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Sexual reproduction in *Aspergillus* species of medical or economical importance: Why so fastidious?

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Abstract

Heterothallism, which is dependent upon the obligatory cross-mating between self-sterile homokaryotic individuals, represents a common pattern of sexuality in yeasts and molds. Heterothallic reproductive cycles have recently been discovered in three *Aspergillus* species of medical and economic importance, namely *Aspergillus fumigatus*, *A. parasiticus* and *A. flavus*. Together with *Aspergillus udagawae* (*Neosartorya udagawae*), heterothallism has now been discovered in a total of four aspergilli that affect human health or economy. These fungi appear to express relatively low-levels of fertility compared to other heterothallic or homothallic aspergilli and require unusually fastidious environmental parameters to complete the sexual cycle. Since the purpose of sex is to reproduce, we favor the hypothesis that while fertility of these species is on the decline it is compensated by their proficiency to reproduce asexually in wider range of environmental conditions. Heterothallism in these species could provide an invaluable tool for recombinational analysis of factors relevant to pathogenicity or toxin production. There is concern, however, whether extensive recombinational analysis can be very practical in light of the fact that formation of ascospores in these species requires a long period of time and construction of genetically marked strains will likely decrease fertility even further.

Significance of sex in *Aspergillus fumigatus*

Aspergillus fumigatus, the principal etiologic agent of life-threatening invasive aspergillosis (IA) in immunocompromised individuals, is one of the most ubiquitous fungi found in soil and organic debris world-wide [1,2]. This species propagates via asexual spores (conidia) that can be dispersed over wide geographic distances by even small air currents, and germinate to grow under a broad range of environmental conditions [2,3]. Due to its thermotolerance, *A. fumigatus* can even grow in compost piles (where temperatures can reach above 50°C) while only a minority of fungal species have the ability to grow at temperatures above 45°C [4]. In general, healthy humans are resistant to *A. fumigatus* despite the daily inhalation of numerous airborne conidia, although allergic or asthmatic reactions can occur [2,5]. However, immunocompromised individuals such as those undergoing organ transplant or chemotherapy for cancer are highly susceptible to life-threatening IA and the incidence of the disease in these patients can be as high as 50% with high mortality [6]. Research to understand why some strains of *A. fumigatus* are inherently more refractory to existing antifungal treatment and investigation into the mutational effects on virulence has been hampered by the lack of a recombination system that would allow

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genetic analysis. The recent discovery of long unknown heterothallic sexual cycle in *A. fumigatus* offers the possibility of such an analysis despite their slow process of sexual reproduction with low fertility. The subject is important and timely since the cases of life threatening aspergillosis due to *A. fumigatus* is on the rise world-wide and genomic data of these fungi is now in the public domain.

Discovery of *Neosartorya fumigata*, the sexual state of *Aspergillus fumigatus*

Until recently, *A. fumigatus* was considered to be an asexual fungus in spite of the fact that its genomic sequence included a complement of genes required for sexual reproduction [7-10]. This is because no investigators have been able to induce the sexual life cycle in crosses between two genetically identified opposite mating type strains of *A. fumigatus* - *MAT1-1* and *MAT1-2* - on various mycological media that induce the sexual cycle in most of other common ascomycetous fungi. Thus, the recent description of heterothallic sexuality in *A. fumigatus* (whose teleomorph was described as *Neosartorya fumigata*) [11] has evoked excitement among mycologists. It has also raised questions as to the role of sexual reproduction in the survival of this microorganism, and its utility as a tool for recombinational analysis.

It appears that *A. fumigatus* is more fastidious in sexual reproduction than most other sexual aspergilli [1]. The *N. fumigata* state with viable ascospores was first reported in strains isolated from five environmental locations in Dublin, Ireland [11]. The media and incubation conditions that induced sex in these populations are notably specific compared to other sexual species with *Aspergillus* anamorphs. Before the recent discovery of sexuality in *A. fumigatus*, *A. parasiticus* and *A. flavus*, teleomorphs had been identified in six heterothallic and many homothallic species with *Aspergillus* anamorphs. The six heterothallic species were: *Emericella heterothallica* [12], *Neosartorya fennelliae* [13], *N. nishimurae* [14], *N. otanii* [15], *N. spathulata* [15] and *N. udagawae* [16]. None of these six heterothallic species nor homothallic *Aspergillus* species require media and incubation conditions as specific as *N. fumigata* to complete the sexual life cycle. Mycological media that are generally poor in nitrogen source (such as corn-meal agar, V-8 juice agar, malt extract agar, alphacell agar, oatmeal agar and soil extract agar) support the formation of mature cleistothecia in most homothallic as well as heterothallic *Aspergillus* species at temperatures between 25 to 35°C within two to four weeks [1,12,13]. The only exception is in *Aspergillus alliaceus* (teleomorph: *Petromyces alliaceus*) which requires 3 to 10 months of incubation at 25°C in order to form mature cleistothecia [1]. In contrast, for *N. fumigata*, only Parafilm sealed oatmeal agar plates induced the formation of cleistothecia. Furthermore, six months were required to produce mature cleistothecia in a cross between *MAT1-1* and *MAT1-2* strains in the dark and only at 30°C [11].

With the combination of such particular requirements, it is not surprising that the sexual state of *A. fumigatus* was not found for decades. One wonders whether *A. fumigatus* encounters such specific conditions in nature that can induce the sexual cycle and whether sexual reproduction is practical for survival of the species. Since strains of the two opposite mating types exist in equal frequency and are found in close proximity to each other [11], it is no longer a question whether the sexually compatible strains can find each other to undergo sexual reproduction. Finding a suitable environment, however, with an appropriate substrate and a constant temperature of 30°C for six months might be rare in nature and yet *A. fumigatus* is one of the most ubiquitous fungi world-wide. It is possible that the fungus undergoes sexual reproduction on substrata in nature yet to be identified where the specific set of parameters that are deemed necessary in the laboratory can largely be circumvented. We used the same environmental conditions described above but failed to induce the

complete sexual cycle while crossing the *A. fumigatus* strain AF293 (*MAT1-2*; used for genomic sequencing [7]) with A1163 (CE10) (*MAT1-1*) or with B-5233 (*MAT1-1*) (our unpublished observations). This might be due to improper regulation of the *MAT* genes [17] or mutation in any mating-associated genes in any of these strains [17].

***Aspergillus fumigatus* is successful despite its low fertility**

It appears that most *A. fumigatus* isolates are not very fertile. Could it be that the sexual fertility of strains from Dublin represent a rare subpopulation [11] in which *MAT* genes have retained functionality with correct regulation? Are the Dublin isolates a result of ecological selection or ecological reproductive isolation of fertile ancestral strains? The evidence of sexuality being ancestral to asexuality in ascomycetes comes from the studies in *Gibberella*/*Fusarium* [18,19] and *Aspergillus* species [20]. There is no information as to the microecology of the fertile Dublin strains since they are reported as isolates from air sampling [11]. It has been known that adaptation to different ecological niches can foster genetic diversity in subsets of populations [21-23]. Remarkably, there is strong evidence suggesting that fertility might be declining even in the Dublin strains [11]. Of the 36 different mating pairs of *MAT1-2* and *MAT1-1* strains, 80 % produced less than 60 cleistothecia while only 8 % produced ≥ 100 cleistothecia on each agar plate (Figure 1A,B). Such low fertility of *N. fumigata* strains from Dublin is in stark contrast to the other heterothallic *Neosartorya* species such as *N. fennelliae* (Figure 1C), *N. nishimurae*, *N. spathulata* and *N. otanii* where the number of cleistothecia produced are usually too high to count. Interestingly, these four highly fertile *Neosartorya* species are not as ubiquitous as *A. fumigatus*, not known to be of medical or economic importance and do not require a specific set of environmental parameters for sexual reproduction as exemplified by *N. fennelliae* in Figure 1C. It is noteworthy that *N. udagawae*, a heterothallic species pathogenic to humans which has often been misidentified as *A. fumigatus* in clinical laboratories based on anamorphic similarity [24], also exhibits low fertility. We studied the mating pattern of nine clinical and two environmental strains of *N. udagawae* under various environmental conditions, including the parameters previously used to induce sexual reproduction for *N. udagawae* or for *N. fumigata*. Only four clinical isolates mated with the *MAT1-1* type strain but produced only abortive cleistothecia or cleistothecia containing sterile ascospores within two months. The remaining strains did not mate with the two mating type reference strains (our unpublished observation). Even the *MAT1-1* $\times$ *MAT1-2* cultures of the type strains failed to form cleistothecia on ordinary mycological media [25]. Similar results as to the lack of mating among clinical isolates of *N. udagawae* have been reported in Japan [26,27] as well as in the United States [28].

It is not uncommon to observe a “decline of fertility” in heterothallic environmental fungi that cause invasive disease in humans. For example, freshly isolated strains of *Ajellomyces capsulatus* (*Histoplasma capsulatum*) and *Ajellomyces dermatitidis* (*Blastomyces dermatitidis*), either from the environment or clinical sources, mate and produce cleistothecia readily in the laboratory [29,30]. However, maintenance of fertility in each of the strains was impractical in the laboratory and made recombinational analysis via heterothallism nearly impossible, since genetically marked strains lost their mating ability even faster than the unmarked strains. This is the primary reason why heterothallism in both species has not been exploited for recombinational analysis (our unpublished observations).

Is there any evidence of recombination among global strains of *A. fumigatus*? Most epidemiological studies that have revealed the high genetic diversity among *A. fumigatus* strains have relied upon the patterns of unstable molecular markers such as retrotransposon-like elements [31,32]. However, multilocus sequence typing (MLST) and other analyses of stable genetic markers such as rDNA and house-keeping genes showed contrary results.

Bain and colleagues showed that MLST of 100 *A. fumigatus* strains, originated from clinical or environmental sources collected on three different continents, formed a single clonal cluster with low levels of sequence dissimilarity [33]. Similar low levels of polymorphism were reported in the sequences of the internal transcribed spacer (ITS) of rDNA and the gene encoding β -tubulin among 70 environmental isolates of *A. fumigatus* from all six continents [34]. However, the fertile Dublin isolates were not included in these studies. The lack of population structure in *A. fumigatus* is in contrast with that of its close teleomorphic relatives such as *N. fischeri* and *N. spinosa* [34]. These studies suggest that sexual recombination is not a regular event in *A. fumigatus*, and the species might be evolving to be functionally asexual [10].

On the other hand, Pringle and colleagues reported a delineation of two phylogenetic species within 63 global *A. fumigatus* strains based on the association analysis of alleles at five loci, and concluded that the species reproduces clonally and appears to recombine [35]. Could discrepancies between the two previously mentioned studies [33,34] and the one by Pringle be due to differences in the identification of *A. fumigatus*? It is noteworthy that, whereas Pringle's report did not characterize the strains analyzed, those used for MLST by Bain and coworkers were all rapidly sporulating typical strains thereby excluding the slow-sporulating sister species of *A. fumigatus* [33]. In the slow sporulating species, such as *A. udagawae* (*N. udagawae*) and *A. lentulus*, the anamorph can not be distinguished from *A. fumigatus* phenotypically, and thus they are often misidentified as *A. fumigatus* [24]. These two species of clinical origin were first differentiated from *A. fumigatus* on the basis of *rodA* and *benA* sequences after the Pringle's report was published [24,28,36].

The 91 Dublin isolates showed genetic diversity based on the PCR patterns of randomly amplified polymorphic DNA (RAPD). If the genetic variability in this population resulted from recombination it is possible that the variation could have been fixed long before their fertility began declining. Since truly asexual organisms are rare [37] and sexual recombination is believed to provide an important long term advantage [38], the global asexual strains might have only recently spread world-wide. Furthermore, clonal populations might owe their success to their wide geographic distributions that far exceed those of their possible sexual progenitors [39,40].

Why do heterothallic species of pathogenic *Neosartorya* manifest low fertility compared to non-pathogenic *Neosartorya* species? Clonal populations could have acquired a survival advantage via pathogenicity that has enabled them to circumvent the toll exacted by sex with respect to nutritional and energy requirement. This hypothesis could be tested by comparing the pathogenicity of clonal populations of environmental strains that do not reproduce sexually with those that have retained sexual reproduction such as the Dublin strains. Furthermore, it should be of interest to compare the fertility among patient strains originating from the same region where the fertile strains were isolated in Dublin.

Since accumulation of deleterious mutations in asexual organisms is likely to result in their eventual extinction [41,42], clonal populations might resort to the parasexual cycle for recombination. Parasexuality has been studied in *A. fumigatus*. The frequency of diploid colony formation in heterokaryotic strains varied between 7×10^{-3} to 2.5×10^{-7} depending on the type of heterokaryons, and haploid segregants from the diploid strains revealed evidence of efficient recombination [43]. While detection of diploid formation in the laboratory is facilitated by crossing strains with complementary auxotrophies [44], it would be difficult to find clear evidence of the importance of parasexual recombination in natural populations of *A. fumigatus*. However, heterokaryon formation has widely been observed between prototrophic strains in many asexual *Aspergillus* species which may increase the fitness of these species via genetic diversity [1].

Progress in recombinational analysis requires the availability of genetically marked isogenic pairs of *MAT1-1* and *MAT1-2* fertile strains. Construction of an isogenic pair will require at least 10 generations of backcrossing with the foundational strain [45]. Therefore, it would be a challenge to construct an isogenic pair of *N. fumigata* due to its declining fertility. Unlike in sexually robust species such as *A. nidulans* or *S. cerevisiae*, introducing genetic markers into *N. fumigata* would be risky since marked strains are likely to be less fertile and eventually lose sex. Six-month long incubation periods for the maturation of meiotic products (ascospores) in *N. fumigata* would be another obstacle for its heterothallism to be a practical tool for extensive recombinational analysis.

Sex in aflatoxin producing aspergilli

Aspergillus flavus and *A. parasiticus* are the two major aflatoxin producing species among the members of Section Flavi [46]. Because aflatoxin is a potent carcinogen [47] produced by these species in corn, peanuts and other agricultural commodities [48], *A. parasiticus* and *A. flavus* have significant economic importance. In addition to aflatoxin production, *A. flavus* is an important cause of aspergillosis in immunocompromised patients [49]. Like *A. fumigatus*, *A. parasiticus* and *A. flavus* were considered asexual species for decades in spite of the presence of *MAT1-1* and *MAT1-2* idiomorphs consistent with heterothallic sexuality [50]. The recent discovery of a heterothallic sexual cycle in these species, therefore, created much excitement in the communities of fungal biology and medical mycology.

Discovery of *Petromyces* teleomorphs in *Aspergillus parasiticus* and *Aspergillus flavus*

Long before the genetic identification of *MAT1-1* and *MAT1-2* idiomorphs in *A. flavus*, Geiger and colleagues observed two reproductively isolated clades among 31 strains of *A. flavus* based on restriction site polymorphism and sequence of various protein encoding genes. One of the clades showed no concordance in gene genealogies which is consistent with the history of recombination [51]. A decade later, Carbone and coworkers confirmed the existence of a recombination system based on the sequence of intergenic regions in the aflatoxin gene cluster of 24 *A. parasiticus* strains isolated from a peanut field in Georgia, USA [52]. Subsequently, Ramirez-Prado and colleagues detected either a *MAT1-1* or *MAT1-2* gene in the genomes of *A. flavus* and *A. parasiticus* [50]. They also found that the two idiomorphs occurred in equal frequency among the populations isolated from the same peanut field in Georgia suggesting the possible existence of a sexual cycle [50]. Eventually, Horn and coworkers discovered heterothallism in *A. parasiticus* [53,54] and in *A. flavus* [55]. Their teleomorphs were described as *Petromyces parasiticus* [54] and *P. flavus* [55], respectively.

Unlike *Neosartorya* (Figure 2A) the *Petromyces* species produces cleistothecia within the sclerotia, a discrete hard structure consisting of thick-walled parenchyma-like cells [1] (Figure 2B,C) that serve as survival structures during adverse environmental conditions [1, 56]. There are currently five species in the genus *Petromyces* among which *P. parasiticus* and *P. flavus* are the only heterothallic species. Interestingly, sexually compatible strains of *P. parasiticus* as well as *P. flavus* belong to different vegetative compatibility groups (VCGs) which would enhance genetic diversity among progenies [53, 55]. Sexual reproduction in the genus *Petromyces* is known to be an extremely slow process, taking up to 10 months, with low fertility as first described in *A. alliaceus* (*P. alliaceus*) [1]. However, low fertility does not appear to be limited to the homothallic species, *P. alliaceus*. In *P. parasiticus*, the sclerotia bearing ascocarps (cleistothecia) produced in the crosses between sexually compatible strains belonging to different VCGs varied from 0 to 82% . Furthermore, the ascocarps bearing ascospores were often below 10%. Even for the most

fertile pairs, only 55% of the ascocarps contained ascospores [51] with variable germination rates (I. Carbone, personal communication). The frequency of ascospore bearing ascocarps produced in *P. flavus* was also low. Less than 50% of the ascocarps bore ascospores in eight of twelve crosses between sexually compatible strains belonging to different VCGs. Germination rate of ascospores in *P. flavus* has not been reported [53]. The slow process of sexual reproduction and low fertility in *P. parasiticus* as well as in *P. flavus* also presents a challenge for recombinational analysis.

Concluding remarks and future directions

In this article, we summarized recent findings on heterothallic sexuality in three *Aspergillus* species of medical and or economic importance, namely *A. fumigatus*, *A. flavus* and *A. parasiticus*. These fungi were considered to be asexual for decades because of their low fertility coupled with the requirement of unusually particular environmental parameters to produce teleomorphs. Genome sequence of these fungi made it clear that they occur in two idiomorphs *MAT1-1* and *MAT1-2*, which invigorated the mycologists in their quest to discover the sexual cycle. The recombination system found in these species undoubtedly will significantly contribute to our understanding of molecular and genetic systems that are associated with pathogenicity or toxin production. However, the sexual cycle in these species may not be as useful as the model fungi due to the fastidious nature of the process unless the obstacles in the process can be eased by molecular and genetic manipulation (Box 1). Even in the Dublin strains, evidence of declining fertility is strong when compared with other heterothallic *Neosartorya* species of no medical or economic importance. Similar situations have been known in *N. udagawe*, another medically important *Neosartorya* species with *A. fumigatus*-like anamorph. This highlights the apparent advantage of asexual reproduction over sexual reproduction in pathogenic aspergilli. Since evidence of sexual reproduction among global strains of *A. fumigatus* isolated from clinical cases remains unclear, there could be yet another set of environmental parameters which could induce the sexual cycle in these strains. Although the low fertility coupled with prolonged periods that are required for sexual reproduction in all three heterothallic *Aspergillus* species discussed in this article can be an obstacle, attempts should be made to construct fertile, isogenic pairs of *MAT1-1* and *MAT1-2* strains that can be used for classic as well as molecular genetic studies.

Box1. Outstanding questions

- Do the sexually fertile and infertile strains belong to different subsets of the *A. fumigatus* population? Phylogenetic analysis based on the sequences of multiple genes using asexual global strains and the fertile strains from Dublin is warranted to answer this question.
- Six to ten months of incubation for the collection of mature ascospores is an obstacle for the progress in genetic analysis, and new environmental parameters should be searched to shorten the waiting period.
- The fertility in *N. fumigata* strains might be increased by genetic manipulation of mating -related genes [11,57]. Strains over- expressing mating-related genes might be constructed in both idiomorphs with the background of strains from Dublin or other fertile strains in order to improve fertility.
- An isogenic set of fertile *MAT1-1* and *MAT1-2* strains will be extremely useful for genetic and molecular studies. Can such strains be constructed by mating the most fertile pair of *MAT1-1* and *MAT1-2* strains and the selection of high-fertility progenies that can be used as the foundational strain for back crossing?

- Are there differences in virulence between the fertile and the infertile strains isolated from the same environment? This would reveal any association between fertility and virulence.
- There are other *Aspergillus* species of medical or economic importance, such as *A. niger*, *A. oryzae* and *A. terreus* that are known to have *MAT* genes but their teleomorphs are not known. Sexual reproduction in these species should be revisited using environmental parameters similar to those used for the sexual reproduction in *A. fumigatus* or *A. parasiticus*.

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Glossary

Anamorph	asexual form
Ascospores	sexual spores produced after meiosis in fungi belonging to Ascomycetes.
Aspergillosis	infection caused by fungal species belonging to the genus <i>Aspergillus</i> .
Cleistothecium (pl. cleistothecia)	a completely enclosed sexual fruiting body produced in Ascomycetes.
Conidium (pl. conidia)	asexual spore.
Heterokaryon	an individual exhibiting genetically different nuclei in the same protoplast or same mycelium.
Heterothallic	self-sterile individuals requiring the union of two compatible thalli for sexual reproduction
Homokaryotic	an individual with genetically alike nuclei in the same protoplast or same mycelium.
Homothallic	self-fertile individuals that reproduce sexually without cross mating.
Idiomorph	fungal mating types which do not show homology between strains of the opposite sex.
<i>MAT1-1</i> and <i>MAT1-2</i>	genetic designation of two idomorphs in heterothallic filamentous Ascomycetes containing single mating type locus.
Parasexual cycle	a process in which fusion of unlike nuclei, mitotic crossing over and haploidization take place in sequence.
Sclerotium (pl. sclerotia)	a hard resting body resistant to unfavorable conditions that can remain dormant for long periods of time.
Section Flavi	a subgroup within the genus <i>Aspergillus</i> that includes mycotoxin producing species represented by <i>Aspergillus flavus</i> .
Stroma	a compact somatic structure (such as sclerotia) in which fruiting bodies are formed.

Teleomorph	sexual form.
Vegetative compatibility group	grouping based on a genetic system that allows fusion between individual mycelia of the same species to form heterokaryons.

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Figure 1.

Sexual reproduction in *Neosartorya* species. **(A)** Paired culture of two compatible mating types of *Neosartorya fumigata* incubated for 6 months at 30°C. **(B)** Sparsely produced cleistothecia (arrows) along the junctions of intersecting colonies of *MAT1-1* and *MAT1-2* strains of *N. fumigata*. Scale bar, 1 cm. **(C)** Paired cultures of two opposite mating types of *Neosartorya fennelliae* incubated for one week on alphacel-yeast extract agar (top), on soil extract agar (left) and on malt extract agar (right), showing numerous cleistothecia along junctions of interacting colonies of the opposite mating type. Images reproduced with permission from refs. [11] **(A, B)** and [13]**(C)**.

Figure 2. Comparison between sexual reproductive structures of *Neosartorya* and *Petromyces*. **(A)** White globose cleistothecia surrounded by loose hyphal envelope produced by *Neosartorya fumigate*. Scale bar, 400 μm (reprinted with permission from ref. [11]). **(B)** Sclerotia nested among green conidial structures in *P. parasitica*. **(C)** Sectioned stroma of *P. parasitica* showing three large and two small ascocarps imbedded in the matrix. **(B, C)** Scale bars: 500 μm (courtesy of Dr. Carbone; reprinted with permission from ref. [53]).