

Reproduction in *Aspergillus fumigatus*: sexuality in a supposedly asexual species?

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Aspergillus fumigatus has long been considered to reproduce only by asexual means. However, accumulating evidence suggest that a sexual stage for *A. fumigatus* may yet be identified. We describe results from published and ongoing studies involving population genetic analyses, genome analysis, studies of mating-type gene presence and distribution, expression of sex-related genes, and taxonomic work which support the assertion that *A. fumigatus* has the potential to reproduce by sexual means. The consequences of sexual reproduction for the population biology and disease management of the species are discussed. The possible mechanisms of evolution of asexuality are then considered. It is proposed that asexual species may arise in one step by mutation or loss of a key gene(s), and/or there may be a 'slow decline' in sexual fertility within the species as a whole. Thus, it is argued that species should not be considered simply as sexual or asexual, but rather as individual isolates being present on a continuum of sexual fertility, with the implications for understanding sexuality/asexuality in *A. fumigatus* discussed.

Keywords asexuality, *Aspergillus*, evolution, mating type, sexuality

Introduction

Filamentous fungi reproduce by asexual and sexual means. Asexual reproduction involves the generation of propagules such as conidia by mitotic processes, whereas sexual reproduction involves nuclear fusion and consequent spore production by meiotic processes. Where species exhibit both asexual and sexual life cycles, the phases of reproduction often occur under different environmental and nutritional conditions [1]. Thus, for example, many pathogens reproduce asexually when conditions are favourable for growth but reproduce sexually at the end of growing seasons when nutrient availability is low and sexual fruit bodies can be formed which are resistant to adverse environmental conditions [1–3].

There are many perceived benefits to a species reproducing by both sexual and asexual means, as opposed to species that reproduce exclusively by

asexual processes. Sexual reproduction can lead to increased genetic variation, as a result of crossing over and recombination of genes, which can allow a species to respond to environmental change and evolve [4]. It also allows evasion from pathogens, avoids genetic 'hitchhiking' and enables removal of deleterious genes [2,5]. The latter is a particular benefit over purely asexual species where mutations can accumulate and become fixed within populations (e.g. the concept of Muller's ratchet) [3,5]. Indeed, it has been argued that the benefits of sex, even if the sexual cycle only occurs very infrequently, are sufficient to mean that most organisms should reproduce sexually and the presence of exclusively asexual species may be viewed as 'an evolutionary scandal' [5,6].

Despite the many advantages of sexual reproduction, almost one-fifth of all fungi are only known to reproduce by asexual means with no sexual stage or 'teleomorph' identified [7]. This is an astonishingly high number of asexual taxa given that truly asexual species are considered to be very rare [6,8]. Typical of the supposedly 'asexual' fungi are many species of *Aspergillus*. The genus comprises some 182 species and includes taxa with homothallic (selfing) and hetero-

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thallic (obligate outcrossing) sexual breeding systems. However, the majority of species are only known to reproduce by asexual means [9,10]. This includes species of key importance in the medical, biotechnology and food sectors such as *A. fumigatus*, *A. niger* and *A. oryzae*. These species are well known and some have been studied for hundreds of years, yet no definite sexual stage has ever been described [11].

Although no telomorph has yet been detected, accumulating evidence suggests that many supposedly 'asexual' fungi may have some undiscovered or 'cryptic' sexual state, or other mechanism(s) to promote recombination. For example, the fungal pathogen responsible for eyespot disease of cereals, *Pseudocercospora herpotrichoides*, was known only as an asexual organism for much of the last century despite being much studied due to its economic importance. However, field surveys in the late 1980s and 1990s belatedly revealed the presence of a sexual stage of the fungus, which was placed in the genus *Tapesia* [12]. The sexual stage may have previously gone unnoticed because it only developed on straw stubble left after harvest, and such stubble was normally burnt or incorporated prior to instigation of set-aside schemes. Meanwhile, indirect population genetic analyses have provided evidence of recombination within several asexual species including the medical pathogen *Coccidioides immitis* and the food spoilage agent *Aspergillus flavus* [13]. Finally, recent genome sequencing projects have provided insights into supposed asexuality in fungi. Most notably was the discovery of a series of genes involved with sexual reproduction within the long assumed asexual pathogen *Candida albicans* [14]. This provided the impetus for further experimental studies that have since established that certain genotypes of *C. albicans* have the ability to undergo sexual mating and have shown evidence of recombination, although meiosis has yet to be demonstrated [15,16].

We now present a review of experimental findings of relevance to understanding the mode of reproduction of the saprophyte and opportunistic pathogen *A. fumigatus*. This species has become one of the most prevalent airborne fungal pathogens over the last decade, causing severe and usually fatal invasive infections in immunocompromised hosts and allergic responses in other patients [17]. The fungus has long been considered an asexual organism, yet recent findings strongly suggest that it may have a latent potential for sexual reproduction. The potential presence of a sexual cycle is highly significant for the population biology, pathogenesis and disease management of *A. fumigatus*, as will be described.

Evidence for sexual potential from population genetic analyses

It may be predicted that species reproducing by purely asexual means will exhibit a clonal population structure, with relatively few well represented genotypes present. Within these lineages mutations (especially neutral mutations) may accumulate over time (the concept behind 'the Meselson Method', see reference [6]). In contrast, species with an outbreeding sexual phase would be predicted to exhibit increased genetic diversity within populations, although a few well adapted genotypes might still dominate. Various tests are available to distinguish whether populations have a clonal structure, consistent with asexuality, or more diverse set of genotypes consistent with recombination and presence of sexual reproduction [13]. Where it is possible to obtain multilocus phenotypes, representatives of each phenotype can be tested for association among loci to differentiate between the null hypothesis of recombination and the alternate hypothesis of clonality, e.g. the index of association (I_A), partition homogeneity, and tree length phylogenetic tests [13]. Such population genetic analyses have already been used successfully to detect a signature of recombination in several supposedly asexual species [13,18].

Various studies have assessed genetic variation within populations of *A. fumigatus*. Debeauvais *et al.* [19] investigated genetic diversity amongst European clinical and environmental isolates and found that 424 of 879 isolates were unique based on retrotransposon fingerprinting, with no clustering of samples from different sources evident. The source of this extreme diversity was unclear [17,19]. A similar study of more than 700 clinical and environmental samples from hospitals in France again detected a high degree of genetic diversity, with 85% of isolates being unique [20]. A further study using multiple typing methods also detected extensive genetic diversity within 52 French clinical isolates [21]. Data from the latter study, together with isoenzyme and SSDP data from 10 other published reports, were reanalysed by Varga and Tóth [22], who concluded there was evidence for recombination within some populations, although clonality was evident in other samples. Clonality was also evident in a recent analysis of 61 worldwide individuals [8]. Meanwhile, an ongoing analysis of worldwide isolates, including subpopulations from Europe and North America, has revealed clear evidence of recombination within the test samples when assessing linkage between three intergenic non-coding regions [C. Rydholm, *et al.* unpublished results].

Taken together, these findings indicate the likelihood of recombination within populations of *A. fumigatus*, consistent with occasional sexual reproduction. However, it should be cautioned that the variation detected might be the result of historical meiotic exchange, before any potential loss of sexuality. Thus, current sexual activity cannot be inferred from these results. Also recombination may arise from parasexual means (i.e. in the absence of mating), although there is little evidence for such phenomena in the wild [17]. Thus being recombinationally proficient is not necessarily synonymous with being sexually proficient.

Evidence for sexual potential from genome analysis

The genomes of four species of aspergilli: *A. fumigatus*, *A. nidulans*, *A. niger* and *A. oryzae* have recently been sequenced, with data from the *A. fumigatus* project made publicly accessible [23]. This has allowed BLAST searching of the genome, with a fully annotated version of the genome to be released imminently [23; W. Nierman, personal communication].

Studies from other filamentous fungi and work based on the yeast model indicate that several hundred genes are involved in sexual reproduction in ascomycete fungi [1]. For simplicity, these may be divided into genes involved with three sequential stages of the sexual cycle: (a) sexual identity and mating processes; (b) signalling pathways for mating response; (c) fruit body development and meiosis. In a preliminary screen, Pöggeler [24] was able to detect genes involved with early stages of sexual development, e.g. genes controlling mating type, pheromone production and certain pheromone receptors within the genome of *A. fumigatus*. A similar preliminary search also detected the same mating-type gene within the genome database [25]. Recently, a more comprehensive screen has been undertaken and confirmed these earlier findings [P.S. Dyer, *et al.* unpublished results]. This latter screen also detected the presence of genes involved in later stages of sexual development. However, no clear clustering of sex-related genes was evident, in contrast to the genome organization of some other fungi [26,27].

The discovery of genes involved specifically in sexual reproduction within the genome of *A. fumigatus* is significant because it points towards a latent potential for sexuality. However, it has yet to be determined whether such genes for the 'sexual machinery' [28] remain functional; they may instead be pseudogenes or have been subverted for some other use. It is also possible that other so far unidentified genes essential for sex have been lost, and the observations are

consistent with the hypothesis that asexual species evolve from sexual ancestors [29]. A putative pheromone receptor gene has also been detected by genome analysis of the apparently asexual pathogen *Penicillium marneffei* [30].

Meanwhile, multiple copies of a repeat sequence *AFUT1* have been found in the genome of *A. fumigatus* [17]. Intriguingly, *AFUT1* has a sequence organization characteristic of a retrotransposon element, although is defective due to multiple stop codons within the putative coding domains. The pattern of nucleotide transition has been observed to be reminiscent of that of the repeat-induced point mutation (RIP) process which occurs specifically during sexual reproduction of *N. crassa* [17]. Therefore these observations suggest that *A. fumigatus*, at least historically, possessed a functional sexual cycle when foreign genetic elements could be inactivated by a RIP-like process. Also, propagation of this element may have relied on fusion of individuals during sexual reproduction [8].

Evidence for sexual potential from mating-type presence and distribution

For sexual reproduction to occur in heterothallic ascomycete fungi it is necessary for isolates of compatible 'mating type' to be present [1]. By convention these are termed 'MAT1-1' and 'MAT1-2' (abbreviated to MAT-1 and MAT-2 for ease of reference). Research over the past decade has revealed that mating-type identity is conferred by mating-type (*MAT*) genes, which reside at a 'MAT' locus. Highly dissimilar stretches of DNA termed 'idiomorphs' are found at the *MAT* locus in isolates of opposite mating type, with MAT-1 identity conferred by a *MAT1-1* gene containing a conserved alpha domain, whilst MAT-2 identity is conferred by a *MAT1-2* gene containing a conserved high mobility group sequence [31,32].

Genome analysis, as described above, has revealed the presence of a *MAT1-2* gene within *A. fumigatus* [24,25; W. Nierman *et al.*, unpublished results]. Thus, one explanation for the observed asexuality of *A. fumigatus* might be that the species evolved from a MAT-2 ancestor, with sexually compatible MAT-1 isolates absent as a consequence. Alternatively, the required MAT-1 isolates might have a limited geographic distribution, precluding sexual reproduction, as has been reported for some other ascomycete fungi [33,34].

The topic of mating-type presence and distribution in *A. fumigatus* is currently being investigated (M. Paoletti & P.S. Dyer, unpublished results), and some insights are already available. We used the known

sequence of a *MAT1-1* alpha-domain gene from *A. nidulans* [28], together with knowledge of the sequence of the *MAT1-2* locus of *A. fumigatus*, to devise a degenerate PCR-based approach to search for *A. fumigatus* isolates containing an alpha-domain gene. And within a preliminary screen of nine isolates we were able to detect isolates with a putative *MAT1-1* alpha-domain gene at the *MAT* locus, i.e. isolates of *MAT-1* genotype. The entire *MAT1-1* idiomorph of *A. fumigatus* is currently being cloned and sequenced. Accumulating sequence information has been used to design a multiplex PCR-based mating-type diagnostic assay [35] to determine whether isolates of *A. fumigatus* are of *MAT-1* or *MAT-2* genotype (Fig. 1). Work is in progress to use this test to survey a series of worldwide clinical and environmental isolates [M. Paoletti, *et al.* unpublished results].

The discovery of both *MAT-1* and *MAT-2* genotypes of *A. fumigatus* is highly significant because the presence of such sexually compatible isolates is one of the key prerequisites for sexual reproduction to occur. However, it is noted that *MAT* genes have been found in other asexual taxa such as *Alternaria*, *Bipolaris* and *Fusarium* species [36–39]. Therefore, the presence of *MAT* genes alone is not sufficient to confer sexuality. Indeed, transformation of asexual *Bipolaris* species with functional mating-type genes from closely related *Cochliobolus* species has failed to restore sexuality – although significantly, the *MAT* genes from asexual *Bipolaris* species were able to alter reproductive mode of the sexual species *C. heterostrophus* [38]. Asexual populations generally show a strong deviation from a 1:1 distribution ratio of compatible mating-types [39,40]. For example, imbalanced ratios of up to 40:1 have been detected in medical pathogens such as *Cryptococcus neoformans* [41]. Thus, it will be of great interest to determine the *MAT-1*:*MAT-2* distribution in a large worldwide sampling of *A. fumigatus*.

Evidence for sexual potential from expression of sex-related genes

As described above, BLAST searching of the genome of *A. fumigatus* revealed the presence of a series of genes associated with sexual reproduction in yeasts and filamentous fungi. This hinted at latent sexuality within *A. fumigatus*, although it was not clear whether these genes are functional or are perhaps pseudogenes. However, it is hoped that insights will be available from studies underway to investigate whether genes with putative roles in sexual identity and mating processes are expressed at the RNA level by isolates of *A. fumigatus* [M. Paoletti & P.S. Dyer, unpublished results]. We are currently using an RT-PCR approach to assess expression of the identified mating-type genes (*MAT1-1* and *MAT1-2*), an α -factor like pheromone-precursor gene (termed *ppgA*), and the α - and α -factor like pheromone-receptor genes (termed *preA* and *preB*, equivalent to *Saccharomyces cerevisiae* STE3 and STE2 respectively). Apparent expression of some of these genes has already been detected in one isolate, although confirmation by sequencing of the PCR products is required and further isolates need to be tested.

Should expression of such ‘sex-related’ genes be detected and confirmed, then results would again be consistent with a sexually reproducing species. It will be of interest to compare patterns of mating-type, pheromone-precursor and pheromone-receptor gene expression to those seen in the heterothallic ascomycete fungi *Neurospora crassa*, *Cryphonectria parasitica* and *Podospora anserina* [42–45]. The investigation of expression of genes involved with the early stages of sexual reproduction is especially intriguing. From an evolutionary point of view it might be argued that to express genes involved with sexuality in a truly asexual species is metabolically wasteful. Therefore the genes most likely to be selectively lost (or not expressed) would be those genes at the start of the sexual process, rather than those further downstream in sexual development, as this would minimize needless gene expression.

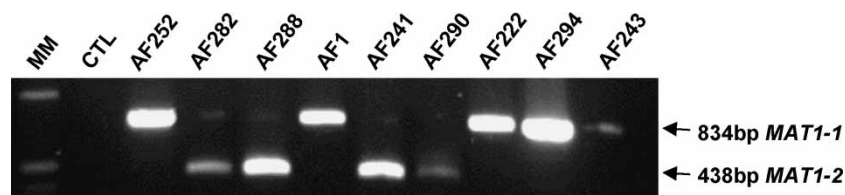


Fig. 1 Results from multiplex-PCR assay to determine mating type (genotype) of isolates of *Aspergillus fumigatus*. The PCR assay involves one common primer, and two mating-type specific primers, with amplicons of either 834 or 438 bp generated by *MAT1-1* or *MAT1-2* isolates respectively. Lanes 1–2 = molecular weight marker (MM) and water control (CTL); lanes 3–11 = amplicons from *A. fumigatus* isolates, code number indicated above each lane.

However, it is noted that the expression of genes relating to sexual reproduction has been described in some other supposedly asexual species. For example, *MAT* gene expression has been detected in filamentous *Alternaria* and *Fusarium* species [36,39], whilst expression of a putative pheromone receptor has been detected in the lung pathogen *Pneumocystis carinii* (*P. jiroveci*) [46]. Meanwhile, an STE12 homolog has been discovered in the asexual *Penicillium marneffei* which is able to complement defects in sexual development in *A. nidulans* mutants with the equivalent *steA* mutation [47].

Evidence for sexual potential from taxonomic considerations

There are some taxonomic groupings of Aspergilli within which most species are only known to reproduce by asexual means [e.g. see 48], and it has been proposed that the reproductive strategy of related taxa might result from a shared historical event that was carried forward during evolution of that group [49]. However, *A. fumigatus* has many close taxonomic relatives with described sexual states within the genus *Neosartorya* [50]. The homothallic species *N. fischeri* is particularly closely related according to molecular analysis of β -tubulin gene sequence [51]. Furthermore, some isolates of *A. fumigatus* have apparently produced abortive cleistothecia when paired with mating-type testers of the heterothallic *N. fennelliae* [25]. These studies with related taxa clearly demonstrate that *A. fumigatus* exists within a group of species able to undergo sexual reproduction, so has at least originated from a sexual ancestor. Thus, if *A. fumigatus* has truly become asexual, then it is most likely a relatively recent evolutionary event. This would support the hypothesis that asexuality has arisen from multiple independent losses of sexuality within the *Aspergillus* section *Fumigati* [50,51], unlike a single evolutionary event influencing reproductive lifestyle as proposed for certain *Sordariaceae* [49].

Significance of possible sexual reproduction in *Aspergillus fumigatus*

The above findings provide evidence that sexual reproduction may occur within populations of *A. fumigatus*. Although a teleomorph has yet to be detected, it is important to note that the presence of such a sexual state would have profound implications for our understanding of the biology and management of the species. Sexual reproduction through outbreeding would be expected to maintain genetic variation

within populations, with the constant opportunity to generate novel genotypes. This is critical to allow response to environmental change, and enable long-term survival and evolution of the species. The presence of genetic variation has associated implications for disease management of *A. fumigatus* because sexual crossing might produce offspring with increased virulence or resistance to antifungal agents. For example, sexual crossing between isolates of the phytopathogen *Tapesia yallundae* has been shown to result in progeny with markedly increased resistance to azole fungicides compared to the original parental isolates [52]. Sexual reproduction can also increase gene flow within populations, ensuring the spread of favourable mutations [5].

A further implication for disease management is that sexual reproduction may confound diagnostic tests based on the assumption of a clonal population structure, since sexual recombination may break up the markers on which such diagnostics are based. Similarly, the occurrence of sex may invalidate studies using DNA variation to identify fungi and track clinically relevant markers [13]. However, there are potential benefits to the discovery of a sexual stage of *A. fumigatus*. For example, if it were possible to devise a method to cross isolates under controlled conditions then the sexual cycle could be used in classical genetic studies. This might involve the analysis of inheritance of genes of interest such as putative virulence genes or those involved with resistance to antifungals. Similar *in vitro* crosses have been used to determine the basis of resistance (whether mono- or polygenic) to azole fungicides in *T. yallundae*, with consequences for assessing the risk of evolution of fungicide resistance and designing management strategies for effective fungicide usage [52].

Asexuality or sexuality in *A. fumigatus*?

Taken as a whole, the findings described above provide support for the hypothesis that there is a latent potential for sexual reproduction in *A. fumigatus*. However, it should be stressed that there may be genuine short-term advantages to an asexual lifestyle. These include preservation of well-adapted combinations of genes, which might be broken up during sexual recombination. Also there are metabolic costs to sexual reproduction due to mating processes and investment in fruit body production. In addition it has been argued that sexual reproduction might perversely prevent rapid evolution whereas this is inevitable in asexual populations in response to dramatic change [5]. Furthermore, synergism between genes might overcome the problem of accumulation of deleterious mutations as described

by Muller's ratchet [5]. Indeed, there is compelling evidence for the existence of a few truly ancient asexual lineages in nature, examples including the bdelloid rotifers, Darwinulid ostracods and the Glomeromycota (arbuscular mycorrhizal fungi) [6,53,54].

Thus, we are left with the question – has *A. fumigatus* truly evolved to asexuality or does sexual reproduction still occur in the 'wild', involving some as yet unidentified teleomorph? It is still not possible to definitively answer this question. However, some final insights may come by considering the possible stages in evolution of an asexual species. Given the short-term advantages of asexuality [5] it is conceivable that asexuality might arise due to a single mutation in, or loss of, a key gene involved in sexual development (or control element of that gene, be it promoter or repressor). Given that many hundred genes are required for sexual development [1] this might be achieved fairly easily. Indeed, sexuality is often one of the first traits lost when fungal isolates are subjected to mutagenic treatment, as sex is reliant on so many genes working in concert.

However, we here wish to propose an additional/alternative hypothesis – that of a 'slow decline' in sexual fertility as a cause for the evolution of asexuality. It has been observed with many ascomycete species that when field isolates are crossed with laboratory tester strains that the resulting crosses exhibit a wide range of fertility with few to abundant fertile ascospores formed [1]. Well known examples include *Magnaporthe grisea* and some *Fusarium* species in which most field isolates may be infertile, but a low proportion retain the ability to cross with tester strains [55,56]. There are various possible reasons for this variation in fertility as discussed fully elsewhere [1]. These include the presence of male/female sexuality (referring to the ability to act as a fertilizing agent or form ascospores respectively) superimposed on a basic two-allele sexual compatibility system; variable expression of genes required for sexual reproduction; variable synthesis of key sexual morphogens or hormonal factors; the presence of secondary sexual incompatibility systems or heterogenic incompatibility; and possible mismatch in karyotype or cytoplasmic factors. Meanwhile, it is a common observation that when sexual species are cultured in the vegetative state over extended periods then the sexual fertility of isolates may slowly decrease, as seen for example with classic studies with *Aspergillus glaucus* [57], which might give the final impression of an 'asexual' species. Thus, an alternative way for asexuality to arise within a species may be by the 'slow decline' of sexual fertility within the species as a whole, although some isolates within populations may

still remain relatively fertile. Loss of sexual fertility might arise by any one (or more) of the mechanisms described above. For example, in the case of *M. grisea*, most field isolates in a worldwide sampling of 476 isolates were found to be female sterile, with many isolates also male sterile [55]. Thus, this species may conceivably be viewed as slowly evolving away from sex as a result of a loss of ability to mate or form ascospore tissues. Similarly, it has been argued that there is a definite mutational trend away from sex in *Fusarium* species, with a pattern of homothallic strains becoming heterothallic and the arising strains then developing impaired mating systems [56]. It is noted that an evolution in mating systems has also been described in hemiascomycete yeasts, in which a trend from obligate heterothallism to homothallic switching is apparent [58]. A specific example of possible 'slow decline' of sexual fertility is that of *Fusarium* species with sexual states within the *Gibberella fujikuroi* complex. Here female sterile strains may accumulate to such a proportion at local field sites that the population effectively appears asexual [59]. The 'slow decline' process may be due to short-term selection pressure for asexuality and/or against sexuality, particularly under artificial conditions such as those encountered in modern agriculture [59].

So as an overall perspective *species should not be viewed as either sexual OR asexual but rather being present on a continuum of sexual fertility* – with the potential presence of isolates within a species with a range of abilities to undergo sexual reproduction from high to low fertility to even apparent 'asexuality'. Indeed, given the argument that 'a little sex goes a long way' [5] (i.e. that a very low rate of sexual recombination is sufficient to overcome evolutionary problems associated with asexuality), then it may only be necessary to retain a low proportion of isolates that retain the ability to reproduce sexually. Of relevance is the observation that only certain genotypes of *C. albicans* have the ability to undergo sexual mating [15].

So what does this mean in the case of *A. fumigatus*? One possibility is that most isolates are indeed sexually fertile and that a sexual stage (presumably resembling a *Neosartorya* species [9,60]) does occur in nature but has simply remained overlooked – and that the sexual cycle has not been induced in the laboratory as it is not amenable to *in vitro* studies. Alternatively, the sexual stage may require exceptional environmental or nutrient conditions to occur, which are rare in nature. However, another explanation is that there is a 'slow decline' in fertility amongst populations of *A. fumigatus* and that it will be necessary to screen many isolates to identify a small (and ever decreasing?) subset of

isolates which retain the ability to reproduce sexually. But such sexually fertile isolates would have the ability to profoundly effect the population structure and influence the evolution of the species, so are likely to be retained albeit at a low proportion.

Conclusions

There is evidence from population genetic analyses, genome analysis, studies of mating-type gene presence and distribution, expression of sex-related genes, and taxonomic studies to indicate that a sexual stage for *A. fumigatus* may yet be identified. This may require the screening of numerous isolates and assays on many different nutritional media. But the development of the mating-type diagnostic test has allowed the identification of potentially sexually compatible MAT-1 and MAT-2 isolates, which will allow the design of 'targeted' crosses. Also there is now the potential to genetically manipulate mating-type, and other sex-related genes in order to attempt to induce sexuality as has been performed elsewhere [38]. It is hoped that findings from *A. fumigatus* will provide useful insights into the biology of other supposedly 'asexual' fungal species.

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