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Aspergillus: Sex and Recombination

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Abstract The genus *Aspergillus* is one of the most widespread groups of fungi on Earth, comprised of about 300–350 species with very diverse lifestyles. Most species produce asexual propagula (conidia) on conidial heads. Despite their ubiquity, a sexual cycle has not yet been identified for most of the aspergilli. Where sexual reproduction is present, species exhibit either homothallic (self fertile) or heterothallic (obligate outcrossing) breeding systems. A parasexual cycle has also been described in some *Aspergillus* species. As in other fungi, sexual reproduction is governed by mating-type (*MAT*) genes, which determine sexual identity and are involved in regulating later stages of sexual development. Previous population genetic studies have indicated that some supposedly asexual aspergilli exhibit evidence of a recombining population structure, suggesting the presence of a cryptic sexual cycle. In addition, genome analyses have revealed networks of genes necessary for sexual reproduction in several *Aspergillus* species, again consistent with latent sexuality in these fungi. Knowledge of *MAT* gene presence has then successfully been applied to induce sexual reproduction between *MAT1-1* and *MAT1-2* isolates of certain supposedly asexual aspergilli. Recent progress in understanding the extent and significance of sexual reproduction is described here, with special emphasis on findings that are relevant to clinically important aspergilli.

Keywords *Aspergillus* · Population structure · Recombination · Sexual reproduction · Mating-type genes

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Introduction

The genus *Aspergillus* is one of the most widespread groups of fungi on Earth, comprised of about 300–350 species assigned to various subgenera and sections [1], whose lifestyle can be very diverse. Regarding their mode of reproduction, most aspergilli are known to produce asexual propagula (conidia) from branching conidiophores borne on characteristic conidial heads. Indeed, the majority of *Aspergillus* species (approximately 64 %) are only known to reproduce by asexual means [2]. For the minority of species where a sexual cycle has been described, these exhibit either homothallic (self fertilising) or heterothallic (obligate outcrossing) sexual reproduction. A parasexual cycle has also been observed in some species [3]. The sexual stages of the aspergilli were previously assigned to

Table 1 Taxonomic outline of the genus *Aspergillus* [2, 4–6]

Subgenus	Section	Associated teleomorphic (sexual) stage
Aspergillus	*Aspergillus*	*Eurotium*
	Restricti	*Eurotium*
Fumigati	*Fumigati*	*Neosartorya*
	Clavati	*Neocarpenteles, Dichotomomyces*
	Cervini	–
Circumdati	*Circumdati*	*Neopetromyces*
	Nigri	*Saitoa*
	Flavi	*Petromyces*
	Cremei	*Chaetosartorya*
Candidi	*Candidi*	–
Terrei	*Terrei*	–
	Flavipedes	*Fennellia*
Nidulantes	*Nidulantes*	*Emericella*
	Usti	*Emericella*
	Sparsi	–
	Aenei	*Emericella*
	Versicolores	*Emericella*
	Bicolor	–
	Raperi	–

different teleomorphic genera such as *Eurotium, Neosartorya* or *Emericella* (Table 1) [2, 4–6]. However, according to the new rules of the Melbourne Code, adopted by the 18th International Botanical Congress in 2011, only one name can be used for one fungus [7], and the International Commission on *Penicillium* and *Aspergillus* decided to use the anamorph name *Aspergillus* in 2012 (Samson et al. in press; http://www.aspergilluspenicillium.org/).

Here, we wish to give an overview of recent progress in understanding the extent and significance of sexual reproduction and recombination in the aspergilli, with a special emphasis given to clinically important *Aspergillus* species.

Reproductive Strategies in the Genus *Aspergillus*

Aspergillus species can spread by a variety of methods, including via vegetative propagation and sexual and asexual sporulation (see example in Fig. 1; for greater detail, see references [12, 24]). The majority of *Aspergillus* species have traditionally been considered to reproduce only by asexual means. However, there has been accumulating evidence that gene flow as a result of sexual outcrossing and the associated recombination (shuffling) of genes has occurred, or is occurring, in natural populations of various *Aspergillus* species that were once thought to be strictly asexual, including, for example, *A. flavus* [8] and *A. fumigatus* [9–11].

Both sexual and asexual development within the aspergilli are preceded by the vegetative growth of hyphae, which emerge from either a single conidium (a mitotic product of asexual reproduction) or an ascospore (a meiotic product resulting from sexual reproduction). The growth phase includes the germination and development of a network of hyphae, which aggregate to form the mycelium. After a period of vegetative growth, some hyphal elements start to develop aerial hyphae, which can differentiate into asexual reproductive structures called conidiophores. During this stage of differentiation, some cells form an L- or T-shaped foot cell with a thickened wall producing a single conidiophore. The apical end of the conidiophore enlarges forming the vesicle, and the upper layer of the vesicle gives rise to the phialides. In the case of uniseriate species, these phialides emerge directly from the surface of the vesicle, whilst the vesicle of biseriate species bud twice forming two layers of phialides with the first layer called metulae [12–14]. The phialides produce chains of mitotic asexual spores called conidia.

Purely asexual reproduction results in the formation of exact clones of the parental organism, which can lead to the accumulation of deleterious mutations, as postulated by the concept of 'Muller's ratchet', and has been argued to represent an evolutionary 'dead end' by some [15, 16]. However, it is possible that in the absence of any sexual recombination, some asexual aspergilli are able to generate novel genetic diversity via the 'parasexual cycle', which was first described by Pontecorvo et al. [17]. The first step in the parasexual cycle is the fusion of genetically compatible haploid hyphae to form a heterokaryon. Within the heterokaryotic hyphae, two haploid nuclei may fuse together (karyogamy), resulting in the formation of a diploid nucleus. This then undergoes mitotic crossing over and subsequent mitotic division without replication to return to the original haploid state. Occasionally chromosomal non-disjunction can also occur during the steps of haploidisation. In some cases, diploid nuclei are very unstable and undergo

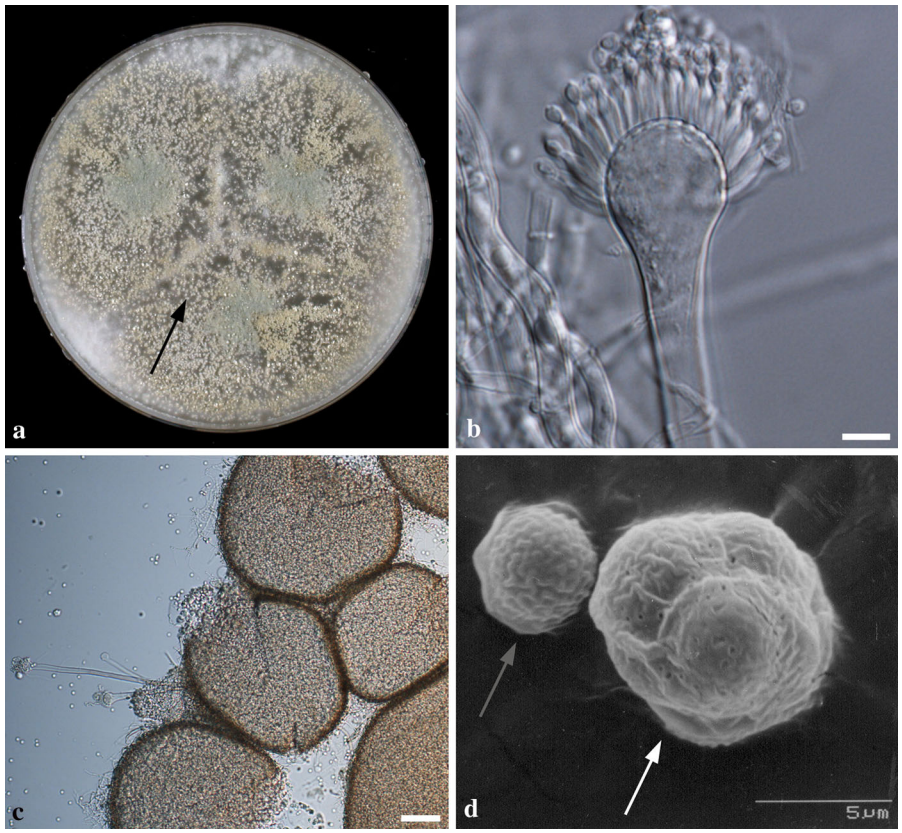

Fig. 1 Reproductive structures in *Aspergillus hiratsukae*. **a** Numerous yellow cleistothecia visible on oatmeal agar as yellow points (*black arrowed*) against dark background. **b** Conidial heads (*scale bar* 10 µm). **c** Crushed cleistothecia (*scale bar* 30 µm). **d** Ascus (*white arrowed*) and ascospore (*grey arrowed*)

rapid haploidisation without the formation of diploid segregants. This phenomenon has been described by Ball and Hamlym [18] in *Acremonium strictum* (=*Cephalosporium acremonium)* and was also later found in *A. niger* and *A. nidulans* [19, 20].

Meanwhile, the main source of genetic recombination and gene flow in the genus *Aspergillus* is thought to arise from sexual reproduction, with approximately one-third (36 %) of the accepted species having a known sexual cycle (Table 1; [2, 21]). During the sexual cycle, four meiotic ascospore progeny are produced, and a subsequent mitotic division yields eight ascospore progeny within each ascus. The ascospores normally have a significantly different morphology from conidia, often exhibiting species-specific ornamentation of the outer ascospore wall [22]. In general, the two hyphae that fuse to make the dikaryotic stage are undifferentiated, so male and female elements (e.g. ascogonia and antheridia) have not been distinguished for most species except for the genus *Fennellia* [14, 21]. However, this might be an artefact because of the overwhelming number of homothallic aspergilli, which might be expected to exhibit reduced mating apparatus concomitant with other genomic adaptations [23, 24]. Asci are produced in fruiting bodies called cleistothecia that develop from fused hyphae, and which may be surrounded by external tissues such as Hülle cells, or can be enclosed within sclerotia [24]. Where a sexual state is present, aspergilli have either heterothallic or homothallic breeding systems. Homothallic species can enter into the sexual cycle without the need to cross with a compatible partner, but it is important to emphasise that homothallic species such as *A. nidulans* retain the ability to outcross so are not restricted to self-fertilisation [23, 25]. Homothallism is more prevalent

amongst the aspergilli and only 12 heterothallic species had been reported by 2012 compared with over 150 homothallic species [24].

As with other filamentous ascomycetes, so-called mating-type (*MAT*) genes have a key role in controlling the sexual cycle in both heterothallic and homothallic *Aspergillus* species [26]. These *MAT* genes encode transcription factor proteins, which determine sexual identity and appear to regulate later stages of sexual development. Two distinct classes of *MAT* genes can be recognised: firstly, *MAT1-1* family genes, which encode an α-domain protein, and secondly, *MAT1-2* family genes, which encode a high-mobility group (HMG) box protein [27]. In heterothallic species, a single *MAT* locus is present, which contains either *MAT1-1* or *MAT1-2* family genes. By definition, *MAT1-1* isolates contain a *MAT1-1* locus with a *MAT1-1* family gene, whereas *MAT1-2* isolates contain a *MAT1-2* locus with a *MAT1-2* family gene [27]. The *MAT1-1* and *MAT1-2* loci are highly divergent in sequence and have therefore been termed 'idiomorphs'. Gene deletion studies with *A. fumigatus* have shown that *MAT* genes are required for sexual development [28]. Meanwhile, homothallic aspergilli possess both *MAT* genes within the same genome, either adjacent to each other at a single *MAT* locus, or at two distinct loci within the genome termed *MAT1* and *MAT2* in accordance with standard terminology for *MAT* genes (also co-termed *matB* and *matA*, respectively, by some authors) [22, 23, 29, 30]. A functional analysis in *A. nidulans* revealed that the mating-type genes are essential determinants of sexual development. In the absence of *MAT1* or *MAT2* genes, there was a significant decrease in the number and size of cleistothecia and ascospores failed to be produced. However, the deletion of the *MAT* genes did not have any obvious effect on vegetative growth or asexual sporulation [23].

Approaches to Detect Sex and Recombination in Fungal Species and Populations

There are two fundamental means by which fungi and other organisms transmit genes to the next generation: through clonal reproduction or by reproduction involving outcrossing and gene recombination. In the case of asexual reproduction, each progeny is a clonal descendant of only one parent, and therefore, its genome is an exact mitotic copy of its parent. By contrast, recombining populations exhibit genetic variation that has arisen either as a result of sexual reproduction and meiosis following mating or through a parasexual cycle (mitotic recombination). It is noted that self-fertilisation via homothallism is considered a form of clonal reproduction, despite the presence of meiosis, because the genomes of the progeny are identical to the parental genome in the absence of any outcrossing event [31].

It is important to understand the reproductive nature of any living organism since reproduction has implications for the population dynamics and evolution of the respective species. This is particularly of importance for fungal pathogens where the presence of recombining populations might increase the rate at which fungi evolve resistance to antifungal drugs and fungicides and increase the speed of evolution of traits linked to virulence and pathogenicity [32]. This understanding is also critical in a clinical setting for strain typing and monitoring applications [31]. Concerning the specific issue of asexuality in the aspergilli, a series of different methods can be used to assess whether supposed 'asexual' species might have a cryptic, or previously unidentified sexual stage that could result in a recombining population structure [2]. A classical way is to use morphological observation to directly seek evidence of the formation of sexual reproductive structures (e.g. cleistothecia and ascospores) in either pure culture or on natural materials. However, several indirect tests can also be used to assess the potential for sexual reproduction. For example, several methods have been developed to examine the population structure of fungi (Table 2). An excellent summary of these methods is provided by Taylor et al. [31]. The population structures of a number of pathogenic *Aspergillus* species have been

Table 2 Some methods to detect recombination in fungal populations [9, 31]

Examination of the distribution of mating-type genes
Mosaic gene structure
Incongruence between trees based on different data sets
Presence of all possible combinations of alleles at two loci
Index of association test
Parsimony tree length permutation test (PTLPT)
Partition homogeneity test (PHT)
Homoplasy test

examined using a combination of these classical and molecular techniques to seek evidence of recombination. One of the first uses of this methodology was to investigate the reproductive mode of the homothallic species *A. nidulans.* A lack of association was found between certain genetic markers and vegetative compatibility groups (VCGs) in UK populations, indicating that VCGs arose as a result of recombination. Thus, although populations were propagating primarily in a clonal fashion, it was concluded that there were sufficient recombination events to disrupt the stable maintenance of clonal genotypes [33], although the population structure might have been due to ancestral recombination.

Another test that can give an insight into possible sexuality concerns the identification of sex-related genes in the genome of the species in question [2]. The ease with which whole genome sequencing can now be accomplished means that genomes of supposedly asexual fungi can be screened for the presence of genes known to be required for sexual development. For example, over 70 candidate genes are known to be involved in sexual reproduction of *Aspergillus* species [22, 24]. If these sex-related genes are absent or mutated, then this would provide evidence for asexuality, whereas the presence of apparently functional sets of genes would be consistent with latent sexuality. This approach has been applied both to filamentous and yeast-like ascomycete fungi [34, 35]. The third test involves subsequent experimental analysis to determine whether such genes associated with sex are expressed or can be shown to be functional in heterologous systems [2]. If a gene specifically related to sexual processes can be shown to be expressed at the mRNA level, then this would be consistent with latent sexuality; likewise, if genes encoding for a given protein can complement the homologous defective (or deleted) gene in a known sexual species, then this also provides evidence of latent sexuality.

A final category of evidence for possible sexuality concerns the presence and distribution of isolates of different mating types within populations [2]. The development of PCR diagnostics now allows the rapid identification of the mating type of isolates from a variety of *Aspergillus* species using either degenerate or specific primer sets designed to anneal to conserved regions of the *MAT* genes [23, 30, 36]. It would be expected that sexual recombination would maintain a near 1:1 ratio of *MAT1-1*:*MAT1-2* isolates, whereas a clonally reproducing population would show marked divergence from this ratio due to genetic drift or selection [37].

Sexual Reproduction in Clinically Important *Aspergillus* Species

In the following sections, the results of investigations into the reproductive status of a variety of *Aspergillus* species of clinical relevance will be reviewed. This will include a description of how the various methods and categories of evidence described above [2] have been applied to evaluate the reproductive status of various aspergilli and how in certain cases this has led to the exciting discovery of a previously unknown sexual cycle.

Aspergillus Section *Fumigati*

Members of the *Aspergillus* Section *Fumigati* are characterised by uniseriate conidial heads bearing conidia, in shades of green, borne on flask-shaped vesicles [38]. The group includes species reported to cause opportunistic invasive infections in humans—most notably *A. fumigatus*. This fungus can give rise to various forms of the disease aspergillosis, which can prove fatal, particularly in immunocompromised hosts [39]. In addition, members of section *Fumigati* can produce a range of mycotoxins, which may also present a serious health hazard [38]. Conversely, certain species have beneficial properties for mankind, some producing a range of pharmaceutically active compounds whilst others have been used for bioabsorption of toxic chemicals [38, 40]. Members of section *Fumigati* are found worldwide, particularly in soil and rotting vegetation, and propagules of certain species such as *A. fumigatus* are commonly found in air samples [41]. Regarding their mode of reproduction, the majority of species are able to reproduce sexually, with the teleomorphs assigned to the genus *Neosartorya* [39]. All of the *Neosartorya* species produce heat-resistant ascospores, which might reflect selection in a common ancestor of *Neosartorya* for survival in ecological niches where high temperatures might be encountered, such as composting vegetation [42]. The vast majority of *Neosartorya* species reported to date exhibit homothallic reproduction, with an approximate 33:7 ratio of homothallic:heterothallic species [24].

For most of its 150 years described history, *A. fumigatus* was considered to reproduce exclusively by asexual means. However, there had been accumulating evidence for the presence of a cryptic sexual cycle based on the different categories of evidence for sex described above [2]. Firstly, evidence for recombination came from population genetic studies. There had been many reports of a high degree of genetic variation within natural populations of *A. fumigatus* consistent with sexual reproduction and recombination. For example, Debeaupuis et al. [43] found over 400 genetically unique isolates (based on retrotransposon fingerprinting) in samples from European clinical and environmental samplings, whilst a similar study of over 700 samples from hospitals in France found 85 % of isolates to be genetically unique [44]. These data, together with other reported observations of isoenzyme and sequence-specific DNA primer analysis data gathered from the literature, were pooled and analysed by Varga and Tóth [9], who concluded that there was evidence for recombination in some populations. This involved the application of the index of association test and the parsimony tree length permutation test (Table 2). Network methods were also used successfully to visualise the recombining structures of *A. fumigatus* populations. In a subsequent study, Pringle et al. [45] sequenced five polymorphic loci from 53 worldwide samples of *A. fumigatus* and found sufficient variation to conclude that recombination was occurring, although clonality dominated and recombination was rejected by certain statistical tests. Meanwhile, Paoletti et al. [11] sequenced three intergenic loci from 106 isolates spanning five subpopulations from Europe and North America and again found sufficient variation to conclude that recombination had occurred within the test samples. By contrast, Rydholm et al. [46] reported markedly lower sequence variation in 103 global samples of *A. fumigatus* than in the known sexual species *N. fischeri* and *N. spinosa*.

Further evidence for the presence of a cryptic sexual cycle in *A. fumigatus* came from bioinformatic analysis of the presence of sex-related genes. BLAST searching of the genome of *A. fumigatus* revealed the presence of over 200 genes associated with sexual reproduction, which lacked any obvious mutation so appeared to be functional [34]. This included the presence of a *MAT* locus containing a *MAT1-2* family HMG gene, confirming an earlier preliminary report by Pöggeler [47]. In a related study, Paoletti et al. [11] were able to identify isolates of *A. fumigatus* that contained the complementary *MAT1-1* (alpha domain) idiomorph and demonstrated that elements of a mating pheromone signalling pathway were expressed at the mRNA level—again consistent with potential sexuality. It was soon after demonstrated by Pyrzak et al. [94] and Groβe and Krappmann [95] that the *MAT1-2*, *MAT1-1* and *nsdD* genes, respectively, from *A. fumigatus* were functional based on their ability to drive sexual reproduction in *A. nidulans* gene deletant strains. Additional evidence for sexual potential then came from field surveys of the presence of isolates of compatible mating type. Paoletti et al. [11] designed a multiplex PCR-based mating-type diagnostic to determine the mating type of isolates of *A. fumigatus* and applied this to 290 worldwide clinical and environmental isolates. This revealed the presence of *MAT1-1* and *MAT1-2* genotypes in similar proportions (43 and 57 %, respectively), again consistent with latent sexuality. Bain et al. [48] also detected *MAT1-1* and *MAT1-2* isolates in a survey of 100 worldwide *A. fumigatus* isolates from clinical and environmental sources, although a bias towards the *MAT1-2* genotype was evident. Most recently, O'Gorman et al. [49] collected a population of *A. fumigatus* isolates from locations around Dublin, Ireland and showed an exact 1:1 distribution of *MAT1-1* and *MAT1-2* isolates. Furthermore, phylogenetic analysis showed that *MAT1-1* and *MAT1-2* isolates were interleaved together when represented graphically on a phylogenetic tree, consistent with recent recombination and the presence of a sexual cycle.

This mounting evidence led O'Gorman et al. [49] to set up directed crosses between *MAT1-1* and *MAT1-2* isolates from the Dublin population on a range of growth media and under a variety of different environmental conditions. And a major breakthrough was then made when it was discovered that a sexual cycle could be induced when isolates of compatible mating type were crossed in a barrage formation and incubated at 30 °C on oatmeal agar for 6–12 months in the dark. At this point, light-yellow cleistothecia typically 150–500 μm in diameter, resembling those of other *Neosartorya* species, formed singly or in small clusters mainly along the junction where hyphae of the parental isolates came into contact, and to a lesser extent within mycelium on either side of the barrage zone. When crushed, the cleistothecia were

found to contain asci within which were heat-resistant ascospores characteristic of the genus *Neosartorya.* Given these observations and the species phylogenetic affinity, the teleomorph was named *Neosartorya fumigata* [49]. Analysis using DNA fingerprint markers and the *MAT* locus demonstrated recombination amongst the ascospore offspring, confirming the presence of a heterothallic sexual cycle. The discovery of the sexual cycle was deemed to be of major medical significance as it suggested that, if prevalent in nature, sexual recombination could result in the appearance of progeny with increased virulence or resistance to antifungal agents and confound diagnostic tests based on the assumption of clonality. However, the sexual cycle was also deemed to offer a valuable laboratory tool with which to determine the genetic basis of traits of interest for this fungus [49].

A major question arising from the study of O'Gorman et al. [49] is whether sexual fertility is evident more broadly in worldwide populations of *A. fumigatus*, beyond the original sampled population from Dublin, Ireland. This is an area of ongoing study, but preliminary reports indicate that the majority of worldwide isolates might be sexually fertile in terms of the ability to produce cleistothecia with viable ascospores. An extensive sampling of *A. fumigatus* from Europe, Asia, Africa and the Americas has recently demonstrated that approximately 85 % of isolates are sexually fertile with Irish tester strains (O'Gorman CM, Swilaiman S and Dyer PS, unpublished results). This is consistent with other recent findings. Szewczyk and Krappmann [28] and Camps et al. [50] reported the presence of continental European isolates of *A. fumigatus* that were able to produce cleistothecia in crosses, whilst Sugui et al. [51] demonstrated that many clinical isolates from the USA could produce viable ascospore offspring. Interestingly, these findings differ from those with the emerging agent of aspergillosis *Neosartorya* (*Aspergillus*) *udagawae*, in which crosses either failed to produce cleistothecia or produced ascospores which did not germinate [52].

In follow-up work, Sugui et al. [51] screened a variety of *A. fumigatus* isolates for fertility and discovered two 'supermater' isolates, AFB62 (*MAT1-1*) from a case of invasive aspergillosis and AFIR928 (*MAT1-2*) from the environment, that exhibited high sexual fertility relative to other isolates tested and which could complete the sexual cycle and produce viable ascospores within 4 weeks (although highest ascospore viability was not reached until 20 weeks of incubation). The supermater isolates were also shown to be highly virulent in two different murine models and to have a high recombination frequency, thus providing a valuable tool for genetic studies [51].

The discovery of a sexual cycle in *A. fumigatus* then prompted investigations into possible sexuality in the related species *A. lentulus*, a member of the *Aspergillus* section *Fumigati* which had previously only been described to reproduce asexually [53]. This species had been identified as a causal agent of aspergillosis and is of especial clinical relevance because it exhibits decreased sensitivity to antifungal drugs commonly used to treat *Aspergillus* infections [54] and can be easily misidentified as *A. fumigatus* [55]. Swilaiman et al. [42] found that it was possible to successfully apply the *A. fumigatus* multiplex mating-type diagnostic of Paoletti et al. [11] to *A. lentulus,* revealing a ratio of *MAT1-1:MAT1-2* worldwide isolates of 38 versus 62 %, respectively. *MAT1-1* and *MAT1-2* idiomorph regions were also analysed, revealing the presence of characteristic alpha and HMG domain genes. Using similar crossing conditions to *A. fumigatus*, it was then possible to induce a sexual cycle between *MAT1-1* and *MAT1-2* isolates, with mature cleistothecia (containing heat-resistant ascospores) being produced after only 3 weeks of incubation. Recombination consistent with a heterothallic sexual cycle was confirmed using molecular markers. Previously, there were claims of abortive crossings between *A. fumigatus* and *N. fennelliae* [56], so pairings between *A. lentulus* and *A. fumigatus* were also tested. However, isolates of *A. lentulus* failed to cross with fertile Irish tester strains of *A. fumigatus,* demonstrating probable reproductive isolation between these sibling species [42]. This finding is of clinical significance as it indicates a possible lack of gene flow between the species and therefore reduced risk of antifungal drug resistance spreading between the species. Of especial relevance to the latter finding is that Camps et al. [50] found evidence that sexual reproduction within *A. fumigatus* field populations might have contributed to early genetic diversification of a pool of isolates containing a TR_{34}/L98H mutation in the CYP51A gene, which confers resistance to certain azole antifungal drugs. Thus, the sexual cycle provides a means by which gene flow can occur within

populations and might allow further spread of the resistance mutation in the future [50].

One other intriguing observation has been an apparent possible association between virulence and mating type in *A. fumigatus*. Alvarez-Perez et al. found an almost fourfold higher frequency of *MAT1-1* compared with *MAT1-2* isolates in cases of invasive aspergillosis from a hospital with Spain and also found a statistically significant correlation between increased elastase activity and isolates of the *MAT1-1* genotype [96]. Similarly, Cheema and Christians found that *MAT1-1* isolates exhibited higher virulence than *MAT1-2* isolates in a wax moth *Galleria mellonella* model system, although a relatively low number of isolates were tested [97].

Aspergillus Section *Nigri*

Members of the *Aspergillus* section *Nigri*, also called the 'black aspergilli', represent an important group of fungi. They are widely distributed in nature and extensively used in biotechnology for the production of hydrolytic enzymes such as lipases, amylases [57] and organic acids like citric acid and gluconic acid [58]. They also play a role in the bioremediation of contaminated soil [59] and the biosorption of substances from industrial effluent [60]. Besides their beneficial properties, these fungi can cause several forms of aspergillosis disease in humans, primarily in immunocompromised patients [61], but also infections in the ear canal of otherwise healthy individuals [13]. Furthermore, they can also be important pathogens of plants, and commonly contaminate food and feed crops at both pre- and post-harvest stages [62], which is of particular importance since some of the species may produce mycotoxins such as ochratoxin A and fumonisins [63, 64]. Black aspergilli are amongst the more difficult groups concerning species identification. Some closely related species belonging to this taxon cannot be distinguished from each other based solely on their morphological characters. In these cases, DNA-based molecular identification is useful. The sequences of partial calmodulin and β-tubulin genes have been found to be particularly suitable for species delimitation [65].

Regarding their mode of reproduction, until recently, the black aspergilli were almost exclusively known to reproduce only by asexual means. The one exception was a report of sexual reproduction in *A. japonicus*, which was described to form hard, white to cream-coloured sclerotia containing asci and ascospores when incubated on malt extract agar. The teleomorph was named *Saitoa japonica* [4]. However, there has also been mounting evidence for the potential for sexual reproduction more broadly amongst the black aspergilli. There has been evidence, albeit limited, for recombination from population genetic studies. Van Diepeningen [66] examined the population structure of a number of black aspergilli using partial sequences of three genes coding for non-essential extracellular enzymes. Comparing the genealogies of these genes, she found evidence for a very low level of nuclear recombination in *A. niger*. Additionally, recombination in the mitochondrial DNA has been observed [67]. Secondly, genome analysis of two *A. niger* strains has revealed the presence of a full complement of functional genes related to sexual reproduction, *inter alia* the *MAT 1-1* mating-type gene in both strains [68]. A *MAT 1–2* gene homologue was not detected in either of the isolates (compared with the homothallic aspergilli where both *MAT* genes are present in the same genome [23]), indicating that *A. niger* is either heterothallic or has lost its *MAT1-2* gene and is now truly asexual. Thirdly, it is known that dioxygenase genes are involved in the production of oxylipins, chemicals which are linked to reproduction as they regulate the balance between sexual and asexual sporulation [69]. Wadman et al. [70] found that *A. niger* has similar dioxygenase genes and produces the same oxylipins as *A. nidulans*, where a sexual reproductive mode is known. This finding points to the possible existence of sexual reproduction in *A. niger*, unless these compounds have evolved to have a broader function in the physiology of this species.

Finally, results from studying the ratio of *MAT1-1:MAT1-2* isolates in the field have provided evidence for sexual potential in the black aspergilli. We have ongoing work in which over 125 environmental black *Aspergillus* isolates from different origins including onions, dates, raisins, indoor air and *Welwitschia* plants have been screened for the presence of mating-type genes, using a PCR diagnostic approach. A total of 34 isolates from onions and 13 from *Welwitschia* plants from the Namibian desert were all found to belong to the species *A. welwitschia*, which is closely

related to *A. niger* [71, 72], whilst all 38 isolates from dates were *A. tubingensis*. From raisins, we were able to isolate both *A. niger* and *A. welwitschia*, whilst from indoor air *A. niger*, *A. welwitschia* and *A. tubingensis* were isolated (J Varga et al. unpublished data). A PCR-based diagnostic test was used, which revealed that *MAT 1-1* or *MAT 1-2* fragments were detected in all strains. In the case of *A. niger* and *A. tubingensis*, a ratio of *MAT1-1*:*MAT1-2* isolates close to 1:1 was detected, but in the case of *A. welwitschia* a divergent 6:1 ratio was observed. Notably, all *A. welwitschia* strains isolated from *Welwitschia* seeds, and most of the isolates from indoor air and onions, were of the *MAT1-1* mating-type indicating a prevalence of clonal reproduction in these environmental niches.

In related work, Horn et al. [73] analysed 34 randomly selected *A. tubingensis* isolates from North Carolina, USA for the presence of mating-type genes. Two isolates were found to be of *MAT1-2* genotype, whilst the remaining were all of the *MAT1-1* genotype. Two isolates of opposite mating type were crossed on mixed cereal agar and incubated for 5–6 months. This resulted in the induction of a sexual cycle involving the production of sclerotia that contained ascospore-bearing cleistothecia. Ascospores were found to have reticulate ornamentation and the presence of two crests that formed an equatorial furrow. However, no evidence was presented for recombination in the offspring, so outbreeding remains to be confirmed. In parallel work, Darbyshir et al. [74] similarly reported the discovery of a sexual stage for *A. sclerotiocarbonarius* which again involved the production of ascospores borne in cleistothecia formed within sclerotia following mating of *MAT1-1* and *MAT1-2* isolates, indicative of a heterothallic breeding system.

Although there have been very few reports so far of sexual reproduction in the black aspergilli, the initial formation of sclerotia as a medium for fruiting body development seems to be a common prerequisite for production of the teleomorph stage. This is also observed in *Petromyces* teleomorphic species from the phylogentically related section *Flavi* [2, 24]. So far there has been no proof of successful induction of sexual reproduction in *A. niger* although reports often mistakenly identify its teleomorphic state as *Sterigmatocystis nigra* (e.g. see http://www.life-worldwide.org/fungal-diseases/aspergillus-niger/).

Aspergillus Section *Flavi*

Members of *Aspergillus* section *Flavi* are well known as contaminants of crops and food products that can cause food spoilage. Certain species can also pose a serious health risk, due to the production of carcinogenic aflatoxins, which can contaminate foodstuffs, and also the fact that *A. flavus* is the second most common agent of aspergillosis after *A. fumigatus*, and a prominent cause of disease in eye infections and wounds [13]. Regarding their mode of reproduction, until recently, sexual reproduction had only been observed in *A. alliaceus* and *A. albertensis* (teleomorph *Petromyces*), involving the formation of sclerotia within which physical evidence of sexual reproduction were observed [75, 76]. These species are homothallic, and in *A. alliaceus*, the mating-type genes are tightly linked [30]. The most important species in this section are *A. flavus* and *A. parasiticus,* both in terms of their prominence, and clinical and economic impact. Much work has therefore focused on their mode of reproduction, especially in the context of biocontrol measures.

The first evidence for possible sexuality in *A. flavus* came from population genetic analyses. Geiser et al. [8] compared genealogies of five partial gene sequences and concluded that two cryptic species were present. In one of these species, lack of concordance of gene genealogies was interpreted as an evidence for recombination. In a related study, Peterson et al. [77] compared phylogenies of five nuclear genes and observed a recombining population structure for *A. nomius*, but could not find evidence for recombination in *A. bombycis*, both members of the *Aspergillus* section *Flavi.* Secondly, genome analysis of *A. flavus* and the closely related *A. oryzae* revealed the presence of a full complement of functional genes related to sexual reproduction, consistent with a potential for sexual reproduction [34] (Dyer PS and Eagle C, unpublished results). Thirdly, Wada et al. [36] were able to show by *MAT* gene replacement that the mating-type genes of *A. oryzae* differentially regulated expression of a series of target genes including those involved with pheromone signalling, indicating that the *MAT* genes had a functional role, most likely in sexual reproduction.

Finally, results from studying the ratio of *MAT1-1:MAT1-2* isolates in the field provided clear evidence of the potential for sexual reproduction in *A. flavus* and

A. parasiticus. Ramirez-Prado et al. [30] designed oligonucleotide primers targeting conserved regions of the *MAT* genes and were able to detect the existence of both *MAT* idiomorphs in a field population of *A. flavus* and *A. parasiticus*. They also demonstrated that the *MAT* genes were expressed at the mRNA level, indicating a potential functional role of these genes and the possible existence of an extant sexual state for these fungi [30]. Whilst *A. alliaceus* was found to be homothallic, both *A. flavus* and *A. parasiticus* appeared functionally heterothallic with each isolate containing a single mating-type gene [30]. Crossing individuals of the opposite mating-type on mixed cereal agar was then found to result in the development of sexual structures in *A. flavus* similar to those of *A. alliaceus* after incubation for 6–11 months [78]. It was speculated that recombination between aflatoxin gene clusters might account for variation in mycotoxin production, but unfortunately no evidence for recombination amongst the offspring was provided. Horn et al. [79] soon afterwards also reported that *A. parasiticus* undergoes a complete sexual cycle, which results in the development of ascospore-bearing ascocarps embedded within stromata. Strains with opposite MAT loci (*MAT1-1* and *MAT1-2*) from different (VCGs) were crossed by inoculating mixed conidial suspensions on mixed cereal agar, and cultures were incubated at 30 °C in the dark for 6–9 months. Multilocus sequence typing analysis of three genetic markers in 57 progeny from three crosses demonstrated in this case that recombination occurred during the sexual cycle.

The discovery of sexual cycles for both *A. flavus* and *A. parasiticus* provides an invaluable tool for genetic analyses to better understand the biology and evolution of these species. The basal placement of *A. alliaceus* in the section *Flavi* clade [80] as well as the conserved synteny and orientation of the mating-type genes and their flanking regions for all species in this clade suggest that gene loss in a homothallic ancestor may be responsible for the transition to heterothallism, as has been proposed previously for aspergilli [22, 34]. Besides, results from experimental matings indicate that sexual recombination is driving genetic and functional hyperdiversity in *A. flavus* and may lead to recombination within the aflatoxin biosynthesis gene cluster through the meiotic processes of independent assortment and crossing over. For example, Olarte et al. [81] presented direct genetic evidence of crossovers influencing the aflatoxin-producing phenotype of *A. flavus* strains. The authors also stated that 'the vertical transmission of cryptic alleles indicates that whilst an *A. flavus* deletion strain is predominantly homokaryotic, it may harbour aflatoxin cluster genes at a low copy number', meaning that there is the risk that non-aflatoxigenic biocontrol strains used in agriculture might be able to become aflatoxin producers in the field due to sexual recombination [81].

Vegetative incompatibility amongst strains of these species gives rise to different VCGs that limit genetic exchange through the parasexual cycle and may eventually lead to isolation and homogeneity in toxin phenotype [82]. Aflatoxin production and morphology (sclerotium size and number; conidial colour) were found to be highly similar within a given VCG [83]. By contrast, sexual reproduction in *A. flavus* and *A. parasiticus* occurs between individuals that belong to different VCGs and often differ in their toxicity [79, 84]. The correlation between toxin chemotype profile and VCG suggests that asexual reproduction fixes diverse toxin chemotypes in populations, whereas sexual reproduction creates new VCGs with different toxin profiles [85].

Regarding other species in the Section *Flavi*, Peterson et al. [77] reported possible cryptic sexual reproduction and recombination in *A. nomius* (see above). Later, Horn et al. [86] identified mating-type genes for *A. nomius* and successfully induced sexual reproduction. Ascospores were detected in some crosses, which were similar to those of *A. flavus* and *A. parasiticus*. Unusually, some isolates were found to harbour both mating-type genes [86]. Similar heterokaryosis was also observed in *A. flavus* [81].

Moore et al. [85] explored the contributions of asexual and sexual reproduction to mycotoxin diversity in global populations of *Aspergillus* section *Flavi*. They examined natural genetic variation in 758 isolates of *A. flavus, A. parasiticus* and *A. minisclerotigenes* sampled from single peanut fields in different countries. To assess the contributions of asexual and sexual reproduction to fixation and maintenance of toxin chemotype diversity in populations from each locality/species, they tested the null hypothesis of an equal number of *MAT1-1* and *MAT1-2* mating-type individuals. Different distributions were observed in the examined populations, for example, 98 % of the *A. parasiticus* isolates from Argentina were of *MAT1-1* genotype, whilst 81 % of the *A. flavus* isolates with

large (L) scelrotia were the *MAT1-2* mating-type. Sequence analysis of several genes of the aflatoxin gene cluster led to the conclusion that the reproductive nature of the given population is predictive of aflatoxin chemotype diversity. In another study, differences were not observed in the virulence of *A. flavus* isolates carrying the different idiomorphs in *Drosophila melanogaster* [87]. Recently, sexual recombination was also observed in ascospore progeny from sclerotia produced naturally on maize [88].

Recently, we examined the ratio of *MAT* idiomorphs in clinical ($n = 46$) and environmental ($n = 90$) isolates of *A. flavus* sampled from Hungary, Serbia and India. In agreement with previous studies [89], we found the *MAT1-1* mating-type exclusively (100 %) in clinical samples from human keratitis cases in India. However, isolates carrying the *MAT1-2* idiomorph were prevalent in environmental samples (representing mainly isolates from maize cobs) collected in Hungary (96 %, data not shown). This is in agreement with the findings of Sweany et al. [90] who also observed a skewed ratio of the mating-type genes isolated from maize, with isolates carrying the *MAT1-2* idiomorph dominating. Further studies are in progress to examine the possible reasons for these observations.

In summary, recombination within the aflatoxin gene cluster, expression of *MAT* genes at the mRNA level, and an equal distribution of mating types in populations together provide strong evidence for sexual reproduction in aflatoxigenic fungi in nature. Individuals within *A. flavus* and *A. parasiticus* populations vary widely in their ability to produce aflatoxins, ranging from those that are non-aflatoxigenic to those that are potent producers of aflatoxins [91]. Sexual recombination could account for much of this natural variation in toxicity [79]. A minireview has recently been published that details the nature of sex and recombination in aflatoxin-producing fungi from *Aspergillus* section *Flavi* [92].

Other Sections

The presence of mating-type genes has also recently been demonstrated in other aspergilli of clinical relevance that were previously described as asexual. Degenerate PCR primers have been developed to allow the identification of mating types within a diverse range of aspergilli. In *A. clavatus*, a 1:1 distribution was observed between the *MAT1-1* and *MAT1-2* mating-type genes of 20 isolates, although the sexual cycle has not yet been observed. Similarly, in *A. terreus*, a near 1:1 ratio of *MAT1-1:MAT1-2* isolates was detected (Eagle C and Dyer PS, unpublished results). Both of these species can cause opportunistic infections in immunocompromised patients. Indeed, Arabatzis and Velegraki [93] went on to cross *MAT1-1* and *MAT1-2* isolates of *A. terreus* on mixed cereal agar. One *MAT1-2* isolate was found to produce hyphal masses when crossed with four *MAT1-1* strains after 3 weeks incubation at 37 °C. Cleistothecia were reported to form inside the hyphal masses and produce asci containing smooth-walled ascospores with an equatorial protuberance, in contrast to the conidia of *A. terreus*, which have a striate ornamentation [62].

Conclusions

In conclusion, several aspergilli of clinical relevance, previously thought to propagate only by asexual means, have now been shown to be capable of sexual reproduction. Since almost all of the species have heterothallic breeding systems, there is the potential for recombination which might lead to problems both in clinical and agricultural settings, e.g. evolution of increased resistance to antifungal drugs, transfer of mycotoxin biosynthetic genes and increased virulence. Further studies are now needed to determine the extent of sexual reproduction within the remaining 'asexual' aspergilli and to clarify the possible effects of sexual recombination on the evolution of and gene flow within *Aspergillus* species of medical and economic importance.

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