

Sexual structures in *Aspergillus*: morphology, importance and genomics

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The genus *Aspergillus* comprises a few hundred species sharing a common asexual spore forming structure, the aspergillum. Approximately one-third of these species also produce a sexual stage, all but five of which are known to be homothallic. Sexual stages associated with *Aspergillus* fall into approximately ten different genera, reflecting a tremendous degree of phylogenetic and biological diversity. Sexual stages in *Aspergillus* are plectomycetous, typical for the order in which it resides, the Eurotiales. Theoretically, a homothallic *Aspergillus* species can produce both asexual conidia and sexual ascospores in both clonal and recombinant fashion, although the actual significance of these potential modes of reproduction is unclear. *Aspergillus* species with known sexual stages tend to be minor players in infections of humans, perhaps because of their tendency to produce fewer asexual spores compared to their non-teleomorphic congeners. The discovery of population genetic and genomic evidence for sex in species with no known sexual stage indicates that no assumptions can be made about the clonal versus recombinant life histories of a species based on its known mitotic and/or meiotic reproductive modes.

Keywords *Neosartorya*, *Emericella*, cleistothecium

Sexual and asexual reproduction in *Aspergillus*: pleomorphism

The genus *Aspergillus* is phylogenetically aligned with the Order Eurotiales (Eurotiomycetidae/Eurotiomycetes/Pezizomycotina/Ascomycota [1]. Like many members of the Pezizomycotina, a subphylum previously known as 'Euascomycetes' or 'the filamentous Ascomycota' [2,3] members of the genus tend to produce both mitotic ('asexual') and meiotic ('sexual') spores, a condition known as pleomorphism [4]. The genus *Aspergillus* refers to its asexual propagative structure, the distinctive aspergillum, by which it produces conidiospores ('conidia') via mitosis. This 'anamorph' genus is connected to approximately ten different 'teleomorph' genera (Table 1), which correspond to the production of ascospores via meiosis. Collectively,

the entirety of the anamorph and teleomorph is known as the holomorph, in which the teleomorphic stage receives taxonomic precedence, such that scientists are expected to refer to species with known sexual stages by their teleomorph names. In practice, and following the lead of Raper and Fennell [5], this rule is often ignored among *Aspergillus* geneticists.

The morphology of the sexual stage

Sexual stages associated with *Aspergillus* are typical for the Eurotiales, and follow the classic morphological characteristics of 'Plectomycetes.' [1,3,6] (Fig. 1). The cells that fuse to form the dikaryotic stage, the gametangia, are hyphal and often undifferentiated into clear male and female elements. One exception is found in the genus *Fennellia*, where a relatively large female gametangium is surrounded by and fertilized by a smaller, coiled male gametangium [7]. Mating gametangia tend to take the form of coils, or tightly knotted hyphal structures [8]. Asci possess a thin wall that deliquesces at maturity ('prototunicate') to release the

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Table 1 Sexual stages associated with the genus *Aspergillus*

Genus	Some phylogenetically associated species ¹	Notes
<i>Neosartorya</i>	<i>A. fumigatus</i> , <i>N. fischeri</i> , <i>A. lentulus</i>	Cleistothecial wall composed of flattened hyphae
<i>Emericella</i>	<i>E. nidulans</i> , <i>E. echinulata</i> , <i>E. quadrilineata</i> , <i>E. rugulosa</i> , <i>A. ustus</i> , <i>A. calidoustus</i> , <i>A. versicolor</i>	Dark cleistothecia with walls composed of flattened cells; red ascospores. Cleistothecium surrounded by a stromatic layer of hülle cells
<i>Fennellia</i>	<i>A. terreus</i> , <i>F. flavipes</i>	Cleistothecial wall composed of thick-walled cells
<i>Petromyces</i>	<i>P. alliaceus</i> , <i>A. flavus</i> , <i>A. parasiticus</i> , <i>A. niger</i> (?)	Multiple cleistothecia enclosed in a usually dark sclerotial stroma
<i>Neopetromyces</i>	<i>A. ochraceus</i> , <i>A. sclerotiorum</i>	1–2 cleistothecia enclosed in a pale sclerotial stroma
<i>Eurotium</i>	<i>E. amstelodami</i> , <i>E. herbariorum</i> , <i>E. glaucum</i>	Bright red, orange and yellow cleistothecia. Wall composed of a single layer of flattened cells. Often associated with osmo/xero/psychrotolerant species
<i>Chaetosartorya</i>	<i>C. chrysellia</i> , <i>A. wentii</i> , <i>C. cremea</i>	Uncommon. Cleistothecial wall with sterile hyphae
<i>Scleroeleista</i>	<i>A. ornatus</i>	Uncommon. Cleistothecium produced in a multilayered stromatic structure. Placement in <i>Aspergillus</i> in doubt.
<i>Warcupiella</i>	<i>A. spinulosus</i>	Rare. Cleistothecia composed of thin layers of hyphae. Placement in <i>Aspergillus</i> in doubt.
<i>Hemicarpenteles</i>	<i>A. paradoxus</i>	Rare. Placement in <i>Aspergillus</i> in doubt.
<i>Neocarpenteles</i>	<i>A. clavatus</i>	Rare. Stromatic ascomata containing a single cleistothecium.
<i>Dichotomomyces</i>	Weak phylogenetic association with <i>A. clavatus</i> and <i>Neosartorya</i>	Uncommon. Does not produce an <i>Aspergillus</i> anamorph.

¹See Peterson *et al.*, 2008 [21]; Geiser *et al.*, 2008[22], and references therein.

ascospores free inside the fruiting body. Asci almost always contain eight ascospores, which are unicellular and lens-shaped, often with two or more equatorial rings around the periphery. The equatorial rings, as well as the convex surfaces of the ascospore, may be ornamented in ways that are diagnostic for species.

Asci are produced in enclosed, globose fruiting bodies called cleistothecia, which often can be seen in the fungal colony produced among conidia and conidiophores. Asci are not produced from a single, fertile layer within the fruiting body, and instead are scattered throughout the centrum (the cavity of the ascoma).

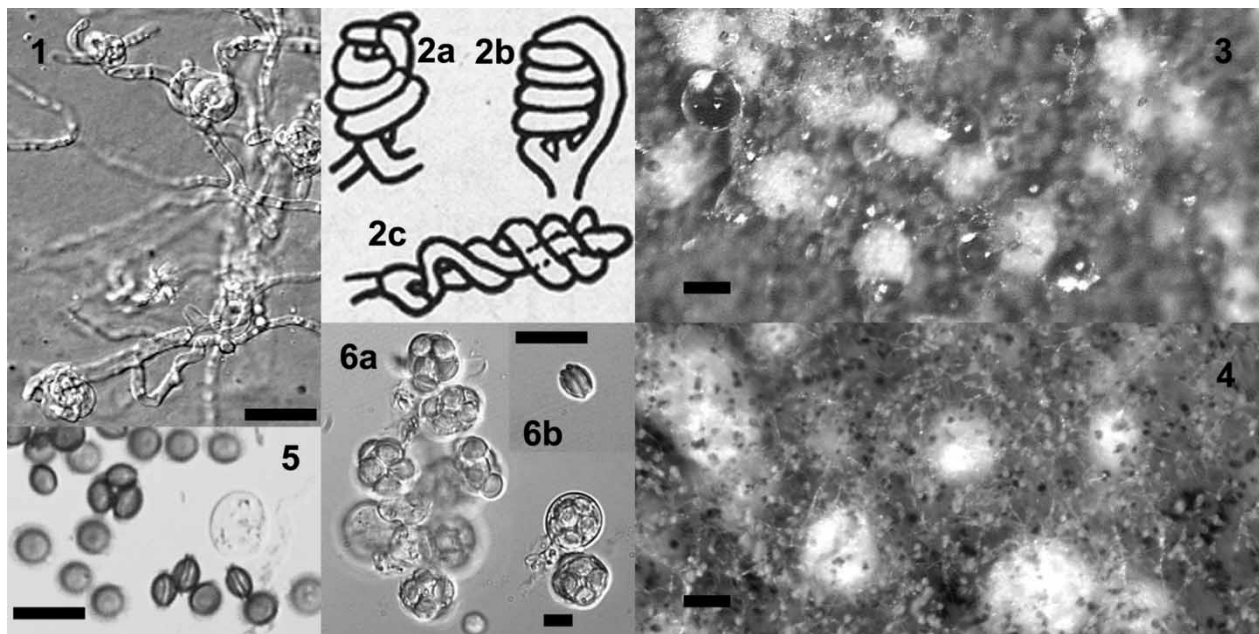


Fig. Representative sexual structures in *Aspergillus*. Bar = ~10 µm in Figs 1, 5 and 6; ~100 µm in Figs 3 and 4. **Fig. 1** Coiled hyphal gametangia in *Eurotium*. **Fig. 2** Line drawings of gametangia produced in *Eurotium*, reproduced from Benjamin, 1955 [8]. **Fig. 3** *Emericella* cleistothecia produced among *Aspergillus nidulans* conidiophores. The red cleistothecia are surrounded by gold-colored hülle cells. **Fig. 4** *Neosartorya* cleistothecia produced among *Aspergillus fischerianus* conidiophores. **Fig. 5** Red *Emericella* ascospores. **Fig. 6a** *Eurotium* asci. **Fig. 6b** *Eurotium* ascospore, reproduced from Geiser *et al.*, 2006 [1].

There are no interascal elements such as paraphyses and periphyses. Cleistothecial walls vary, from layers of variably shaped cells to somewhat loosely aggregated hyphae. The cleistothecium may be surrounded by a supporting structure called a stroma. In the genera *Petromyces* and *Neopetromyces*, the sexual stages associated with *Aspergillus alliaceus* and *A. muricatus*, the stroma takes the form of what would be considered a sclerotium, except that it houses multiple cleistothecia containing asci and ascospores [9–11]. Based on their morphological similarity and their phylogenetic connection, sclerotia produced by species such as *A. flavus* and *A. niger* should be considered ascomatal primordia. In the genus *Emericella*, which includes the sexual stage of *A. nidulans*, the cleistothecium is surrounded by a layer of thick-walled cells of unknown function called hülle cells, which have been interpreted as stromatic cells [12].

The importance of the sexual stage – genetic and ecological consequences

About one-third of the described species of *Aspergillus* have a known sexual stage, and all but five of those are homothallic. Homothallic species have the potential to produce both mitotic and meiotic spores in both clonal and recombinant fashion. In *E. nidulans*, conidia can be produced both asexually and via the parasexual cycle, where genetically distinct individuals fuse vegetatively to form a heterokaryon, and then produce recombinant conidia following diploidization, mitotic recombination and re-segregation to the haploid state [13]. The significance of the parasexual cycle in nature is in doubt, however, because heterokaryon incompatibility blocks its initiation, and genetically distinct isolates are almost always heterokaryon incompatible [14,15]. Homothallics also have the potential to produce ascospores in both clonal and recombinant fashion. *Aspergilli* are almost always haploid, although wild *E. nidulans* isolates are occasionally diploid and produce sterile cleistothecia. In a haploid system, the genetic consequences of selfing are apomictic, that is, clonal and the same as those of asexual mitosis. While homothallics almost always self-fertilize, it is incorrect to assume that homothallic species undergo less recombination than their obligately outcrossing, heterothallic counterparts. Multilocus analyses of *E. nidulans* isolates from Great Britain showed low levels of linkage disequilibrium consistent with recombination [15]. In other ascomycetes, the homothallic plant pathogen *Fusarium graminearum* showed high levels of mixis on wide geographic scales [16] but others such as *Sclerotinia sclerotiorum* appear to exhibit a

strong clonal dynamic [17]. Indeed, in *Aspergillus* outcrossing could easily be more frequent in homothallics, because they are constantly producing gametangia. While those gametangia will be self-fertilized almost all of the time, the frequency of cross-fertilization could still be higher than what occurs in heterothallics. In many cases, including *Aspergillus*, the tendency toward homothallic reproduction may reflect the ecological advantages of producing an ascospore rather than any sort of genetic consequences. Ascospores in *Aspergillus* species may be adaptive in a number of ways, including dispersal and survival in harsh environments. Indeed, ascospores of *Neosartorya fischeri* are highly resistant to heat, and are an important cause of food spoilage because of their ability to survive heat treatment, including pasteurization [18].

Why is there so much homothallism in Aspergillus – or is there?

An early phylogenetic study in ‘strictly mitotic’ biverticillate *Penicillium* species and their teleomorphic *Talaromyces* relatives revealed multiple, independent origins of species with no known sexual stage [19]. Across the genus *Aspergillus*, a similar pattern was noted [20] which has been solidly confirmed in a wide variety of subsequent phylogenetic studies [21,22]. Early on, these patterns were hypothesized to reflect the effects of Mueller’s ratchet, which dictates that asexual organisms are doomed to extinction, accumulating deleterious mutations over time with no way to get rid of them [23]. The origins of asexual species were interpreted to be recent, with relics of lost sexual states seen in the presence of hülle cell masses in species such as *A. versicolor* and *A. ustus*, both of which are related to *Emericella* species, or in hyphal masses such as those seen in *A. wentii*, which is related to *Chaetosartorya* species [20]. In a study of the molecular phylogenetics of *Aspergillus* section *Fumigati*, it was observed that the hypothetical tree where homothallic species were derived from heterothallic ancestors was significantly less likely than the unconstrained best tree for the group, suggesting that heterothallic species evolved from homothallic ancestors [24].

These early studies were conducted with no knowledge of the genetic basis of homothallism in Eurotiales. The molecular genetic basis of homothallism in Pezizomycotina (a filamentous ascomycete) was first discovered in the Dothideomycete genus *Cochliobolus*, where patterns were revealed that strongly indicated multiple independent transitions from heterothallism to homothallism [25]. Individuals of homothallic *Cochliobolus*

species possess both *MAT* idiomorphs, usually arranged in a single *MAT* locus, with each homothallic species exhibiting a different pattern, suggesting a unique origin. The molecular basis of homothallism in *Aspergillus*, however, was more difficult to dissect and took several years to be discovered. With the release of complete genome sequences of various homothallic and strictly anamorphic *Aspergillus* species, mating-type loci were identified and comparisons have been made between homothallic species such as *E. nidulans* and *N. fischeri* [26,27] and species with no known sexual stage such as *A. fumigatus* and *A. oryzae* [28]. In *A. fumigatus* and *A. oryzae*, a single *MAT* locus was identified, with individuals within both species possessing either a *MAT1* or *MAT2* idiomorph in nearly equal ratios [26,28; P. Dyer, pers. comm.]. This leads to the intriguing possibility that both *A. fumigatus* and *A. oryzae* are actually heterothallic, although attempts to mate them in the lab have not been successful. In both *E. nidulans* and *N. fischeri*, two *MAT* loci have been identified, on different chromosomes, with each harboring one idiomorph or the other. The *MAT2* locus is arranged differently in *N. fischeri* than in *E. nidulans*, consistent with independent origins of the two homothallic systems [27]. While this two-locus arrangement may be more genetically stable than the typical homothallic placement of both *MAT* idiomorphs in a single locus, it remains a mystery why no ancient homothallic gene arrangements have been identified anywhere in Ascomycota, and raises questions about their overall long-term evolutionary stability.

Are Aspergillus species with no known sexual stage actually heterothallic?

The discovery of *MAT* idiomorphs within the species *A. fumigatus* and *A. oryzae* raises the question of whether most, if not all *Aspergillus* species have a sexual stage [29]. If the *MAT* locus were not under balancing selection due to the lack of a heterothallic sexual stage, one would expect genetic drift to lead to aberrant ratios of *MAT* idiomorphs in populations, or to the extinction of one mating-type or the other, very much unlike observations in *A. fumigatus* and *A. oryzae*. It could be that the *MAT* locus is under balancing selection for a reason other than sex, and/or that the loss of sexuality in this species occurred so recently that *MAT* idiomorphs have not had enough time for genetic drift to render one or the other extinct. Because the fairly even distribution of *MAT* idiomorphs is observed on multiple continents [26], and because it exists in a background of a high degree of genetic homogeneity in this species [30], it seems likely

that there is strong balancing selection on the *MAT* locus, and the best candidate force behind that is an active sexual cycle. Finding that sexual stage, however, may prove difficult in *Aspergillus* species where none is known. Identifying and pairing compatible mating-types is only the first challenge; creating laboratory conditions appropriate for mating and sexual development may be entirely another. While many *Aspergillus* species with no known sexual stage exhibit some degree of pre-sexual development in culture [20], *A. fumigatus* shows virtually none under standard laboratory culture conditions. A wide variety of factors, including temperature and other environmental factors, the availability of nutrition, and appropriate substrates for the production of ascospores, may be required for sexual development. Indeed, the homothallic sexual stage of *A. alliaceus*, *Petromyces*, was only discovered when sclerotia from year-old cultures were opened; sexual development typically takes three months or more in this species [9]. The expression of a heterothallic sexual stage in sclerotial species such as *A. flavus*, *A. oryzae* and *A. niger* may require a similar degree of patience, if not serendipity. The fact that the first two heterothallic *Aspergillus* species were identified and described by the same person serves as further evidence of this [31,32].

The relevance of the sexual stage in human infections

Aspergillus species with known sexual stages tend to be minor players in the clinical realm. Infections by species with *Emericella*, *Neosartorya*, *Eurotium* and *Petromyces* sexual stages are generally considered occasional, if not rare. *Emericella nidulans* is somewhat frequently identified in aspergillosis associated with chronic granulomatous disease [33]; whether this has anything to do with the production of its sexual stage is unknown. One hypothesis explaining this relative rarity is that people are far less likely to encounter infective propagules, either conidia or ascospores, from a species with a known sexual stage, because they produce fewer spores overall than their anamorphic, perhaps heterothallic relatives. A cursory observation of most homothallic species indicates that conidiation is drastically reduced, and it is generally understood that there is a genetically and environmentally determined balance between asexual and sexual sporulation in *Aspergillus* species. In *E. nidulans*, the expression of the *velvet* gene decreases conidiation and increases cleistothecium production [34] and does so in response to red light captured via a cytoplasmically localized phytochrome [35,36]. In upcoming years, comparative genomic studies of *E. nidulans*, *N. fischeri*, *A. fumigatus* and

other species should greatly increase our understanding of the developmental genetics of the sexual cycle.

While there is reason to hypothesize that the presence of a homothallic sexual stage is a negative factor in predisposing an *Aspergillus* species to human infection, the genetic consequence of sex, recombination, is still an important consideration [37]. Recombination generates genotypic variation that may underlie novel virulence phenotypes, resistance to antifungal treatment, and response to vaccines. The response to any strategy to prevent, control or cure any fungal disease will depend on the population dynamics of the pathogen, and as we discover more about the secret sexual habits of fungi such as *A. fumigatus*, we will gain a better understanding of how it circumvents our best efforts to stop it.

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