 5' region of the *pyrG* gene, whilst the forward primer used for amplification of the downstream flanking region had some extra-nucleotides complementary to the 3' region of the *pyrG* gene.

The *pyrG* gene was amplified using a forward primer (GATTTCAGTAACGTTAAGTGGAT) and reverse primer (PR) (GACAGAAGATGATATTGAAGGAGC) with a predicted product size of 1732 bp (Schoch *et al.*, 2009), using a deletion cassette from Fungal Genetic Stock centre as a DNA template, which contained the *pyrG* gene of *A. fumigatus*.

Upstream flanking regions of *MAT1* were amplified using a forward (GCATCTGAGTGATCCTACAC) and reverse primer (ATCCACTTAACGTTA-CTGAAATCACGCTGAAGAGGTGAGAGT) that had extra complementary nucleotides for the 5' region of the *pyrG* gene, with a predicted PCR product of 701 bp. For downstream amplification, a forward primer with overlapping nucleotides with the 3' sequence of the *pyrG* gene (CTCCTTCAATATCA-TCTTCTGTCATCTAGGTCTCGGTTGGTTC) and reverse primer (ACAGAAGAAGACCTGTAGCC) were used, with an expected product size of 819 bp; genomic DNA of *A. nidulans* 2-259 was used as a template.

The same strategy was applied to amplify the upstream and downstream of flanks of the AN4734 (*MAT2*) gene. For the upstream amplification a forward (GAAGGAGCATA-GTCTTGAGC) and reverse primer (ATCCACTTAAC-GTTACTGAAATCTAGCAATCGATACAGCAGCC) were used with predicted product size (811 bp); for the downstream amplification a forward (CTCCTTCAATATCATCTTCTGTCGCAGCCGATTACTTCTGAG) and reverse primer (CTCTCTCCCTATATTCGC) were used with a predicted product size 954 bp; genomic DNA of *A. nidulans* 2-259 again served as the template.

All individual fragments were amplified using a Phusion High Fidelity kit according to manufacturer's instruction with an annealing temperature of 63 ^{0}C. Fusion (overlapping) of all three fragments to obtain a final product was then performed in one step. An equal amount of 100 ng of the three fragments was used in PCR. For 50µl PCR reaction: 1 µl of each fragment was mixed with 10 µl of 5X phusion buffer, 1 µl deoxynucleotide, 0.5 µl Phusion High Fidelity polymerase and 2.5 µl of each primer (final concentration of 400 nM), which was then made up to 50 µl with ultra purified water. The primer pairs used for amplification of the *MAT1* deletion cassette were general forward (GCATCTGAGTGATCCTACAC) and reverse primer (ACAGAAGAAGA-CCTGTAGCC) yielding a predicted product of 3451 bp; also nested primers were designed within the predicted deletion cassette and used for amplification which were nested forward (TCTGGTTGTCTTCGGGATG) and nested reverse primer (GTAGCCCCAGCAATCAATG) predicted size 3396 bp. Similarly for

amplification of the *MAT2* deletion cassette a general forward (GAAGGAGCATAGTCTTGAGC) and general reverse primer (CTCTCTCCCT-ATATTCGC) were used with predicted product size of 3204 bp; then a nested forward primer (AGGAGCATAGTCTTGAGCC) and nested reverse primer (GCATACAGTCGTCCCATAC) predicted size 3146 bp. The same PCR cycling parameters were used as recommended by the Oakley lab for fusion PCR, involving a gradual increase in the annealing temperature to 57 ^{0}C (**Appendix 8**). The final product was analysed on a 1% agarose gel and gel purified using a Nucleospin ® gel and PCR clean up kit according to manufacturer's instructions before being used in transformation work.

4.3.3 Transformation and screening of transformants by PCR

The purified HMG gene deletion cassettes were transformed into *A. nidulans* strain 2-258 by PEG mediated transformation as described earlier (**Sections 2.2-8 and 2.2.9**). Putative HMG transformant colonies were selected and screened by PCR (using primers listed in **Appendix 9**) to confirm the deletion of the target HMG gene in transformant strains. Also the correct integration of the *pyrG* gene was verified by positional PCR (using primers listed in **Appendix 10**) as described previously (**Section 2.2.10**).

4.3.4 Sexual fertility test for HMG deletion strain

All HMG gene deletion strains were tested for sexual fertility to determine the effect of gene deletion on sexual development compared to that of the parental and transformation control strains. Sexual fertility tests were performed as described previously (**Section 2.2.11**).

4.3.5 Complementation test

Any HMG deletion strains (all with green spored conidia) which exhibited a significant change in sexual fertility compared to the parental strain were crossed

with *A. nidulans* strain 2-259 [*paba A1-*, *yA2* (yellow spore), *veA+*, *pyro+*] to restore back the deleted HMG gene and to ensure that the observed phenotype was due to target gene deletion rather than any other factor. Gene restoration was achieved as previously described (**Section 2.2.12**); strains with the restored target gene were tested for sexual fertility (**Section 2.2.11**) and checked for the restoration of the parental wild-type phenotype.

4.3.6 Overexpression of *A. nidulans* putative HMG genes

Cloning vectors were built up for both putative HMG genes AN1962 and AN3667 as described in **Section 2.2.16**. Three separate cloning steps were performed per gene to construct the final vector using either the NEBuilder Gibson or NEBuilder HiFi cloning kits according to manufacturer's instruction. The same pAN52.1 plasmid containing the *A. nidulans gpdA* promoter was used as described previously (**Section 2.2.16**).

Specifically, the vector for AN1962 was constructed by inserting both the *pyrG* gene and the 3' flank of AN1962 at the site after the terminator in pAN52.1 by opening the plasmid using *Hin*dIII endonuclease. The primers used for amplification of each fragment used in the construction of the vector were designed to have overlapping nucleotide sequences for the site of insertion. For example, regarding the *pyrG* primers, the forward primer (**acctgcaggcatgca**GATTTCAGTAACGTTAAGTGG**)** had overlapping nucleotides (in red) with the terminator region of the pAN52.1 plasmid and the reverse primer (**acccttttt**GACAGAAGATGATATTGAAGG) had complementary nucleotides (in green) for the starting nucleotides of the 3' flanking region of AN1962. In contrast, regarding the primers used for amplification of the 3' flanking region of AN1962, the forward primer (AN1962-3FF) (**cttctgtc**AAAAAGGGTGTTTTGATAAATTTTTC) contained nucleotide sequence overlapping with the end of the *pyrG* gene whilst the reverse primer (AN1962-3FR) (**cgacggccagtgcca**AAAGGCCGTCAGAATGATC) contained complementary nucleotides to the other side of the pAN52.1 plasmid following digestion with *Hin*dIII. The two fragments were assembled into pAN52.1 plasmid

using the NEBuilder Gibson or NEBuilder HiFi cloning kits; then 2 µl of the assembled mixture was used for transformation to *E. coli* by the heat shock method (**Section 2.2.16)**. The correct integration of both fragments was confirmed by extraction of plasmid DNA from putative transformants and digestion with *Sac*I endonuclease enzyme.

The vector constructed in the first step of cloning was then opened by digestion with the *Nco*I endonuclease and used for construction of the second cloning vector. In this stage the AN1962 gene was added to the vector after the *gpdA* promoter. The primers used for amplification of AN1962 were designed to contain overlapping nucleotides for both sides of the opened plasmid; the forward primer (AN1962F) (**cttgagcagacatcac**ATGTCTTACGATCGAGTTC) had overlap with the *gpdA* promoter whilst the reverse primer (AN1962R) (**gtaacgttaagtggatc**AGATTGTGTAATGTAATCTTCAATTTC) had overlap with the other sides of the first step vector. After assembly and transformation into *E. coli*, plasmid was extracted from representative transformants and was digested with *Msc*I to check for insertion of AN1962 at the correct site.

Finally, to insert the 5' -flanking region of AN1962, the vector from the second cloning step was opened with *Eco*RI at the site before *gpdA* promoter. The primers used for amplification of the 5' flank of AN8184 were designed to contain extra nucleotides for the linear vector; thus the forward primer (AN8184_5FF) (**ctatgaccatgattacg**AGCCGGGCCCCAGTCCGG) had overlap with the starting nucleotides of the *gpdA* promoter whilst the reverse primer (AN8184_5FR) (**gtgtagagatacaaggg**CTATTTAATGCGAAATTCAGTGAGTA-GAGAGGCGAAGGTGAGC) had overlap with the other side of the vector. The final vector was screened by digestion with *Stu*I to confirm the correct insertion of the 5' flank of AN1962.

Meanwhile, the overexpression vector for AN3667 was constructed in a slightly different order. In the first step the 5' flank of AN3667 was added, then AN3667 itself was added in the second stage, and in the last step it was attempted to add both *pyrG* and the 3' flank of the gene. This was attempted several times.

However, it was only possible to insert the *pyrG* gene. The primers used for this cloning are presented in **Appendix 4**.

Final overexpression vectors were transformed into *A. nidulans* 2-258 (pyrG89-, pyro A4-, *nkuA*□*::*argB, veA^{+}) by PEG-mediated transformation (**Sections 2.2.8** and **2.2.9**), using *pyrG* as a selective marker for transformants. After transformation, some colonies were selected and screened by PCR to confirm the integration of the vector within the genome of *A. nidulans,* For such screening a forward primer was designed in the 5' flank of the gene and a reverse primer was designed within the gene. For example to confirm the integration of the AN1962 overexpression vector within the genome both AN1962_5FF and AN1962R were used (**Appendix 5**). Transformant strains with the correct integration were selected for further study. Firstly, expression levels of the cloned gene were quantified by qRT-PCR (**Section 2.2.15**) in all *A. nidulans* transformant strains (containing the desired cloning vector) and in the parental strain. RNA was extracted from cultures grown under conditions favourable for sexual reproduction (**Section 2.2.13**) using TRIzol (**Section 2.2.13**). Secondly, *A. nidulans* transformant and parental strains were tested for sexual fertility as described previously (**Section 2.2.11**) by comparison of the number and diameter of cleistothecia.

4.3.7 Determination of transcript levels of HMG genes and other key sex genes in the HMG deletion strains

qRT-PCR was used to quantify the expression level of all the HMG genes and some other key genes with important roles in sexual development (*ppgA*, *preA*, *preB*, and *steA*) in each of the HMG gene deletion strains compared to expression levels in the *A. nidulans* parental strain 2-258, using primer pairs listed in **Appendix 3**. Strains were grown on ACM agar, supplemented with uridine and uracil and pyridoxine covered with a Nylon filter membrane (pore size 11µm) as described previously (**Section 2.2.13**). RNA was extracted using TRIzol (**Section 2.2.13**), cDNA synthesised (**Section 2.2.13**), and qRT-PCR was then performed in

three independent biological replicates using duplicate technical repeats (**Section 2.2.14**). The housekeeping gene AN5993 orthologue for *Podospora anserine (AS1* (Pa_1_16650)) was used for normalization of results.

4.4 Results

4.4.1 Identification of putative HMG genes

Putative HMG genes were identified in *A. nidulans* by using sequences of the HMG box core (80 amino acids) from both *A. nidulans* and *P. anserina* in BLASTP searches against a protein database of *A. nidulans* fungal transcription factors and a superfamily database (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html). Eight putative HMG genes were identified (**Table 4-2**). In addition two other genes (AN0879 and AN5073) were included in this study that showed weaker similarity from the BLAST searching, as was the divergent *MAT1* MATα_HMG gene (AN2755) The chromosomal location of these genes was checked in the *Aspergillus* genome database (AspGD). The cellular localization and type of HMG genes were determined via PSort (http://psort.hgc.jp/form2.html) and structural prediction (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), respectively (**Table 4-2**). Most of the putative HMG genes showed nuclear localization except for AN3549, AN3580 and AN3667, which were predicted as cytoplasmic proteins. NB. Note that AN3667 was not subject to subsequent gene deletion as this had already been conducted by Ashour (2014), who demonstrated that three replicate ΔAN3667 transformant strains were completely sterile in terms of lack of production of cleistothecia and ascospores, but did show prolific production of Hülle cells [see **Figure 6-4** in Ashour (2014)]. AN3667 was termed Aspni2c by Benkhali *et al.* (2013) and is a member of the HMG_UBF_HMG (H) clade (**Figure 4-1**).

Table 4-2. BLAST search results for putative HMG genes in *Aspergillus nidulans*

Gene ID	E-value*	Region of HMG core	Type of HMG	Chromosomal location	Cellular localization	Description of the gene
AN 1267	3.27e-16	234-314	HMGB	chr VIII	Nucleus	High-mobility group (HMG) protein with a predicted role in sequence-specific DNA binding, RNA polymerase III transcriptional preinitiation complex assembly and chromatin remodeling; required for germination of condidia and ascospores.
AN 1962	1.83e-21	91-181	MATA_HMG	chr VII	Nucleus	Uncharacterized
AN 2885	1.06e-30	18-98	HMGB	chr VII	Nucleus	High-mobility group (HMG) protein with a predicted role in sequence-specific DNA binding, RNA polymerase III transcriptional preinitiation complex assembly and chromatin remodeling.
AN 3549	4.97e-20	8-87	MATA_HMG	chr II	Cytoplasm	Protein of unknown function; transcript repressed by nitrate
AN 3580	8.06e-09	227-321	HMG	chr II	Cytoplasm	Putative polyamine oxidase with a predicted role in aromatic amino acid biosynthesis
AN 3667	1.06e-18	206-285	MATA_HMG	chr II	Cytoplasm	Uncharacterized

AN 4734	8.77e-25	120-199	MATA_HMG	chr III	Nucleus	HMG domain MAT2 mating-type protein; regulator of sexual development; acts with MAT1 alpha-domain protein; null mutant cleistothecia are sterile and Hulle cells show abnormal aggregation; gene expression is induced during sexual development
AN 10103	4.19e-18	88-172	HMGB	chr VIII	Nucleus	High-mobility group (HMG) protein with a predicted role in sequence-specific DNA binding, RNA polymerase III transcriptional preinitiation complex assembly and chromatin remodeling
AN 0879	0.0471	139-188	Weak hit to HMG-box	chrVIII		Ortholog(s) have cytosol, nucleus localization
AN 5073	0.00279	16-63	Weak hit to HMG-box	chr III		Uncharacterized
AN 2755			α-1 MAT1-1	chr VI	Nucleus	Alpha-domain mating-type protein; regulator of sexual development; acts with MAT2 HMG domain protein; null mutant cleistothecia are sterile; gene expression is induced during sexual development

* The e-value corresponds to values obtained with *A. nidulans* BLASTP searches

4.4.2 Amplification of HMG deletion cassettes

All HMG gene deletion cassettes were successfully amplified except for AN0879 and AN3549, which produced secondary products (**Figure 4-3**).

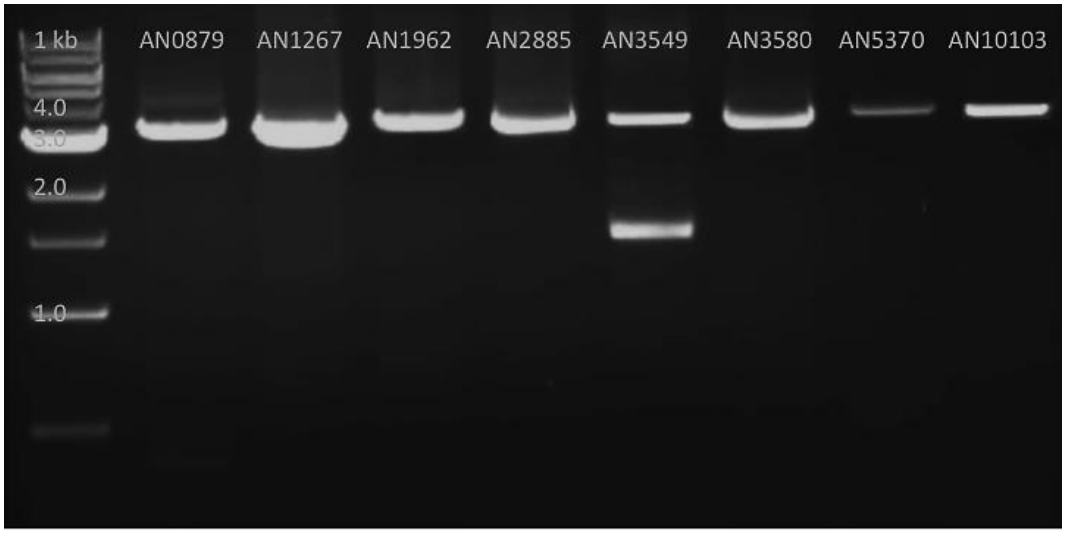


Figure 4-3. 1.2 % Agarose gel showing representative results of amplification of HMG deletion cassettes of *Aspergillus nidulans*.

4.4.3 Construction of deletion cassettes for *A. nidulans* AN4734 and AN2755 genes

Deletion cassettes had to be constructed for both mating-type genes (*MAT1* and *MAT2*) by double joint PCR. The *pyrG* gene of *A. fumigatus* was used as a selective marker and approximately 1kb of the flanking region of each gene were used. Both general and nested primers were used to amplify the final construct for both genes. As shown in **Figure 4-4** the nested primers produced better products for both genes, with amplicons of the expected size of (3146 and 3396 bp)

obtained for both AN4734 and AN2755 deletion cassettes, respectively. The gel-purified products from nested primers were used for later transformations.

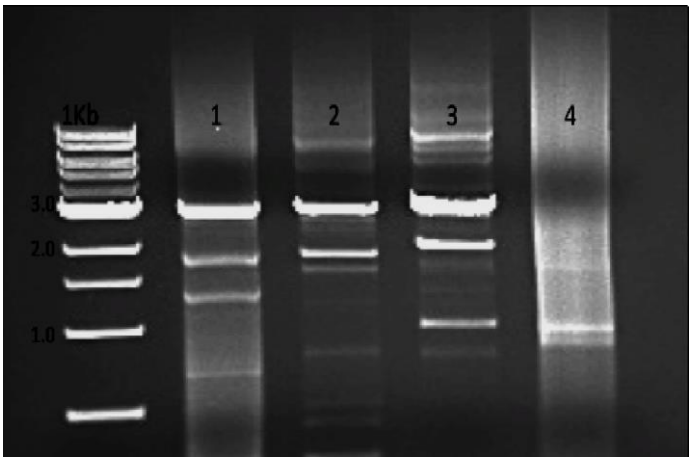


Figure 4-4. 1.2% agarose gel of fusion PCR for construction of the deletion cassettes for AN2755 and AN4734. Lane 1 is 1 kb DNA ladder, lane 2 fusion PCR of AN4734 using nested primers, lane 3 fusion PCR of AN4734 using general primers, lane 4 fusion PCR of AN2755 using nested primer and lane 5 fusion PCR of AN2755 using general primers.

4.4.4 Transformation and screening of transformants by PCR

A total of 1-3 µg of purified PCR product of the HMG deletion cassettes were transformed into *A. nidulans* 2-258 parental strain (*pyrG*$^{-}$, *pyro-*, *ve+* and *Ku*$^{-}$) by PEG mediated transformation. Transformant clonies were selected on AMM agar given that the parental strain has a mutation in *pyrG* so could not grow on AMM without supplementation of uridine and uracil. The transformant colonies were screened by PCR to confirm deletion of the respective HMG genes (**Appendix 18**). Representative results are presented in **Figure 4-5.**

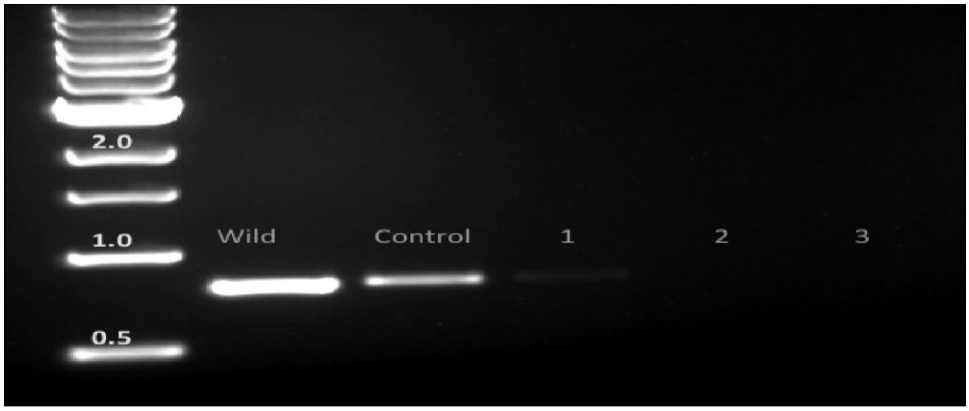
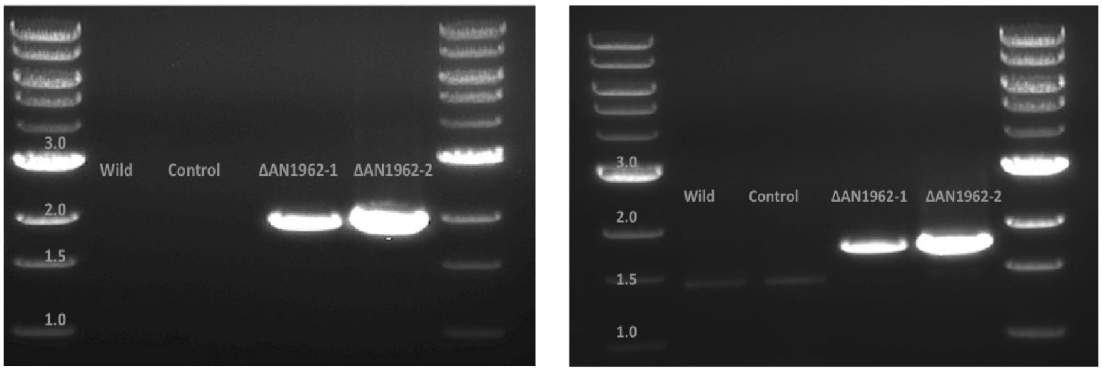


Figure 4-5 1.2% agarose gel showing representative results of gene deletion screening in AN1962 by PCR (using primers within the target gene; predicted size of product 809 bp). Lanes show amplification from the wild-type (parental) and protoplast control (no DNA transformation), in which both show amplification of the target gene, compared to transformant strains (2 and 3) in which the target gene was deleted and failed to amplify and produce any product.

Finally, positional PCR was performed for all HMG gene deletion strains to confirm the correct integration of the *pyrG* selective marker gene in the place of the target HMG gene within the genome of *A. nidulans*. Both upstream and downstream regions were amplified by using the primer pairs of one primer annealing to the outside of flanking region and another being within the *pyrG* gene. It was expected that there would be no product in both parental and control strains because they did not have *pyrG* gene, whereas in both deletion strains of AN1962 the *pyrG* gene was integrated in the place of AN1962 gene, which was confirmed by amplifications of both upstream and downstream of the genes (**Appendix 19**) (**Figure 4-6**). If the *pyrG* marker gene had integrated somewhere else within the genome, there would again be no product, a result the same as that with the control strains.



Upstream flanking region

Downstream flanking region

Figure 4-6 1.2% agarose gel showing representative results of positional PCR to confirm integration of the *pyrG* marker gene in place of the AN1962 target gene in deletion strains compared to parental (wild) and control strains. For amplification upstream (FP1 and TP4; expected size 1884 bp) and downstream (TP3 and FP2; predicted size 1852 bp) primers pairs were used.

4.4.5 Sexual fertility

The HMG deletion strains, parental and –DNA protoplast control strains were tested for sexual fertility as judged by the number and size of cleistothecia formed, and the presence and absence of ascospores. Duplicate deletion strains were obtained for 6 of the 10 HMG genes under investigation (AN1962, AN2755, AN3549, AN3580, AN4734 and AN10103), with single deletion strains for the other investigated genes (AN0879, AN1267, AN2885, and AN5073).

In terms of fruiting body formation, most of the putative HMG genes under study (7 out of 10) were found to have an important role in sexual development as they were all necessary for normal formation of cleistothecia (**Figures 4-7 and 4-9**). At the most extreme, deletion of AN3549 blocked fruiting body formation at a very early stage; two ΔAN3549 strains both failed to produce any macroscopic sexual structures and even Hülle cells were not produced. Meanwhile, deletion of AN1267, AN1962, AN2755, AN2885, AN4734 and AN5073 did not result in a complete loss of fertility but led to a significant reduction in the number of cleistothecia formed to various degrees (**Figures 4-7 and 4-9**) i.e these genes

appeared to act as activators of sexual development. By contrast, deletion of AN0879, AN3580 and AN10103 had no effect on sexual fertility, with similar numbers of cleistothecia produced to the parental strain. Non-Parametric Kruskal-Wallis test were used to analyse data due to failure to satisfy the Levene's test of homogeneity of variance for One-Way ANOVA (**Appendix 11a**).

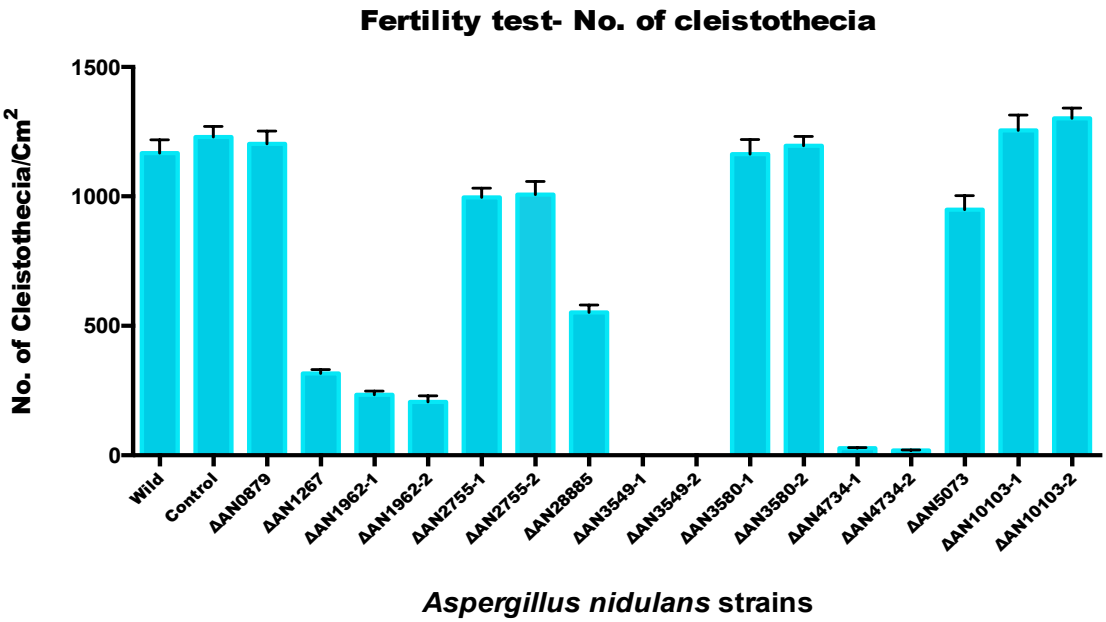


Figure 4-7 Number of cleistothecia produced by *A. nidulans* HMG gene deletion strains compared to parental (wild) and –DNA protoplast (control) strains. Error bars represent SEM (standard error of the mean).

In terms of the second parameter assayed, the size of cleistothecia, there were no significant differences in the size of cleistothecia produced by all the HMG gene deletion strains compared to the parental control except for AN1962, AN2755, AN2885 and AN4734 (also note that cleistothecia were absent from the ΔAN3549 strains). Deletion of both AN1962 and AN4734 resulted in the formation of cleistothecia markedly larger than the control strains (**Figures 4-8 and 4-9**). A

non-Parametric Kruskal-Wallis test was again used to analyse data because of failure of the Levene's test of homogeneity of variance (**Appendix 11b**).

Finally in terms of the last parameter, being presence or absence of ascospores, most HMG deletion strains that formed cleistothecia also produced ascospores. However, ΔAN2755 and ΔAN4734 strains were sterile with no ascospores detected.

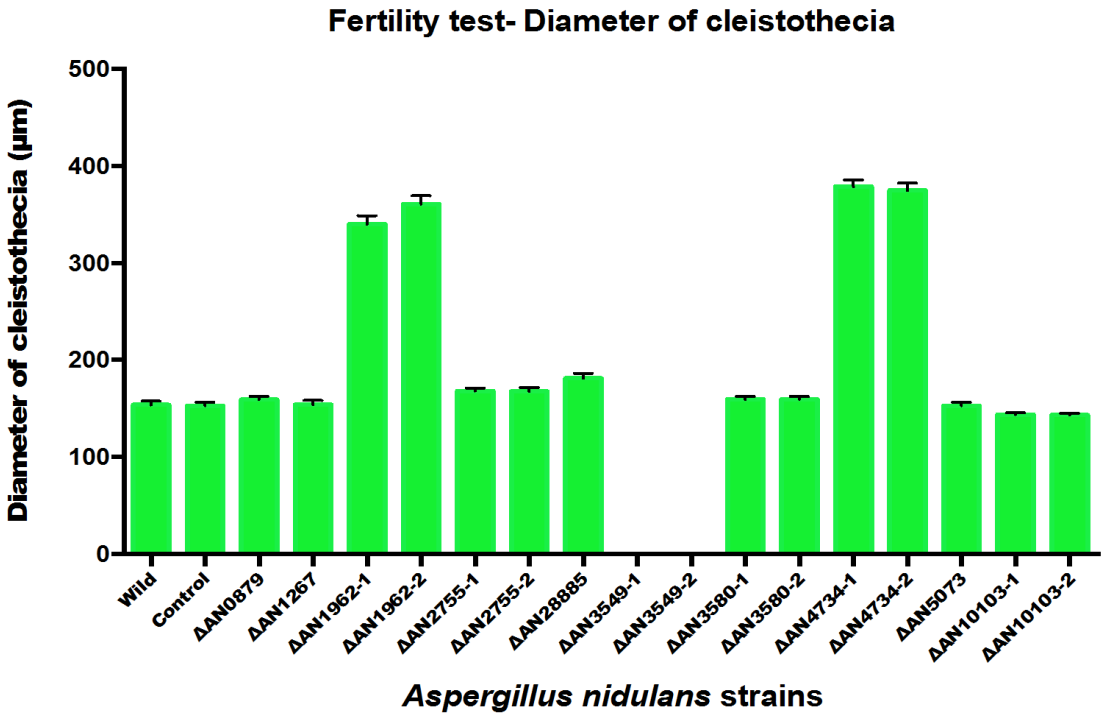


Figure 4-8 Diameter of cleistothecia produced by various *A. nidulans* HMG gene deletion strains compared to parental (wild) and –DNA protoplast (control) strains. Error bars represent SEM.

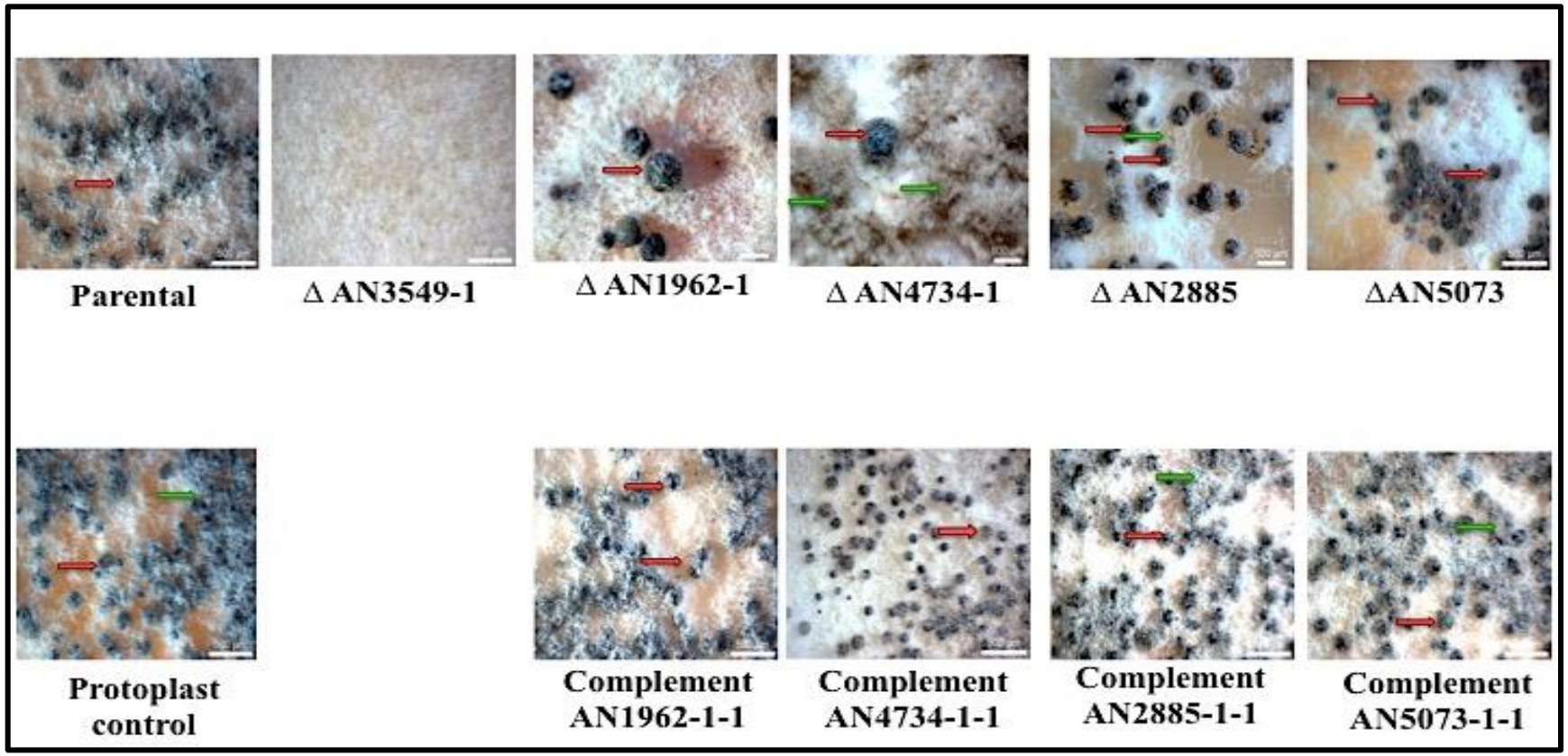


Figure 4-9 Representative images showing sexual fertility of some HMG deletion strains and their complemented strains. Red arrows show the cleistothecia produced by test strains and green arrows indicate Hülle cells surrounding the developing cleistothecia. Scale bar indicates 500 µm.

4.4.6 Complementation of transformants

The HMG deletion strains (*pyro-*, *pyrG*$^{+,}$ *ku*$^{-}$, green spored) that showed significant phenotypic changes in fertility compared to the parental strain were crossed with *A. nidulans* strain 2-259 [*paba A1-*, *yA2* (yellow spore), *veA+*, *pyro+*] to restore the target deleted HMG gene. Sexual crossing and restoration of genotype was performed as described earlier (**Section 2.2.12**). Green colonies that were only able to grow on AMM supplemented with pyridoxine were selected and further tested by PCR to confirm the restoration of the target gene by amplifying a region of gene (**Appendix 20**) and (**Figure 4-10**).

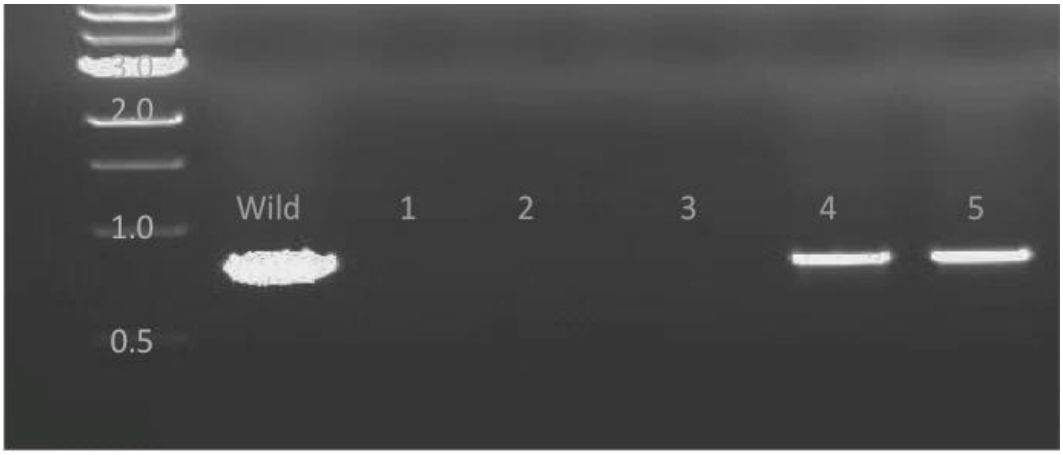


Figure 4-10 1.2 % agarose gel showing representative results of PCR amplification of the target gene in complement strains of AN1962 and parental (wild-type) strains using primer sets amplifying within the gene with a predicted size of 809 bp. The target gene was restored in complement strains 4 and 5.

After reinserting back the target gene, all complement and control strains were tested for sexual fertility (**Section 2.2.11**) to confirm whether the observed phenotypes exhibited by the gene deletion strains were due to the specific gene deletion or other artefacts. The same parameters were taken into account as in the

fertility tests. There were no significant differences in the number and size of cleistothecia produced by all complemented strains compared to the parental control strain (**Figures 4-9, 4-11 and 4-12**). All of the test strains showed restoration of the control sexual phenotype after restoration of the target gene, which confirmed that observed changes were due to the deletion of the putative HMG genes and not any other factor. Non-parametric test –Kruskal-Wallis H test was used to detect statistical differences in the number and size of diameter of cleistothecia that were produced by complement strains compared to parental strain. The statistical test revealed that there were no significant differences in the number and size of cleistothecia formed by tested strains (data not shown).

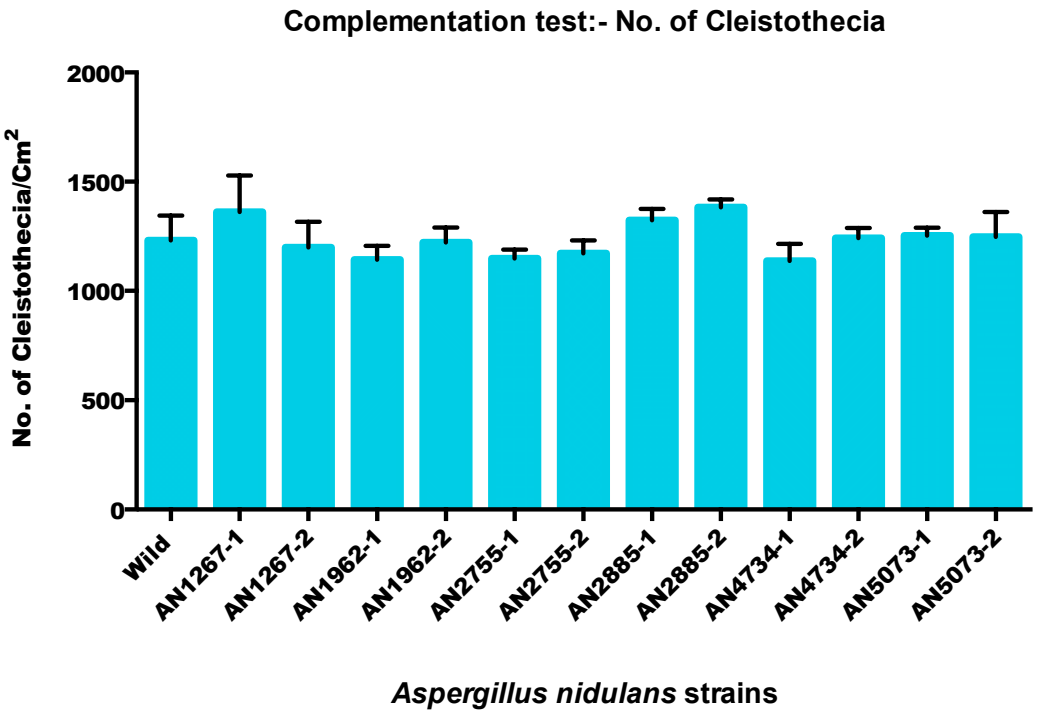


Figure 4-11 Number of cleistothecia produced by complemented strains of *A. nidulans* compared to the parental (wild-type) control. Error bars represent SEM.

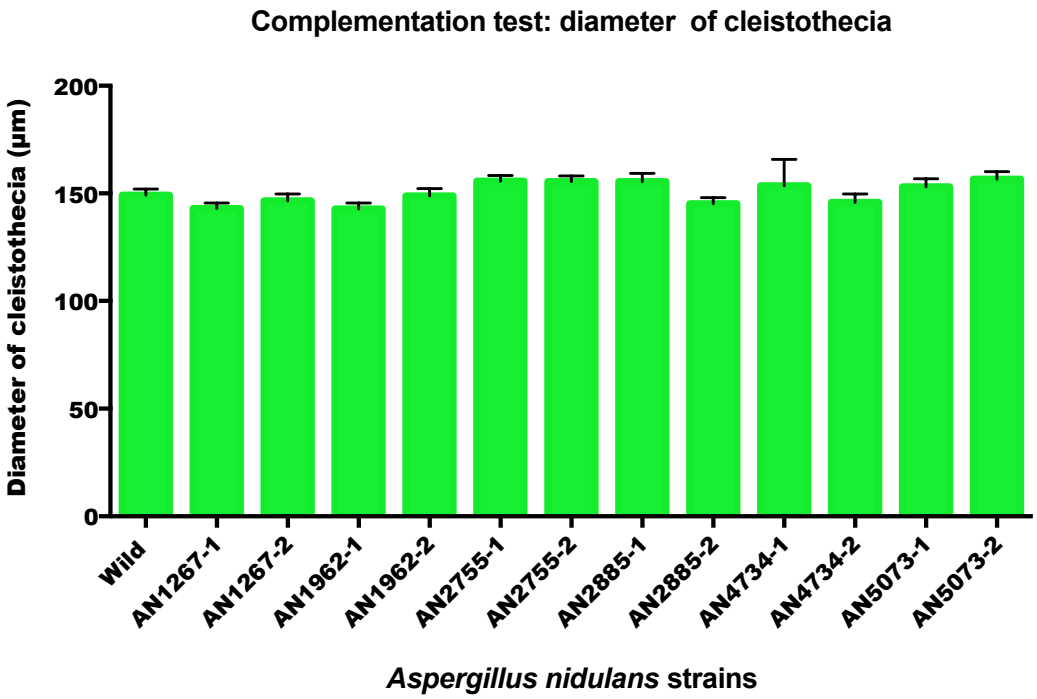


Figure 4-12 Diameter of cleistothecia produced by complement strains compared to the parental (wild-type) control. Error bars represent SEM.

4.4.7 Overexpression of HMG genes

Attempts were made to overexpress two of the HMG genes, AN1962 and AN3667 (Ashour 2014), which appeared to act as particularly significant promoters of sexual development.

1. **Amplification of DNA fragments for cloning**

Cloning was preformed for both AN1962 and AN3667 in three different steps by using either Gibson or HiFi NEBuilder cloning kits. For construction of cloning

vectors 1kb of both flanking region of target gene wherever possible, the target gene and the *pyrG* marker gene of *A. fumigatus* were amplified by PCR. The primer used for amplification of each of these fragments contained complementary nucleotides specific to the site of insertion in the pAN52.1 plasmid. **Figure 4-13** shows a representative example of amplification of fragments used for construction of the overexpression-cloning vector for AN1962.

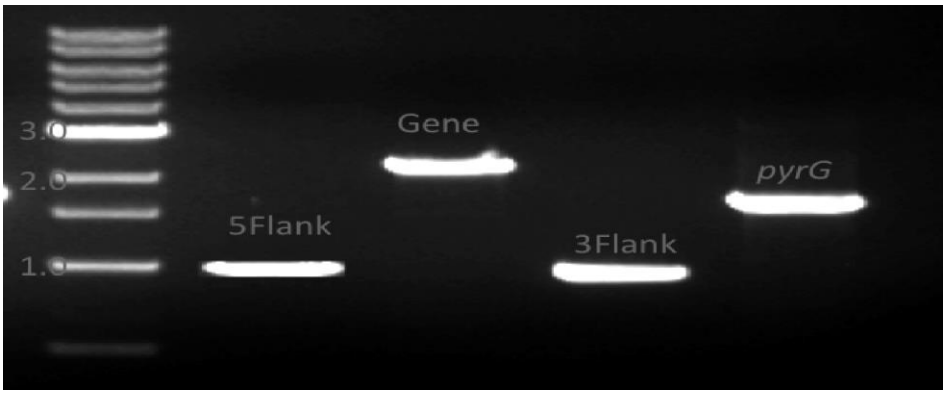


Figure 4-13. 1.2% agarose gel showing amplification of DNA fragments used for cloning of AN1962 gene. The expected size for the PCR product of both upstream and downstream flanking region is 1000 bp, for AN1962 gene is 2258 bp and for *pyrG* gene is 1732 bp.

2. Assembling of DNA fragments into vector

The DNA fragments were assembled into the pAN52.1 vector containing the *A. nidulans gpdA* promoter in three different cloning steps. In the first step both *pyrG* gene and the 3' flank of the AN1962 gene were inserted into the linearized vector by *Hind*III endonuclease; these three fragments were assembled by using NEBuilder Gibson cloning kit, the predicted map is presented in **Figure 4-14**.

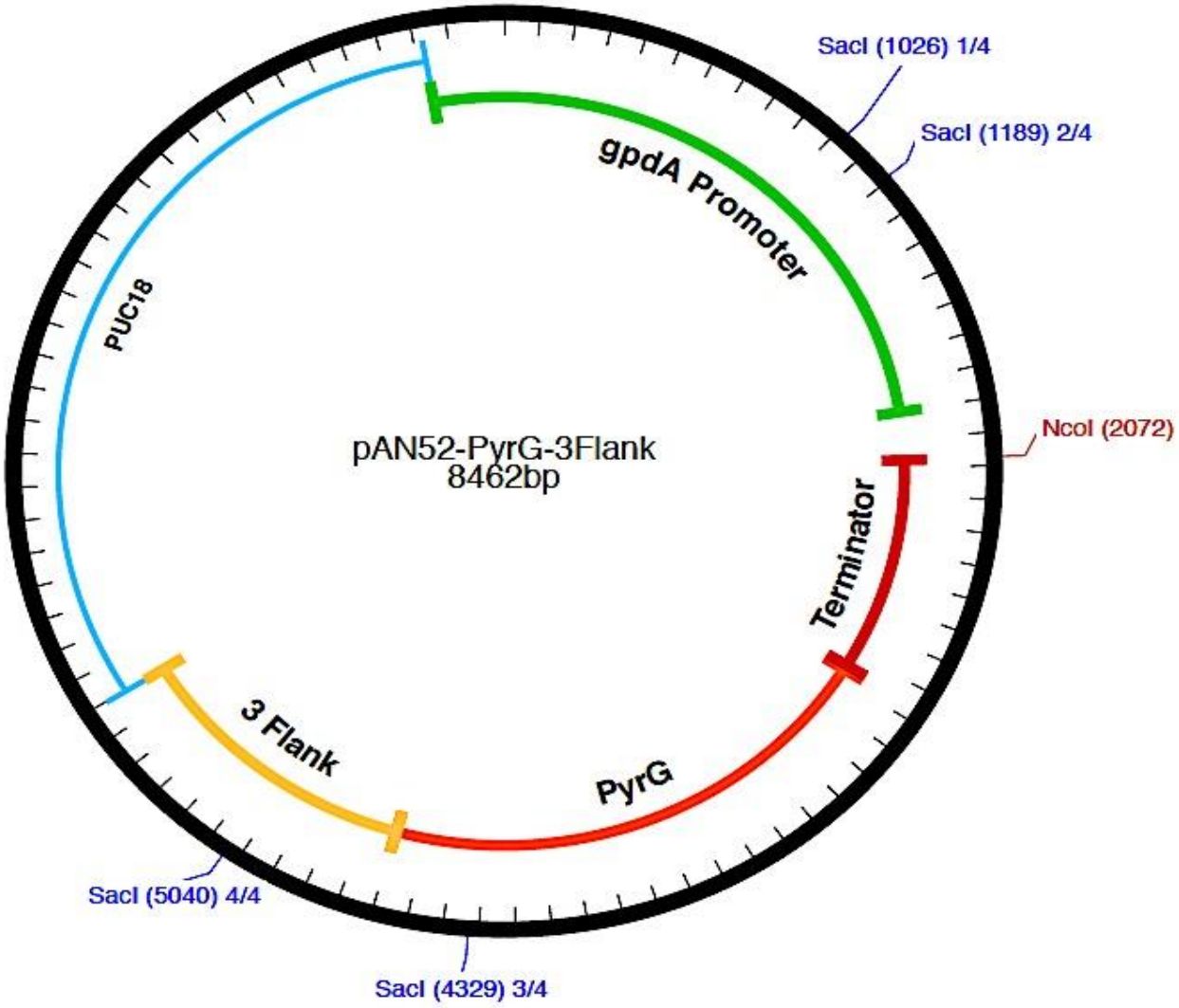


Figure 4-14 Predicted map of the pAN52.1 with *pyrG* and 3 flank of AN1962 gene correctly inserted.

After assembly, 2 µl of the assembling mixture were transferred into chemical competent *E. coli* by heat shock. Transformant colonies of *E. coli* were selected on LB media containing ampicillin. Correct integration of the two fragments was checked by extracting and digesting plasmid with *Sac*I (**Figure 4-15**).

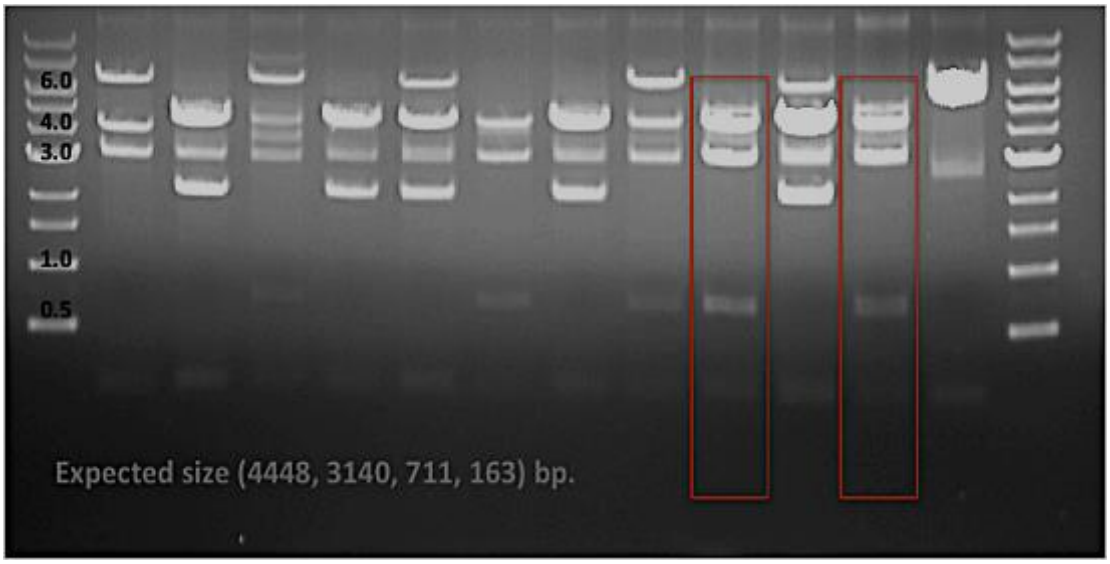


Figure 4-15 Screening of the pAN52.1 plasmid containing both pyrG and 3 flank of the AN1962 gene digested with *Sac*I restriction enzyme.

The second step of cloning was performed by using the product vector in the first step (pAN52.1-PyrG-3Flank), which was opened with the *Nco*I restriction enzyme. In this step AN1962 gene was integrated into the vector at the site between *gpdA* promoter and the terminator (**Figure 4-16**).

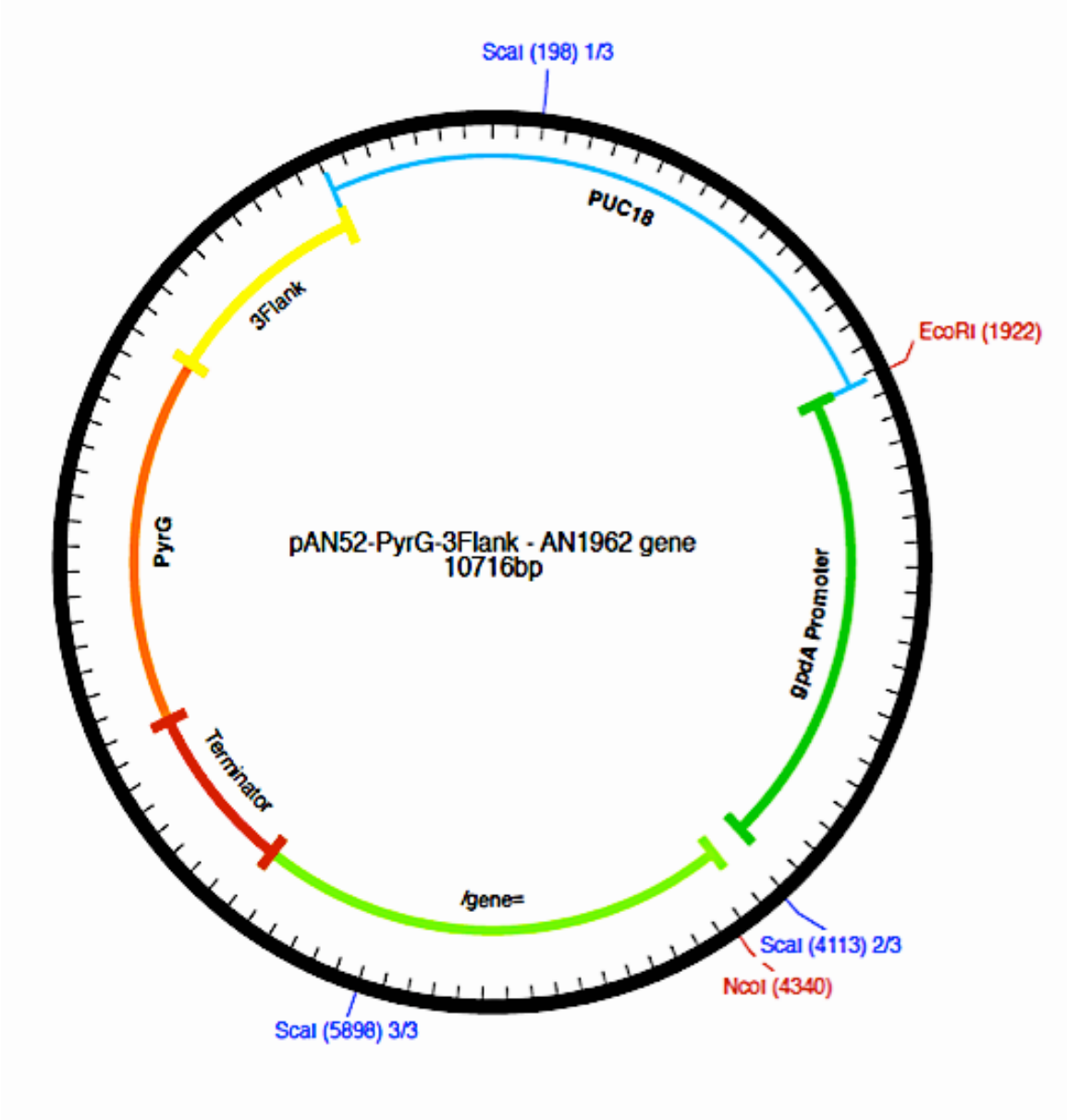


Figure 4-16 Predicted map of the pAN52-PyrG-3 flank with AN1962 correctly inserted after the *gpdA* promoter.

The assembling mixture was transferred as in the first step to *E. coli* and some colonies were selected and plasmid extracted. The plasmid was screened using *Sca*I to confirm the correct insertion of the AN1962 gene into pAN52-PyrG-3 flank vector (**Figures 4-17 and 4-18**).

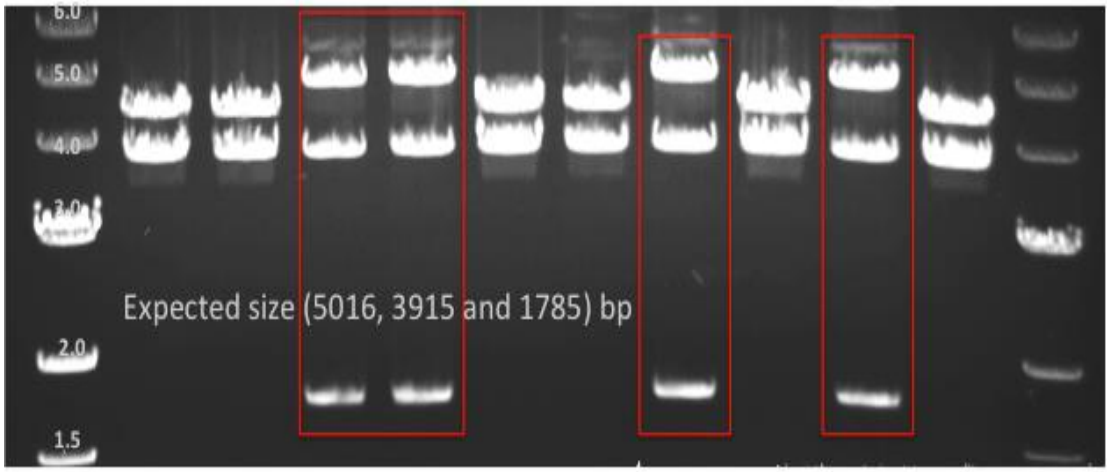


Figure 4-17 Screening of the pAN52-PyrG-3 flank plasmid containing AN1962 in the correct site following digestion with *Sca*I.

Finally, the last fragment was inserted into the vector pAN52-PyrG-3Flank-AN1962 gene by opening it with *Eco*RI restriction enzyme at the site where the *gpdA* promoter started (**Figure 4-18**). The final vector was screened by digesting the plasmid with *Stu*I to verify correct insertion of the 5'flank DNA fragment (**Figure 4-19**).

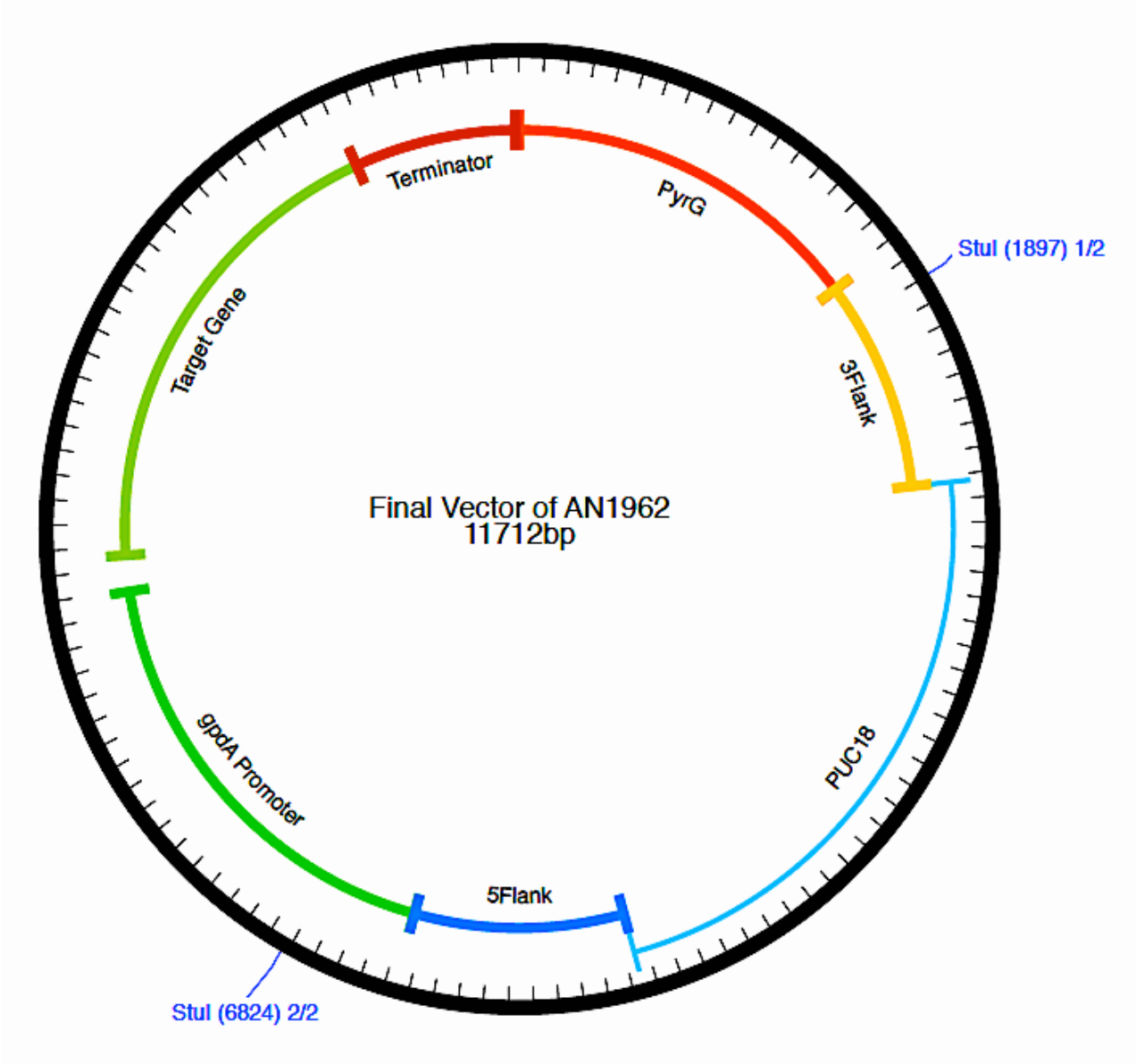


Figure 4-18 Predicted map of final overexpression vector for AN1962 gene.

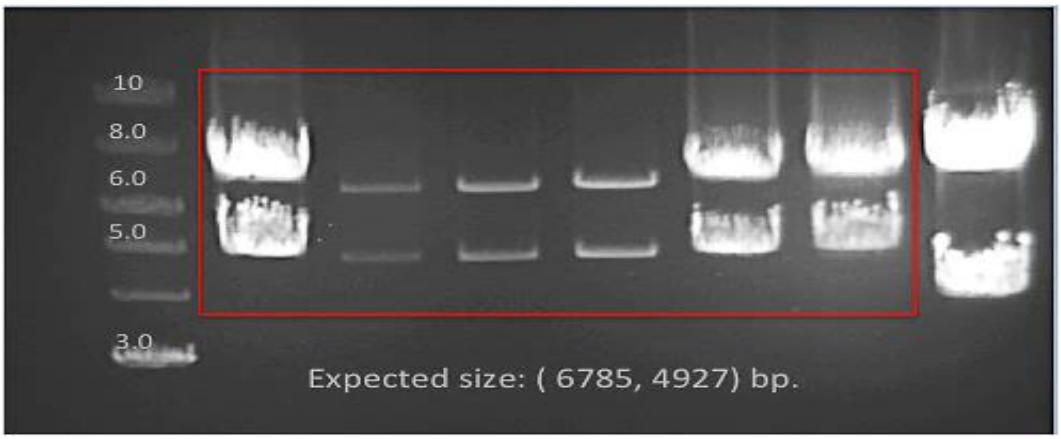


Figure 4-19 0.7% agarose gel showing screening the final vector of AN1962 with *Stu*I to confirm the insertion of the 5'flank.

3. Transformation into *A. nidulans* and screening of transformants

The overexpression vectors for AN1962 and AN3667 were transferred to *A. nidulans* strain 2-258 by PEG mediated transformation using the *pyrG* selective marker. Some transformant colonies were selected and screened for correct integration of the cloning vectors within the genome using a forward primer within the 5' flank of the target gene and a reverse primer within the target gene. **Figure 4-20** shows a representative result of PCR screening for insertion of the overexpression vector of AN1962 within the genome of *A. nidulans*. If the plasmid had inserted in the correct site to replace the resident target gene there should only be one PCR product which should be larger than that of the parental strain because of incorporation of the *A. nidulans gpdA* promoter between the 5' flank and the target gene.

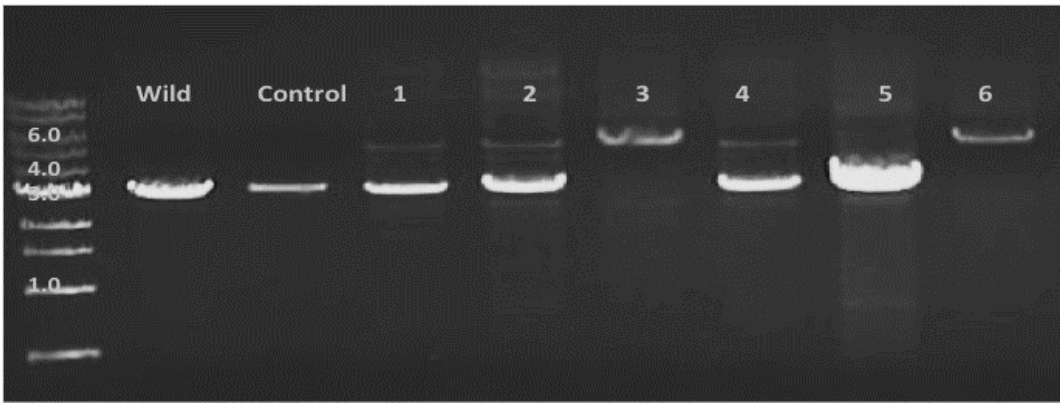


Figure 4-20 % 1.2 agarose gel showing screening of transformant colonies by PCR to check for integration of the overexpression vector of AN1962 within the genome. In transformant strains 3 and 6 the cloning vector replaced the resident AN1962 gene as there is a single band of the predicted size (5553bp); whereas the other three transformant strains (1, 2 and 4) show ectopic integration of the vector as there is amplification of two copies of gene - the original site (3258bp as in parental control) and the ectopic cloned gene (5553bp).

4.4.8 Determination of transcript level of overexpressed genes by qRT-PCR

The expression level of AN1962 was quantified by qRT-PCR in two transformant strains of *A. nidulans* (OvEAN1962-1 and OvEAN1962-3) compared to parental strain (**Figure 4-21**). The first strain contained a single copy of AN1962 (with the *gpdA* promoter) integrated at the homologous location of the original copy of AN1962; while the second strain had two copies of the gene, being the original resident gene and an extra transformant copy integrated ectopically within the genome expressed under the control of the *gpdA* promoter. One-way ANOVA-LSD revealed that there were significant differences in the expressions of AN1962 gene between wild-type strain and transformant strains using log transformed data ($F(2,6) = 1558.36$, $P < 0.0001$).

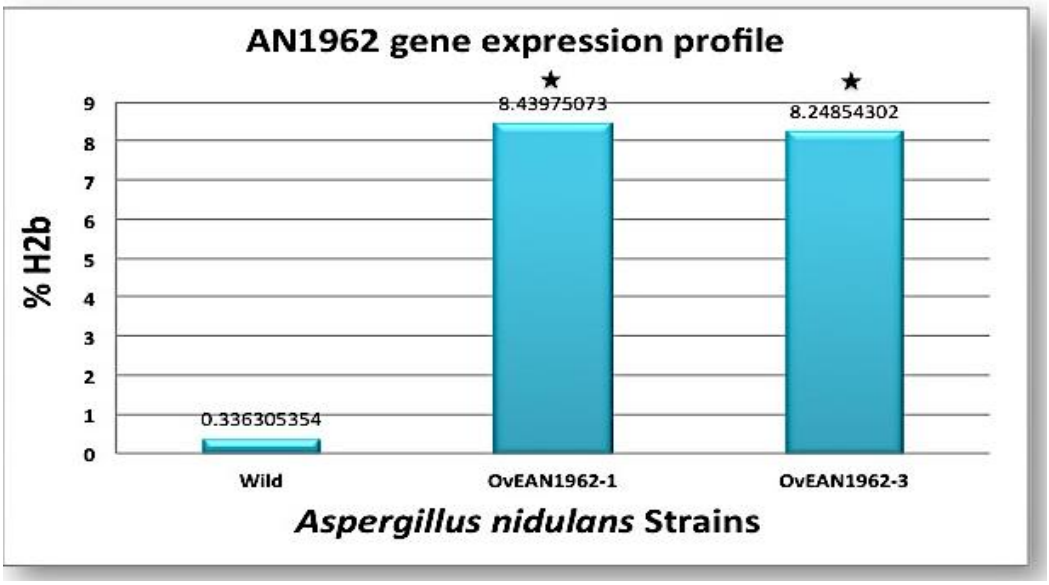


Figure 4-21 qRT-PCR showing expression profile of AN1962 gene in the parental (wild-type) control strain and two transformant strains containing the AN1962 overexpression vector. ★ indicates statistically significant differences in the expression between relevant test and control strains.

4.4.9 Fertility test for overexpressed strain

Aspergillus nidulans strains that contained either a single copy of the AN1962 overexpression vector integrated homologously (OvEAN1962-1 and OvEAN1962-2) or a copy of the AN1962 overexpression vector integrated ectopically together with the resident AN1962 gene (OvEAN1962-3 and OvEAN1962-4) and four overexpression strains of AN3667 were tested for fertility. The effect of gene overexpression on fertility was determined by comparing the number and size of cleistothecia produced by these strains compared to parental strain (**Figures 4-22, 4-23 and 4-24**). All four strains of OvEAN3667 resulted in complete loss of fertility, being unable to produce fruiting bodies or Hülle cells. In contrast, the OvEAN1962 strains produced cleistothecia but there were statistically significant differences between the number of cleistothecia produced by all of the OvEAN1962 strains and the

parental (wild-type) strain (**Figure 4-22**). Perhaps surprisingly all of these overexpression strains produced fewer cleistothecia compared to the parental strain. Square root transformed data was used for One-way ANOVA-LSD ($F(5,60) = 104.493$, $P < 0.0001$).

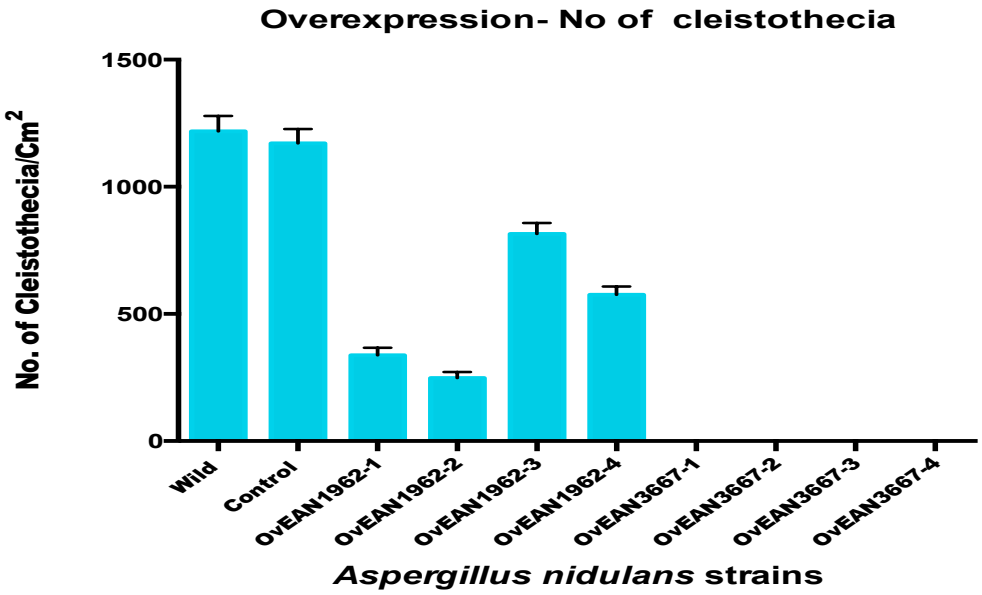


Figure 4-22 Number of cleistothecia produced by AN1962 and AN3667 overexpression strains compared to parental (wild) and –DNA protoplast (Control) strains. Error bars represents SEM.

By contrast, there was a significant variation in the size of cleistothecia produced by some of the AN1962 overexpression strains compared to the wild-type strain (**Figure 4-23**). Both strains (OvEAN1962-1 and OvEAN1962-2), which contained only the overexpressed AN1962 gene produced significantly larger cleistothecia than the parental strain; while the other strains (OvEAN1962-3 and OvEAN1962-4) that contained two copies of AN1962 produced similar size

cleistothecia to the parental strain. Log transformed data was used for One-way ANOVA-LSD ($F(5,594) = 74.136$, $P < 0.001$).

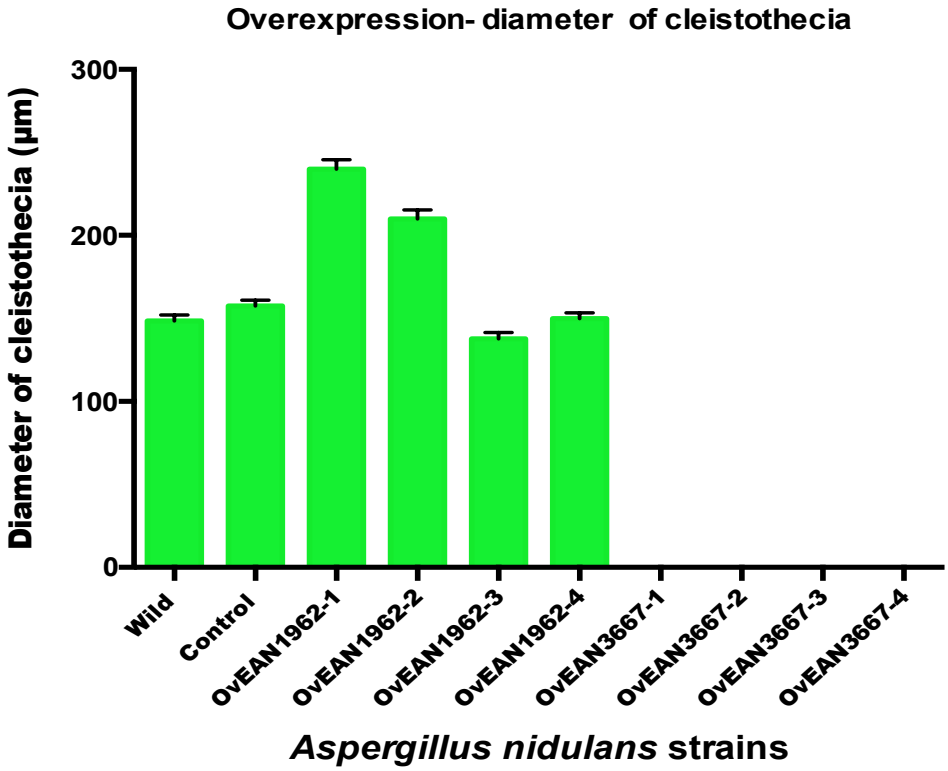


Figure 4-23 Size of cleistothecia (diameter) produced by several of AN1962 and AN3667 overexpression strains compared to the parental (wild) and –DNA protoplast (Control) strains. Error bars represent SEM.

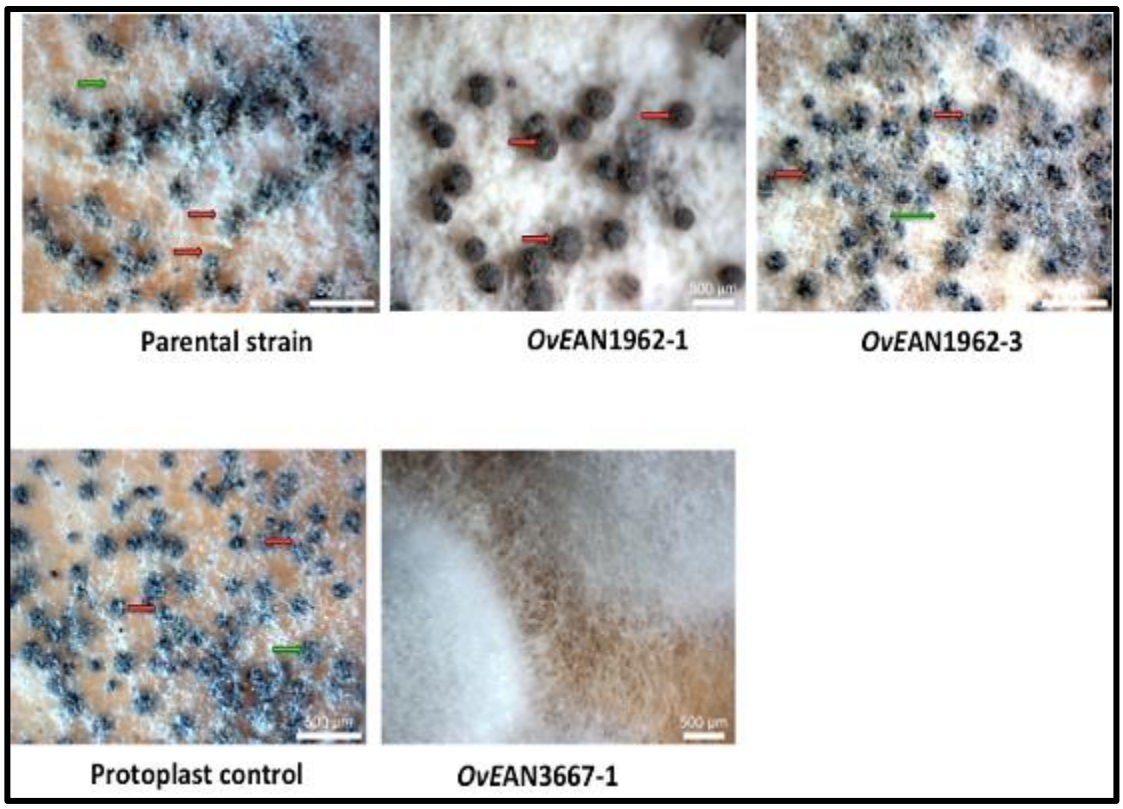


Figure 4-24 Representative images showing sexual fertility of AN1962 and AN3667 overexpression strains compared to parental (wild) and –DNA protoplast (control) strains. Red arrows indicate cleistothecia produced by various test strains and green arrows indicate Hülle cells which surround the developing cleistothecia. Scale bar indicates 500 µm.

4.4.10 Transcriptional expression of HMG genes and other key sex genes in the HMG deletion strains

Deletion of most of the putative HMG genes resulted in defects of fertility, especially both AN3549 and AN3667 that led to blockage of fruiting body formation. qRT-PCR was used to determine the effect of each HMG gene deletion on the expression of other genes and also to detect the relationship between these genes. The fold change (FC) data were expressed as a heat map (the fold change calculated as a relative quantity of normalized cDNA in a mutant

strain to the amount normalized cDNA of parental strain). The gene is considered to be downregulated if the 0< FC<1 and the P-value < 0.05 while gene was defined as upregulated if FC >1 and the P-value was < 0.05. As shown in the **Figure 4.25** expression of almost all of the tested gene was downregulated in the mutant strains of (AN3549, AN2755 (MAT1) and AN1267. These two genes (AN3549 and AN1267) appeared to act as the main regulator of mating; both mating type genes, and pheromone signaling were downregulated in these mutant strains (**Figure 4-25** and **Figure 4.26**)

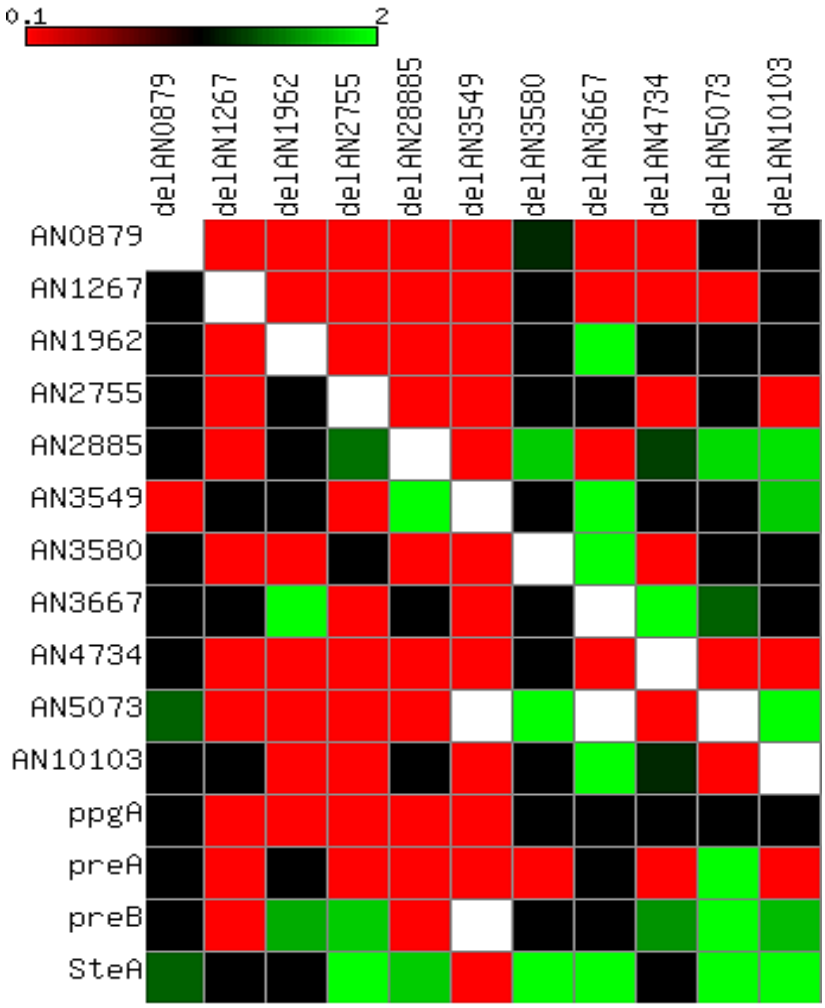


Figure 4-25 A heat map showing expression of HMG genes and some other sex-related genes in HMG mutant strains. The data in **Appendix 13** were used to create a heat map by using Materix2png (Pavlidis and Nobel, 2003). The black

squares represent non-significant values, and white squares stand for non-applicable values (the gene deleted or not expressed). The red and green colours represent down and upregulation of genes, respectively.

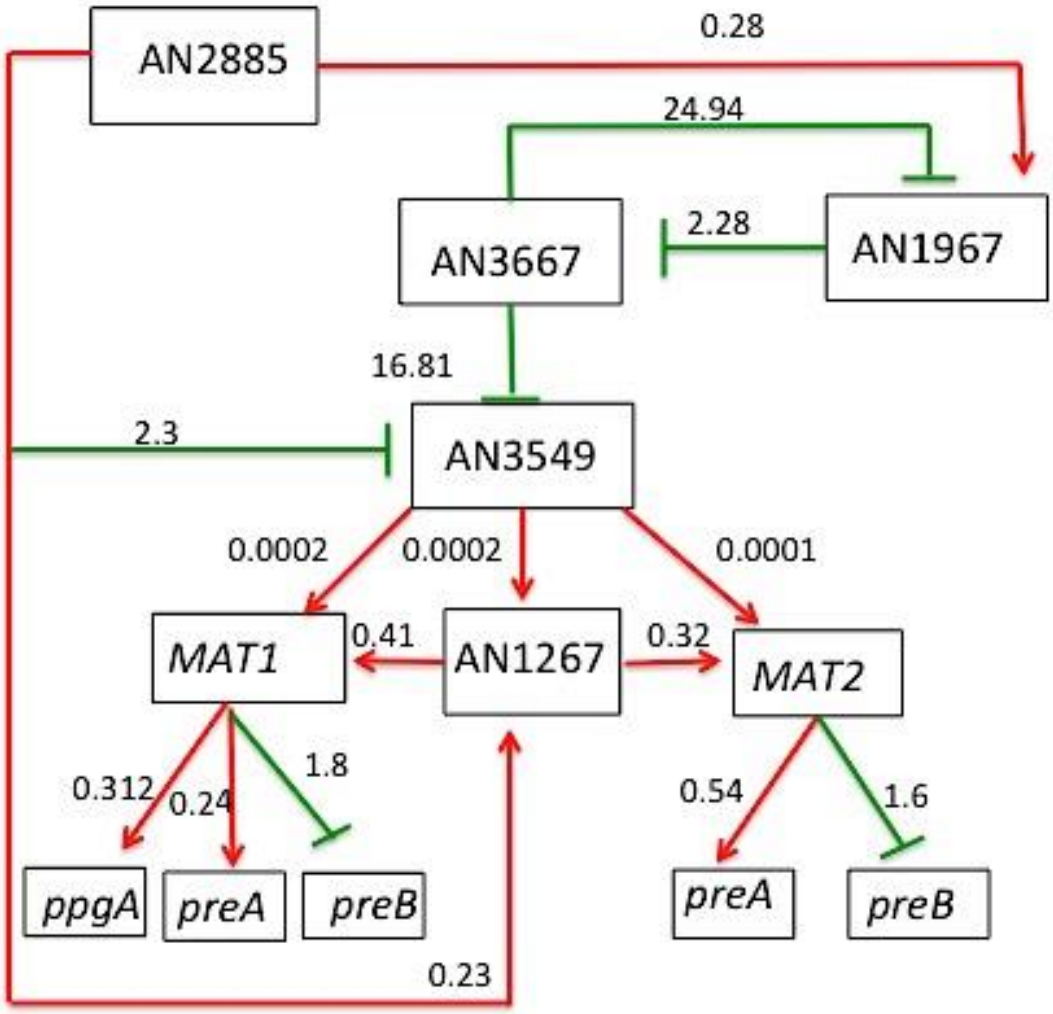


Figure 4-26 Schematic diagram showing the genetic interaction of some HMG genes that regulate mating in *A. nidulans.* The red arrows indicated that the genes were induced while the green arrows indicate a suppressive effect of the genes. Numbers represent the fold change in gene transcript level in the mutant strains relative to the parental strain.

4.5 Discussion

Fruit body formation in ascomycete fungi requires the coordinated regulation of several external and internal factors. A network of genes has been identified that is involved in this process in the aspergilli (Dyer and O'Gorman 2012). Among these genes, transcription factors have pivotal roles in the regulation and balancing of transition between asexual and sexual reproduction. For example *veA*, *rosA*, *nosA* and *nsdA* encode transcriptional factors that have essential roles in the sexual development (**Section 1.8.4**). In the current chapter work focussed on high mobility group (HMG) domain transcription factors that bind and bend DNA (Ueda and Yoshida, 2010) and which control the regulation of genes involved in developmental and metabolic processes (Bianchi and Agresti, 2005). HMG genes are considered as ancestral genes within the fungal kingdom with an essential role in the determination of sex (Idnurm *et al.*, 2008). In fungi, two members of the HMG family have been well-characterized (AN4734 or *MAT2*, and AN2755 or *MAT1*) and have been shown to be necessary for determination of mating-type identity and are required for sexual reproduction in the aspergilli (Paolettii *et al.*, 2007). Other members of the HMG family have been less well studied. Preliminary investigations were undertaken by Ashour (2014) who found that deletion of the putative HMG gene AN3667 in *A. nidulans* resulted in the loss of fruiting body formation and increased Hülle cell formation. Therefore work in the current chapter aimed to identify and characterize other putative HMG genes in *A. nidulans*, and to determine if they have any role in sexual development.

4.5.1 Identification of HMG homologues

Eight HMG genes were identified within the genome of *A. nidulans* by BLASTP searching using sequences from both *A. nidulans* and *P. ansinera* HMG core proteins. In addition two other genes (AN0879 and AN5073) were detected with weak homology. Furthermore, the *MAT1* gene (AN2755) was also included in this study despite its apparent low similarity (**Table 4-2**), given recent findings that *MAT1* and *MAT2* are evolutionary related and both are members of the HMG

superfamily. The *MAT1* alpha domain clustered as a monophyletic group in the MATA-HMG domain grouping, showing closer relationship to the MATA-HMG domain than either SOX or HMGB-lineages (**Figure 4-1**) (Martin *et al.*, 2010; Benkhali *et al.*, 2013).

4.5.1 Functional characterization of HMG homologues

To functionally characterize the putative HMG genes and determine their role in sexual development in *A. nidulans*, gene deletion was carried out using deletion cassettes provided by fungal genetics stock center (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm) or deletion cassettes made during the present studies using overlap PCR. Transformant strains were screened by PCR for target gene deletion and correct homologous integration of the deletion cassette; at least one deletion strain for each gene was obtained for all putative HMG genes, which were then tested for sexual fertility to detect any change in the fertility compared to the parental (wild-type) strain.

Most of the HMG genes were found to act as inducers of sexual development given that deletion of most of them resulted in a reduction in fruit body formation (**Figures 4-7, 4-8 and 4-9**). Notably, deletion of AN3549 completely abolished sexual reproduction; even Hülle cells were not produced. AN3549 belongs to MATA-HMG box group (the same as *MAT2*). It is orthologous to *Candida albican RFG1*, *Schizosacharomyces pombe mat3-MC* and *Saccharomyces cerevisiae ROX1*; all of these genes were placed in the same clade as *PaHMG8* of *Podospora anserina* [**Figure 4-1**; termed Aspni2d by Benkhali *et al.* (2013)]. Deletion of *PaHMG8* in *P. anserina* similarly resulted in female sterility and loss of fruit body (perithecium) formation (Benkhali *et al.*, 2013). In contrast both orthologous genes in *C. albican* and *S. cerevisiae* have different roles in the cell rather than mating; RFG1 represses filamentous growth and has an essential role controlling certain genes involved in virulence (Kadosh and Johnson, 2001) while Rox1 in *S. cerevisiae* is one of the genes that acts as a repressor of hypoxic genes (Deckert *et al.*, 1995).

Surprisingly the AN1962 putative HMG of *A. nidulans* is also orthologous to the same genes of *C. albicans, S. pombe* and *S. cerevisiae* as AN3549. Both AN1962 and AN3549 belong to the MATA-HMG box group but whereas PSort analysis indicated that AN1962 is localized in the nucleus, AN3549 instead appeared to localise to the cytoplasm. Similar to AN3549, AN1962 acted as an activator of sexual reproduction but deletion of this gene did not result in the complete loss of fertility (unlike AN3549); instead there was a drastic decrease in the number of cleistothecia formed, which were larger in size compared to the parental strain (**Figures 4.7, 4.8 and 4.9**).

In contrast to AN3549, deletion of three HMG genes (AN0879, AN3580 and AN10103) had no obvious effect of sexual reproduction, all deletion strains producing cleistothecia similar to those produced by the parental strain. AN0879 is likely not to be an HMG gene and has no known function; orthologues have cytosol and nuclear localization. However, both AN10103 and AN3580 do belong to the HMG box family. AN3580 belongs in the same clade as *Pa_HMG3* of *P. anserina* and *lsd*1 of *S. pombe*. A deletion mutant strain of *Pa_HMG3* was found to be male and female fertile and produced normal fruiting bodies but with perithecia distributed over an unusual 'wide ring' on crossing plates (Benkhali *et al.*, 2013). The *S. pombe lsd*1 homologue has histone demethylase activity; mutation of this gene resulted in decreased growth (Nicolas *et al.*, 2006). Similarly, deletion of AN10103 had no obvious effect on sexual fertility in *A. nidulans*. AN10103 (HmbC) is homologous to *S. cerevisae Hmo1* and *S. pombe Sp-Hmo1* (Karacsony *et al.*, 2014), and belongs to the same clade as *Pa_HMG4* of *P. ansinera* [**Figure 4-1**; termed Aspni5b by Benkhali *et al.* (2013)]. Mutant strains of *Pa_HMG4* are male and female fertile and develop normal perithecia (Ait Benkhali *et al.*, 2013). Mutant strains of *S. cerevisae Hmo1* are characterized by decreased growth rate and other growth defects but show normal sporulation (Lu *et al.*, 1996).

The remaining HMG genes (AN1267, AN2755, AN2885, AN4734 and AN5073) were all essential for normal sexual reproduction; deletion of these genes resulted in a significant decrease in the number of cleistothecia formed compared to the

parental wild-type strain to various degrees. Both mating-type genes AN2755 and AN4734 [*MAT1* in the MATα_HMG clade, and *MAT2* in the MATA_HMG clade, termed Aspni1a and Aspni2a, respectively, by Benkhali *et al.* (2013); **Figure 4-1**] had previously been deleted by Paoletti *et al.*, (2007) in the *veA1* background; in this study both genes were deleted in the in *veA*$^{+}$ background. The results obtained in this study were similar to those of Paoletti *et al.* (2007) in that gene deletion resulted in a significant decrease in numbers of cleistothecia formed compared to the parental strain and both ΔAN2755 and ΔAN4734 strains failed to form ascospores. However, one result was markedly different, namely that both mutant strains produced larger cleistothecia compared to the parental strain, especially the ΔAN4734 mutant strain in which the cleistothecia were two-fold larger (**Figures 4-8 and 4-9**). By contrast, Paoletti *et al.* (2007) found a significant decrease in size of cleistothecia formed following *MAT* gene deletion. These findings confirm the requirement of both mating-type genes for self-fertility and for ascosporogenesis (Miller *et al.*, 2005; Paoletii *et al.*, 2007). The difference in the cleistothecia size might be due to the effect of the *VeA1* mutation. Similarly, *MAT* genes have been show to be necessary for sexual development in the homothallic ascomycete species *Gibberella zeae* and *Sordaria macrospora* (Lee *et al.*, 2003; Pöggeler *et al.*, 2006).

In parallel to the present study, the function of the HMG gene AN1267 was investigated by Karacsony *et al.* (2014) in the *A. nidulans veA1* background strian. They reported that *A. nidulans* AN1267 [termed Aspni5c by Benkhali *et al.* (2013)] is a member of the mitochondrial high mobility group domain HMGB family (in the HMGB-UBF_HMG clade, **Figure 4-1**), and was found to have diverse functions, being essential for germination of both sexual and asexual spores, mitochondrial DNA copy number and sterigmatocystin production (Karacsony *et al.*, 2014). In addition, the present study demonstrated that this gene is required for the formation of cleistothecia, deletion of AN1267 resulting a decrease in the number of cleistothecia formed compared to the parental strain, although those formed were of the same size as the parent (**Figures 4.7 and 4-8**). The HMG gene AN1267 is structurally similar to *mtHMG1* of *Podosprora anserina* (Karacsony *et al.*, 2014). *mtHMG1* is also a mitochondrial HMG,

deletion of this gene led to shortening of lifespan, slowdown of ascospore germination and female sterility in *P. anserina* (Dequard-Chablat and Alland, 2002). Another important role of AN1267 might be involvement of in the balancing the redox state of the cell through its role in the ROS (reactive oxygen species) protective process (Karacsony *et al.*, 2015).

The AN2885 gene [termed Aspni5a by Ait Benkhali *et al.* (2013)] also belongs to to the HMGB family (in the HMGB-UBF_HMG clade, **Figure 4-1**), being a homologue of *S. cerevisiae NHP6B* which contains a single HMG domain (Karacsony *et al.*, 2014). Deletion of this gene in *A. nidulans* resulted in reduced fertility compared to the parental strain with a reduction in the number of cleistothecia formed but with those cleistothecia, which were produced having a significantly larger diameter (**Figures 4-7, 4-8 and 4-9**). This phenotype resembles the orthologous *Pa_HMG6* of *P. anserina* in which a deletion mutant strain was male fertile but showed considerably reduced female fertility with a 50-fold decrease in perithecial formation compared to the wild-type strain, and with a modified perithecial shape - the neck of the perithecium being larger than that of the body (Benkhali *et al.*, 2013).

The final gene included in this study was AN5073. In reality it is considered that this was unlikely to be a member of the HMG family, as it has very little similarity to HMG core box It was a previously uncharacterized gene with no known orthologue in other ascomycete fungi. However, this gene was found to have a role in sexual development, deletion of this gene resulting in a slight but significant decrease in fertility, although cleistothecia were of the normal diameter compared to the parental strain (**Figures 4-7 and 4-8**).

The mutant strains (ΔAN1267, ΔAN1962, ΔAN2755, ΔAN2885, ΔAN3549, ΔAN4734 and ΔAN5073) that produced significant changes in sexual fertility in *A. nidulans* were crossed *A. nidulans* 2-259 to reinsert the deleted gene back to the genome as described by Todd *et al.*, (2007). Two complement strains were obtained for almost all the of *A. nidulans* transformant strains. When tested for fertility, all of the strains showed a restoration of phenotype, producing

cleistothecia similar to the parental strain, after restoring the target gene thereby confirming that the observed phenotype was due to gene deletion of the target gene. The only exception was for the ΔAN3549 mutant. Several crosses were set up between this mutant strain and *A. nidulans* 2-259 but all attempts to obtain hybrid cleistothecia failed, only very small cleistothecia were produced and most of the time these were sterile or contained very few ascospores that only belonged to the yellow spore *A. nidulans* 2-259 parent. This suggests that AN3549 has a role in hyphal anastomosis that cannot be restored by this crossing method, and that alternative methods will have to be used.

4.5.2 Overexpression of HMG genes

Deletion of the putative HMG genes AN1962 (the present study) and AN3667 (Ashour 2014) resulted in particularly interesting phenotypes with deletion of both leading to a very marked decrease in fertility. Thus, these genes appeared to act as strong inducers of sexual development and it was postulated that up-regulation/overexpression of these genes might result in an increase in sexual fertility. AN3667 had previously been deleted in the *VeA* background (Ashour 2014) and the ΔAN3667 mutant failed to form cleistothecia, only a massive aggregation of Hülle cell was produced.

To determine and study the up regulation of expression of these genes and the resultant phenotype overexpression plasmids were constructed for both AN1962 and AN3667, with the genes under the expression of the highly expressed constitutive *gpdA* (glyceraldehde-3 phosphate- dehydrogenase) promoter of *A. nidulans.* Unfortunately despite several attempts it was not possible to create an overexpression cassette for AN3549 both *pyrG* and the 3'flank of the gene were cloned into a plasmid but further steps of cloning failed, perhaps because of the large sizes of the gene and plasmid (3386 and 8846 bp, respectively). The constructed plasmids for both genes (complete for AN1962 and partial for AN3667) were transformed into *A. nidulans* 2-258 (*PyrG-*, *PyroA4-*, green spored colour and *paba+*, *veA+*). Transformant colonies were screened by PCR to confirm the insertion of the clone of gene within the genome. An increase in gene

expression of AN1962 was confirmed by qRT-PCR compared to wild-type strain under condition favourable for sex (**Figure 4-21**), but for the putative transformant strain of AN3667 after 10 days of incubation under these conditiosn only a very small colony was formed (compared to the parental strain) and there was insufficient growth for RNA extraction and quantification of gene expression. It is hypothesised that overexpression of AN3667 might have resulted in the decreased vegetative growth rate under these conditions. An inhibition of vegetative growth rate was also observed following overexpression of the HMG *MAT1* and *MAT2* genes in *A. nidulans* (Paoletti *et al.*, 2007).

To detect any possible effect of increased gene expression on sexual development in the successfully obtained OvEAN1962 transformants, strains containing the overexpression vectors were tested for fertility and the resulting phenotype compared to the parental wild-type strain. Surprisingly, the *gpdA::AN1962* OvEAN1962-1 and OvEAN1962-2 transgenic strains that contained a single overexpressed copy of the gene that replaced the resident target gene produced a phenotype similar to the ΔAN1962 deletion strain, showing a reduction in the number of cleistothecia but at the same time an increase in diameter of cleistothecia. Whereas the OvEAN1962-3 and OvEAN1962-4 strains that contained two copies of the AN1962 gene, one transgenic (overexpressed and integrated ectopically within the genome) and one wild-type gene (supposedly normal expression at the resident locus) also produced a significant decrease in the number of cleistothecia but which were of normal size compared to parental strain. These phenotypes suggested that the genomic position and exact tightly control of expression levels of this gene are required for normal sexual reproduction. Similarly, overexpression of two HMG genes in *P. anserina* (*Pa_HMG5* and *mtHMG1*) did not result in an increase in fertility (Dequard-Chablat and Alland, 2002; Benkhali *et al.*, 2013). A transgenic *Pa_HMG5* strain exhibited female sterility in a cross with the parental strain as seen in the deletion mutant strain (Benkhali *et al.*, 2013).

4.5.3 Genetic interaction of HMG genes and some sex key genes

Possible interactions between the HMG genes were determined by quantification of the transcript level of the HMG, and also some other sex-related, genes in each of the HMG mutant strains under conditions suitable for sex. Results are presented in **Figure 4.25** showing that AN3549 appreared to be a main regulator controlling the expression of all of the tested genes; this result is consistent with the phenotype of the ΔAN3549 strain, deletion of AN3549 resulting in complete loss of fertility. In contrast, in *Podospora anserina* PaHMG5, which is an orthologue of AN3667, was reported to be the central regulator that controlled mating, whilst PaHMG8 (an orthologue of AN3549) was only a secondary regulator (Benkhali *et al.*, 2013).

4.6 Conclusion

High mobility group proteins belong to superfamily of transcription factors, the core HMG-box being conserved throughout the Eukaryota. They have diverse structures and roles within eukaryotic organism, one of the most prominent being sex determination including in fungi where HMG genes determine mating-type identity. The *A. nidulans* genome contains eight HMG genes; six of them were found to have an essential role in normal sexual development of *A. nidulans*, all of the HMG genes acting as inducers of sex. Deletion of two of these HMG genes (AN3667 and AN3549) resulted in a complete loss of fruiting body development, whereas deletion of other genes (AN1267, AN1962, AN2885, AN4734 and AN2755) resulted in a decrease in the number of cleistothecia formed compared to the parental strain. In addition it seems that AN3549 acts as a main regulator of mating in *Aspergillus nidulans*. This is an important finding as it confirms that HMG genes other than the previously characterised *MAT* genes are also required for sexual development of *Aspergillus* species.

Chapter 5 Potential for Sexual Development in Asexual Aspergilli

5.1 Introduction

The Phylum Ascomycota is the largest phylum of filamentous fungi in terms of number of species. Members were originally defined by the characteristic that they produced ascospores in asci during sexual development. However, the group is now defined on phylogenetic grounds. As a result only about 55% of the Ascomycota are known to have the ability to undergo sexual reproduction, a sexual state has not yet been detected for the rest of the species (Horwitz *et al.*, 2010). The lack of a sexual state in the remaining 45% of species is perhaps surprising given the many supposed evolutionary benefits of sex. One major advantage of sex over purely asexual reproduction is that sex between two genetically distinct partners results in genetic recombination through recombination and crossing over. This results in the production of offspring with increased genetic variation with the likelihood that some will be better adapted to harsh conditions and also will be able to get rid of deleterious mutation (Dyer *et al.*, 1992; Ene and Bennett, 2014). In contrast, the accumulation of deleterious mutations in asexually reproducing species can result in the extinction of these species (Ene and Bennett, 2014; Dyer *et al.*, 2016).

Although 45% of the Ascomycota are considered as asexual species, it is not certain that these species are entirely asexual or whether sexual reproduction has simply not yet been detected (Taylor *et al.*, 1999). Indeed, many population studies have provided evidence for the occurrence of limited genetic recombination in some of these asexual reproducing fungi i.e. they exhibit 'cryptic sexuality' (Taylor *et al.*, 1999; Paoletti *et al.*, 2005). Many researchers have tried to answer the question: why are these species asexual? Were they previously sexual species that have lost sex, or is sex occurring in nature but it has

gone unnoticed? In pioneering molecular studies a functional *MAT2* homologue of the heterothallic *Cochliobolus heterostrophus* was found in the asexual reproducing fungus *Bipolaris sacchari*, a close relative according to phylogenetic studies (Sharon *et al.*, 1996). Similarly, mating-type genes have since been identified in a number of other asexual reproducing species. For example in *Candida albicans*, a pathogenic fungal species considered to reproduce only by asexual means, a complete set of mating-type genes resembling those of *Saccharomyces cerevisiae* were identified by Hull and Johnson (1999). This led to the final induction of mating in *C. albicans* under certain condition, achieved by the construction of strains expressing either *MTLa* (mating type like) a or *MTLα* because the laboratory strain of *C. albicans* is diploid and heterozygous in MTL loci; after mating a tetraploid cell was produced (Hull *et al.*, 2000).

5.1.1 Asexuality in the genus *Aspergillus*

The genus *Aspergillus* belongs to the phylum Ascomycota and consists of approximately 300-350 species divided into a series of sections (Varga *et al.*, 2014). It contains species with a cosmopolitan distribution in nature and having diverse ecological roles; some of them beneficial and other having deterimental effects on human, plant and animal life (Dyer, 2007; Perrone *et al.*, 2007; Benoit *et al.*, 2013). For example *A. sydowii*, a soil saprophytic fungus, is an opportunistic pathogen that can infect sea fan corals (*Gorgonia ventalina*) (Nagelkerken *et al.*, 1997; Ellner *et al.*, 2007; Bruno *et al.*, 2011). *Aspergillus clavatus* (section *Clavati*) is an economic and medically important species, it produces various toxins including patulin which can contaminate with apple juice (Varga *et al.*, 2007) and it also causes extrinsic allergic alveolitis known as farmer's or malter's lung (Lopez-Diaz and Flannigan, 1997; Mckenzie *et al.*, 2004). Conversely many members of the black aspergilli, a group of *Aspergillus* species characterized by the formation of black conidia, are widely used in industry (Abarca *et al.*, 2004). For example, *Aspergillus niger* has the ability to secrete various type of enzymes to the external environment and this feature, and

its safe use status, has been industrially exploited for the production of various chemical components and enzymes (Schuster *et al.*, 2002; Pel *et al.*, 2007). However, another member of the black aspergilli *Aspergillus carbonarius* produces a carcinogenic and nephrotoxic mycotoxin called ochratoxin A that can contaminate a variety of foodstuffs (Fungaro *et al.*, 2004; Perrone *et al.*, 2007).

About one-third of *Aspergillus* species have the ability to propagate sexually, the majority are only known to reproduce by asexual mean involving the production of conidiospores (Dyer and O'Gorman, 2012; Krijgsheld *et al.*, 2013; Varga *et al.*, 2014). Sexually reproducing *Aspergillus* species are either homothallic (self compatible) or heterothallic (self sterile) (Dyer *et al.*, 2007; Dyer and O'Gorman, 2012). Despite the lack of known sexual cycle in these asexual species, there is evidence from phylogenetic analysis of both mitochondrial and nuclear genomes confirming the evolution of asexual species from a sexual ancestor (Geiser *et al.*, 1996). The availability of complete genome sequences for many fungal species has subsequently provided a valuable tool for genetic analysis and to evaluate the potential of sexuality in supposedly asexual species (Kück and Pöggeler, 2009; Dyer and O'Gorman, 2011). Various tests have been used to determine the possibility of a cryptic or undiscovered sexual state for supposedly asexual species such as evidence of recombination; discovery of previously unnoticed sexual structures, and/or the presence and expression of sex-related genes (Varga *et al.*, 2014). For example, population genetic analysis of several asexual fungi has revealed genetic recombination as detected in *Cryptococcus neoformans* serotype A via multilocus genotypes analysis (Litvintseva *et al.*, 2003). Similarly, in *Penicillium roqueforti* the detection of RIP-like (repeat induced point) mutations has indicated a possible sexual state because this gene silencing phenomenon occurs in the dikaryotic phase during sexual reproduction (Ropars *et al.*, 2012).

Aspergillus fumigatus is a saprophytic fungus in the soil, which can also cause invasive infections in immunosuppressed patients (Pöggeler, 2002). It previously had no known sexual cycle but genome analysis revealed the presence of homologues of the *Neurospora crassa MAT1-2* HMG mating-type gene, *ppg1*

pheromone precursor and both pheromone receptors within the genome of *A. fumigatus* (Pöggeler, 2002; Varga, 2003; Galagan *et al.*, 2005). The complementary *MAT1-1* gene was later identified, consistent with a heterothallic breeding system. The distribution of both mating types was then investigated within clinical and environmental isolates and a near equal distribution of *MAT1-1* and *MAT1-2* isolates detected. Also, transcription of the mating-type, pheromone precursor and pheromone receptor genes was detected (Paoletti et al., 2005). Finally a sexual stage was discovered in *A. fumigatus*, which required an extended incubation for six month on oatmeal agar media (O'Gorman *et al.*, 2009). Similarly, sexual stages have recently been identified in the aflatoxin producing fungi *Aspergillus flavus* and *Aspergillus parasiticus*, which also required prolonged incubation for 6-9 months; both species were previously only known to propagate by asexual means (Horn *et al.*, 2009a; Horn *et al.*, 2009b). These results suggest that many supposedly 'asexual' *Aspergillus* species might have the potential for sexual reproduction.

5.2 Aims of the work

The genus *Aspergillus* contains several species of economic and medical importance. The majority of these species is only known to reproduce by asexual means. It would be of major benefit to discover sexual reproduction in these species because it would provide a tool to allow strain improvement for industry and allow classical genetic analysis. The aim of work in the present chapter was therefore to evaluate the possibility of sexual reproduction in a range of asexual *Aspergillus* species being studied as part of an international genome consortium (R De Vries, PS Dyer pers. comm). This was achieved by BLAST searching the newly available genomes of these species for the presence and absences of key sex genes encoding mating-type, pheromone precursor and pheromone receptor proteins. If these genes were found to be present, RT-PCR experimental studies were then conducted to determine the possible expression of these genes under conditions thought to be conducive for sexual reproduction.

5.3 Materials and methods

5.3.1 Materials

Oatmeal Agar

Oatmeal agar was prepared as described by O'Gorman *et al.* (2009). 40 g/ L of Quaker porridge Oats (for UK Oatmeal agar) was mixed with tap water, and heated till boiling with continuous stirring; then the heater was lowered and left to simmer for a further 45 min. The mixture was filtered through two layers of muslin (cheese cloth) to remove the solid particles; the filtrate had a creamy colour with a slightly viscous texture. The filtrate was made up to a of 1 litter volume and 20 g of agar (Oxoid, UK) was added. The mixture was then autoclaved at 117 ^{0}C for 30 min with the cooling jacket turned off.

CTAB-RNA extraction buffer

Stocks of 5 M of Sodium chloride, 1 M Tris-HCl pH 8 and 0.5 M of EDTA pH 8 were prepared in DEPC-treated water and autoclaved at 121 ^{0}C for 15 min. A CTAB-RNA extraction buffer was then prepared by mixing 1.4 M sodium chloride, 25 mM Tris-HCl pH 8, 100 mM EDTA pH 8, 2% PVP (polyvinylpyrrolidone, molecular weight 360,000), 2% v/v of hexadecyl trimethyle ammonium bromide (CTAB) and 0.5 g/L of L-spermatine, which were then made up to final volume of 200 ml and autoclaved at 121 ^{0}C for 15 min. 2% ß-mercaptoethanol (v/v) was then added directly before use (Jaakola *et al.*, 2001).

5.3.2 Methods

5.3.2.1 Fungal strains and culture maintenance

Several *Aspergillus* species were used in this study, including both asexually and sexually reproducing species. These were provided as part of an international genome consortium study (R De Vries, PS Dyer pers. comm). Sexually reproducing (control) species were *A. flavus* strain 57-6 (CBS 128202), *A. fumigatus* 47-5 (Af293) and *A. tubingensis* 76-46 (CBS 134.48); while asexual species included *A. clavatus* 65-21 (CBS513.65), *A. sydowii* 96-1 (CBS 593.65), *A. versicolor* 95-1 (CBS 795.97), *A. wentii* 97-1 (DTO 134-E9) and *A. zonatus* 94-1 (CBS 506.65). In addition some asexual black apergilli were included in studies, namely *A. aculeotus* strain 87-5 (CBS 172.66), *A. brasiliensis* 81-10 (CBS 101740), *A. carbonarius* 82-15 (DTO 115-B6), *A. feotidus* 83.4 (CBS 106.47) and two *A. niger* strains 8-1 (N402) and 8-152 (CBS 113.46). All of these species were obtained from the University of Nottingham BDUN culture collection and grown ACM agar slopes (**Section 2.1.1**) before storage at 4 ^{0}C for subsequent experiments.

5.3.2.2 BLAST searches

BLAST searching of newly available *Aspergillus* genome databases was performed to try and identify the presence of some essential genes for sexual reproduction. Protein sequence of previously identified mating-type, pheromone precursor and pheromone receptor genes of *Aspergillus nidulans* were used as search terms (**Table 5-1**). BLASTP searches were performed of protein databases available at both the JGI (http://genome.jgi.doe.gov/) and/or AspGD (http://www.aspergillusgenome.org/) websites. Hits that had a minimum expectation value of e^{-10-70} were considered significant. Synteny analysis (SYBIL) was then performed using the Jaccard Orthologous cluster option at AspGD or the synteny option at the JGI website. Based on high levels of similarity and gene

synteny, predicted proteins were annotated as the corresponding functional proteins.

Table 5-1. Reference sequences from *A. nidulans*

Role	Gene	Protein Accession
Mating type	*MAT1*	AN2755
	MAT2	AN4734
Pheromone precursor	*ppgA*	AN5791
Pheromone receptor	*preA* (*gprB*)	AN7743
	preB (*gprA*)	AN2520

5.3.2.3 Extraction of DNA

DNA was extracted from all of strains of *Aspergillus* species using a Nucleospin® plant II kit (Macherey-Nagel GmbH & Co. KG, Germany) according to manufacturer's instruction. The cultures were grown in *Aspergillus* complete media (ACM) broth (Paoletti *et al.,* 2007), incubated at 28 ^{O}C with shaking for two days. The culture was harvested by filtration through a sterile miracloth and washed with Phosphate buffer solution pH 7.5, then freeze dried and ground into a powder by motor and pestle. The freeze-dried powder was used for DNA extraction using Nucleospin® plant II kit as previously described (**Section 2.2.3**). 50 ng of extracted DNA was used as a template for PCR.

5.3.2.4 Extraction of RNA

RNA was extracted from mycelia grown on solid rather than liquid cultures. 50 µl of spore suspension containing 1x 10^5 were spread over a Nylon filter membrane (pore size 0.20 µm) placed over the surface of a 5 cm Petri dish containing 10 ml of UK Oat meal agar (OMA). This media was used for all species except for *A. versicolor*, which did not grow well on OMA; instead ACM was used for growth of this latter species. Cultures were incubated in darkness at 32 ^{O}C, and the plate was sealed with two layer of parafilm after 15-18hr of incubation. RNA was extracted after 4 and 8 days of incubation [as per previous work with *A. fumigatus* and *A. nidulans* (Paoletti et al. 2005, 2007)] by harvesting mycelium from the surface of the Nylon filter membranes (normally 4 plates were used per extraction), and snap-frozen in liquid nitrogen. Two different methods were used for RNA extraction. For non-black aspergilli the trizole method was used as previously described (**Section 2.213**) whereas for black aspergilli the following CTAB method was used.

RNA extraction by CTAB method

The method used to extract RNA from the black aspergilli was as described by Darbyshir (2013). 100 mg of frozen powder mycelium was transferred into a 2 ml Eppendorf tube containing 750 ul of CTAB extraction buffer (**Section 5.3.1**), vigorously vortexed then incubated at 65 ^{0}C for 15 min. An equal amount of chloroform was added to the mixture and mixed by inverting the tube, then the mixture was spun down at 11000 x **g** at 4 ^{0}C for 10 min. The aqueous layer was transferred into a new 2 ml Eppendorf tube then 1/3 volume of 8 M of lithium chloride was added to the tube and incubated overnight at 4 ^{0}C to precipitate the RNA. The precipitated RNA was collected by centrifugation at 11000 x **g** at 4 ^{0}C for 60 min then the pellet was washed with 200 µl of 70% of ethanol prepared in DEPC water and air dried in a laminar flow cabinet before being dissolved in 100 µl of DEPC water.

The extracted RNA was further purified using a NucleoSpin® RNA kit (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's

protocol. Briefly, 600 µl of buffer RA1- ethanol-premix (300 µl of RA1 mixed with 300 µl of ethanol 96-100% previously) was added to 100 µl of the RNA sample, mixed by pipetting and 700 µl were loaded onto a NucleoSpin® RNA clean up column (light blue ring) placed in a 2 ml collection tube which was then spun at 11,000 x **g** for 30 sec. The flow through was discarded and 350 µl of MDB was added to the membrane to desalt the silica membrane. Next followed a DNase treatment step. 10 µl of DNase (provided in the kit) was premixed with 90 µl of rDNase buffer and 95 µl of this mixture was added to the membrane and left at room temperature for 1h. The membrane was then washed in three steps: 200 µl of RAW2 and 600 µl of RA3 were added respectively for first and second steps, each time being spun at 11,000 x **g** for 30 sec. and the flow through discarded. Finally 250 µl of RA3 was added and centrifuged for 2 min at 11,000 x **g** to wash and dry the membrane. The column was transferred to a 1.5 ml tube provided with the kit; 60 µl of Elution buffer was added to the membrane and centrifuged for 1 min, with the flow through containing the RNA stored in ice.

The extracted and purified RNA was quantified by a Nanodrop ND-1000 (Labtech International); the purity and integrity of RNA was checked by PCR and gel electrophoresis, respectively. To confirm the RNA purity and absence of DNA, standard PCR was performed using 1 µl of RNA sample as a template (and genomic DNA as a control), trying to amplify the house-keeping gene actin. To check RNA integrity, 3 µl of RNA sample were mixed with an equal amount of RNA loading dye (**Section 2.1.2**) and resolved on 1.2% TBE agarose gels at 120v for 30 min before final analysis under UV light using a Bio-Rad chemidoc XRS+, with Quantity one 4.6.6 program (BioRad, Hemel, Hempstead, Hertfordshire, UK). Lanes were checked for the appearance of 40S and 60S ribosomal sub units RNA bands as an indication of RNA quality. RNA samples were then stored at -80 ^{o}C.

5.3.2.5 cDNA synthesis

cDNA was synthesized using 500 ng of total RNA from both 4 day and 8 day extractions from all of the tested strains. SuperScript III kit (Invitrogen) was used

to reverse transcribe the RNA and produce the first-strand cDNA (**Section 2.2.14**). cDNA were stored at -20 ^{o}C with 1 µl being used as a template for later RT-PCR.

5.3.2.6 Amplification of key sex genes

The identified sex genes from each tested *Aspergillus* species were amplified by PCR using both genomic and cDNA as a template for PCR, with the identified sequences being used to design primers. Primer sets were designed to span a putative intron(s) if possible to allow the differentiation of genomic and cDNA by differential size products (**Appendix 14**). The Phusion High Fidelity PCR kit was used according to manufacturer's instruction for amplifications. 1 µl of each genomic DNA and 4 day and 8 day cDNA were used as templates for amplification of *MAT1*, *MAT2*, *ppgA*, *preA*, *preB* and *ActA* (Actin A), the latter used as a 'house keeping' control gene. PCR products were analysed on 2.0-2.5% agarose gels (**Section 2.2.4**).

5.4 Results

5.4.1 BLAST search

Results of BLAST searches are presented in **Table 5.2**. All of the asexual species (*A. clavatus*, *A. sydowii*, *A. versicolor*, *A. wentii*, *A. zonatus*, *A. aculeotus*, *A. brasiliensis*, *A. carbonarius*, *A. feotidus* and *A. niger*) were found to contain either a *MAT1-1* or *MAT1-2* gene encoding an alpha or high-mobilty group domain protein, respectively, consistent with heterothallism. All of the asexual species were also found to contain *ppgA* homologues encoding a pheromone precursor, and *preA* and *preB* homologues encoding pheromone receptors. Furthermore, the gene synteny was found to be conserved across all species including known sexual species (**Figure 5.1**).

Table 5.2 Summary of BLASTP searches of putative genes related to mating and pheromone signalling

Species	Strain	Repro-ductive Mode[1]	Protein counts	Source	Mating		Pheromone signalling		
					MAT1	MAT2	PpgA[2]	PreA (GprB)[4]	PreB (GprA)[4]
Aspergillus acidus *(A. foetidus)*	CBS 106.47	A	13530	JGI	Aspfo1_0180958	Absent	Aspfo1_0035576	Aspfo1_0331625	Aspfo1_0197156
A. aculeatus	ATCC16872	A	10828	JGI	Absent	Aacu16872_042173	Aacu16872_060395	Aacu16872_036541	Aacu16872_047726
A. brasiliensis	CBS 101740	A	13000	JGI	Absent	Aspbr1_0167991	Aspbr1_0031661	Aspbr1_0077623	Aspbr1_0657476
A. carbonarius	ITEM 5010	A	11624	JGI	Absent	Acar5010_001991	Acar5010_003535 (Acar5010_045261)[3]	Acar5010_016080	Acar5010_508024
A. clavatus	NRRL 1	A	9121	AspGD	ACLA_034110	Absent	ACLA_088140	ACLA_012620	ACLA_041790
A. flavus	NRRL 3357	S-HE	12604	AspGD	Absent	AFL2G_11189	AFL2G_02931	AFL2G_06286	EQ963477.1[5]
A. nidulans	FGSC A4	S-HO	29005	AspGD	AN2755	AN4734	AN5791	AN7743	AN2520
A. niger	CBS 513.88	A	42912	AspGD	An11g10180	Absent	An18g06770	An03g03890	An09g04180
A. sydowii		A	13620	JGI	Absent	Aspsy_0087884	Aspsy1_0850417	Aspsy1_0058761	Aspsy1_0731872

A. terreus	NIH2624	S-HE	10406	AspGD	ATEG_08812	Absent	ATEG_07407	ATEG_08338	ATEG_03500
A. tubingensis	CBS 134.48	S-HE	12322	JGI	Asptu1_0124452	Absent	Asptu1_0162956	Asptu1_0195628	Asptu1_0135593
A. versicolor	CBS 583.65	A	13228	JGI	Absent	Aspve1_0082222	Aspve1_0711099	Aspve1_0132409	Aspve1_0081374
A. wentii	DTO 134E9	A	12442	JGI	Absent	Aspwe1_0184745	Aspwe1_0026227	Aspwe1_0147264	Aspwe1_0108900
A. zonatus		A	11362	JGI	Absent	Aspzo1_0146122	Aspzo1_0064544	Aspzo1_0061636	Aspzo1_0153857

[1]A=Asexual; S-HO = Sexual homothallic; S-HE = Sexual heterothallic

[2]E values for the relatively short PpgA protein (approx. 100-110 amino acids) were in the region 1 x 10^{-6} to a x 10^{-30}

[3]The same gene annotated twice

[4]Gene synonyms

[5]Gene present but not auto-annotated when searched for using AO090701000605. See https://www.aspergillusflavus.org/genomics/

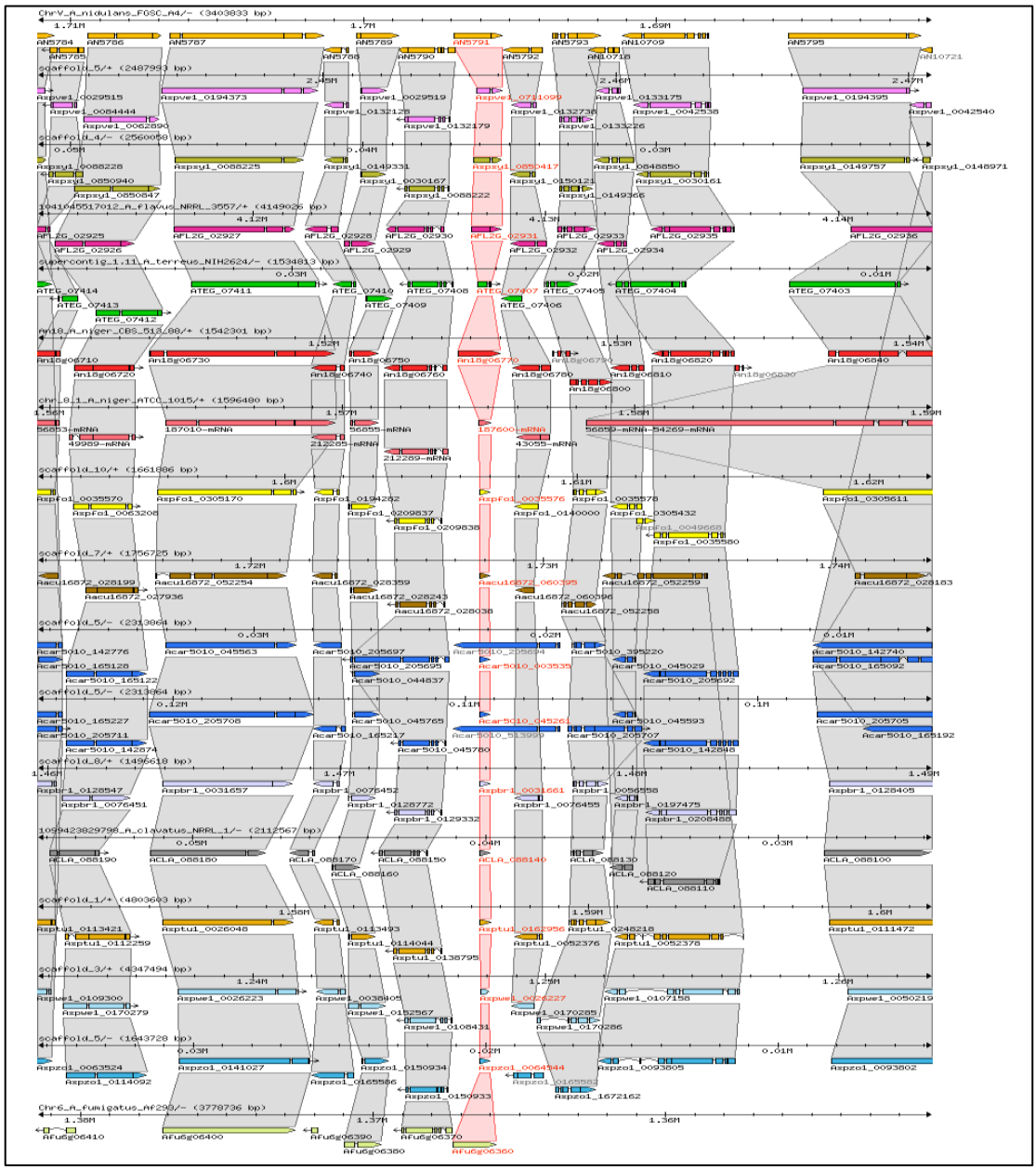


Figure 5-1. Representative example showing conserved synteny of the alpha pheromone precursor *ppgA* gene (AN5791) of *Aspergillus nidulans* and other *Aspergillus* species (shown as vertical pink track).

5.4.2 RT-PCR analysis

Semi-quantitative RT-PCR analysis revealed that almost all of the *MAT1-1* or *MAT1-2* genes together with *ppgA*, *preA* and *preB* were expressed in all of the asexual species (*A. clavatus*, *A. sydowii*, *A. versicolor*, *A. wentii*, *A. zonatus*, *A. aculeotus*, *A. brasiliensis*, *A. carbonarius*, *A. feotidus* and *A. niger*) in a similar manner as in control sexual species (*A. flavus*, *A. fumigatus* and *A. tubingensis*) after both 4 and 8 days growth (**Figure 5.2;** non-black aspergilli) (**Figure 5.3;** black aspergilli), demonstrating that key genes linked to sexual reproduction appear to be functionally active in all of the test asexual species. However, it was noted that there were some double bands for some cDNA amplifications corresponding to both genomic DNA and the processed RNA product (e.g. *A. clavatus preA*; **Figure 5.2**) and that some amplification products were very weak corresponding to low expression (e.g. *A. niger MAT*; **Figure 5.3**). Also no *MAT* gene expression could be detected for *A. versicolor (*even with genomic DNA). (**Figure 5.2**) and strangely the *A. fumigatus MAT* gene showed a band corresponding to the processed cDNA even in the genomic control (**Figure 5.2**).

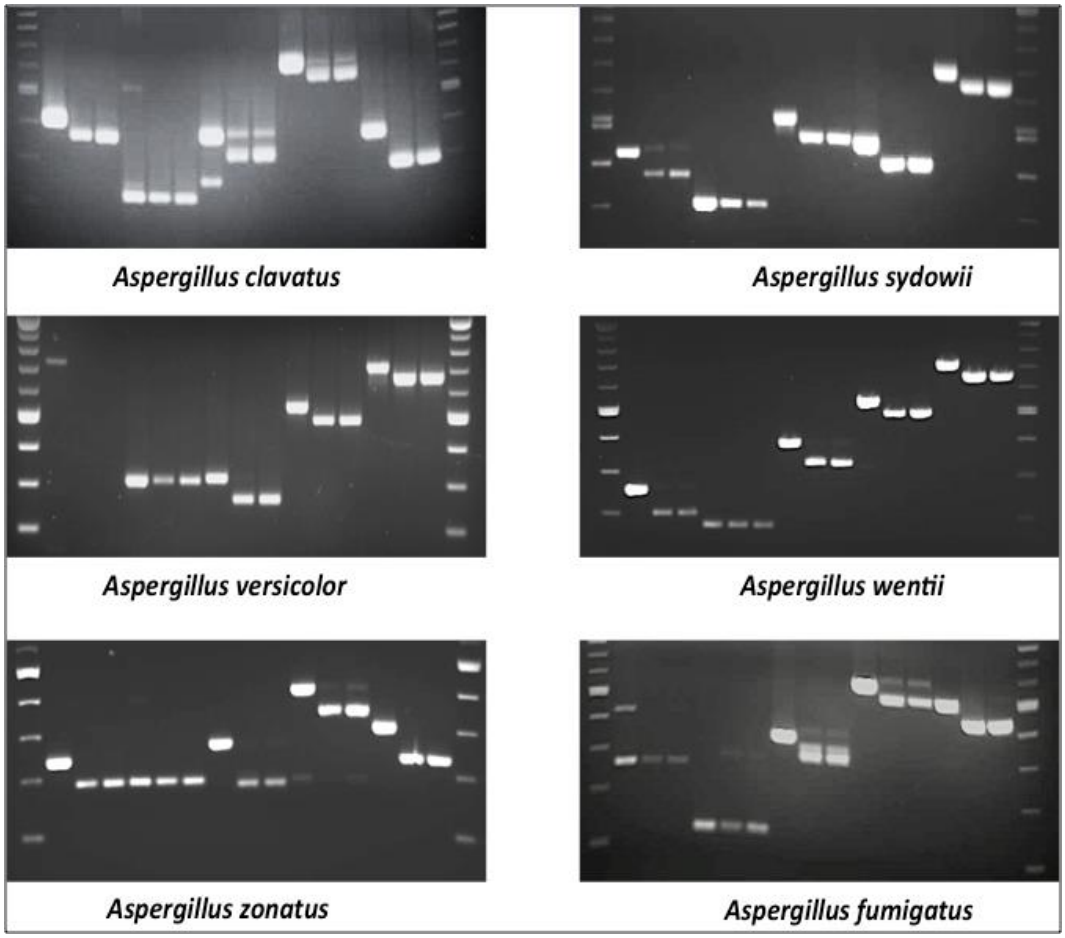


Figure 5-2 2.5% agarose gels showing expression of mating and pheromone signalling pathway genes in some non-black asexual aspergilli compared to *A. fumigatus* as a control sexual species. For all panels: Lanes 1 and 17, 100 bp DNA ladder; lane 2, PCR of either *MAT1-1* or *MAT1-2* from genomic DNA; lanes 3 and 4, PCR of either *MAT1-1* or *MAT1-2* from cDNA from 4 and 8 days growth, respectively; lane 5, PCR of *ppgA* from genomic DNA; lanes 6 and 7, PCR of *ppgA* from cDNA from 4 and 8 days growth, respectively; lane 8, PCR of *preA* from genomic DNA; lanes 9 and 10, PCR of *preA* from cDNA from 4 and 8 days growth, respectively; lane 11, PCR of *preB* from genomic DNA; lanes 12 and 13, PCR of *preB* from cDNA from 4 and 8 days growth, respectively; lane 14, PCR of *actA* from genomic DNA; lanes 15 and 16, PCR of *actA* from cDNA from 4 and 8 days growth, respectively. Note that the presence of a smaller size amplicon from the cDNA template indicates the excision of an intron.

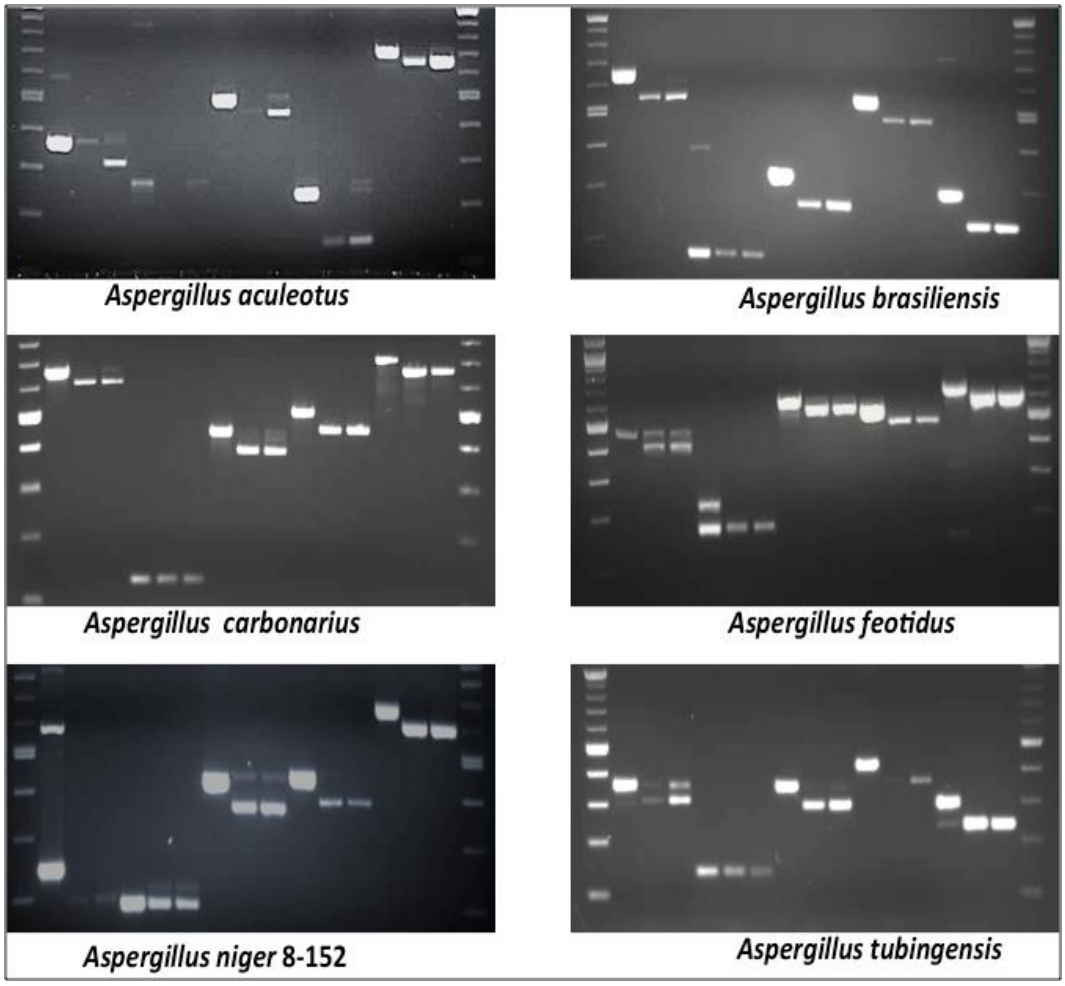


Figure 5-3 2.5% agarose gels showing expression of mating and pheromone signalling pathway genes in some black asexual aspergilli compared to *A. tubingensis* as a control sexual species. For all panels: Lanes 1 and 17, 100 bp DNA ladder; lane 2, PCR of either *MAT1-1* or *MAT1-2* from genomic DNA; lanes 3 and 4, PCR of either *MAT1-1* or *MAT1-2* from cDNA from 4 and 8 days growth, respectively; lane 5, PCR of *ppgA* from genomic DNA; lanes 6 and 7, PCR of *ppgA* from cDNA from 4 and 8 days growth, respectively; lane 8, PCR of *preA* from genomic DNA; lanes 9 and 10, PCR of *preA* from cDNA from 4 and 8 days growth, respectively; lane 11, PCR of *preB* from genomic DNA; lanes 12 and 13, PCR of *preB* from cDNA from 4 and 8 days growth, respectively; lane 14, PCR of *actA* from genomic DNA; lanes 15 and 16, PCR of *actA* from cDNA from 4 and 8 days growth, respectively. Note that the presence of a smaller size amplicon from the cDNA template indicates the excision of an intron.

5.5 Discussion

Members of the fungal kingdom exhibit diverse modes of reproduction. Most of them have the ability to reproduce by both sexual (selfing or outcrossing) and asexual means. About 20% of them are only known to reproduce asexually, with no sexual state described (Dyer and Paoletti, 2005), and have been traditionally classified as imperfect fungi in the phylum Deuteromycotina. The genus *Aspergillus* contains species that propagate by both asexual and sexual reproduction, but the majority (about 64%) of them have no known sexual state (Geiser, 2009; Lee *et al.,* 2010a), although there is accumulating evidence that some of these species may have the ability to undergo sex (Dyer & O'Gorman 2011, 2012). As mentioned in the introduction to this chapter, the genus *Aspergillus* includes many species with important roles in food production, pathogenicity and industrial useage. Unfortunately, many of these species only have an asexual cycle, which has hindered the possibility of developing improved strains, with desirable characters for industrial purposes, via the sexual cycle (Dyer *et al.*, 2003).

Work in the current chapter aimed to try and detect the presence and assess the possible functionality of some key sex genes necessary for the onset of sexual reproduction (mating-type, pheromone precursor and pheromone receptor genes) in some medically, ecologically and biotechnologically important asexually reproducing *Aspergillus* species being studied as part of an international genome consortium project. Blast searches indicated that all of the asexual species under investigation (*A. clavatus*, *A. sydowii*, *A. versicolor*, *A. wentii*, *A. zonatus*, *A. aculeotus*, *A. brasiliensis*, *A. carbonarius*, *A. feotidus* and *A. niger*) contained an apparently intact mating-type gene (i.e. lacking any stop codon or frameshift mutation), and that this was either of the *MAT1-1* or *MAT1-2* genotype, consistent with a heterothallic breeding system. In such heterothallic breeding systems, either a *MAT1-1* or *MAT1-2* mating-type gene occupies the *MAT* locus, a compatible partner of the complementary mating type is necessary for sexual reproduction to occur (Dyer *et al.*, 1992; Coppin *et al.*, 1997). Pheromone

precursor and receptor genes (both **a** and alpha types) were also found to be present in all of the test species.

Semi-quantitative RT-PCR was then used to check whether these mating-type and pheromone signalling genes were expressed as mRNA as an indication of possible functionality of these genes. Expression of all genes was detected similar to levels seen in some control species (*A. flavus, A. fumigatus* and *A. tubingensis*) with known sexual states at 4 and 7 d growth (**Figure 5.2 and Figure 5.3**). The existence and expression of these genes in these asexual species is of great significance as the findings are indicative of cryptic sexuality or that at least the species are evolved from sexual ancestors and have only recently lost the ability to reproduce sexually. Ni *et al.* (2011) postulated that the existence of pheromone and their cognate receptors within the genome of any species might be indicative of sexual capacity of that species. However, Turrà et al. (2015) have very recently shown that fungal pheromone receptors might also be involved with chemotropic sensing of host plant signals.

Previous studies have identified over 200 putative genes with potential roles in fungal sexual development. Of these, more than 70 genes have been experimentally characterized in the aspergilli and shown to be involved in sexual reproduction, having roles in cell identity, environmental signalling and the development of fruiting bodies and meiospores (reviewed by Dyer and O'Gorman, 2012). Lack of and/or mutation in any of these genes might result in the loss of a sexual cycle. However, all studies to date have failed to detect such gene deletion or mutations; instead supposedly asexual species appear to contain intact sets of sexual genes (Varga *et al.*, 2014). In addition there is other evidence supporting the latent sexual potential in supposedly asexual species (Dyer and Paoletti, 2005). Genome sequencing of *A. niger* revealed the presence of RIP-like (repeat induced point mutation) within the genome of *A. niger*, consistent with the presence of sex (Braumann *et al.*, 2008). Indeed, recently Frisvard and coworkers (2014) were able to induce sclerotial formation in a strain of *A. niger* grown on Czapek yeast extract agar containing raisins. Previously *A. niger* was not reported to produce sclerotia. This finding is very important because sclerotia formation is

considered as a prerequisite for sexual reproduction in the section *Nigri* (Frisvad *et al.*, 2014). These findings further confirm the possibility of cryptic sex in *A. niger*, and under the correct environmental condition it might be possible to induce the sexual cycle in *A. niger* as seen recently in the other related species *A. tubingensis*, *A. flavus*, *A. parasiticus* and *A. fumigatus* (O'Gorman *et al.*, 2009; Horn *et al.*, 2009a; Horn *et al.*, 2009b; Horn *et al.*, 2013).

Despite these findings, sexual reproduction has not been yet observed in several asexual aspergilli and it is possible that mutation in some other sex genes not yet characterized might be the cause of loss of sexual fertility (Dyer and O'Gorman, 2011). In chapters 3 and 4 of the present work, additional genes have been identified with a potential role in sexual development, most of them have not been characterized previously, so it is possible that mutation or loss of any of these genes might have resulted in asexuality. Other possible reasons for asexuality might be a lack of compatible partners of the correct mating type in nature (all of the tested species being self-incompatible), simple failure to notice fruiting body structures in the environment, and/or a slow decline of sexual reproduction as a result of prolonged period of asexual propagation in the laboratory (Dyer and Paoletti, 2005).

One further final implication of work from the present chapter is that it suggests that the majority of 'asexual' aspergilli might in fact have heterothallic breeding systems. This indicates that heterothallism is likely to be the ancestral reproductive state of the aspergilli, rather than homothallism as proposed by Geiser *et al.* (1998) based on the apparent dominance of homothallism among *Aspergillus* species. This matter will be discussed further in the final general discussion (**Section 6.3**).

5.6 Conclusion

Genes required for early sexual development (*MAT*, *ppgA*, *preA* and *preB*) were detected in the genomes of a number of asexual *Aspergillus* species by BLAST search analysis. All of these genes seem to be functional as they are expressed at the mRNA level under conditions suitable for sexual reproduction. Also the asexual reproducing species in the study appear to exhibit heterothallic breeding systems, each species containing either a *MAT1-1* or *MAT1-2* mating-type gene. This supports the presence of a heterothallic ancestral state in the aspergilli.

Chapter 6 General Discussion

Reproduction is one of the most variable processes in living organisms. Within the fungal kingdom, species can reproduce by both sexual and asexual means, with some species exhibiting both forms of reproduction whereas others are reported to be restricted to either asexual or sexual propagation. Having an understanding of the reproductive mode of an organism is of great importance for gaining insights into the potential for the evolution of species such as pathogens (Dyer *et al.,* 2012), while the sexual cycle also provides a valuable tool for improving strains used in industrial applications such as in *Penicillium chrysogenum* where sexual crossing was used to generate a strain with new traits beneficial for penicillin production (Böhm *et al.,* 2013). Work in the present thesis focused on an investigation of the genetic basis of sexual reproduction in ascomycete fungi using *Aspergillus* as a model organism. The genus *Aspergillus* contains both sexual and asexually reproducing species, but the majority of them propagate asexually, which provided an excellent system to study the genetic basis of reproductive modes in fungi.

6.1 Identification of novel genes regulating sexual development

One primary aim of the current study was to identify and characterise novel genes involved in sexual reproduction of *Aspergillus* species, a topic of both fundamental and applied importance. More than 75 genes have previously been experimentally characterized in the aspergilli and shown to be involved in sexual reproduction (reviewed by Dyer and O'Gorman, 2012). However, it has been suggested that more than 400 genes might participate in different stages of sexual development (Dyer *et al.,* 1992). Therefore, it was considered likely that there are additional genes, which participate in sexual reproduction but had not yet been characterized; the discovery of such additional genes would provide insight into

the control of sexual development and possible evolution of asexuality. To achieve this aim, this study exploited data from previous work in which a set of genes had been identified that were differentially expressed in complementary *MAT1-1* and *MAT1-2* mating-type strains of *A. oryzae*, most of which were of unknown function.

Thirty-three genes had been identified which were differentially expressed according to mating type. Out of these 20 homologous genes were found by BLAST searching of the genome of *A. nidulans*, which was used as an experimental model to investigate the role of these genes because *A. oryzae* only reproduces by asexual means (Kobayashi *et al.*, 2007). In contrast, *A. nidulans* has the ability to reproduce sexually and asexually, and furthermore despite being homothallic it retains the ability to outcross, which proved advantageous for restoring deleted genes by crossing with other *A. nidulans* strains. A systematic gene deletion programme was performed, depending on the availability of deletion cassette for homologous gene replacement in *A. nidulans* from the FGSC in the USA, to investigate their role in sexuality. A total of thirteen genes were successfully deleted. Significantly, 11 of these genes were found to have a role in sexual development. Especially notable were genes AN3562 and AN8656, whose deletion resulted in a complete loss of sexual fertility. These results may provide additional insights into the genetic basis of asexuality in aspergilli, as it is possible that these genes might have become inactive in asexual species due to the accumulation of deleterious mutation(s) as a result of continuous propagation by asexual reproduction (Dyer *et al.*, 1992; Dyer and O'Gorman, 2011; Ene and Bennett, 2014). Therefore in any future genomic screens for asexuality in the aspergilli (and beyond) it is argued that it is vital that genes AN3562 and AN8656 should be included in the list of 'essential sex genes' used to interrogate asexual species. On other hand, deletion of most of these genes resulted in a moderate change in fertility. This in itself was an important finding as it indicates that such genes may represent a class of genes which moderate the fertility of sexual crosses, helping to explain the wide variation in sexual fertility seen in many sexual crosses despite mating-type compatibility (Dyer *et al.*, 1992). By altering

the expression of such genes it might be possible to increase the fertility of some isolates otherwise having low fertility.

Among the genes shown to have a significant effect on fertility of crosses, the *ppgA* gene, which encoded an alpha pheromone precursor protein, was of especial interest. Pheromone signalling has previously been shown to be necessary for sexual identity and development in a range of ascomycete species. As perhaps expected, deletion of *ppgA* resulted in a dramatic decrease in fruit body formation, similar to other fungi. Therefore our expectation was that if pheromone expression could be increased that this might lead to an increase in fruit body formation. Unfortunately, overexpression of the *ppgA* alpha pheromone gene under the constitutive *gpd*A promoter of *A. nidulans* resulted in a more severe phenotype. Under conditions suitable for sex only a few small colonies were formed with cleistothecia absent. These finding indicate that tight expression levels of pheromones within a limited range are required for normal fruit body formation.

6.2 Role of genes encoding high-mobility group in sexual development of *Aspergillus nidulans*

High mobility group (HMG) domain proteins belong to a superfamily of transcription factors that are conserved through the Eukaryota, being involved in the regulation of a variety of cellular process including sexual reproduction. Previous work had already shown that sexual identity in fungi is determined by HMG genes located at the *MAT* locus with two complementary mating-type genes found in the Ascomycota; *MAT1-1* encodes an α-domain and *MAT1-2* encodes an HMG- box domain both with an important role in sexual development. But additional HMG domain genes are now known to be present in the genomes of filamentous fungi. For example, 12 HMG genes were found in *Podospora anserina*, with 11 of them found to have important roles in sexual development (Benkhali *et al.*, 2013). Also in *Fusarium graminearum* several HMG genes were found to have a role in sexual fertility (Son *et al.*, 2011).

Work in the present study was started before the pioneering work of Benkhali *et al.* (2013) was published. A key aim of the present study was to investigate the presence of other HMG gene in the genome of *A. nidulans* and if HMG genes were found, to then investigate any role in sexuality using a gene deletion approach. A total of nine HMG genes (including the *MAT1-1* α-domain) were found by BLAST searching using the conserved core HMG- box. Most of these newly identified HMG (7 out of 9 the genes) were found to have an important role in sexual development, with deletion of these genes reducing sexual fertility [one of these HMG genes, AN3667, was previously characterized by Ashour (2014)]. This was a key finding as it demonstrates the important and wider roles of HMG genes in fungal sexual development, beyond merely the previously identified *MAT1-1* and *MAT1-2* mating-type genes. These results may again provide addition insights into the genetic basis of asexuality in aspergilli, as it is possible that any of these HMG genes might have become inactive in asexual species due to the accumulation of deleterious mutation(s). Therefore in any future genomic screens for asexuality in the aspergilli (and beyond) it is again argued that it is vital that all of the HMG genes with roles in sexual fertility should be included in the list of 'essential sex genes' used to interrogate asexual species. And there is again the possibility that by altering the expression of such genes it might be possible to increase the fertility of some isolates otherwise having low fertility.

6.3 Potential for sexual development in asexual aspergilli

The sexual cycle has recently been discovered in some *Aspergillus* species that were previously thought to be strictly asexual. This has resulted in increased research interest in the study of asexual species and fungal sexual development, and the investigation of factors that might control sex at the molecular genetic level and factors that might be responsible for asexuality.

Several 'sex tests' are now available to investigate the potential of sex in supposedly asexual species (Dyer and O'Gorman 2011). One of these tests is the presence and functional expression of sex genes. This approach was applied in the

current study to assess the potential of sex in a number of supposedly 'asexual' *Aspergillus* species. To investigate whether the asexual species might have a cryptic sexual cycle, the presence and expression of firstly mating-type (*MAT*) genes conferring sexual identity (Dyer et al. 2016), and secondly genes from a pheromone signalling pathway involved with mating (Debuchy et al. 2010) was explored. All of the asexual species were found to contain either a *MAT1-1* or *MAT1-2* gene encoding an alpha or high-mobilty group domain protein, respectively, with local gene synteny conserved across all species. This is consistent with heterothallism, involving the presence of idiomorphs with either a *MAT1-1* or *MAT1-2* gene (Dyer *et al.*, 2016). All of the asexual species were also found to contain *ppgA* homologues encoding a pheromone precursor, and *preA* and *preB* homologues encoding pheromone receptors. The existence of mating-type genes in asexual species is of key importance, as it suggests not only that these species are derived from the sexual ancestor, but also that they might have some latent potential for sexual reproduction.

All of these asexual species appeared to have heterothallic breeding systems. This may explain the supposed asexuality of these species because heterothallic *Aspergillus* species are often much more 'fastidious' in requirements for sexual reproduction than homothallic species (Kwon-Chung and Sugui, 2009), as shown by the sexual requirements of *A. fumigatus*, *A. flavus*, and *A. parasiticus*, which were all previously considered asexual until precise conditions were found to induce the sexual cycle. All of these species required special nutrient and environmental conditions for sex to occur unlike related homothallic *Apergillus* species. The lack of isolates of compatible mating type in natural environments may be another reason for asexuality as shown in both *A. niger* and *A. carbonarius* where a bias in *MAT1-1* and *MAT1-2* isolates, respectively, has been detected (Darbyshir, 2013). Therefore the introduction of compatible mating type isolates could induce sex as has been reported for *Cryptococcus neoformans* (Lin *et al.*, 2005), *Fusarium* (*Hypomyces*) *solani* (Snyder *et al.*, 1975) and *Didymella* (*Ascochyta*) *rabiei* (Barve *et al.*, 2003).

A further interesting point raised by the present work was the heterothallic nature of all asexual species included in the current study, because all of them contained either *MAT1-1* or *MAT1-2* mating type genes. These finding may have an impact on the theory of the evolution of breeding system in the genus *Aspergillus*. There have been continued arguments over whether heterothallism is the ancestral state and homothallic species evolved from heterothallism or vice versa (Dyer, 2007). One of the key pieces of evidence to support a homothallic ancestral state is the predominance of homothallic species within the genus *Aspergillus*, as most of sexual reproducing species are homothallic and only a few species were heterothallic (Geiser *et al.*, 1998; Galagan *et al.*, 2005). However, results from the current study together with a discovery of sexual cycles in some previously considered asexual aspergilli are consistent with heterothallic breeding systems being dominant within *Aspergillus* species, which would instead support a heterothallic ancestor. Furthermore, the conserved heterothallic organisation with idiomorphs and synteny of the mating-type (*MAT*) locus observed in the current study also contrasts with recent reports showing variation in the organisation of *MAT* loci in homothallic *Aperrgillus* species (Galagan *et al.*, 2005; Paoletti *et al.*, 2007; Rydholm *et al.*, 2007; Dyer 2007; Ramirez-Prado *et al.*, 2008). Given these findings, the results overall strongly support the hypothesis that the aspergilli are evolved from a heterothallic ancestor, with homothallic species derived from this showing divergent forms of *MAT* gene arrangement as previously seen in *Cochliobolus* species (Yun *et al.*, 1999). This conclusion is of significance for future attempts to induce sexual reproduction in asexual species and possible genetic manipulation of *MAT* genes to induce sexuality (Dyer *et al.*, 2016).

6.4 Concluding remarks and further work

The combined results of chapters 3 and 4 have identified 13 and 10 extra genes, respectively**;** most of them have roles in sexual development of *Aspergillus* species **Table 6-1**.

Table 6-1 phenotypes of the *A. nidulans* mutant and over-expressed strains

Strain	Sexual fertility test		
	No. of cleistothecia	Diameter of cleistothecia	Ascospore formation
ΔAN1356	Decrease	Normal	-
ΔAN3239	Normal	Normal	+
ΔAN3275	Decrease	Large	+
ΔAN3562	No cleistothecia	No cleistothecia	-
ΔAN4686	Decrease	Large	+
ΔAN5791	Decrease	Large	+
ΔAN5993	Normal	Normal	+
ΔAN6881	Increase	Normal	+
ΔAN7818	Decrease	Normal	+
ΔAN8184	Increase	Normal	+
ΔAN8656	No cleistothecia	No cleistothecia	–
ΔAN11253	Decrease	Normal	+
ΔAN1958	Increase	Normal	+
ΔAN0879	Normal	Normal	+
ΔAN1267	Decrease	Normal	+
ΔAN1965	Decrease	Large	+
ΔAN4734	Decrease	Large	-
ΔAN2885	Decrease	Large	+
ΔAN2755	Decrease	Large	-
ΔAN3549	No cleistothecia	No cleistothecia	-
ΔAN3580	Normal	Normal	+
ΔAN5073	Decrease	Normal	+
ΔAN10103	Normal	Normal	+
OvEAN8184	Normal	Normal	+
OvEAN5791	No cleistothecia	No cleistothecia	-
OvEAN1962	Decrease	Large	+
OvEAN3667	No cleistothecia	No cleistothecia	-

+ Indicates ascospore formation, while – indicates no ascospore formation.

Dyer and O'Gorman (2012) previously published a schematic diagram showing the different stages of sexual development at which 75 previously identified genes acted in *Aspergillus* species as included as **Figure 1.11.** This figure has now been updated to include the information about the new genes as shown in **Figure 6.1.** These will require naming according to standard *A. nidulans* nomenclature in due course.

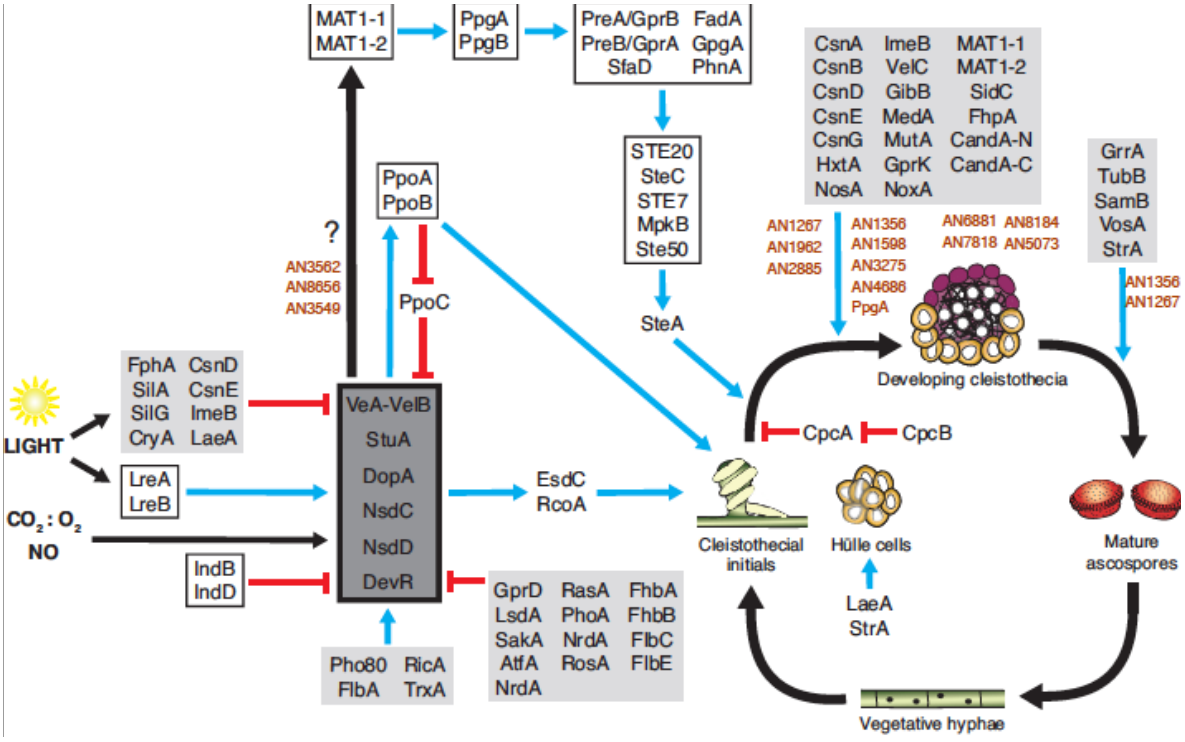


Figure 6-1 Suggested placements of the new proteins relative to other sex related proteins in aspergilli.

This raises some final suggestions for future work concerning these newly identified genes:

- Both the essential genes and those moderating sexual identity could be deleted in model heterothallic species to see if there was any effect on sexual fertility.
- Both the essential genes and those moderating sexual identity could be differentially expressed in model heterothallic species to see if there was any effect on sexual fertility i.e. whether it was possible to increase the productivity of sexual crosses.
- The genes could be used to revisit previous screens of asexual species such as *A. niger*, to verify if these genes were present and expressed in the supposedly asexual species – another indicator of latent sexuality.
- The natural environment could be screened for the presence of compatible mating-type strains of the asexual species studied in Chapter 5 and the distribution of mating-type gene determined within the natural environment. If partner strains of the other mating type were found, crosses could then be set up between different isolates using a variety of different culture conditions to see if it might be possible to induce sex under certain specific environmental parameters.
- The modern technology of ChIP- seq (chromatin immunoprecipitation) could be used to identify the binding site of DNA associated with HMG transcription factor protein.
- Epistatic analysis can be used to detect the genetic interaction between these identified genes, to determine if they are working on the same pathway or in different pathway.

References

Abarca ML, Accensi F, Cano J, Cabañes J (2004). Taxonomy and significance of black aspergilli'. Antonie Van Leeuwenhoek 86: 33-49.

Abe K, Gomi K, Hasegawa F, Machida M (2006). Impact of *Aspergillus oryzae* genomics on industrial production of metabolites. *Mycopathologia* 162: 143-153.

Adams TH, Wieser jK, Yu JH (1998). Asexual sporulation in *Apergillus nidulans. Microbiology and Molecular Biology Reviews* 62: 35-54.

Agresti A, Bianchi ME (2003). HMGB proteins and gene expression. *Current Opinion in Genetics and Development* 13: 170-178.

Aguirre J, Rios-Momberg M, Hewitt D, Hansberg, W (2005). Reactive oxygen species and development in microbial eukaryotes. *Trends in Microbiology* 13: 111-118.

Amaike S, Keller NP (2009). Distinct roles for vea and laea in development and pathogenesis of *Aspergillus flavus. Eukaryotic Cell* 8: 1051-1060.

Amaike S, Keller NP (2011). *Aspergillus flavus*. *Annual Review Phytopathology* 49: 107-133.

Anwar MM, Caoft JH, Dales RBG (1993). Analysis of heterokaryon incompatibility between heterokaryon-compatibility (h-c) groups R and GL provides evidence that at least eight het loci control somatic incompatibility in *Aspergillus nidulans. General Microbiology* 139: 1599-1603.

Araujo-Bazan L, Dhingra S, Chu J, Fernandez-Martinez J, Calvo AM, Espeso EA (2009). Importin alpha is an essential nuclear import carrier adaptor required for proper sexual and asexual development and secondary

metabolism in *Aspergillus nidulans. Fungal Genetics and Biology* 46: 506-515.

Archer DB, Dyer PS (2004). From genomics to post-genomics in *Aspergillus*. *Current Opinion Microbiology* 7: 499-504.

Arnaise S, Zickler D, Glass NL (1993). Heterologous expression of mating-type genes in filamentous fungi. *Proceedings of the National Academy of Sciences of the United States of America* 90: 6616-6620,

Arratia-Quijada J, Sanchez O, Scazzocchio C, Aguirre J (2012). Flbd, a Myb transcription factor of *Aspergillus nidulans*, is uniquely involved in both asexual and sexual differentiation. *Eukaryotic cell* 11: 1132-1142.

Ashour AO (2014). Control of sexual reproduction in *Aspergillus* species. *PhD Thesis Univeristy of Nottingham.*

Astell CR, Ahistrom-Jonasson L, Smith ML (1981).The sequence of the dnas coding for the mating-type loci of *Saccharomyces cerevisiae. Cell* 27: 15-23.

Baidya S, Cary JW, Grayburn WS & Calvo AM (2011). Role of nitric oxide and flavohemoglobin homologous genes in *Aspergillus nidulans* sexual development and mycotoxin production. *Applied Environmental Microbiology* 77: 5524-5528.

Baker LG, Specht CA, Lodge JK (2009). Chitinases are essential for sexual development but not vegetative growth in *Cryptococcus neoformans. Eukaryotic cell* 8: 1692-1705.

Baker SE (2006). *Aspergillus niger* genomics: Past, present and into the future. *Medical Mycology* 44: S17-21.

Bardwell L (2004). A walk-through of the yeast mating pheromone response pathway. *Peptides* 25: 1465-1476.

Barve MP, Arie T, Salimath SS, Muehlbauer FJ, Peever TL (2003). Cloning and characterization of the mating type (mat) locus from *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) and a mat phylogeny of legume-associated *Ascochyta* spp. *Fungal Genetics and Biology* 39: 151-167.

Bayram O, Bayram OS, Ahmed YL, Maruyama J, Valerius O, Rizzoli SO, Ficner R, Irniger S, Braus GH (2012). The *Aspergillus nidulans* MAPK module anste11-ste50-ste7-fus3 controls development and secondary metabolism. *PLoS Genetics* 8: e1002816.

Bayram O, Biesemann C, Krappmann S, Galland P, Braus GH (2008a). More than a repair enzyme: *Aspergillus nidulans* photolyase-like CryA is a regulator of sexual development. *Molecular Biology of the Cell* 19: 3254-3262.

Bayram O, Braus GH (2012). Coordination of secondary metabolism and development in fungi: The velvet family of regulatory proteins. *FEMS Microbiology Reviews* 36: 1-24.

Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, Braus-Stromeyer S, Kwon NJ, Keller NP, Yu JH, Braus GH (2008b). Velb/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* 320: 1504-1506.

Bayram Ö, Sari F, Braus GH & Irniger S (2009). The protein kinase ImeB is required for light-mediated inhibition of sexual development and for mycotoxin production in *Aspergillus nidulans*. *Molecular Microbiology* 71: 1278-1295.

Benjamin CR 1955. Ascocarps of *Aspergillus* and *Penicillium*. *Mycologia* 47:669-687.

Benkhali AJ, Coppin E, Brun S, Peraza-Reyes L, Martin T, Dixelius C, Lazar N, van Tilbeurgh H, Debuchy R (2013). A network of HMG-box

transcription factors regulates sexual cycle in the fungus *Podospora anserina*. *PLoS Genetics* 9: e1003642.

Bennett (2010). An overview of the genus *Aspergillus*. In: *Aspergillus* Molecular Biology and Genomics (ed. Machida, M. and Gomi, K.); Caister Academic Press, Norfolk.

Bennett JW (2009). *Aspergillus*: A primer for the novice. *Medical Mycology* 47: S5-S12.

Benoit I, Malavazi I, Goldman GH, Baker SE, de Vries R P (2013). *Aspergillus*: Genomics of a cosmopolitan fungus. In: Genomics of Soil -and Plant-associated fungi (ed. Horwitz, B A, Mukherjee, P K, Mukhrerijee, M, Kubicek, C.), ©Springer-Verrlag Berlin Heidelberg: 89-126.

Bianchi ME, Agresti A (2005). HMG proteins: Dynamic players in gene regulation and differentiation. *Current Opinion Genetics and Development* 15: 496-506.

Billiard S, Lopez-Villavicencio M, Devier B, Hood ME, Fairhead C, Giraud T (2011). Having sex, yes, but with whom? Inferences from fungi on the evolution of anisogamy and mating types. *Biological Reviews of the Cambridge Philosophical Society* 86: 421-442.

Billiard S, Lopez-Villavicencio M, Hood ME, Giraud T (2012). Sex, outcrossing and mating types: Unsolved questions in fungi and beyond. *Journal of Evolutionary Biology* 25: 1020-1038.

Bistis GN (1998). Physiological heterothallism and sexuality in Euascomycetes: A partial history. *Fungal Genetics and Biology* 23: 213–222.

Blumenstein A, Vienken K, Tasler R, Purschwitz J, Veith D, Frankenberg-Dinkel N, Fischer R (2005). The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. *Current Biology* 15: 1833-1838.

Bobrowicz P, Pawlak R, Correa A, Pedersen DB, Eboole DJ (2002). The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. *Molecular Microbiology* 45: 795-804.

Böhm J, Hoff B, O'Gorman CM, Wolfers S, Klix V, Binger D, Zadra I, Kurnsteiner H, Poggeler S, Dyer PS, Kuck U (2013). Sexual reproduction and mating-type-mediated strain development in the penicillin-producing fungus *Penicillium chrysogenum*. *Proceedings of the National Academy of Sciences of the United States of America* 110: 1476-1481.

Bölker M, Kahmann R (1993). Sexual pheromones and mating responses in fungi. *The Plant Cell* 5: 1461-1469.

Braumann I, van den Berg M, Kempken F (2008). Repeat induced point mutation in two asexual fungi, *Aspergillus niger* and *Penicillium chrysogenum*. *Current Genetics* 53: 287-297.

Braus GH, Krappmann S, Eckert SE (2002). Sexual development in ascomycetes fruit body formation of *Aspergillus nidulans. In: Molecular biology of fungal development* (ed. Osiewacz HD), pp. 215–244. Marcel Dekker, New York.

Brodhun F, Schneider S, Gobel C, Hornung E, Feussner I (2010). Ppoc from *Aspergillus nidulans* is a fusion protein with only one active haem. *The Biochemical Journal* 425: 553-565.

Bruno JF, Ellner SP, Vu I, Kim K, Havell D (2011). Impact of aspergillosis on sea fan coral demography modeling a moving target. *Ecological Monographs* 81: 123-139.

Busby TM, Miller KY & Miller BL (1996). Suppression and enhancement of the *Aspergillus nidulans* medusa mutation by altered dosage of the bristle and stunted genes. *Genetics* 143: 155-163.

Busch S, Eckert SE, Krappmann S & Braus GH (2003). The COP9 signalosome is an essential regulator of development in the filamentous fungus *Aspergillus nidulans. Molecular Microbiology* 49: 717-730.

Busch S, Schwier EU, Nahlik K, Bayram Ö, Helmstaedt K, Draht OW, Krappmann S, Valerius O, Lipscomb WN & Braus GH (2007). An eight-subunit COP9 signalosome with an intact JAMM motif is required for fungal fruit body formation. *Proceedings of the National Academy of Sciences of the United States of America* 104: 8089-8094.

Bussink HJ, Osmani SA (1998). A cyclin-dependent kinase family member (phoA) is required to link developmental fate to environmental conditions in *Aspergillus nidulans*. *The EMBO Journal* 17: 3990-4003.

Bustin M (2001). Revised nomenclature for high mobility group (HMG) chromosomal proteins. *Trends in Biochemical Sciences* 26: 152-153.

Bustin M (2010). High mobility group proteins. *Biochimica et Biophysica Acta* 1799(1-2): 1-2.

Butler G (2007). The evolution of mat: The ascomycetes. In Sex in Fungi: Molecular Determination and Evolutionary Implications, (ed. Heitman J, Kronstad JW, Taylor JW, Casselton LA). Washington DC: ASM Press pp. 3-18.

Butler G (2010). Fungal sex and pathogenesis. *Clinical Microbiology Reviews* 23: 140-159.

Butler G, Kenny C, Fagan A, Kurischko C, Gaillardin C, Wolfe KH (2004). Evolution of the MAT locus and its HO endonuclease in yeast species. *Proceedings of the National Academy of Sciences of the United States of America* 101:1632-1637.

Caddick MX, Dobson C, Morozov IY, Jones MG (2006). Gene regulation in *Aspergillus*: From genetics to genomics. *Medical Mycology* 44: 13-16.

Calvo AM (2008). The VeA regulatory system and its role in morphological and chemical development in fungi. *Fungal Genetics and Biology* 45: 1053-1061.

Calvo AM, Bok J, Brooks W, Keller NP (2004). VeA is required for toxin and sclerotial production in *Aspergillus parasiticus. Applied and Environmental Microbiology* 70: 4733-4739.

Calvo AM, Cary JW (2015). Association of fungal secondary metabolism and sclerotial biology. *Frontiers in Microbiology* 6:1-16.

Calvo AM, Gardner HW, Keller NP (2001). Genetic connection between fatty acid metabolism and sporulation in *Aspergillus nidulans. The Journal of Biological Chemistry* 276: 25766-25774.

Calvo AM, Wilson RA, Bok JW, Keller NP (2002). Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* 66: 447-459.

Cary JW, Harris-Coward PY, Ehrlich KC, Mack BM, Kale SP, Larey C, Calvo A M (2012). NsdC and NsdD affect *Aspergillus flavus* morphogenesis and aflatoxin production. *Eukaryotic cell* 11: 1104-1111.

Casselton L (2002). Mat recognition in fungi, *Heredity* 88:142-147.

Casselton LA (2008). Fungal sex genes-searching for the ancestors. *BioEssays* 30: 711-714.

Casselton, L. and Zolan, M. 2002. The art and design of genetic screens: Filamentous Fungi. *Nature Reviews Genetics,* 3**,** 683-697.

Chamilos G, Bignell EM, Schrettl M, Lewis RE, Leventakos K, May GS, Haas H, Kontoyiannis DP (2010). Exploring the concordance of *Aspergillus fumigatus* pathogenicity in mice and Toll- deficient flies. *Medical Mycology* 48:506-510.

Champer SP, El- Zayat AE (1989). Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. *Bacteriology* 171: 3982-3988.

Champer SP, Rao P, Chang A (1987). An endogenous inducer of sexual development in *Aspergillus nidulans*. *General Microbiology* 133: 1383-1387.

Chang PK, Ehrlich KC (2010). What does genetic diversity of *Aspergillus flavus* tell us about *Aspergillus oryzae*? *International Journal of Food Microbiology* 138: 189-199.

Chikashige Y, Ding D, Imai Y, Yamamoto M, Haraguchi T, Hiraoka Y (1997). Meiotic nuclear reorganization: Switching the position of centromeres and telomeres in the fission yeast *Schizosaccharomyces pombe*. *The EMBO Journal* 16: 193-202.

Coelho MA, Goncalves P, Sampaio JP (2011). Evidence for maintenance of sex determinants but not of sexual stages in red yeasts, a group of early diverged basidiomycetes. *BMC Evolutionary Biology* 11: 249.

Coppin E, de Renty C, Debuchy R (2005). The function of the coding sequences for the putative pheromone precursors in *Podospora anserina* is restricted to fertilization. *Eukaryotic Cell* 4: 407-420.

Coppin E, Debuchy R, Arnaise S, Picard M (1997). Mating types and sexual development in filamentous ascomycetes. *Microbiology and Molecular Biology Reviews* 61:411-428

Corcoran P, Jacobson DJ, Bidartondo MI, Hickey PC, Kerekes JF, Taylor JW, Johannesson H (2012). Quantifying functional heterothallism in the pseudohomothallic ascomycete *Neurospora tetrasperma*. *Fungal Biology* 116: 962-975.

Czaja W, Miller KY, Miller BL (2011). Complex mechanisms regulate developmental expression of the matA (HMG) mating type gene in homothallic *Aspergillus nidulans*. *Genetics* 189: 795-808.

Darbyshir HL (2013). Mating type and sexual reproduction in *Aspergillus* section Nigri. *PhD Thesis Univeristy of Nottingham.*

de Groot PWJ, Brandt BW, Horiuchi H, Ram A FJ, de Koster CG, Klis FM (2009). Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans. Fungal Genetics and Biology* 46: S72-S81.

Debuchy R, Berteaux-Lecellier V, Silar P (2010). Mating systems and sexual morphogenesis in ascomycetes. In: Cellular and Molecular Biology of Filamentous Fungi (ed. Borkovich KA and Ebbole DJ) pp. 501-535. ASM Press, Washington.

Debuchy R, Turgeon BG (2006). Mating-type structure, evolution, and function in Euascomycetes. In The Mycota, vol. I: growth, differentiation and sexuality (ed. Kues U, Fischer R). Germany, Springer, Berlin.

Deckert J, Rodriguez Torres AM, Simon JT, Zitomer RS (1995). Mutational analysis of Rox1, a DNA-bending repressor of hypoxia gene in *Saccharomyces cerevisiae. Molecular and Cellular Biology* 15: 6109-6117.

Deflorio R, Brett ME, Waszczak N, Apollinari E, Metodiev MV, Dubrovskyi O, Eddington D, Arkowitz RA, Stone DE (2013). Phosphorylation of Gbeta is crucial for efficient chemotropism in yeast. *Journal of Cell Sciences* 126: 2997-3009.

Dequard-Chablat M, Alland C (2002). Two copies of mthmg1, encoding a novel mitochondrial HMG-like protein, delay accumulation of mitochondrial DNA deletions in *Podospora anserina. Eukaryotic cell* 1: 503-513.

Dohlman HG, Thorner JW (2001). Regulation of G protein-initiated signal transduction in yeast: Paradigms and principles. *Annual Review of Biochemistry* 70: 703-754.

Dupont S, Lemetais G, Ferreira T, Cayot P, Gervais P, Beney L (2012). Ergosterol biosynthesis: A fungal pathway for life on land? *Evolution* 66: 2961-2968.

Duran RM, Cary JW, Calvo AM (2007). Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by VeA, a gene necessary for sclerotial formation. *Applied Microbiology and Biotechnology* 73(5): 1158-1168.

Dyer PS (2007). Sexual reproduction and significance of mat in the aspergilli. In sex in fungi: molecular determination and evolutionary implications ((ed. Heitman J, Kronstad JW, Taylor JW, Casselton LA). Washington DC: ASM Press pp. 123-142.

Dyer PS (2008). Evolutionary biology: Genomic clues to original sex in fungi. *Current Biology* 18: R207-R209.

Dyer PS, Inderbitzin P, Debuchy R (2016). Mating-type structure, function, regulation and evolution in the Pezizomycotina. In: Growth, Differentiation and Sexuality, The Mycota I (3rd edition) (ed. J Wendland) pp. 351-385. Springer International Publishing, Switzerland.

Dyer PS, Ingram DS, Johnstone K (1992). The control of sexual morphogenesis in the Ascomycotina. *Biological Reviews of the Cambridge Philosophical Society* 67: 421-458.

Dyer PS, O'Gorman CM (2011). A fungal sexual revolution: *Aspergillus* and *Penicillium* show the way. *Current Opinion in Microbiology* 14: 649-654.

Dyer PS, O'Gorman CM (2012). Sexual development and cryptic sexuality in fungi: Insights from *Aspergillus* species. *FEMS Microbiology Reviews* 36: 165-192.

Dyer PS, Paoletti M (2005). Reproduction in *Aspergillus fumigatus*: Sexuality in a supposedly asexual species? *Medical Mycology* 43: 7-14.

Dyer PS, Paoletti M, Archer DB (2003). Gemonics reveals sexual secrets of *Aspergillus. Microbiology* 149: 2301-2303.

Eisendle M, Schrettl M, Kragl C, Müller D, Illmer P & Haas H (2006). The intracellular siderophore ferricrocin is involved in iron storage, oxidative-stress resistance, germination and sexual devlopment in *Aspergillus nidulans. Eukaryotic Cell* 5: 1596-1603.

Ellner SP, Jones LE, Mydlarz LD, Harvell CD (2007). Within-host disease ecology in the sea fan *Gorgonia ventalina*: Modeling the spatial immunodynamics of a coral-pathogen interaction. *American Soceity of Naturalists* 170: E143-E161.

Ene IV, Bennett RJ (2014). The cryptic sexual strategies of human fungal pathogens. *Nature Reviews Microbiology* 12: 239-251.

Etxebeste O, Garzia A, Espeso EA, Ugalde U (2010a). *Aspergillus nidulans* asexual development: Making the most of cellular modules. *Trends in Microbiology* 18: 569-576.

Etxebeste O, Ugalde UA, Espeso E (2010b). Adaptative and developmental responses to stress in *Aspergillus nidulans. Current Protein and Peptide Science* 11: 704-718.

Ferreira AVB, Zhiqiang AN, Metzenberg RL, Glass NL (1998). Characterization of mat a-2, mat a-3 and dalta matA mating-type mutants of *Neurospora crassa. Genetics* 148: 1069-1079.

Fraser JA, Heitman J (2003). Fungal mating type loci. Current Biology 13: R792-R795.

Fraser JA, Heitman J (2004). Evolution of fungal sex chromosomes. *Molecular Microbiology* 51: 299-306.

Fraser JA, Heitman J (2005). Chromosomal sex-determining regions in animals, plants and fungi. *Current Opinion and Genetic Development* 15:645-651.

Frisvad JC, Petersen LM, Lyhne EK, Larsen TO (2014). Formation of sclerotia and production of indoloterpenes by *Aspergillus niger* and other species in section Nigri. *PLoS One* 9: e94857.

Fungaro MH, Vissotto PC, Sartori D, Vilas-Boas LA, Furlaneto MC, Taniwaki MH (2004). A molecular method for detection of *Aspergillus carbonarius* in coffee beans. *Current Microbiology* 49: 123-127.

Futagami T, Nakao S, Kido Y Oka T, Kajiwara Y, Takashita H, Omori T, Furukawa K, Goto M (2011). Putative stress sensors WscA and WscB are involved in hypo-osmotic and acidic ph stress tolerance in *Aspergillus nidulans. Eukaryotic Cell* 10: 1504-1515.

Futagami T, Seto K, Kajiwara Y, Takashita H, Omori T, Takegawa K, Goto M (2014). The putative stress sensor protein MtlA is required for conidia formation, cell wall stress tolerance, and cell wall integrity in *Aspergillus nidulans. Bioscience, Biotechnology and Biochemistry* 78: 326-335.

Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee, SI, Basturkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D'Enfert C, Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, Selker EU, Archer DB, Penalva MA, Oakley BR, Momany M, Tanaka T, Kumagai T, Asai K, Machida M, Nierman WC, Denning D W, Caddick M, Hynes M, Paoletti M, Fischer R, Miller B, Dyer P, Sachs MS, Osmani SA, Birren B W (2005). Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae. Nature* 438: 1105-1115.

Garzia A, Etxebeste O, Herrero-Garcia E, Fischer R, Espeso EA, Ugalde U (2009). *Aspergillus nidulans* FlbE is an upstream developmental activator of conidiation functionally associated with the putative transcription factor FlbB. *Molecular Microbiology* 71: 172-184.

Garzia A, Etxebeste O, Herrero-Garcia E, Ugalde U, Espeso EA (2010). The concerted action of bZip and cMyb transcription factors FlbB and FlbD induces brlA expression and asexual development in *Aspergillus nidulans*. *Molecular Microbiology* 75: 1314-1324.

Geiser D M (2009). Sexual structures in *Aspergillus*: Morphology, importance and genomics. *Medical Mycology* 47: S21-S26.

Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA (2007). The current status of species recognition and identification in *Aspergillus*. *Studies in Mycology* 59: 1-10.

Geiser DM, Timberlake WE, Arnold ML (1996). Loss of meiosis in *Aspergillus*. *Molecular Biology and Evolution* 13: 809-817.

Geiser, D. M., Frisvad, J. C. and Taylor, J. W. 1998. Evolutionary relationships in *Aspergillus* section *Fumigata* inferred from partial beta-tubulin and hydrophobin DNA sequences. *Mycologia,* 90**,** 831-845.

Gioti A, Mushegian AA, Strandberg R, Stajich JE, Johannesson H (2012). Unidirectional evolutionary transitions in fungal mating systems and the role of transposable elements. *Molecular Biology and Evolution* 29: 3215-3226.

Glass NL, Grotelueschen J, Metzenberg RL (1990). *Neurospora crassa* a mating-type region. *Proceedings of the National Academy of Sciences of the United States of America* 87: 4912-4916.

Glass NL, Jacobson DJ, Shiu PKT (2000). The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. *Annual Review of Genetics* 34:165–86.

Glass NL, Kuldau CA (1992). Mating type and vegetative incompatibility in filamentous ascomycetes. *Annual Review of Phytopathology* 30:201-24.

Große V, Krappmann S (2008). The asexual pathogen *Aspergillus fumigatus* expresses functional determinants of *Aspergillus nidulans* sexual development. *Eukaryotic Cell* 7: 1724-1732.

Grunbacher A, Throm T, Seidel C, Gutt B, Rohrig J, Strunk T, Vincze P, Walheim S, Schimmel T, Wenzel W, Fischer R (2014). Six hydrophobins are involved in hydrophobin rodlet formation in *Aspergillus nidulans* and contribute to hydrophobicity of the spore surface. *PLoS One* 9: e94546.

Guzmán-de-Peńa D, Aguirre J, Ruiz-Herrera J (1998). Correlation between the regulation of sterigmatocystrin biosynthesis and asexual and sexual sporulationin *Emericella nidulans. Antonie Van Leeuwenhoek* 73: 199-205.

Han K, Han K, Yu J, Chae K, Jahng KDH (2001). The *nsdD* gene encodes a putative gata-type transcription factor necessary for sexual development of *Aspergillus nidulans. Molecular Microbiology* 41: 299-309.

Han KH, Kim JH, Moon H, Kim S, Lee SS, Han DM, Jahng KY & Chae KS (2008b) The *Aspergillus nidulans esdC* (early sexual development) gene is necessary for sexual development and is controlled by *veA* and a heterotrimeric G protein. *Fungal Genetics and Biology* 45: 310-318.

Han KH, Lee DB, Kim JH, Kim M, Han KY Kim WS, Park YS, Kim HB, Han DM (2003). Environmental factors affecting development of *Aspergillus nidulans. Microbiology* 41: 34-40.

Han KH, Seo JA, Yu JH (2004). A putative g protein-coupled receptor negatively controls sexual development in *Aspergillus nidulans. Molecular Microbiology* 51: 1333-1345.

Han SY, Ko JA, Kim JH, Han KY, Han KH & Han DM (2008). Isolation and functional analysis of the *silA* gene that controls sexual development in response to light in *Aspergillus nidulans. Korean Journal of Mycoogyl* 36: 189-195.

Han, K (2009). Molecular genetics of *Emericella nidulans* sexual development. *Microbiology* 37: 171-182.

Harley VR (2003). The molecular action and regulation of the testis-determining factors, SRY (sex-determining region on the y chromosome) and SOX9 [SRY-related high-mobility group (HMG) box 9]. *Endocrine Reviews* 24: 466-487.

Harris SD (2009). The spitzenkorper: A signalling hub for the control of fungal development? *Molecular Microbiology* 73: 733-736.

Harris SD, Turner G, Meyer V, Espeso EA, Specht T, Takeshita N, Helmstedt K (2009). Morphology and development in *Aspergillus nidulans*: A complex puzzle. *Fungal Genetics and Biology* 46: S82-S92.

Hatakeyama R, Nakahama T, Higuchi Y, Kitamoto K (2007). Light represses conidiation in koji mold *Aspergillus oryzae. Bioscience, Biotechnology, and Biochemistry* 71: 1844-1849.

Heitman J, Carter DA, Dyer PS, Soll DR (2014). Sexual reproduction of human pathogens. *Cold Spring Harbor Prospective in Medicine* 4: a019281.

Heitman J, Sun S, James TY (2013). Evolution of fungal sexual reproduction. *Mycologia* 105: 1-27.

Helmstaedt K, Schwier EU, Christmann M, Nahlik K, Westermann M, Harting R, Grond S, Busch S & Braus GH (2011). Recruitment of the inhibitor Cand1 to the cullin substrate adaptor site mediates interaction to the neddylation site. *Molecular Biology of the Cell* 22: 153-164.

Hermann TE, Kurtz MB, Champe S P (1983). Laccase localized in hulle cells and cleistothecial primordia of *Aspergillus nidulans. Bacteriology* 154: 955-964.

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lucking R, Thorsten Lumbsch H,

Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Koljalg U, Kurtzman CP, Larsson KH, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo JM, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schussler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N (2007). A higher-level phylogenetic classification of the fungi. *Mycological Research* 111: 509-547.

Hirsch JP, Cross FR (1992). Pheromone response in yeast. *BioEssays* 14: 367-373.

Hoffmann B, Wanke C, Lapaglla SK, Braus GH (2000). c-Jun and RACK1 homologues regulate a control point for sexual development in *Aspergillus nidulans. Molecular Microbiology* 37: 28-41.

Horn BW, Moore GG, Carbone I (2009a). Sexual reproduction in *Aspergillus flavus. Mycologia* 101: 423-429.

Horn BW, Olarte RA, Peterson SW, Carbone I (2013). Sexual reproduction in *Aspergillus tubingensis* from section Nigri. *Mycologia* 105: 1153-1163.

Horn BW, Ramirez-Prado JH, Carbone I (2009b). Sexual reproduction and recombination in the aflatoxin-producing fungus *Aspergillus parasiticus. Fungal Genetics and Biology* 46: 169-175.

Hornok L, Waalwijk C, Leslie JF (2007). Genetic factors affecting sexual reproduction in toxigenic *Fusarium* species. *International Journal of Food Microbiology* 119: 54-58.

Horwitz HR, Berteaux-Lecellier V, Silar P (2010). Mating systems and sexual morphogenesis in ascomycetes. In cellular and molecular biology of filamentous fungi (ed. Borkowich KA, Ebbole DJ). ASM press Washington, DC: 5011-5035.

Hosid E, Grishkan I, Yusim E, Frenkel Z, Wasser SP, Nevo E, Korol A (2010). The mode of reproduction in natural populations of ascomycetous fungus, *Emericella nidulans*, from Israel. *Genetics Research* 92: 83-90.

Hull C, Johnson AD (1999). Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. *Science* 285: 1271-1275.

Hull CM, Raisner RM, Johnson A D (2000). Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. *Science* 289: 307-310.

Idnurm A, Walton FJ, Floyd A, Heitman J (2008). Identification of the sex genes in an early diverged fungus. *Nature* 451: 193-196.

Imai Y,Yamamoto M (1994). The fission yeast mating pheromone P-factor: Its molecular structure, gene structure, and ability to induce gene expression and G1 arrest in the mating partner. *Genes and Development* 8: 328-338.

Jaakola L, Pirttila AM, Halonen M, Hohtola A (2001). Isolation of high quality RNA from bilberrry (*Vaccinium myrtillus L*.) fruit. *Molecular Biotechnology* 19: 201-203.

Jeon MH, Hagiwara D, Yoshimi A, Abe K, Han DM & Chae SK (2009). Analysis of *nrdA*, a negative regulator of differentiation in *Aspergillus nidulans. Fungal Genetics Reports* 56 (Suppl): 138.

Jin FJ, Takahashi T, Matsushima K, Hara S, Shinohara Y, Maruyama J, Kitamoto K, Koyama Y (2011). Sclr, a basic helix-loop-helix transcription factor, regulates hyphal morphology and promotes sclerotial formation in *Aspergillus oryzae*. *Eukaryotic Cell* 10: 945-955.

Jin H, McCaffery JM, Grote E (2008). Ergosterol promotes pheromone signaling and plasma membrane fusion in mating yeast. *Journal of Cell Biology* 180: 813-826.

Jones SK, Jones JR, Bennett RJ (2011). Fungal mating pheromones: Choreographing the dating game. *Fungal Genetics and Biology* 48: 668-676.

Jun SC, Lee SJ, Park HJ, Kang JY, Leem YE, Yang TH, Chang MH, Kim JM, Jang SH, Kim HG, Han DM, Chae KS, Jahng KY (2011).The mpkb map kinase plays a role in post-karyogamy processes as well as in hyphal anastomosis during sexual development in *Aspergillus nidulans. Journal of Microbiology* 49: 418-430.

Kadosh D, Johnson AD (2001). Rfg1, a protein related to the *Saccharomyces cerevisiae* hypoxic regulator Rox1, controls filamentous growth and virulence in *Candida albicans. Molecular Cell Biology* 21: 2496-2505.

Kale SP, Milde L, Trapp MK, Frisvad JC, Keller NP, Bok JW (2008). Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus. Fungal Genetics and Biology* 45: 1422-1429.

Karacsony Z, Gacser A, Vagvolgyi C, Hamari Z (2015). Further characterization of the role of the mitochondrial high-mobility group box protein in the intracellular redox environment of *Aspergillus nidulans. Microbiology* 161: 1897-1908.

Karacsony Z, Gacser A, Vagvolgyi C, Scazzocchio C, Hamari Z (2014). A dually located multi-HMG-box protein of *Aspergillus nidulans* has a crucial role in conidial and ascospore germination. *Molecular Microbiology* 94: 383-402.

Kashimada K, Koopman P (2010). Sry: The master switch in mammalian sex determination. *Development* 137: 3921-3930.

Kato N, Brooks W, Calvo AM (2003). The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by *veA*, a gene required for sexual development. *Eukaryotic Cell* 2: 1178-1186.

Kawasaki L, Sánchez O, Shiozaki K, Aguirre J (2002). Saka map kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans. Molecular Microbiology* 45: 1153-1163.

Keszthelyi A, Jeney A, Kerenyi Z, Mendes O, Waalwijk C, Hornok L (2007). Tagging target genes of the mat1-2-1 transcription factor in *Fusarium verticillioides (Gibberella fujikuroi* mp-a). *Antonie Van Leeuwenhoek* 91: 373-391.

Khaldi NA, Wolfer KH (2008). Elusive origins of the extra genes in *Aspergillus oryzae. PLoS ONE* 3: e3036.

Kim H, Borkovich KA (2004). A pheromone receptor gene, *pre-1*, is essential for mating type-specific directional growth and fusion of trichogynes and female fertility in *Neurospora crassa. Molecular Microbiology* 52: 1781-1798.

Kim H, Borkovich KA (2006). Pheromones are essential for male fertility and sufficient to direct chemotropic polarized growth of trichogynes during mating in *Neurospora crassa. Eukaryotic Cell* 5: 544-554.

Kim HR, Chae KS, Han KH, Han DM (2009b). The *nsdC* gene encoding a putative C2H2-type transcription factor is a key activator of sexual development in *Aspergillus nidulans. Genetics* 182: 771-783.

Kim HS, Han KY, Kim KJ, Han DM, Jahng KY & Chae KS (2002). The *veA* gene activates sexual development in *Aspergillus nidulans. Fungal Genetics and Biology* 37: 72-80.

Kim HY, Han KH, Lee M, Oh M, Kim HS, Zhixiong X, Han DM, Jahng KY, Kim JH, Chae KS (2009a). The *veA* gene is necessary for the negative

regulation of the *veA* expression in *Aspergillus nidulans. Current Genetics* 55: 391-397.

Kirk KE, Morris NR (1991). The *tubB* α-tubulin gene is essential for sexual development in *Aspergillus nidulans. Genes and Development* 5: 2014-2023.

Klich MA (2009). Health effects of *Aspergillus* in food and air. *Toxicology and Industrial Health* 25: 657-667.

Kobayashi T, Abe K, Asai K, Gomi K, Juvvadi PR, Kato M, Kitamoto K, Takeuchi M, Machida M (2007). Genomics of *Aspergillus oryzae. Bioscience, Biotechnology, and Biochemistry* 71: 646-670.

Krappmann S, Jung N, Medic B, Busch S, Prade RA, Braus GH (2006). The *Aspergillus nidulans* F-box protein GrrA links SCF activity to meiosis. *Molecular Microbiology* 61: 76-88.

Krijgsheld P, Bleichrodt R, van Veluw GJ, Wang F, Muller WH, Dijksterhuis J, Wosten HA (2013). Development in *Aspergillus. Studies in Mycology* 74: 1-29.

Kronstad JW, Staben C (1997). Mating type in filamentous fungi. *Annual Review Genetics* 31: 245-276.

Kück U, Pöggeler S (2009). Cryptic sex in fungi. *Fungal Biology Reviews* 23: 86-90.

Kwon NJ, Garzia A, Espeso EA, Ugalde U, Yu JH (2010a). FlbC is a putative nuclear C2H2 transcription factor regulating development in *Aspergillus nidulans. Molecular Microbiology* 77: 1203-1219.

Kwon NJ, Shin KS & Yu JH (2010b) Characterization of the developmental regulator FlbE in *Aspergillus fumigatus* and *Aspergillus nidulans. Fungal Genetics and Biology* 47: 981-993.

Kwon-Chung KJ, Bennett JE (1978). Distribution of α and a mating types of *Cryptococcus neoformans* among natural and clinical isolates. *American Journal of Epidemiology* 108: 337-340.

Kwon-Chung KJ, Sugui JA (2009). Sexual reproduction in *Aspergillus* species of medical or economical importance: Why so fastidious? *Trends in Microbiology* 17(11): 481-487.

Lara-Ortíz T, Riveros-Rosas H, Aguirre J (2003). Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans. Molecular Microbiology* 50: 1241-1255.

Lara-Rojas F, Sánchez O, Kawasaki L & Aguirre J (2011). *Aspergillus nidulans* transcription fcator AtfA interacts with the MAPK SakA to regulate general stress responses, development and spore functions. *Molecular Microbiology* 80: 436-454.

Laudet V, Stehelin D, Clevers H (1993). Ancestry and diversity of the HMG box superfamily. *Nucleic Acid Research* 21: 2493-2501.

Lee BY, Han SY, Choi HG, Kim JH, Han KH & Han DM (2005). Screening of growth- or developmental-related genes by using genomic library with inducible promoter in *Aspergillus nidulans. Journal of Microbiology* 43: 523-528.

Lee DW, Kim S, Kim SJ, Han DM, Jahng KH, Chae KS (2001). The *IsdA* gene is necessary for sexual development inhibition by a salt in *Aspergillus nidulans. Current Genetics* 39: 237-243.

Lee J, Lee T, Lee YW, Yun SH, Turgeon BG (2003). Shifting fungal reproductive mode by manipulation of mating type genes: Obligatory heterothallism of *Gibberella zeae. Molecular Microbiology* 50: 145-152.

Lee J, Leslie JF, Bowden RL (2008). Expression and function of sex pheromones and receptors in the homothallic ascomycete *Gibberella zeae. Eukaryotic Cell 7*: 1211-1221.

Lee S, Ni M, Li W, Shertz C, Heitman J (2010a). The evolution of sex: A perspective from the fungal kingdom. *Microbiology and Molecular Biology Reviews* 74: 298-340.

Lee SH, Lee S, Choi D, Lee YW, Yun SH (2006). Identification of the down-regulated genes in a mat1-2-deleted strain of *Gibberella zeae*, using cDNA subtraction and microarray analysis. *Fungal Genetics and Biology* 43: 295-310.

Leslie JF (1993). Fungal vegetative compatibility. *Annual Review Phytopathology* 31: 127-150.

Li S, Bao D, Yuen G, Harris SD, Calvo AM (2007). *basA* regulates cell wall organization and asexual/sexual sporulation ratio in *Aspergillus nidulans. Genetics* 176: 243-253.

Lin X, Hull CM, Heitman J (2005). Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans. Nature* 434: 1015-1017.

Litvintseva AP, Marra RE, Nielsen K, Heitman J, Vilgalys R, Mitchell TG (2003). Evidence of sexual recombination among *Cryptococcus neoformans* serotype a isolates in Sub-Saharan Africa. *Eukaryotic Cell* 2: 1162-1168.

Lopez-Diaz TM, Flannigan B (1997). Production of patulin and cytochalasin E by *Aspergillus clavatus* during malting of barley and wheat', *International Journal of Food Microbiology* 35: 129-136.

Loubradou G, Turcq B (2000). Vegetative incompatibility in filamentous fungi: Aroundabout way of understanding the phenomenon. *Research in Microbiology* 151: 239-245.

Lu J, Kobayashi R, Brill SJ (1996). Characterization of a high mobility group 1/2 homolog in yeast. *Biological Chemistry* 271: 33678-33685.

Lu SW, Yun SH, Lee T, Turgeon BG (2011). Altering sexual reproductive mode by interspecific exchange of *MAT* loci. *Fungal Genetics and Biology* 48: 714-724.

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, Abe K, Gomi K, Horiuchi H, Kitamoto K, Kobayashi T, Takeuchi M, Denning DW, Galagan JE, Nierman WC, Yu J, Archer DB, Bennett JW Bhatnagar D, Cleveland TE, Fedorova ND, Gotoh O, Horikawa H, Hosoyama A, Ichinomiya M, Igarashi R, Iwashita K, Juvvadi PR, Kato M, Kato Y, Kin T, Kokubun A, Maeda H, Maeyama N, Maruyama J, Nagasaki H, Nakajima T, Oda K, Okada K, Paulsen I, Sakamoto K, Sawano T, Takahashi M, Takase K, Terabayashi Y, Wortman JR, Yamada O, Yamagata Y, Anazawa H, Hata Y, Koide Y, Komori T, Koyama Y, Minetoki T, Suharnan S, Tanaka A, Isono K, Kuhara S, Ogasawara N, Kikuchi H (2005). Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* 438: 1157-1161.

Maeder CI, Hink MA, Kinkhabwala A, Mayr R, Bastiaens PI, Knop M (2007). Spatial regulation of Fus3 MAP kinase activity through a reaction-diffusion mechanism in yeast pheromone signalling. *Nature Cell Biology* 9: 1319-1326.

Malagnac F, Lalucque H, Lepere G, Silar P (2004). Two NADPH oxidase isoforms are required for sexual reproduction and ascospore germination in the filamentous fungus *Podospora anserina*. *Fungal Genetics and Biology* 41: 982-997.

Malarkey CS, Churchill ME (2012). The high mobility group box: The ultimate utility player of a cell. *Trends in Biochemistry Sciences* 37: 553-562.

Martin SH, Wingfield BD, Wingfield MJ, Steenkamp ET (2011). Causes and consequences of variability in peptide mating pheromones of ascomysetes fungi. *Molecullar Biology and Evolution* 28:1987-2003.

Martin T, Lu SW, van Tilbeurgh H, Ripoll DR, Dixelius C, Turgeon BG, Debuchy R (2010). Tracing the origin of the fungal α1 domain places its ancestor in the HMG-box superfamily: Implication for fungal mating-type evolution. *PLoS One* 5: e15199.

Mather K, Jinks JL (1958). Cytoplasm in sexual reproduction. *Nature* 182: 1188-1190.

Mayrhofer S, Weber JM, Poggeler S (2006). Pheromones and pheromone receptors are required for proper sexual development in the homothallic ascomycete *Sordaria macrospora. Genetics* 172: 1521-1533.

Mckenzie RA, Kelly MA, Shivas RG, Gibson JA, Cook PJ, Widderick K, Guilfoyle AF (2004). *Aspergillis clavatus* tremorgenic neurotoxicosis in the cattle fed sprouted grain. *Australian Veterinary Journal* 28: 635-638.

Metzenberg RL, Glass NL (1990). Mating type and mating stategies in *Neurospora. BioEssays* 12: 53-59.

Moore-Landecker M (1992). Physiology and biochemistry of ascocarp induction and development. *Mycological Research* 96: 705-716.

Moye-Rowley WS (2003). Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences. *Eukaryotic Cell* 2: 381-389.

Nagelkerken I, Buchan K, Smith GW, Bonair K, Bush P, Garzón-Ferreira J, Botero L, Gayle P, Harvell CD, Heberer C, Kim K, Petrovic C, Pors L,Yoshioka P (1997). Widespread disease in Caribbean sea fans: II. Patterns of infection and tissue loss. *Marine Ecology Progress Series* 160: 255-263.

Nahlik K, Dumkow M, Bayram Ö et al. (2010). The COP9 signalosome mediates transcriptional and metabolic response to hormones, oxidative stress protection and cell wall rearrangement during fungal development. *Molecular Microbiology* 78: 964–979.

Naider F, Becker JM (2004). The α-factor mating pheromone of *Saccharomyces cerevisiae*: A model for studying the interaction of peptide hormones and G protein-coupled receptors. *Peptides* 25: 1441-1463.

Nakai K, Horton P (1999). Psort: A program for detecting sorting signals in protien and predicting their subcellular localization. *Trends in Biochemical Science* 24: 34-35.

Nauta MJ, Hoekstra RF (1996). Vegetative incompatibility in ascomycetes highly polymorphic but selectively neutral. *Journal of Theoretical Biology* 183: 67-76.

Nelson MA (1996). Mating systems in ascomycetes: A romp in the sac. *Trends in Genetics* 12: 69-74.

Ni M, Feretzaki M, Sun S, Wang X, Heitman J (2011). Sex in fungi. *Annual Review of Genetics* 45: 405-430.

Nicolas E, Lee MG, Hakimi MA, Cam HP, Grewal SI, Shiekhattar, R (2006). Fission yeast homologs of human histone H3 lysine 4 demethylase regulate a common set of genes with diverse functions. *Journal of Biological Chemistery* 281: 35983-35988.

Notteghem JL, Silue D (1992). Distribution of the mating type alleles in *Magnaporthe grisea* population pathogenic on rice. *Phytopathology* 82: 421-424.

Nygren K, Strandberg R, Wallberg A, Nabholz B, Gustafsson T, Garcia D, Cano J, Guarro J, Johannesson H (2011). A comprehensive phylogeny of *Neurospora* reveals a link between reproductive mode and molecular evolution in fungi. *Molecual Phylogenetics and Evolution* 59: 649-663.

O'Gorman CM, Fuller HT, Dyer PS (2009). Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus. Nature* 457: 471-474.

Pál K, van Diepeningen AD, Varga J, Hoekstra RF, Dyer PS, Debets AJ (2007). Sexual and vegetative compatibility genes in the aspergilli. *Studies in Mycology* 59: 19-30.

Paoletti M, Rydholm C, Schwier EU, Anderson MJ, Szakacs G, Lutzoni F, Debeaupuis JP, Latge JP, Denning DW, Dyer PS (2005). Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus. Current Biology* 15: 1242-8.

Paoletti M, Seymour FA, Alcocer MJ, Kaur N, Calvo AM, Archer DB, Dyer PS (2007). Mating type and the genetic basis of self-fertility in the model fungus *Aspergillus nidulans*. *Current Biology* 17: 1384-9.

Park HS, Yu JH (2012). Genetic control of asexual sporulation in filamentous fungi. *Current Opinion Microbiology* 15: 669-677.

Pascon RC, Miller BL (2000). Morphogenesis in *Aspergillus nidulans* requires Dopey (DopA), a member of a novel family of leucine zipper-like proteins conserved from yeast to humans. *Molecular Microbiology* 36: 1250-1264.

Pavlidis P, Noble WS (2003) MAterix2png: a utility for visualizing materix data. Bioinformatics 19: 295-296.

Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van Dijk A, Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der

Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN, Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wosten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology* 25: 221-231.

Perrone G, Susca A, Cozzi G, Ehrlich K, Varga J, Frisvad JC, Meijer M, Noonim P, Mahakarnchanakul W, Samson RA (2007). Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology* 59: 53-66.

Philley M, Staben C (1994). Functional analysis of the *Neurospora crassa* MT a-1 mating type polypeptide. *Genetics* 137: 715-722.

Pitt JI, Taylor JW (2014). *Aspergillus*, its sexual states and the new international code of nomenclature. *Mycologia* 106: 1051-1062.

Pöggeler S (2000). The pheromone precursor genes are transcriptionally expressed in the homothallic ascomycetes *Sordaria macrospora. Current Genetics* 37:403-411.

Poggeler S (2001). Mating-type genes for classical strain improvements of ascomycetes. *Applied Microbiology and Biotechnology* 56: 589-601.

Pöggeler S (2002). Genomic evidence for mating abilities in the asexual pathogen *Aspergillus fumigatus. Current Genetics* 42: 153-160.

Pöggeler S, Kück U (2001). Identification of trnscriptionally expressed pheromone receptor genes in filamentous ascomycetes. *Gene* 280: 9-17.

Pöggeler S, Nowrousian M, Kück U (2006b). Fruiting-body development in ascomycetes. In The Mycota I Growth, Differentation and Sexuality (ed. Kües, Fischer) Springer-Verlag Berlin Heidelberg.

Pöggeler S, Nowrousian M, Ringelberg C, Loros JJ, Dunlap JC, Kück U (2006a). Microarray and real-time PCR reveal mating type-dependent gene expresion in a homothallic fungus. *Molecular Genetics and Genomics* 275: 495-503.

Pöggeler S, Risch S, Kück U, Osiewacz HD (1997). Mating-type genes from the homothallic fungus sordaria macrospora are functionally expressed in a heterothallic ascomycete. *Genetics* 147: 567-580.

Pontecorvo G (1956). The parasexual cycle in fungi. *Annual Review of Microbiology* 10: 393-400.

Pontecorvo G, Roper JA, Chemmons LM, Macdonald KD, Bufton AWJ (1953). The genetics o*f Aspergillus nidulans*. *Advance in Genetics* 5: 141-248.

Purschwitz J, Muller S, Kastner C, Schoser M, Haas H, Espeso EA, Atoui A, Calvo AM, Fischer R (2008). Functional and physical interaction of blue- and red-light sensors in *Aspergillus nidulans. Current Biology* 18: 255-259.

Ramirez-Prado JH, Moore GG, Horn BW, Carbone I (2008). Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus. Fungal Genetics and Biology* 45: 1292-1299.

Reeves R (2015). High mobility group (HMG) proteins: Modulators of chromatin structure and DNA repair in mammalian cells. *DNA Repair* 36: 122-136.

Robellet X, Oestreicher N, Guitton A, Velot C (2010). Gene silencing of transgenes inserted in the *Aspergillus nidulans alcM* and/or *alcS* loci. *Current Genetics* 56: 341-8.

Ropars J, Dupont J, Fontanillas E, Rodriguez de la Vega C, Malagnac F, Coton M, Giraud T, Lopez-Villavicencio M (2012). Sex in cheese: Evidence for sexuality in the fungus *Penicillium roqueforti. PLoS One* 7: e49665.

Rosén S, Yu JH & Adams TH (1999) The *Aspergillus nidulans sfaD* gene encodes a G protein β subunit that is required for normal growth and repression of sporulation. *European Molecular Biology Organization Journal* 18: 5592-5600.

Ruger-Herreros C, Rodriguez-Romero J, Fernandez-Barranco R, Olmedo M, Fischer R, Corrochano LM, Canovas D (2011). Regulation of conidiation by light in *Aspergillus nidulans. Genetics* 188: 809-822.

Rydholm C, Dyer PS, Lutzoni F (2007). DNA sequence characterization and molecular evolution of *MAT1* and *MAT2* mating-type loci of the self-compatible ascomycete mold *Neosartorya fischeri*. *Eukaryotic Cell* 6: 868-874.

Sarikaya Bayram O, Bayram O, Valerius O, Park HS, Irniger S, Gerke J, Ni M, Han KH, Yu JH, Braus GH (2010). LaeA control of velvet family regulatory proteins for light-dependent development and fungal cell-type specificity. *PLoS Genetics* 6: e1001226.

Saupe S (2000). Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. *Microbiology and Molecular Biology Reviews* 62: 489-502.

Saupe SJ, Clave C, Bégueret J (2000). Vegetative incompatibility in filamentous fungi: *Podospora* and *Neurospora* provide some clues. *Current Opinion in Microbiology* 3: 608-612.

Scazzocchio C (2006). *Aspergillus* genomes: Secret sex and the secrets of sex. *Trends in Genetics* 22: 521-525.

Scherer M, Wei H, Liese R, Fischer R (2002). *Aspergillus nidulans* catalase-peroxidase gene (*cpeA*) is transcriptionally induced during sexual

development through the transcription factor StuA. *Eukaryotic Cell* 1: 725–735.

Schmoll M, Seibel C, Tisch D, Dorrer M, Kubicek CP (2010). A novel class of peptide pheromone precursors in ascomycetous fungi. *Molecular Microbiology* 77: 1483-1501.

Schoch CL, Sung GH, Lopez-Giraldez F, Townsend JP, Miadlikowska J, Hofstetter V, Robbertse B, Matheny PB, Kauff F, Wang Z, Gueidan C, Andrie RM, Trippe K, Ciufetti LM, Wynns A, Fraker E, Hodkinson BP, Bonito G, Groenewald JZ, Arzanlou M, de Hoog GS, Crous PW, Hewitt D, Pfister DH, Peterson K, Gryzenhout M, Wingfield MJ, Aptroot A, Suh SO, Blackwell M, Hillis DM, Griffith GW, Castlebury LA, Rossman AY, Lumbsch HT, Lucking R, Budel B, Rauhut A, Diederich P, Ertz D, Geiser DM, Hosaka K, Inderbitzin P, Kohlmeyer J, Volkmann-Kohlmeyer B, Mostert L, O'Donnell K, Sipman H, Rogers JD, Shoemaker RA, Sugiyama J, Summerbell RC, Untereiner W, Johnston P R, Stenroos S, Zuccaro A, Dyer PS, Crittenden PD, Cole MS, Hansen K, Trappe JM, Yahr R, Lutzoni F, Spatafora JW (2009). The ascomycota tree of life: A phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Systematic Biology* 58: 224-239.

Schoustra SE, Debets AJM, Slakhorst M, Hoekstra RF (2007). Mitotic recombination accelerates adaptation in the fungus *Aspergillus nidulans*. *PLoS Genetics* 3: e68.

Schuster E, Dunn-Coleman N, Frisvad JC, Van Dijck PW (2002). On the safety of *Aspergillus niger*-a reveiw. *Applied Microbiology and Biotechnology* 59: 426-435.

Seo JA & Yu JH (2006). The phosducin-like protein *PhnA* is required for Gβγ-mediated signaling for vegetative growth, developmental control, and toxin biosynthesis in *Aspergillus nidulans. Eukaryotic Cell* 5: 400-410

Seo JA, Han KH, Yu JH (2004). The *gprA* and *gprB* genes encode putative G protein-coupled receptors required for self-fertilization in *Aspergillus nidulans. Molecular Microbiology* 53: 1611-1623.

Seo JA, Han KH, Yu JH (2005). A multiple role of a heterotrimeric G-protein γ-subunit in governing growth and development of *Aspergillus nidulans. Genetics* 171:81-89.

Sessa L, Bianchi ME (2007). The evolution of high mobility group box (HMGB) chromatin proteins in multicellular animals. *Gene* 387: 133-140.

Sewall TCM, Mims CW, Timberlakea WE (1990). *abaA* controls phialide differentiation in *Aspergilus nidulans. The Plant Cell* 2: 731 -739.

Sharon A, Yamaguchi K, Christiansen S, Horwitz BA, Yoder OC, Turgeon BG (1996). An asexual fungus has the potential for sexual development', *Molecular and General Genetics* 251: 60-68.

Shui PK, Glass NL (2000). Cell and nuclear recognition mechanisms mediated by mating type in filamentous ascomycetes. *Current Opinion in Microbiology* 3:183–188.

Silva CC, Cruz RC, Rodriguez MB, Boas AV (2009). *Neurospora crassa* mat A-2 and mat A-3 protiens weakly interact in the yeast two-hybrid system and affect yeast growth. *Genetics and Molecular Biology* 32: 354-361.

Skaggs BA, Alexander JF, Pierson CA, Schweitzer KS, Chun KT, Koegel C, Barbuch R, Bard M (1996). Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome p-450 involved in ergosterol biosynthesis. *Gene* 169: 105-109.

Skory CD, Chang PK, Cary J, Linz JE (1992). Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin a to sterigmatocystin in aflatoxin biosynthesis. *Applied and Environmental Microbiology* 58: 3527-3537.

Smith ML, Micali OC, Hubbard SP, Rashed NM, Jacobson DJ, Glass NL (2000). Vegetative incompatibility in the het-6 region of *Neurospora crassa* is mediated by two linked genes. *Genetics* 155: 1095-1104.

Snyder WC, Georgopoulos SG, Webster RK, Smith SN (1975). Sexuality and genetics behavior in the fungus *Hypomyces (Fusarium) solani* f.Sp. *Cucurbitae*. *Hilgardia* 43: 161-185.

Sohn KT, Yoon KS (2002). Ultrastructral study on the cleistothecium development in *Aspergillus nidulans. Microbiology* 30: 117-127.

Son H, Seo YS, Min K, Park A, Lee JM, Jin J, Lin Y, Cao P, Hong SV, Kim EK, Lee SH, Cho A, Lee S, Kim MG, Kim Y, Kim JE, Kim JC, Choi G, Yun SH, Lim J, Kim M, Lee YH, Choi YD, Lee YW (2011). A phenome-based functional analysis of transcription factors in the cereal head blight fungus, *Fusarium graminearum. PLoS Pathogens* 7: e1002310.

Soullier S, Jay P, Poulat F, Vanacker J, Berta P, Laudet V (1998). Diversification pattern of the HMG and SOX family members during evolution. *Journal of Molecular Evolution* 48: 517-527.

Spellig T, Bölker M, Lottspeich F, Frank R W Kahmann R (1994). Pheromones trigger filamentous growth in *Ustilago maydis. The EMBO Journal* 13: 1620-1627

Staben C, Yanofsky C (1990). *Neurospora crassa* a mating-type region. *Proceedings of the National Academy of Sciences of the United States of America* 87: 4917-4921.

Stinnett SM, Espeso EA, Cobeno L, Araujo-Bazan L, Calvo AM (2007). *Aspergillus nidulans* VeA subcellular localization is dependent on the importin α carrier and on light. *Molecular Microbiology* 63: 242-255.

Stros M, Launholt D, Grasser KD (2007). The HMG-box: A versatile protein domain occurring in a wide variety of DNA-binding proteins. *Cellular and Molecular Life Sciences* 64: 2590-2606.

Sugimoto A, Iino Y, Maeda T, Watanabe Y, Yamamoto M (1991). *Schizosaccharomyces pombe ste11* + encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. *Gene* 5: 1990-1999.

Sun S, Heitman J (2011). Is sex necessary? *BMC Biology* 9: 56-60.

Swilaiman SS (2013). Sexual potential and population biology of *Aspergillus* and *Penicillium* species. *PhD Thesis Univeristy of Nottingham*.

Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR (2007). Fusion PCR and gene targeting in *Aspergillus nidulans. Nature Protocols* 1: 3111-3121.

Taylor JW, Jacobson DJ, Fisher MC (1999). The evolution of asexual fungi reproduction, speciation and classification. *Annual Review of Phytopathology* 37: 197-246.

Thon M, Al-Abdallah Q, Hortschansky P, Brakhage AA (2007). The thioredoxin system of the filamentous fungus *Aspergillus nidulans*: Impact on development and oxidative stress response. *Journal of Biological Chemistry* 282: 27259-27269.

Todd RB, Davis MA, Hynes MJ (2007). Genetic manipulation of *Aspergillus nidulans:* Meiotic progeny for genetic analysis and strain construction. *Nature Protocols* 2: 811-821.

Todd RB, Hynes MJ, Andrianopoulos A (2006). The *Aspergillus nidulans rcoA* gene is required for *veA*-dependent sexual development. *Genetics* 174: 1685-1688.

Trail F, Mahanti N, Rarick M, Mehigh R, Liang SH, Zhou R, Linz JE (1995). Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway. *Applied and Environmental Microbiology* 61: 2665-2673.

Tsitsigiannis DI, Kowieski TM, Zarnowski R, Keller NP (2004b). Endogenous lipogenic regulators of spore balance in *Aspergillus nidulans*. *Eukaryotic Cell* 3: 1398-1411.

Tsitsigiannis DI, Kowieski TM, Zarnowski R, Keller NP (2005). Three putative oxylipin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans. Microbiology* 151: 1809-1821.

Tsitsigiannis DI, Zarnowski R, Keller NP (2004a). The lipid body protein, PpoA, coordinates sexual and asexual sporulation in *Aspergillus nidulans. Journal of Biological Chemistry* 279: 11344-11353.

Tüncher A, Reinke H, Martic G, Caruso ML & Brakhage AA (2004). A basic-region helix-loop-helix protein-encoding gene (*devR*) involved in the development of *Aspergillus nidulans*. *Molecular Microbiology* 52: 227-241

Turgeon BG, Debuchy R (2007). *Cochliobolus* and *Podospora*: Mechanisms of sex determination and evolution of reproductive lifestyle. In Sex in Fungi: Molecular Determination and Evolutionary Implications (ed. Heitman J, Kronstad JW, Taylor JW, Casselton LA). Washington DC: ASM Press. p 93–121.

Turgeon BG, Yoder OC (2000). Proposed nomenclature for mating type genes of filamentous ascomycetes. *Fungal Genetics and Biology* 31: 1-5.

Turrá M, El Ghalid M, Rossi F, Di Pieto M (2015). Fungal pathogen uses sex pheromone receptor for chemotrophic sensing of host plant signals. *Nature* 527:521-524.

Ueda T, Yoshida M (2010). HMGB proteins and transcriptional regulation. *Biochimica et Biophysica Acta* 1799: 114-118.

Vallim A, Miller K, Miller B (2000). *Aspergillus steA* (sterile 12-like) is a hoeodomain-C2/H2-zn +2 finger transcription factor reqiured for sexual reproduction. *Molecular Microbiology* 36: 290-301.

Varga J (2003). Mating type gene homologues in *Aspergillus fumigatus. Microbiology* 149: 816-819.

Varga J, Due M, Frisvad JC, Samson RA (2007). Taxonomic revision of *Aspergillus* section Clavati based on molecular, morphological and physiological data. *Studies in Mycology* 59: 89-106.

Varga J, Szigeti G, Baranyi N, Kocsube S, O'Gorman CM, Dyer PS (2014). *Aspergillus*: Sex and recombination. *Mycopathologia* 178: 349-362.

Verna J, Ballester R, (1999). A noval role for the mating type (*MAT*) locus in the maiantainance of the cell wall integrity in *Saccharomyces cerevisiae*. *Molecular and General Genetics* 261: 681-689.

Vienken K, Fischer R (2006). The $Zn(II)_2Cys_6$ putative transcription factor NosA controls fruiting body formation in *Aspergillus nidulans. Molecular Microbiology* 61: 544-554.

Vienken K, Scherer M, Fischer R (2005). The $Zn(II)_2Cys_6$ putative *Aspergillus nidulans transcription* factor repressor of sexual development inhibits sexual development under low-carbon conditions and in submersed culture. *Genetics* 169: 619-630.

Wada R, Jin FJ, Koyama Y, Maruyama J, Kitamoto K (2014). Efficient formation of heterokaryotic sclerotia in the filamentous fungus *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* 98: 325-334.

Wada R, Maruyama J, Yamaguchi H, Yamamoto N, Wagu Y, Paoletti M, Archer DB, Dyer PS, Kitamoto K (2012). Presence and functionality of mating type genes in the supposedly asexual filamentous fungus *Aspergillus oryzae. Applied and Environmental Microbiology* 78: 2819-2829.

Wang B, Guo G, Wang C, Lin Y, Wang X, Zhao M, Guo Y, He M, Zhang Y, Pan L (2010). Survey of the transcriptome of *Aspergillus oryzae* via massively parallel mRNA sequencing. *Nucleic Acids Research* 38: 5075-5087.

Ward OP, Qin WM, Dhanjoon J, Ye J, Singh A (2005). Physiology and biotechnology of *Aspergillus*. *Advances in Applied Microbiology* 58: 1-75.

Wei H, Scherer KM, Singh A, Liese R & Fischer R (2001). *Aspergillus nidulans* α-1,3 glucanase (mutanase), *mutA*, is expressed during sexual development and mobilizes mutan. *Fungal Genetics and Biology* 34: 217-227.

Wei H, Vienken K, Weber R, Bunting S, Requena N & Fischer R (2004). A putative high affinity hexose transporter, *hxtA*, of *Aspergillus nidulans* is induced in vegetative hyphae upon starvation and in ascogenous hyphae during cleistothecium development. *Fungal Genetics and Biology* 41: 148-156.

Wei HR, Requena N, Fischer R (2003). The MAPKK kinasestec regulates conidiophore morphology and is essential for heterokaryon formation and sexual development in the homothallic fungus *Aspergillus nidulans*. *Molecular Microbiology* 47: 1577-1588.

Wilken PM, Steenkamp ET, Hall TA, De Beer ZW, Wingfield MJ, Wingfield, BD (2012). Both mating types in the heterothallic fungus *Ophiostoma quercus* contain *MAT1-1* and *MAT1-2* genes. *Fungal Biology* 116: 427-437.

Willier M, Hoffmann L, Styrkarsdottir U, Egel R, Davey J, Nielsen O (1995). Two-step activation of meiosis by the *mat1* locus in *Schizosaccharomyces pombe. Molecular and Cellular Biology* 15:4964-4970.

Wu D, Dou X, Hashmi SB, Osmani SA (2004). The Pho80-like cyclin of *Aspergillus nidulans* regulates development independently of its role in phosphate acquisition. *Journal of Biological Chemistry* 279: 37693-37703.

Wu J, Miller BL (1997). *Aspergillus* asexual reproduction and sexual reproducton are differentially affected by transcrptional and translational mechanisms regulating stunted gene expresion. *Molecular Cell Biology* 17:6191-6201.

Xiang Q, Glass NL (2004). The control of mating type heterokaryon incompatibility by *vib-1*, a locus involved in *het-c* heterokaryon incompatibility in *Neurospora crassa. Fungal Genetics and Biology* 41: 1063-1076.

Yu J, Chang PK, Cary JW, Bhatnagar D, Cleveland TE (1997). *avnA*, a gene encoding a cytochrome p-450 monooxygenase, is involved in the conversion of averantin to aveufin in aflatoxin biosynthesis in *Aspergillus parasiticus. Applied and Environmental Microbiology* 63: 1349-1356.

Yu JH (2010). Regulation of development in *Aspergillus nidulans* and *Aspergillus fumigatus. Mycobiology* 38: 229-237.

Yu JH, Hamari Z, Han KH, Seo JA, Reyes-Dominguez Y, Scazzocchio C (2004). Double-joint PCR: A PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genetics and Biology* 41: 973-981.

Yu JH, Keller N (2005). Regulation of secondary metabolism in filamentous fungi. *Annual Review Phytopathology* 43: 437-458.

Yu JH, Mah JH, Seo JA (2006). Growth and developmental control in the model and pathogenic aspergilli. *Eukaryotic Cell* 5: 1577-1584.

Yun SH, Berbee ML, YOder OC, Turgeon BG (1999). Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors. *Proceedings of the National Academy of Sciences of the United States of America* 96: 5592-5597.

Zhang L, Baasiri RA,Van Alfen NK (1998). Viral repression of fungal pheromone precursor gene expression. *Molecular and Cellular Biology* 18: 953–959.

Appendices

Appendix 1 Primer sets for amplification of *Aspergillus oryzae* homologous genes in *A. nidulans*

Gene ID	Forward primer	Reverse primer
AN1356	5'- CTCAATAATGCCTCCTCTACAC -3'	5'- CAGACTGAAAGCGATGAATGTC -3'
AN2960	5'- ATCAACAGATACCCACCCCTGC -3'	5'- AAAGGTCACATCGGAGTGCCAG -3'
AN3239	5'- TATGCCCATTCCGATACGC -3'	5'- GGTAGAAGTTGCTGAAGACAAG -3'
AN3275	5'- TTGCCAACCCTGGAAATCC -3'	5'- AGTGATGAACGCCGAATGG -3'
AN3562	5'- GTCTACTTCGCTTGCTCAC -3'	5'- ATCGGCATCGGTTTCAAC -3'
AN4686	5'- CGCTTTCTGCCTTTGAAGTC -3'	5'- TGACCATCACCAACACTGC -3'
AN5791	5'- CCGAGCCCTTCAAAATCAATAG -3'	5'- CATCCAAAAAAGCCATAGCG -3'
AN5993	5'- CCTTCTCCTCCTTTCAACAG -3'	5'- GGGTTATCGCAGAGTGTATC -3'
AN6257	5'- CCAGTAGCGAAGATTGACAC -3'	5'- TCCACGGAGGGTCTATTTAC -3'
AN6458	5'- TTTGCGTCTGCCTTCACTTGCC -3'	5'- ATTGCTCGGACCACAGCGAATG -3'
AN6881	5'- CCACACTGAAGAACAAAAGC -3'	5'- ATTGCCGACGAGGTTATTC -3'
AN7818	5'- TCTTGTATCTGCTCTCTACGG -3	5'- CCTTTTCTGCTGTGAACTGC -3
AN8184	5'- TGTCCCTTGTTTTGTTGACC -3'	5'- TTGTGCGATGTTTCCAGG -3'
AN8656	5'- GGATAACTACAACGAGTGCTAC -3'	5'- CACGACCTTCTGGAAATACAG -3'

AN11253	5'- AAGCCGACAACGCCGCTAATAC -3'	5'- TAAACACATCTCCCCAGACCGC -3'
AN10820	5'- TCACATTCAGCATCAGCGTCCC -3	5- TTGGAAGTATCCCAGCATCGGC -3'
AN1598	5'- ACCCTCATCCCTTCCGATTCAC -3'	5'- ACACATTGACGCTCTGCCAGTC -3'
AN5039	5'- TCTGTGCTCTGGTAACGGCATC -3'	5'- TCCCTGACGAGTGATTCATTTCTC -3'

Appendix 2 Primer sets for positional PCR designed in flanking regions of *A. nidulans* together with pair primer sets for *pyrG* marker gene of *A. fumigatus.*

Gene	Forward	Reverse
AN1356	5'- GCTGGTTTCGGTCACATACTTTTC -3'	5'- TACTCAACGGACGGAAGGTG -3'
AN3239	5'- CGCTGGGTTTTGGAACTTAG -3'	5'- GGATACTGGGGATGCTACTATG -3'
AN3275	5'- GCTGAATCTGGCTGGTATTG -3'	5'- TCCGCCAGGATTTGTCTTCG -3'
AN3562	5'- AACTAAGGATGGGCAGTGCG -3'	5'- TATCTGTCCGACTCTCTGGGAG -3'
AN4686	5'- AAACGGCGAGAACCCAAAC -3'	5'- TTGTTGGCACGAATCTCCG -.3'
AN5791	5'- TGACATCAACCTGCTCCCAC -3'	5'- TCTTCCACTCCCCTGTTCTG -3'
AN5993	5'- ACGGAGTCACGAGCATTGTC -3'	5'- AACAAGCACCCCGAAAACG -

		3'
AN6881	5'-GCTGCTGGTATTATGATTGAGGAC-3'	5'-GCCAAGAAAAACCACACTCAG-3'
AN7818	5'- AACTGATTCCCACCCCATCC -3'	5'- TGATGAGGTGCTTCGCTTG -3'
AN8184	5'- ATGCGAACAACGACGACAC -3'	5'- TATCTTCCGTGGTTCGTGC -3'
AN8656	5'- ACACCTTGTCACCGTAGTCG -3'	5'- CGGGTGCCTCTTCAATAGTAG -3
AN11253	5'- TAGAATGTGGACCTGCCTGG -3'	5'- AGGGCTTTGGTCAACTTCG -3'
AN1598	5'- ACCATTCACCGACGACATCG -3'	5'- TGGATGTGGTGCGACAACTG -3'
PyrG	5’- TTTGCCAGAGGATTGGGGTG -3’	5’- ATCAGCAGAGACGGTAACG -3’
pyrG	5’- GCTCGCAAATACAAGAACTTCG -3’	5’- ACGGGTGTGAACCTCTCTTC -3’

Appendix 3 Primer sets for qRT-PCR experiments

Gene ID	**Forward**	**Reverse**
AN3667	5'- CCAAGAACGACGGGAAAATC -3'	5-CCTGATGCTGAGGAAACAAC -3'
AN0879	CACGAGCAAGGACACTAGCA	ACCTGGTTGAACGGGTTCTC
AN1267	5'-CCAAGAAGACCAAGAAGAAAGCAG -3	5'- AGGGCAAGGTAGGCAGTAAC -3'
AN1962	5'- TGAAAGCCTGCCAAAGGTC -3'	5'- TGGACTGGACGATACTGAGC -3'
AN2885	5-CTAAGGCCAATCCTACCCGC-3	5-TCACCGAGCATCTTTCCGAC-3

AN3549	5-CTCCATGTGGTTGGCTGGAT-3	5-TTTGCTGCCGGCTTAGATCA-3
AN3580	5'- ATCGCTCTTATGGCAGGTGACG -3	5'-GGAGTATGTCCCTCTGGTGAACTTG -3'
AN5073	5'- CGCCGACAAATACAAACAGATG -3'	5'- CCAAACGCCACTGAAATCC -3
AN10103	5-GATAAGGCAGCCGACCAACT-3	5-AGGGGGAGTGGGTTCCTTAG-3
AN4734	5-GGCGCAGGTTACATCGGATA-3	5-ATGCAGTCCTGCCATAGTCG-3
AN2755	5-GATGTCAAGGCCCAGGAACA-3	5-GTTTCCCATCATGGACCCGT-3
AN5791 (*ppgA*)	5'-GCCGAGCCCTTCAAAATCAATAG -3'	5'-TCCAAAAAAGCCATAGCGTCTG -3'
AN7743 (*preA*)	5-TGACCGTTGGATCCCTGTTG-3	5-GCAGATCCCGAAGTATGGGG-3
AN2520 (*preB*)	5-CCAGGTATGTCCTGCTATGCT-3	5-AGAGGGCAACGATGGTCAAG-3
AN2290 (*steA*)	5-TTCGAGCCTGTTACACCACC-3	5-ATGGAAAGTTCTCGCTGGGG-3
AN 7388 (laccase II)	5- CAAGACCTTTGGGTTTGCCG-3	5-GGGTTGTCGAGTTCTCCGTT-3
AN5997	5- CCTATCGCGGAATCGACCTC-3	TTCTCGTTAGGCTTGGCCTC
AN3469 (H2b)	5- CTGCCGAGAAGAAGCCTAGC-3	5- CTCCTTCCTGGTCTTTCCGC-3
AN (Actin A)	5-CAGAAGGAAATCACCGCCCT-3	5-GGAAGGTGGACAGAGAAGCC-3

Appendix 4 Primer sets used for construction of cloning vectors. Lower case nucleotides indicate overlapping sequence with a vector and/or joining fragment.

Name of primer	Sequence
AN5791F	cttgagcagacatcacATGAAGCTTTTCTTCGTCTC
AN5791R	gtaacgttaagtggatcTTACCCGTCGGAATTGTC
AN5791-FPyrG	ggtatttcacaccgcaGATTTCAGTAACGTTAAGTGG
AN5791-RPyrG	ggcgtaaaGACAGAAGATGATATTGAAGG
AN5791_3FF	cttctgtcTTTACGCCTATCAGTTAGCAC
AN5791_3FR	tactgagagtgcaccaCAGCGCATGTAGCACAGA
AN5791_5FF	ctatgaccatgattacgGAAAACCTGGCGGAGGGA
AN5791_5FR	gtgtagagatacaagggTGCAAGAATAATAAAGACTTGAAGAGTG
AN8184_FPyrG	acctgcaggcatgcaGATTTCAGTAACGTTAAGTGG
AN8184_RPyrG	catcagaaaGACAGAAGATGATATTGAAGG
AN8184_3FF	cttctgtcTTTCTGATGACATTACTCTGC
AN8184_3FR	cgacggccagtgccaACTGACTCGACCATCTATC
AN8184F	cttgagcagacatcacATGTCCCCCATGGTCCTT
AN8184R	gtaacgttaagtggatcTCAGAGATTGACCAGGCG
AN8184_5FF	ctatgaccatgattacgCTTGAAGGATAACGCAGG
AN8184_5FR	gtgtagagatacaagggCGTGAACAATCTCAAACC
AN1962_FPyrG	acctgcaggcatgcaGATTTCAGTAACGTTAAGTGG
AN1962_RPyrG	acccttttGACAGAAGATGATATTGAAGG
AN1962_3FF	cttctgtcAAAAAGGGTGTTTTGATAAATTTTTC
AN1962_3FR	cgacggccagtgccaAAAGGCCGTCAGAATGATC

AN1962F	cttgagcagacatcacATGTCTTACGATCGAGTTC
AN1962R	gtaacgttaagtggatcAGATTGTGTAATGTAATCTTCAATTTC
AN1962_5FF	ctatgaccatgattacgAGCCGGGCCCCAGTCCGG
AN1962_5FR	gtgtagagatacaagggCTATTTAATGCGAAATTCAGTGAGTAGAGAGGCGAAGGTGAGC
AN3667-5FF	ctatgaccatgattacgTAGGAAGATGTCCAGACAGTTAAC
AN3667-5FR	gtgtagagatacaagggGTTTTTGTGACGCAGTAAATTG
AN3667F	cttgagcagacatcacATGCAGCGGCGATTGATC
AN3667R	gtaacgttaagtggatcTCAATACCCAGCGGGTTG
AN3667- FPyrG	acctgcaggcatgcaGATTTCAGTAACGTTAAGTGG
AN3667- RPyrG	gaagacgaagcgtaGACAGAAGATGATATTGAAGG
AN3562-5FF	ctatgaccatgattacgCCCTGCCGGTTATTTTCAG
AN3562-5FR	gtgtagagatacaagggCGTGACGGATGACCAATG
AN3562F	agcagacatcaccatgATGAAGTCTACTTCGCTTG
AN3562R	tttcagtaacgttaagtgTCAATAGTAAAGAGGATTCTCAG

Appendix 5 Primer sets for screening overexpression tranformant colonies. Lower case nucleotides indicate overlapping sequence with a vector and/or joining fragment.

Gene ID	Forward primer	Reverse primer
AN1962	ctatgaccatgattacgAGCCGGGCCCCAGTCCGG	gtaacgttaagtggatcAGATTGTGTAATGTAATCTTCAATTTC
AN5791	ctatgaccatgattacgGAAAACCTGGCGGAGGGA	gtaacgttaagtggatcTTACCCGTCGGAATTGTC

AN3562	ctatgaccatgattacgCCCTGCCGGTTATTTTCAG	tttcagtaacgttaagtgTCAATAGTAAAGAGGATTCTCAG
AN3667	ctatgaccatgattacgTAGGAAGATGTCCAGACAGTTAAC	gtaacgttaagtggatcTCAATACCCAGCGGGTTG
AN8184	ctatgaccatgattacgCTTGAAGGATAACGCAGG	gtaacgttaagtggatcTCAGAGATTGACCAGGCG

Appendix 6a Fertility test- number of cleistothecia, statistical test results for mutant strains of *A. nidulans* compared to parental strain.

***A. nidulans* strains**	**Mean of the number of Cleistothecia/cm^2**	**P-value**
Parental [a]	1341.63	
Parental [b]	1230.08	
Parental [c]	1052.78	
Protoplast control [a]	1378.96	0.671
Protoplast control [b]	1266.14	0.660
Protoplast control [c]	1182.57	0.52
ΔAN1356-1 [a]	1006.12	0.001
ΔAN 1356-2 [a]	10068.9	0.002
ΔAN1958-1 [c]	1320.86	0.0001
ΔAN1958-2 [c]	1409.0	0.0001
ΔAN3239 [a]	1280.56	0.498
ΔAN3275 [b]	944.2	0.001
ΔAN4686-1 [b]	864.88	0.001

ΔAN4686-2 [b]	953.95	0.001
ΔAN5791-1 [a]	170.52	0.0001
ΔAN5791-2 [a]	201.48	0.0001
ΔAN5993-1 [a]	1284.37	0.525
ΔAN5993-2 [a]	1356	0.80
ΔAN6881-1 [a]	1765.81	0.0001
ΔAN6881-2 [a]	1633.47	0.004
ΔAN7818-1 [a]	1012.91	0.0001
ΔAN7818-2 [a]	1079.5	0.003
ΔAN8184-1 [a]	1928.69	0.0001
ΔAN8184-2 [a]	1982.97	0.0001
ΔAN11253-1 [b]	814.82	0.0001
ΔAN11253-2 [b]	1002.73	0.007

- Strains labelled with the same alphabet letter were statistically analysed together.
- For group (a) One-way ANOVA (square root transformed data) was used for Post Hoc test -LSD [F (14, 165) = 105.043, P-value = 0.001].
- For group (b) the result of One-Way ANOVA of Post Hoc test -LSD is [F (6, 83) = 9.058, P-value = 0.001].
- For group (c) the result of One-Way ANOVA of Post Hoc test -LSD is [F (3, 44) = 11.691, P-value = 0.001].

Appendix 6b Fertility test- diameter of cleistothecia, statistical test results for mutant strains of *A. nidulans* compared to parental strain

***A. nidulans* strains**	**Mean of the diameter of Cleistothecia (µm)**	**Chi-Square X2**	**P-value**
Parental [a]	165.9		
Parental [b]	168.7		
Parental [c]	159.14		
Protoplast control [a]	157.51	2.273	0.132
Protoplast control [b]	170.56	0.350	0.554
Protoplast control [c]	156.58		0.578
ΔAN1356-1 [a]	166.45	0.075	0.782
ΔAN 1356-2 [a]	161.47	0.641	0.423
ΔAN1958-1 [c]	157.28	-	0.686
ΔAN1958-2 [c]	159.1	-	0.363
ΔAN3239 [a]	156.81	2.751	0.097
ΔAN3275 [b]	202.47	33.358	0.0003
ΔAN4686-1 [b]	186.4	11.583	0.001
ΔAN4686-2 [b]	183.84	5.34	0.021
ΔAN5791-1 [a]	312	144.926	0.0001
ΔAN5791-2 [a]	283.33	139.43	0.0001
ΔAN5993-1 [a]	164.96	0.036	0.849
ΔAN5993-2 [a]	156.11	3.7	0.054
ΔAN6881-1 [a]	168.23	0.119	0.731

ΔAN6881-2 [a]	161.94	0.591	0.442
ΔAN7818-1 [a]	174.28	3.319	0.068
ΔAN7818-2 [a]	164.73	0.015	0.902
ΔAN8184-1 [a]	157.042	3.299	0.071
ΔAN8184-2 [a]	159.84	1.192	0.275
ΔAN11253-1 [b]	177.55	2.001	0.157
ΔAN11253-2 [b]	164.03	0.625	0.429

- Strains labelled with the same alphabet letter were statistically analysed together.
- For group (a) Kruskal-Wallis test was used to analyse data [Chi-Square (X2) 539.93, df = 14 and Asymp.sig = 0.0001]
- For group (b) Kruskal-Wallis test was used to analyse data [Chi-Square (X2) 59.16, df = 6 and Asymp.sig = 0.0001]
- For group (c) the result of One-Way ANOVA of Post Hoc test -LSD is [F (3, 44) = 0.868, P-value = 0.458].

Appendix 7a Complementation test- number of cleistothecia, statistical test results for complement strains of *A. nidulans* compared to parental strain.

***A. nidulans* strains**	**Mean No. of Cleistothecia/cm^2**	**Chi-Square X2**	**P-value**
Parental [a]	1385.75		
Parental [b]	1298.37		
Parental [c]	1052.78		
Protoplast control [a]	1315.34	0.368	0.544
Protoplast control [b]	1213.54	-	0.304
Protoplast control [c]	1138.89		0.132
AN1356-1-1 [c]	1152.88		0.081

AN 1356-1-2 [c]	1091.38		0.495
AN3275-1-1 [b]	1477.37	1.844	0.174
AN3275-1-2 [b]	1283.53	0.241	0.623
AN4686-1-2 [b]	1263.17	2.806	0.094
AN4686-1-2 [b]	1223.3	2.810.094	
AN5791-1-1 [a]	1188.1	-	0.182
AN5791-1-2 [a]	1157.55	-	0.089
AN6881-1-1 [a]	1248.75	-	0.547
AN7818-1-1 [a]	1183.85	-	0.166
AN7818-1-2 [a]	1169.85	-	0.121
AN8184-1-1 [a]	1256.81	-	0.614
AN8184-1-2 [a]	1224.76	-	0.926
AN8656-1-1	1460	0.964	0.326
AN8656-1-2	1387	0.188	0.665
AN11253-1-1[b]	1432.41	0.441	0.506
AN11253-1-2 [b]	1415.87	0.334	0.564

- Strains labelled with the same alphabet letter were statistically analysed together.
- For group (a) the result of One-Way ANOVA of Post Hoc test -LSD is [F (8, 99) = 0.8902, P-value = 0.518].
- For group (b) Kruskal-Wallis test was used to analyse data [Chi-Square (X2) 18.615, df = 9 and Asymp.sig = 0.029].
- For group (c) the result of One-Way ANOVA of Post Hoc test -LSD is [F (3, 44) = 1.334, P-value = 0.276].

Appendix 7b Complementation test- diameter of cleistothecia, statistical test results for complement strains of *A. nidulans* ccompared to parental strain.

***A. nidulans* strains**	**Mean of the diameter of Cleistothecia (µm)**	**Chi-Square X2**	**P-value**
Parental [a]	163.1		
Parental [b]	157.51		
Parental [c]	160.3		
Protoplast control [a]	170.55	2.64	0.104
Protoplast control [b]	157.28		
Protoplast control [c]	157.28	0.173	0.677
AN1356-1-1 [c]	161.0	0.024	0.87
AN 1356-1-2 [c]	164.5	2.117	0.146
AN3275-1-1 [b]	153.55		0.386
AN3275-1-2 [b]	164.03		0.154
AN4686-1-2 [b]	165.43		0.083
AN4686-1-2 [b]	157.74		0.959
AN5791-1-1 [a]	68.93	0.763	0.382
AN5791-1-2 [a]	171.72	3.194	0.072
AN6881-1-1 [a]	171.26	1.617	0.204
AN7818-1-1 [a]	170.09	2.513	0.113
AN7818-1-2 [a]	161.7	0.119	0.730
AN8184-1-1 [a]	157.74	2.032	0.154
AN8184-1-2 [a]	156.57	3.683	0.055

AN8656-1-1	157.74		0.959
AN8656-1-2	151.22		0.169
AN11253-1-1[b]	156.11		0.76
AN11253-1-2 [b]	11.18		0.61

- Strains labelled with the same alphabet letter were statistically analysed together.
- For group (a) Kruskal-Wallis test was used to analyse data [Chi-Square (X2) 2.006, df = 8 and Asymp.sig = 0.002]
- For group (b) the result of One-Way ANOVA of Post Hoc test -LSD is [F (9, 999) = 1.789, P-value = 0.066].
- For group (c) Kruskal-Wallis test was used to analyse data [Chi-Square (X2) 3.308, df = 3 and Asymp.sig = 0.346].

Appendix 8 PCR cycling parameter used for construction of deletion cassettes for both mating-type genes.

Fusion PCR cycling

Fusion PCR was done according to a protocol described by Szewczyk *et al.* (2007). The following PCR cycling was used to amplify and ligate the three pieces of deletion cassette of each gene (upstream, PyrG and downstream) by using a Phusion high fidelity PCR kit.

94ºC, 2 min
10 cycles:
94 ºC. 20 sec (denaturation)
Ramp down to 70 ºC at maximum rate (most primers should not anneal in this step)
70 ºC, 1 sec
Ramp down to annealing temperature at 0.1 ºC /sec
57 ºC, 30 sec (annealing step, the annealing temp is normally 5 ºC below the Tm.)
Ramp up to extension temperature at 0.2 ºC /sec
68 ºC, 4 min (the extension time is normally1 min/kb of the expected fusion PCR fragment)
Ramp up at maximum rate to 94 ºC
5 cycles
94 ºC. 20 sec (denaturation)
Ramp down to 70 ºC at maximum rate
70 ºC, 1 sec
Ramp down to annealing temperature at 0.1 ºC /sec
57 ºC, 30 sec (annealing)
Ramp up to extension temperature at 0.2 ºC /sec
68 ºC, 4 min extension time for the first cycle. Increase extension time for each subsequent cycle by 5 sec as enzymes are losing potency. The last cycle will be 4 min 20 sec
Ramp up at maximum rate to 94 ºC
10 cycles
94 ºC. 20 sec (denaturation)
Ramp down to 70oC at maximum rate (most primers should not anneal in this step)
70 ºC, 1 sec
Ramp down to annealing temperature at 0.1 ºC /sec
57 ºC, 30 sec (annealing)
Ramp up to extension temperature at 0.2 ºC /sec
68 ºC, 4 min 20 sec extension time for the first cycle. Increase extension time for each subsequent cycle by 20 sec as enzymes are losing potency. The last cycle will be 9 min 20 sec.
Ramp up at maximum rate to 94 ºC

Appendix 9 Primer sets for amplification of HMG target genes in *A. nidulans*

Gene ID	Forward (HTF)	Reverse (HTR)
AN0879	5'TGAAGAGGACAAGCAATGGG C -3'	5'- CGTCGCAAACCAGGATTCTCAG -3'
AN2885	5'- TGTTCTTCGCCAACGACAACC -3'	5'- CCACTTCTCACCGAGCATCTTTC -3'
AN3580	5'CCCTTTAGAGACAATCATCAC ACGC -3'	5'CCTCCTTTCCCATTCCACATTATC -3'
AN5073	5'GGAATGGGAGAGCCTGACACT AAC -3'	5'- CAAACGCCACTGAAATCCGTG -3'
AN 10103	5'- TTGCCCTCTGAACTGTGTC -3'	5'- CCTCCTTGTCCTTCTTAGCC -3'
AN 3549	5'- AACCAAAGCCCAGAATCCC -3'	5'- AACATCATCTCCCTGCTCG -3'
AN 1962	5-TCGCTCAGTATCGTCCAGTC -3	5'- AAGTCCGTCCTGTATGGCAC -3'
AN 1267	5'- ACGCCGAAGTTACTGCCTAC -3'	5'- GCAAGTTTTTCCACTGAACAGC -3'
AN2755	5'AAACCACCAGTCTTCAGAAAC GAG -3'	5'AGTAGCAAATGTCATAACAGGG GC -3'
AN4734	5'TGAAATCACCAACACAGTCGC C -3'	5'GTGCTTCTTTTTGAACTCTTCCGC -3'

Appendix 10 Primer sets for HMG genes flanking region used for positional PCR.

Gene ID	Forward (HFF)	Reverse (HFR)
AN0879	5'-TTCTGCGTGGTGTTTACCCC-3'	5'-GGCTCACTTTGGTTTCTTGATGTC -3'
AN2885	5'- TCGTGTGGAGAAGCCTCTTG -3'	5'- AGCCTATTCAAGTCACCCGC -3
AN3580	5'- ATCACTTCCTGTCGCTTCCG -3'	5'- ACTGGCAGAACGGTATCAGC -3'
AN5073	5'- TCACAGAAACAGTGGCTGGC -3'	5'- ATGTATCCCTCTACCGCAGC -3
AN 10103	5'ACACATACCCCCAGCGATTC -3	5'- TAATCCGCTTCCTTCCCTCG -3'
AN 3549	'- ATTCTCTGCTGGGACGCAAG -3	5'- TTACGGACACGGTCTGAGAG -3
AN 1962	5'AGAAGAACGACCAGCGGTTG -3'	5'- AAAAGAGGGGAGTGAGGCTC -3'
AN 1267	5'AGACTGGGGTGAACTAACGG -3'	5'- AAGAGGAGTGGAACGGCAAG -3
AN4734	'5AATGGGTGGTGCTACTGTGG -3'	5'- AGGGGCTCAGGTAAGAAACC -3'
AN2755	5'-TTGTAGGTCCTGGCACTCTG -3	5'- ACTATTCCTGCTCGCTGTCG -3'

Appendix 11a Fertility test- number of cleistothecia, statistical test results for putative HMG mutant strains compared to parental strain.

***A. nidulans* strains**	**Mean No. of Cleistothecia/cm^2**	**Chi-Square (X2)**	**P-value**
Parental strain	1148.22		
Protoplast control	1229.66	1.08	0.298
ΔAN0879	1203.36	0.752	0.386
ΔAN1267	316	17.31	0.0001
ΔAN1962-1	233.3	17.3	0.0001
ΔAN1962-2	205.72	17.3	0.0001
ΔAN2755-1	995.94	5.62	0.018
ΔAN2755-2	1006.55	5,62	0.018
ΔAN2885	551.84	16.83	0.0001
ΔAN3580-1	1163.1	0.083	0.773
ΔAN3580-2	1195.3	0.61	0.435
ΔAN4734-1	26.72	17.348	0.0001
ΔAN4734-2	18.66	17.41	0.0001
ΔAN5073	948.86	4.577	0.032
ΔAN10103-1	1254.69	0.564	0.453
ΔAN10103-2	1312.8	2.822	0.093

- Non- parametic test (Kruskal – Wallis test) used to analysed data [Chi-Square (X2) = 158.177, df = 15 and Asymp.sig (P-value) = 0.0001].

Appendix 11b Fertility test- diameter of cleistothecia, statistical test results for HMG mutant strains compared to parental strain.

***A. nidulans* strains**	**Mean of the diameter of Cleistothecia (µm)**	**Chi-Square (X2)**	**P-value**
Parental strain	153.78		
Protoplast control	153.1	0.003	0.956
ΔAN0879	158.91	2.296	.13
ΔAN1267	154.25	0.035	0.851
ΔAN1962-1	339.95	139.34	0.0001
ΔAN1962-2	360.68	142.46	0.001
ΔAN2755-1	168.22	11.86	0.006
ΔAN2755-2	168	7.44	0.001
ΔAN2885	181.27	16.33	0.0001
ΔAN3580-1	159.61	3.217	.073
ΔAN3580-2	159.37	3.045	.081
ΔAN4734-1	378.86	149.67	0.0001
ΔAN4734-2	374.9	148.1	0.0001
ΔAN5073	153.1	0.008	0.927
ΔAN10103-1	144	2.42	0.12
ΔAN10103-2	143.3	3.0	.081

- Non- parametic test (Kruskal – Wallis test) used to analysed data [Chi-Square (X2) = 935.62, df = 15 and Asymp.sig (P-value) = 0.0001].

Appendix 12 a Complementation test – number of cleistothecia, statistical test results for putative HMG genes complement strains compared to parental strain.

***A. nidulans* strains**	**Mean No. of Cleistothecia/cm^2**	**Chi-Square (X2)**	**P-value**
Parental strain	1231.35		
AN1267-1-1	1360.72	0.301	0.583
AN1267-1-2	1199.12	0.001	0.977
AN1962-1-1	1142.28	2.25	0.133
AN1962-1-2	1222.87	2.619	.106
AN2755-1-1	1148.64	3.42	0.065
AN2755-1-2	1172.82	2.619	0.106
AN2885-1-1	1323.82	0.013	0.908
AN2885-1-2	1383.20	0.609	0.435
AN4734-1-1	1137.19	2.343	0.126
AN4734-1-2	1242	0.701	0.402
AN5073-1-1	1247.47	1.268	0.260
AN5073-1-2	1235.7	0.001	0.977

- Non- parametic test (Kruskal – Wallis test) used to analysed data [Chi-Square (X2) = 21.10, df = 1 and Asymp.sig (P-value) = 0.049].

Appendix 12b Complementation test – diameter of cleistothecia, statistical test results for putative HMG genes complement strains compared to parental strain.

***A. nidulans* strains**	**Mean of the diameter of Cleistothecia (µm)**	**Chi-Square (X2)**	**P-value**
Parental strain	149.12		
AN1267-1-1	143.1	1.87	0.171
AN1267-1-2	146.56	0.756	0.385
AN1962-1-1	142.363	2.551	0.110
AN1962-1-2	148.89	0.167	0.683
AN2755-1-1	158.9	2.84	0.092
AN2755-1-2	156.6	2.72	0.99
AN2885-1-1	155.41	0.889	0.346
AN2885-1-2	145.12	0.867	0.352
AN4734-1-1	153.55	2.64	0.104
AN4734-1-2	145.86	0.461	0.497
AN5073-1-1	153.1	0.491	0.506
AN5073-1-2	156.6	1.835	0.175

- Non- parametic test (Kruskal – Wallis test) used to analysed data [Chi-Square (X2) = 32.727, df = 12 and Asymp.sig (P-value) = 0.001].

Appendix 13 Relative quantification of HMG-box gene and mating-type target gene transcription.

1. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN3667* to parental strains.

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	0.35	0.01217	0.0135 – 0.076	0.004	down
AN1267	035	0.02774	0.048 – 0.191	0.004	down
AN1962	24.94	0.054	0. 196– 0.372	0.004	up
AN2755	2.71	0.0118	0.012 – 0.014	0.055	N/S
AN2885	0.48	0.144	0.12 – 0.86	0.025	down
AN3549	16.81	0.0012	0.0009 – 0.007	0.004	up
AN3580	2.02	0.0129	0.036 -0.1	0.016	up
AN3667	-	-	-	-	-
AN4734	0.014	0.0048	0.000- 0.000	0.002	down
AN5073	NE	NE	NE	NE	NE
AN10103	12.2	0.116	1.2 – 1.8	0.004	up
ppgA	0.91	0.0224	0.15 – 0.27	0.11	N/S
preA	0.54	0.0123	0.0018 – 0.065	0.068	N/S
preB	0.73	0.0061	0.0028 – 0.022	0.15	N/S
steA	14.34	0.412	0.945 – 3.06	0.004	up

- Fold change calculated as a ratio of gene transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value. NE stand for no expression.

2. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN0879* to parental strains.

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	-	-	-	-	-
AN1267	0.92	0.0174	0.27 – 0.36	0.337	N/S
AN1962	1.55	0.0123	0. 008 – 0.02	0.15	N/S
AN2755	1.2	0.00131	0.013 – 0.016	0.078	N/S
AN2885	1.04	0.072	0.86 – 1.23	0.749	N/S
AN3549	0.49	0.00002	0.0001 – 0.0002	0.037	down
AN3580	1.15	0.0038	0.028 – 0.048	0.423	NS
AN3667	1.23	0.00024	0.006 –0.007	0.078	NS
AN4734	0.88	0.00423	0.002 – 0.024	0.15	NS
AN5073	1.43	0.00035	0.001 – 0.002	0.022	UP
AN10103	0.92	0.0047	0.1 – 0.13	0.200	NS
ppgA	1.03	0.0055	0.221 – 0.25	0.749	N/S
preA	0.96	0.002	0.055 – 0.065	0..465	N/S
preB	0.87	0.0001	0.001 – 0.002	0.200	N/S
steA	1.43	0.0084	0.177 – 0.22	0.025	up

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.
- NE stand for no expression.

3. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN1267* to parental strains.

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	0.27	0.004	0.024 – 0.044	0.004	down
AN1267	-	-	-	-	-
AN1962	0.48	0.0005	0. 0034 – 0.0055	0.025	down
AN2755	0.41	0.001	0.001 – 0.0041	0.004	down
AN2885	0.78	0.029	0.71 – 0.86	0.006	down
AN3549	1.83	0.0002	0.00002 – 0.0009	0.631	N/S
AN3580	0.26	0.0005	0.007 – 0.01	0.004	dwon
AN3667	1.13	0.00046	0.005 –0.007	0.423	N/S
AN4734	0.32	0.0039	0.002 – 0.06	0.004	down
AN5073	0.22	0.00011	0.0001 – 0.0003	0.0001	down
AN10103	0.88	0.0086	0.1 – 0.15	0.522	N/S
ppgA	0.37	0.0061	0.069 – 0.1	0.004	down
preA	0.57	0.0022	0.03 – 0.0419	0.006	down
preB	0.18	0.00024	0.0017 – 0.0029	0.004	down
steA	1.14	0.0096	0.133 – 0.182	0.423	N/S

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.
- NE stand for no expression.

4. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN1962* to parental strains

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	0.314	0.00892	0.0162 – 0.0623	0.004	down
AN1267	0.46	0.0181	0.11 – 0.2	0.004	down
AN1962	-	-	-	-	-
AN2755	0.68	0.00169	0.0017 – 0.0068	0.055	N/S
AN2885	1.01	0.039	0.91 – 1.11	0.749	N/S
AN3549	0.58	0.0000	0.0001 – 0.0002	0.055	N/S
AN3580	0.38	0.0023	0.007 – 0.019	0.006	down
AN3667	2.28	0.0012	0.009 –0.015	0.004	up
AN4734	0.65	0.0096	0.004 – 0.01	0.037	down
AN5073	0.23	0.0001	0.0001 – 0.0003	0.0001	down
AN10103	0.68	0.0031	0.075 – 0.091	0.004	down
ppgA	0.69	0.0091	0.132 – 0.18	0.004	down
preA	1.17	0.012	0.044 – 0.1	0.855	N/S
preB	1.7	0.00014	0.0022 – 0.0029	0.004	up
steA	0.87	0.0039	0.11 – 0.13	0.337	N/S

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.
- NE stand for no expression.

5. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN4734* to parental strains

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	0.55	0.00731	0.05 – 0.088	0.004	down
AN1267	0.37	0.00916	0.1 – 0.51	0.004	down
AN1962	0.86	0.00075	0.006 – 0.01	0.423	N/S
AN2755	0.55	0.11	0.0001 – 0.0007	0.025	down
AN2885	1.3	0.000	1.04 – 1.6	0.004	up
AN3549	1.03	0.0001	0.00002 – 0.0005	0.522	N/S
AN3580	0.2	0.00055	0.005 – 0.008	0.004	down
AN3667	3.54	0.00435	0.008 –0.03	0.004	up
AN4734	-	-	-	-	-
AN5073	0.15	0.00011	0.00004 – 0.0003	0.0001	down
AN10103	1.2	0.0055	0.133 – 0.161	0.01	up
ppgA	0.88	0.0075	0.183 – 0.22	0.11	N/S
preA	0.54	0.00136	0.03 – 0.037	0.006	down
preB	1.6	0.00027	0.0017 – 0.003	0.025	up
steA	1.2	0.02	0.118 – 0.22	0.423	N/S

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.

NE stand for no expression.

6. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN2885* to parental strains.

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	0.31	0.0022	0.033 – 0.044	0.004	down
AN1267	0.23	0.006	0.065 – 0.095	0.004	down
AN1962	0.28	0.0002	0.002 – 0.003	0.004	down
AN2755	0.1	0.00013	0.00005 – 0.00016	0.004	down
AN2885	-	-	-	-	-
AN3549	2.3	0.00034	0.0003 – 0.0008	0.004	up
AN3580	0.32	0.0001	0.01 – 0.012	0.004	down
AN3667	0.83	0.0002	0.004 – 0.005	0.200	N/S
AN4734	0.1	0.0009	0.0004 – 0.0021	0.004	down
AN5073	0.37	0.00013	0.0003 – 0.0005	0.0001	down
AN10103	0.9	0.003	0.102 – 0.117	0.055	N/S
ppgA	0.16	0.0083	0.016 – 0.058	0.004	down
preA	0.283	0.001	0.016 – 0.02	0.006	down
preB	0.22	0.00021	0.0026 – 0.0037	0.004	down
steA	1.82	0.0089	0.23 – 0.28	0.004	up

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.
- NE stand for no expression.

7. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN2755* to parental strains

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	0.713	0.0066	0.0724 – 0.11	0.006	down
AN1267	0.29	0.01277	0.066 – 0.133	0.004	down
AN1962	0.22	0.00045	0. 0008 – 0.0031	0.004	down
AN2755	-	-	-	-	-
AN2885	1.48	0.0016	1.4 – 1.59	0.004	up
AN3549	0.5	0.039	0.00005 – 0.0002	0.037	down
AN3580	0.66	0.0000	0.018 – 0.026	0.078	N/S
AN3667	0.7	0.00023	0.003 –0.004	0.028	down
AN4734	0.035	0.0004	0.0002 – 0.0007	0.004	down
AN5073	0.08	0.0000	0.00004 – 0.0001	0.0001	down
AN10103	0.66	0.005	0.07 – 0.093	0.004	down
ppgA	0.312	0.021	00174 – 0.125	0.004	down
preA	0.243	0.001	0.0135 – 0.0171	0.006	down
preB	1.8	0.00022	0.0021 – 0.0032	0.004	up
steA	2.23	0.043	0.200 – 0.419	0.01	up

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.
- NE stand for no expression.

8. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN4734* to parental strains.

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	0.00002	0.0000	0.0000 – 0.0000	0.004	down
AN1267	0.00002	0.0000	0.0000 – 0.0000	0.004	down
AN1962	0.0079	0.0000	0. 00002– 0.0001	0.004	down
AN2755	0.00019	0.0000	0.000002 – 0.000004	0.004	down
AN2885	0.00012	0.0000	0.0001 – 0.0002	0.004	down
AN3549	-	-	-	-	-
AN3580	0.0002	0.0000	0.000002 – 0.00001	0.004	down
AN3667	0.0003	0.0000	0.0000 –0.0000	0.003	down
AN4734	0.0001	0.0000			down
AN5073	0	-	-	-	N/E
AN10103	0.007	0.0000	0.07 – 0.093	0.004	down
ppgA	0.0002	0.0000	0.00002 – 0.00005	0.004	down
preA	0.00003	0.0002	0.000001 – 0.000003	0.006	down
preB	0	-	-	-	N/E
steA	0.0064	0.0000	0.0004 – 0.0013	0.004	down

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.
- NE stand for no expression.

9. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN3580* to parental strains

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	1.2	0.0064	0.133 – 0.17	0.025	up
AN1267	0.86	0.021	0.24 – 0.35	0.11	N/S
AN1962	0.92	0.0023	0.003 – 0.014	0.749	N/S
AN2755	0.85	0.0023	0.0023 – 0.008	0.749	N/S
AN2885	1.81	0.11	1.49 – 2.05	0.004	up
AN3549	1,27	0.0001	0.0001 – 0.0005	0.873	N/S
AN3580	-	-	-	-	-
AN3667	1.18	0.00051	0.006 – 0.008	0.200	N/S
AN4734	0.77	0.00724	0.003 – 0.017	0.11	N/S
AN5073	2.5	0.00071	0.002 – 0.0034	0.0001	up
AN10103	1.0	0.0021	0.117 – 0.128	0.873	N/S
ppgA	1.11	0.021	0.199 – 0.306	0.749	N/S
preA	0.75	0.0039	0.038 – 0.058	0.028	down
preB	1.33	0.00024	0.0014 – 0.0026	0.15	N/S
steA	2.53	0.062	0.191 – 0.512	0.025	up

- Fold change calculate as a ratio of gene expression of mutant strain to parental strain.
- NS stand for statistically non-significant value.
- NE stand for no expression.

10. Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN5073* to parental strains.

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	0.84	0.008	0.085 – 0.126	0.11	N/S
AN1267	0.47	0.01135	0.132– 0.191	0.004	down
AN1962	0.59	0.0014	0. 0019 – 0.0089	0.11	N/S
AN2755	0.87	0.0035	0.0035 – 0.0053	0.522	N/S
AN2885	1.88	0.11	1.50 – 2.26	0.004	up
AN3549	0.76	0.0001	0.00001 – 0.0004	0.15	N/S
AN3580	0.56	0.0042	0.0075 – 0.0293	0.11	N/S
AN3667	1.42	0.0013	0.004 –0.0185	0.004	up
AN4734	0.71	0.0036	0.0015 – 0.0185	0.004	down
AN5073	-	-	-	-	-
AN10103	0.69	0.0051	0.071 – 0.097	0.004	down
ppgA	0.83	0.023	0.129 – 0.249	0.337	N/S
preA	2.1	0.0053	0.118 – 0.145	0.006	up
preB	4.24	0.0004	0.0053 – 0.0072	0.004	up
steA	2.78	0.057	0.239 – 0.531	0.004	up

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.

NE stand for no expression.

11.Relative quantification of HMG-box gene and mating-type target gene transcription in *ΔAN10103* to parental strains

Gene number	Fold change in mutant	Std. Error	95% C.I.	p-value	Result
AN0879	1.2	0.0121	0.118 – 0.181	0.11	N/S
AN1267	0.92	0.009	0.293– 0.34	0.337	N/S
AN1962	1.2	0.0016	0. 0068 – 0.015	0.522	N/S
AN2755	0.65	0.001	0.0007 – 0.009	0.004	down
AN2885	1.9	0.15	1.002 – 1.28	0.004	up
AN3549	1.8	0.0002	0.0003 – 0.0006	0.016	up
AN3580	0.94	0.0177	0.0264 – 0.0355	0.749	N/S
AN3667	1.2	0.00045	0.0051 – 0.0074	0.262	N/S
AN4734	0.6	0.0045	0.0018 – 0.014	0.004	down
AN5073	2.8	0.0005	0.0024 – 0.0034	0.0001	up
AN10103	-	-	-	-	-
ppgA	1.03	0.0077	0.216 – 0.256	0.749	N/S
preA	0.7	0.0026	0.037 – 0.051	0.011	down
preB	1.74	0.0003	0.0019 – 0.0033	0.004	up
steA	2.12	0.02	0.248 – 0.351	0.004	up

- Fold change calculated as a ratio of transcript level of mutant strain to parental strain.
- NS stand for statistically non-significant value.
- NE stand for no expression.

Appendix 14 Primer sets for amplification of key sex genes (*MAT1, MAT2, ppgA, preA, preB* and actin A) in some *Aspergillus* species.

1. Primer sets for *MAT1* gene

***Aspergillus* species**	**Forward**	**Reverse**
A. clavatus	5-'TACCAGCCCTCTTTCAGA CAACCG -3'	5'-TCAACTGCCAACCCAT CTCGTC -3'
A. flavus	5'-ATGGAAACCACAGTGTCT CCCCTC -3'	5'- AGCGAAGAATGCCCGACTTTG -3'
A. feotidus	5'- GCTTTCAACGCATTCCTC CTCAC -3'	5'- AGACTCCATTATCGTGGTCAT CGC -3'
A. niger	5'- CCCCTCTGGACACCAATA ATGAGTC -3'	5'-TGCCACAAGAAGCGAAGGA TGC -3'
A. tubingensis	5'-CGTGCTTTCAACGCATTCC TCC -3'	5'-GCCCGACTTCGCTTTCTGAGT AAG -3'

2. Primer sets for *MAT2* gene

***Aspergillus* species**	**Forward**	**Reverse**
A. aculeatus	5'- GCATTTTCGGGAACTGCG GTAG -3'	5'- TACTGATAGTCGGGGTGCTCT TCG -3'
***A.** brasiliensis*	5'- GCTACTTTGGCAGGATGC TTTGC -3'	5'- CGCTTCTTTTCCGATGGTTTA CG -3'
***A.** carbonarius*	5'- TGCATCTCACGAAGTGGC AT-3'	5'- TTGTTCGATCAACGAGCCCA-3'
A. fumigatus	5'- TAACTGAGTCCTTCGCAC CTCGTG -3'	5'-TGAAATCAATCTCGCCCACTG -3'

A. sydowii	5'- AGGGCAGCATCCACCAAA TCTAC -3'	5'- CATACTGGGGGAGTGAACAC ATTCG -3'
A. versicolor	5'- CCTCGGGTCAACGAATAA TG -3'	5'-GCTTTCGAGGGGTGTATTGA-3'
A. wentii	5'- CAAGATGCTTTGCGACAC CTCG -3'	5'-AACTGCGGAGCCTGAAAAT GC -3'
A. zonatus	5'- GGAGCATCATCCAAAAAT CAGGGAG -3'	5'- TCAGAAGGCTTGCGAGGAGC ATAC -3’

3. Primer sets for *ppgA* gene

***Aspergillus* species**	**Forward**	**Reverse**
A. aculeatus	5'-CCTATCCTTGATGCTTG CCACTC-3'	5'- AGCGGAGCGTTTGACCTCA T -3'
A.brasiliensis *A. feotidus*	5'TCTTGCTGCCACCACTGT GC -3'	5'CACCATCTCTGAAGGGTCTC GG -3'
A. carbonarius	5'- ATGCCTCCCCCGAAACC TTG -3'	5'- GCCATAGCGTCTGCGAAA GC -3'
A. clavatus	5'- CACTGTCCAGGCAGGTG CTATG -3'	5'- TCACTTCCTCAGCGGCTTC AG -3'
A. flavus	5'GGTGTTTTACAGAAATGG TGCTCC -3'	5'- GCTTTGAGGCATCCCTGA CC -3'
A. fumigatus	5'-TCTCACTTGTTCTTGCTT CCTTCG -3'	5'- TAACTTCATCCGAGGCGTC AGC -3'
A. niger	5'- TTGTCCTCGCTGCTCTTG CT -3'	5'- ACACACCATCTCTGAAGGG TCTCG -3'

A. sydowii	5'- TTGTCGCTGTTACTCTC GCTGCTC -3'	5'- TTTTCTGGAACTCCTCTGG GGC -3
A. tubingensis	5'- TGCAACATGATCAAGCG TGC -3'	5'- TCTTGTTGCAAGGCTGAC CA-3'
A. versicolor	5'- ATCGCTGTTACCATCGC TGCTC -3'	5'- TTTTCTGGAACTCCTCTGG GGC -3'
A. wentii	5'- TGCCACTAATGTCCAGG CTG -3'	5'- CGGGGAGGTAGCACCATT TT-3'
A. zonatus	5'- ACTGGTGAGGTTAAGCG CTC -3'	5'- CTCCTCATCCATGGCAGC AA -3'

4. Primer sets for *preA* gene

***Aspergillus* species**	**Forward**	**Reverse**
A. aculeatus	5'- CGATTCGTGCGGCTTTTCT TG -3'	5'- TGGGTGTTGTGACCTATGG GTTGC -3'
A. brasiliensis	5'- TTGGTGGAATCACGCAACC GTC -3'	5'- TGACTGAAGGATGTCCCCG AACTG -3'
A. carbonarius	5'- GGTGGAATGGTAATGGACT CTGCG -3	5'- GACTTCGGTATTTGGCAAG GCG -3'
A. clavatus	5'- GCATTCTTTGACCGCTGGA TTCC -3'	5'- TGAGCCTTGGTTGTTTCGT GGG -3'
A. flavus	5'- TATTCTGGTCGGCATCTCA GGCTG -3'	5'- AGGAGCACAATGAAGGAA AGGAGG -3'
A. feotidus	5'- CGCCAATAGTAACCTGACC AAGTCG -3'	5'- CCTCGCAAAAACCAAAGC AGTCC -3'
A. fumigatus	5'- GATGCTGCCACGATGCTTT	5'- AAGAGACACCTTGGTAGC

	GAG -3'	GGAACC -3'
A. niger	5'- GCCGTGTTTTCTACGACCG TTG -3'	5'- CTTCGCAGCATCCAAAGCA GTC -3'
A. sydowii	5'- TGTTCCTGGGTGCCTGGTT TGT -3'	5'-TGCCACGGTAATGAT GCCTCTACTT -3'
A. tubingensis	5'- AAACCTCGTTCCAACCCAG TCG -3'	5'- TGGCGGATTGAAGAATGTC CCC -3'
A. versicolor	5'- AGCAGAACGACTCTTGTCC CGAAC -3'	5'-ATACCGCCTGAGACGCAA TAGCAC -3'
A. wentii	5'- TCGGATTCGGCAGAGATGC TATC -3'	5'- TTGACGGGAAGGAGAAAG TGGG -3'
A. zonatus	5'- TGCCAGGTTGCTCTTCCAT AAGCG -3'	5'- ATTCCGAGTGCCCGAAAAC AGACG -3'

5. Primer sets for *preB* gene

***Aspergillus* species**	**Forward**	**Reverse**
A. aculeatus	5'- CCGTCTTCGTTACCAAAC TGGG -3'	5'- CGTTGACTGCGTAGTGGAGA ATG -3
A. brasiliensis	5'- ACCTCTCATCTGGCTTCA AAGTGC -3'	5'- AACCAATGTCAGCATAGCAA GTCG -3'
A. carbonarius	5'- CGAGAACATCGTCTTCAC CATTAGC -3'	5'- TTGGCGGGGGTATTATCAGC GTAG -3'
A. clavatus	5'- GGCTTCCGTCTTGCTTTTA TGGTC -3'	5'- CCGAATGGGCATCCTTTCTG TAG -3'
A. flavus	5'- CTTGGTTGCCCTTATTCCT GTCG -3'	5'- GATGTTTGATTGCTCCTCGT GCC -3'
A. feotidus	5'- CCATCTCGTCTGGCTTCA	5'- TGGAGGAACCATTATCGGCG

	AAGTGC -3'	TAGC -3'
A. fumigatus	5'- TCGCTTGACTTACTCGGT GATGAAC -3'	5'- CTGCTGGGGTTTACTGGTTG ACTG -3'
A. niger	5'- TCGGATTTGCCATTCGTC AGC -3'	5'- GCGTAGCAGGTTGAGACTGG ATTC -3'
A. sydowii	5'- TACCGTCGTGCTTTGCTT TGCGTG -3'	5'- TTGGGTTTGTCAAGGGTTGT GCCG -3'
A. tubingensis	5'- CATCACCGCCACAATCAG CATC -3'	5'- TCCAAAACAGGAAATGAGA GCACG -3'
A. versicolor	5'- TACCGTCGTGCTTTGCTT TGCGTG -3'	5'- TTGCTGGTGTCTGCCCTTGTA TCC -3'
A. wentii	5'- TTCGTGTCGGTGATTGTT GCCCTG -3'	5'- TTGCTCCTTGTAGTGTTGGTG TTCG -3
A. zonatus	5'- GCGGGTATTAGACGACAT CCAGTG -3'	5'- GGCGGAAGAAGAAGAAGAG GAGTG -3'

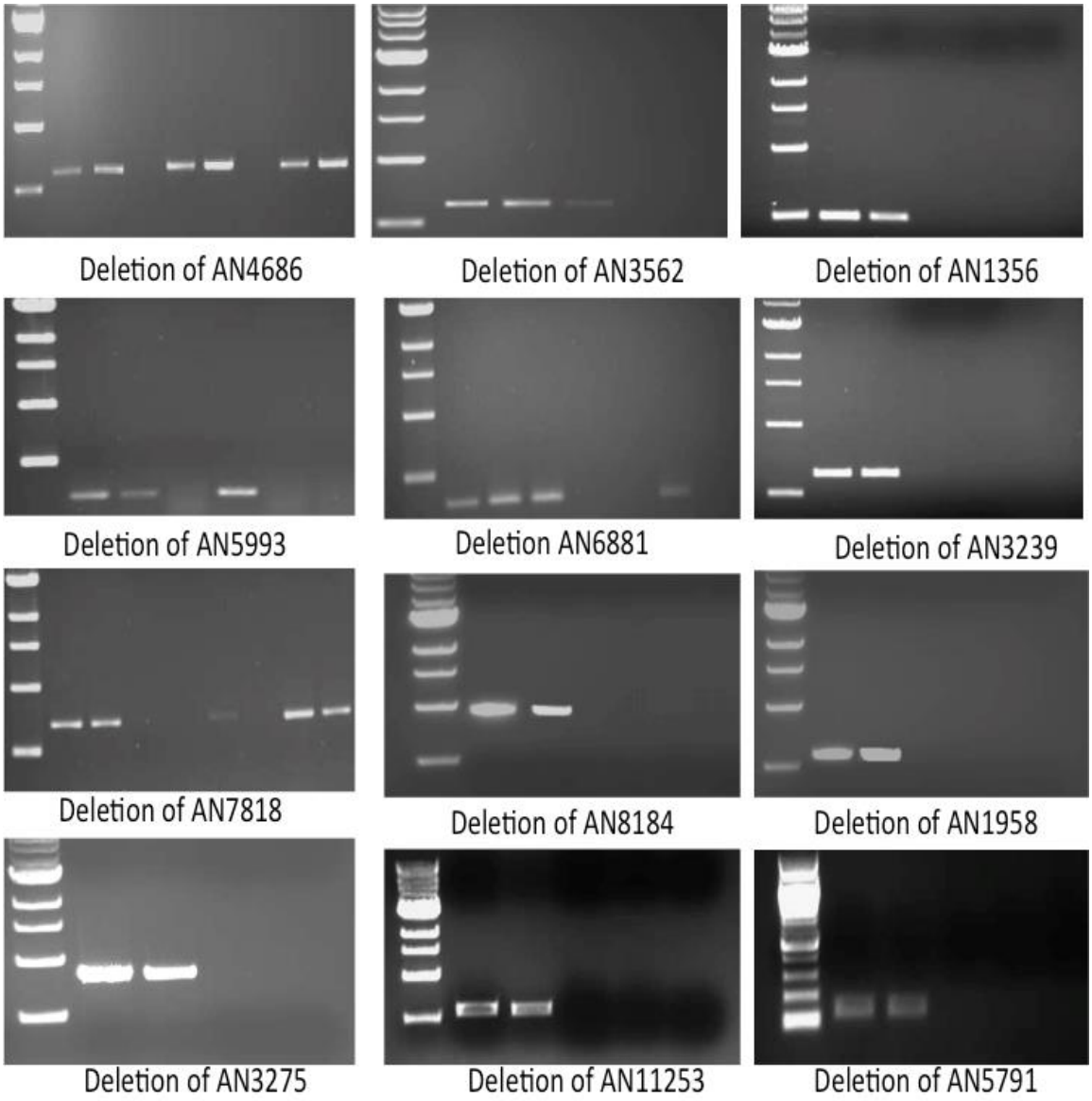
6. Primer sets for actin A (housekeeping) gene

***Aspergillus* species**	**Forward**	**Reverse**
A. aculeatus	5'- TTCTGGACTCTGGTGACG GTGT -3'	5'- AAGCACTTGCGGTGGACGAT -3'
A. brasiliensis	5'- GGTCATCACCATTGGCAA CGAG -3'	5'- TGGAAGAAGGAGCAAGAGCA GTG -3'
A. clavatus	5'- ACAGGTCATCACCATCG GCAAC -3'	5'- CCAATCCACACGGAGTATTTA CGC -3'
A. flavus	5'- GCCGTGATTTGACCGATT ACCTC -3'	5'- TGGACAGGGAAGCCAAGATG GAAC -3'

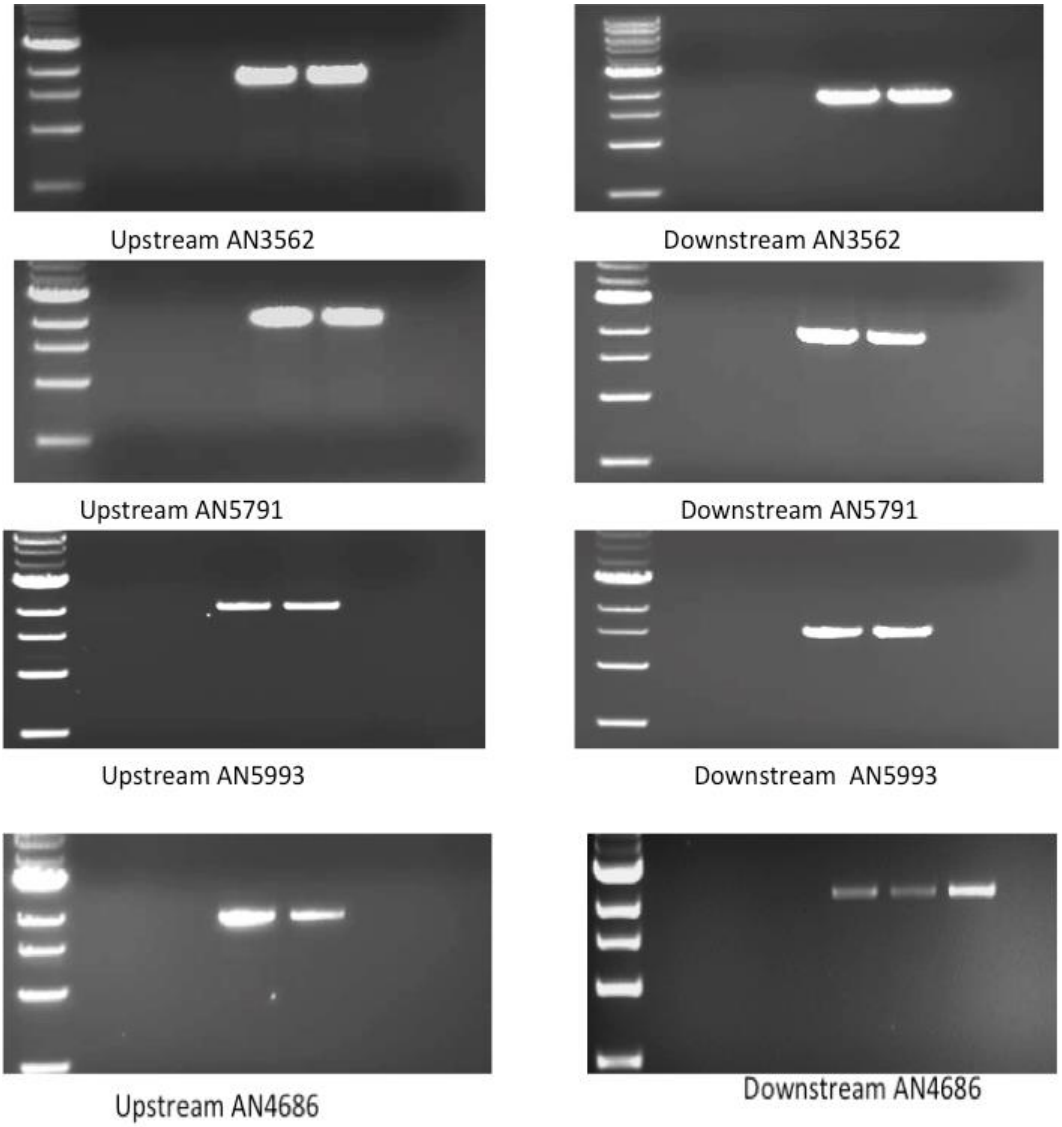
A. feotidus	5'- TTCTGGACTCTGGTGACG GTGT -3'	5'- ACATCTGCTGGAAGGTGGAC AG -3'
A. fumigatus	5'- TGTTCGTGACATCAAGG AGAAGC -3'	5'- ACATCTGCTGGAAGGTGGAC AG -3'
A. sydowii	5'- TTCAACGCTCCCGCCTTC TA -3'	5'- AGCCAAGATGGAACCACCGA -3'
A. versicolor	5'- CGACTCTGGTGATGGTGT TACCCA -3'	5'- AGAAGCACTTGCGGTGGACG AT -3'

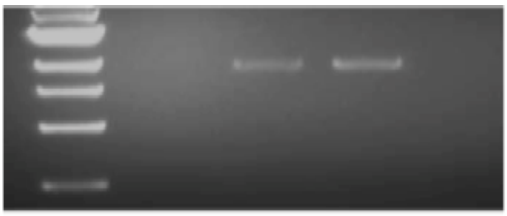
- Note that some primer pairs are used for multiple species as mention bellow, because the same primer annealing sites, such as for the actin A gene, are highly conserved between species.
- *A. brasilliensis, A. tubingensis* and *A. zonatus*
- *A. feotidus* and *A. niger.*
- *A. aculeotus* and *A. carbonarius.*
- *A. sydowii* and A. *wentii*

Appendix 15: 1.2% agarose gel pictures showing PCR screening of target gene deletion in transformant strains compared to parental strain and – DNA protoplast control. Lane 1 is DNA ladder, Lane 2 parental strain, lane 3 is – DNA protoplast control and other lanes are transformant colonies.



Appendix 16: 1.2% agarose gel pictures showing positional PCR screening to confirm the integration of *pyrG* marker gene in place of deleted target gene compared to parental strain and – DNA protoplast control. Lane 1 is DNA ladder, Lane 2 parental strain, lane 3 is – DNA protoplast control and other lanes are deleted strains.

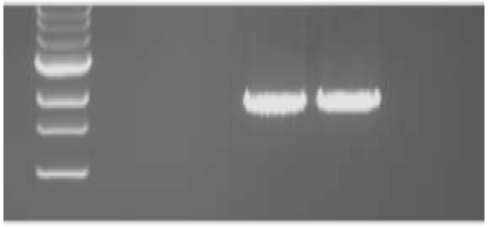




Upstream AN1356

Downstream AN1356

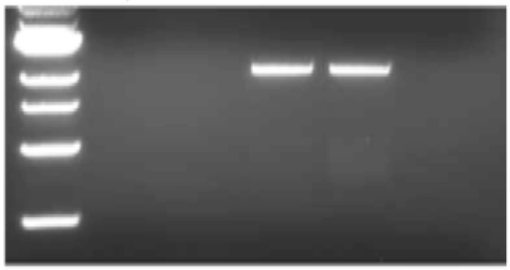
Upstream AN3239



Downstream AN3239

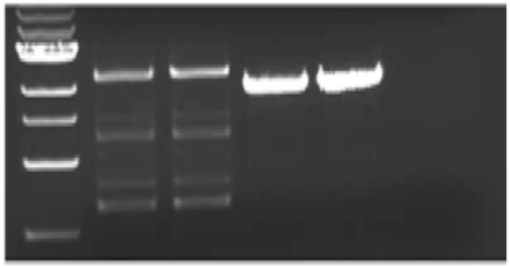
Upstream AN8184

Downstream AN8184



Upstream AN11253

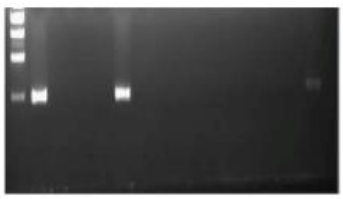
Downstream AN11253



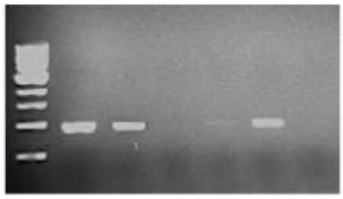
Downstream AN1958

Downstream AN3275

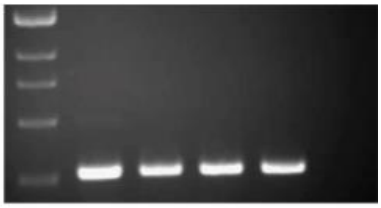
Appendix 17: 1.2% agarose gel pictures showing PCR screening to confirm the restoration of target deleted gene compared to parental strain Lane 1 is DNA ladder, Lane 2 parental strain and other lanes are complement strains.



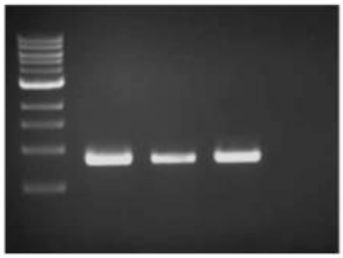
Complement AN 1356



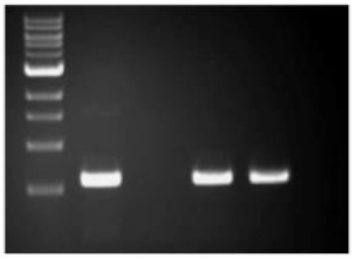
Complement AN8184



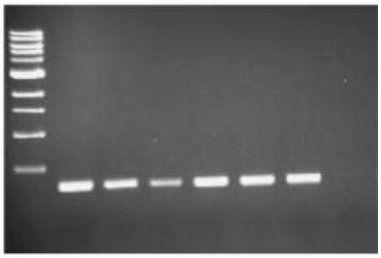
Complement AN 11253



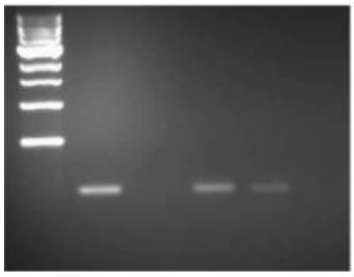
Complement AN3275



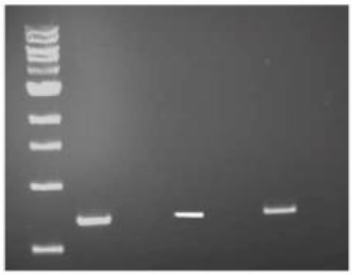
Complement AN4686



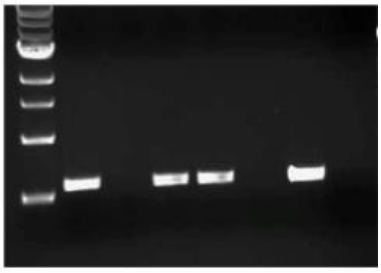
Complement AN6881



Complement AN5791

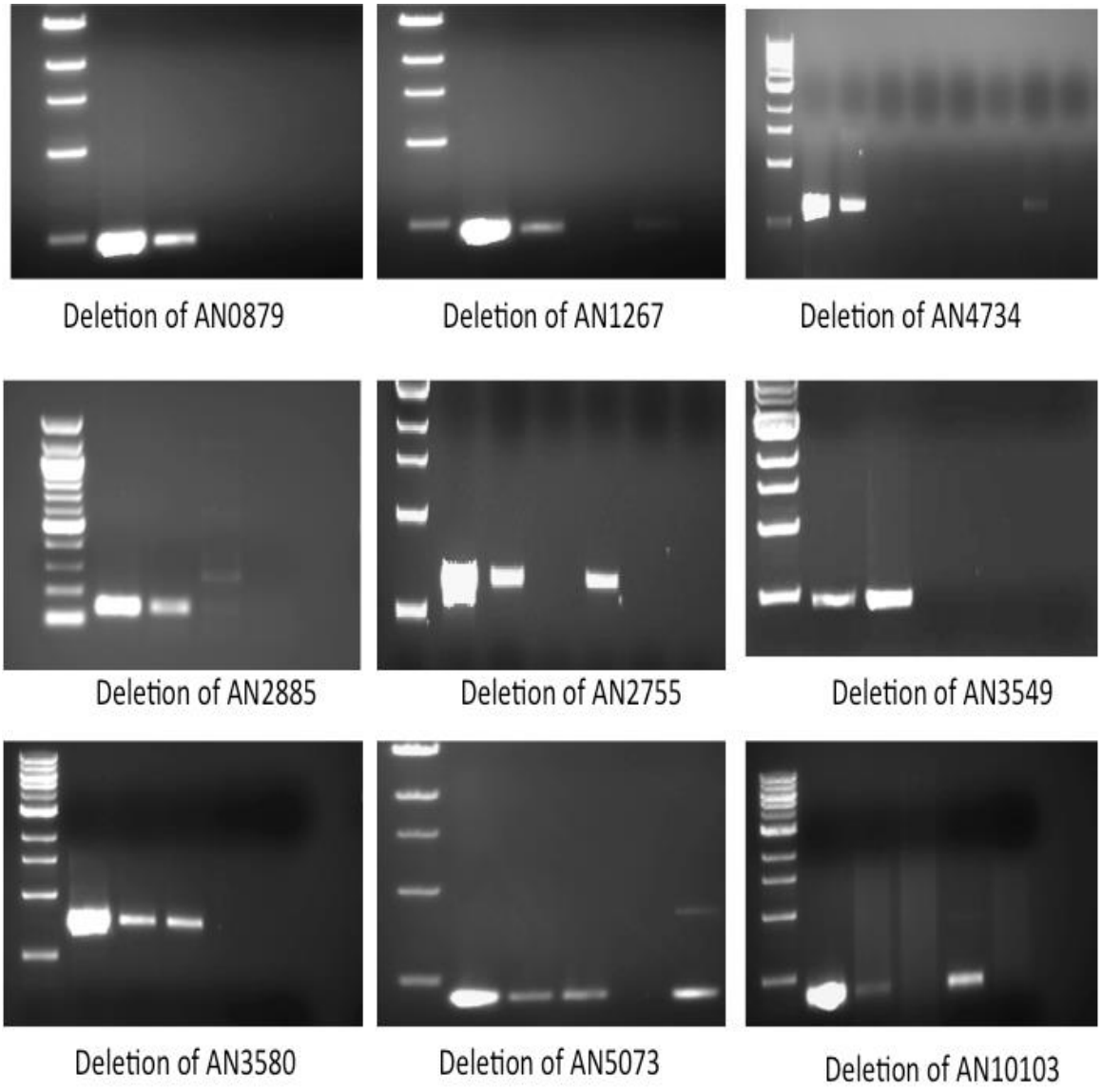


Complement AN7818

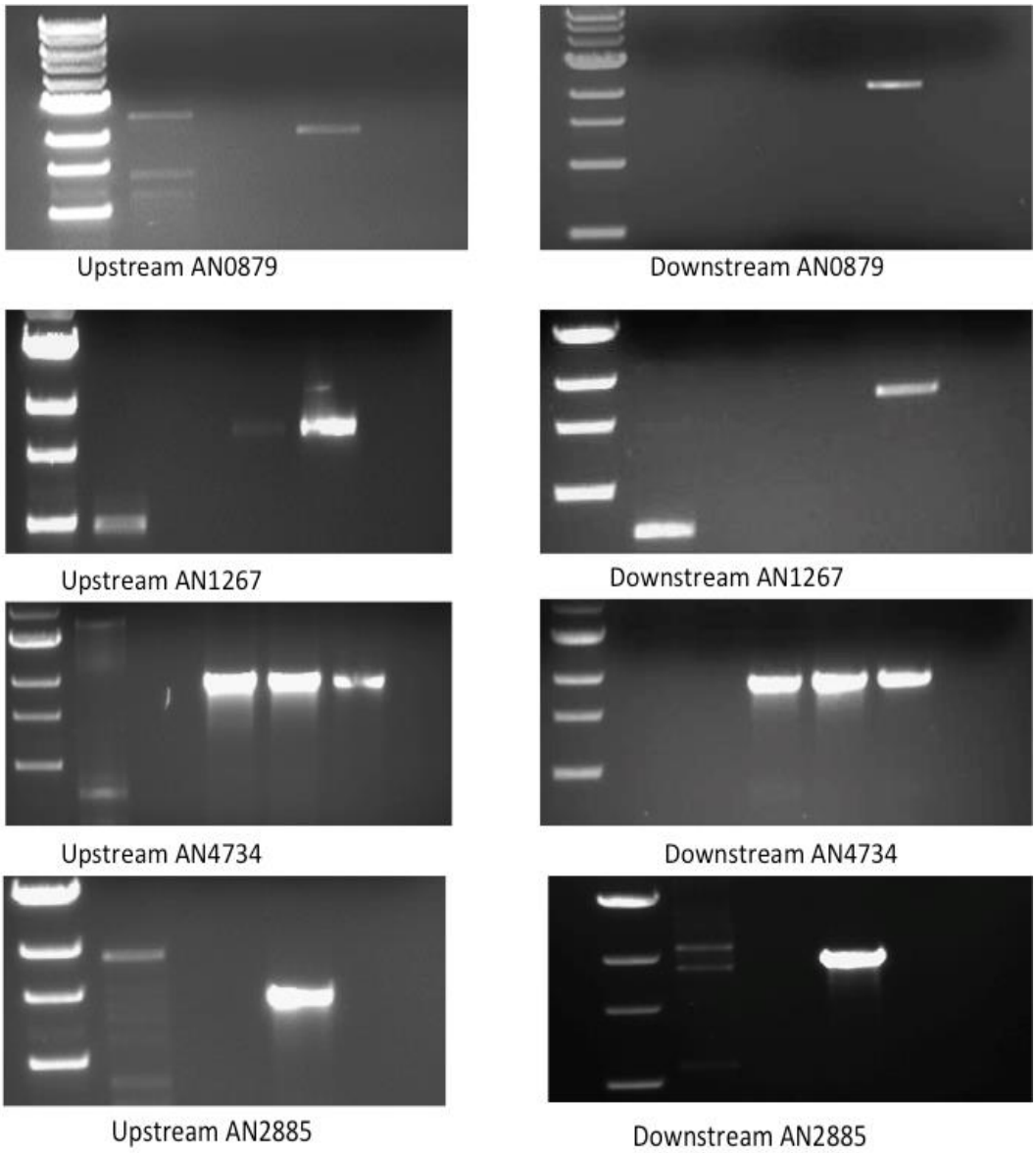


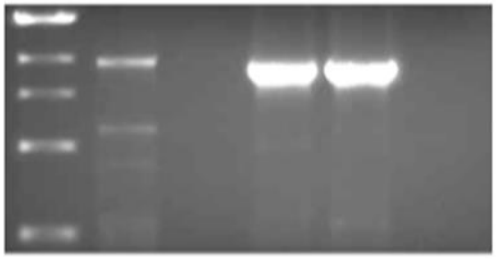
Complement AN1598

Appendix 18: 1.2% agarose gel pictures showing PCR screening of HMG gene deletion in the transformant strains compared to parental strain and – DNA protoplast control. Lane 1 is DNA ladder, Lane 2 parental strain, lane 3 is – DNA protoplast control and other lanes are transformant colonies.

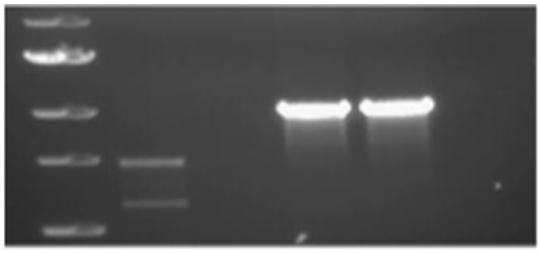


Appendix 19: 1.2% agarose gel pictures showing positional PCR screening to confirm the integration of *pyrG* marker gene in place of deleted HMG target gene compared to parental strain and – DNA protoplast control. Lane 1 is DNA ladder, Lane 2 parental strain, lane 3 is – DNA protoplast control and other lanes are deleted strains.

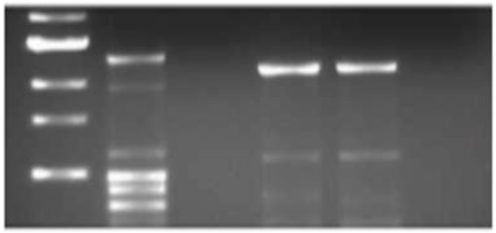




Upstream AN2755

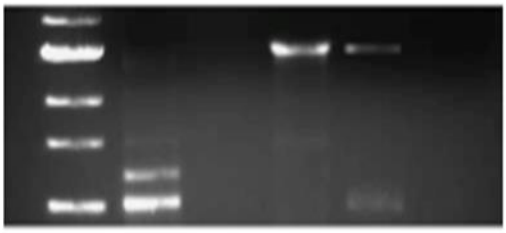


Downstream AN2755

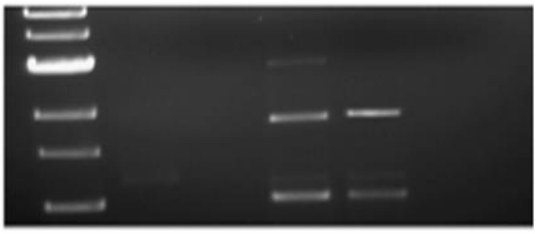


Upstream AN3549

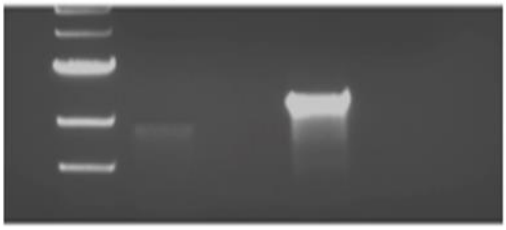
Downstream AN3549



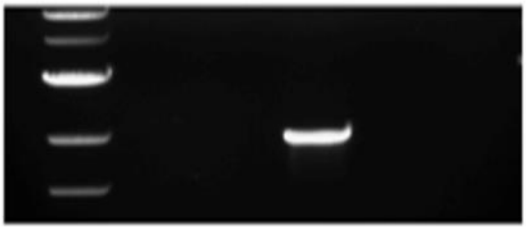
Upstream AN3580



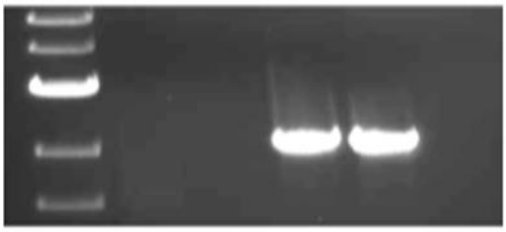
Downstream AN3580



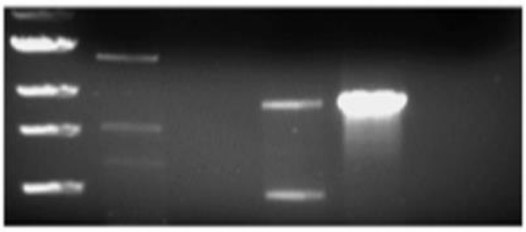
Upstream AN5073



Downstream AN5073

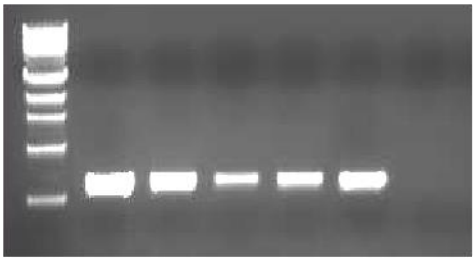


Upstream AN10103

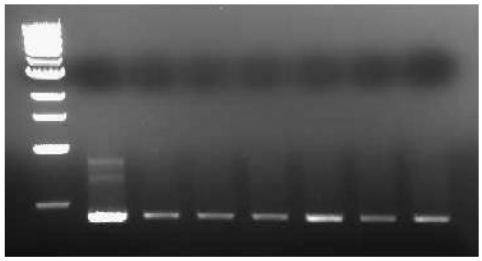


Downstream AN10103

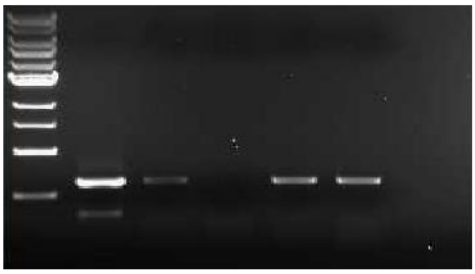
Appendix 20: 1.2% agarose gel pictures showing PCR screening to confirm the restoration of target HMG deleted gene compared to parental strain Lane 1 is DNA ladder, Lane 2 parental strain and other lanes are complement strains.



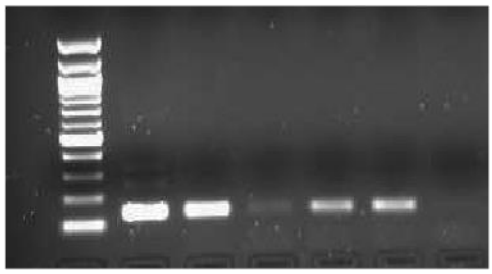
Complement AN2755



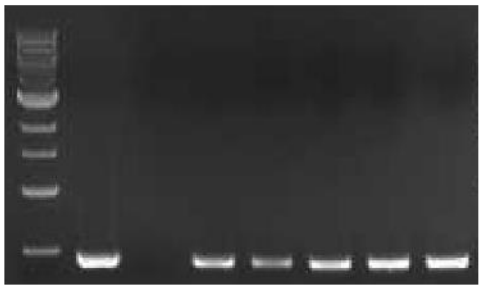
Complement AN5073



Complement AN4734



Complement AN2885



Complement AN1267