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Evidence for Sexuality in the Opportunistic Fungal Pathogen *Aspergillus fumigatus*

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Summary

Aspergillus fumigatus **is a medically important opportunistic pathogen and a major cause of respiratory allergy [1]. The species has long been considered an asexual organism. However, genome analysis has revealed the presence of genes associated with sexual reproduction, including a *MAT-2* high-mobility group mating-type gene and genes for pheromone production and detection (Galagan et al., personal communication; Nierman et al., personal communication; [2, 3]). We now demonstrate that *A. fumigatus* has other key characteristics of a sexual species. We reveal the existence of isolates containing a complementary *MAT-1* α box mating-type gene and show that the *MAT* locus has an idiomorph structure characteristic of heterothallic (obligate sexual outbreeding) fungi [4, 5]. Analysis of 290 worldwide clinical and environmental isolates with a multiplex-PCR assay revealed the presence of *MAT1-1* and *MAT1-2* genotypes in similar proportions (43% and 57%, respectively). Further population genetic analyses provided evidence of recombination across a global sampling and within North American and European subpopulations. We also show that mating-type, pheromone-precursor, and pheromone-receptor genes are expressed during mycelial growth. These results indicate that *A. fumigatus* has a recent evolutionary history of sexual recombination and might have the potential for sexual reproduction. The possible presence of a sexual cycle is highly significant for the population biology and disease management of the species.**

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Results and Discussion

Aspergillus fumigatus Fresenius is a thermotolerant, saprophytic fungus with a worldwide distribution. Over the past decade, it has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in immunocompromised hosts all over the world [1, 6]. Airborne conidia are also significant allergens, contributing to asthma, allergic sinusitis, and extrinsic allergic bronchoalveolitis [1, 6]. Despite the importance of the species, many aspects of the biology of *A. fumigatus* remain unclear. Of particular relevance to the current study is the observation of high genetic diversity within this supposed asexual species, which might be explained by past meiotic exchanges or a cryptic sexual state [1, 7]. Indeed, there is accumulating evidence that many supposedly asexual organisms may have a latent potential for sex. Within the fungal kingdom, field surveys have revealed teleomorphs for what were previously thought to be asexual plant pathogenic species [8], and population genetic analyses have detected evidence of recombination within several "asexual" species [9, 10]. Most notably, genome analysis and subsequent experimental manipulation and haplotype analysis have provided proof of sexuality in the opportunistic pathogenic yeast *Candida albicans*, which had long been considered asexual [11–14]. Meanwhile, evidence for sexuality has recently been presented for the protist *Giardia*, indicating an early eukaryotic origin of meiosis [15].

In related work, Galagan et al. and Nierman et al. describe the discovery of a series of genes associated with sexual reproduction in the genome of *A. fumigatus* (personal communication). This includes genes involved in mating processes, pathway signaling, fruit-body development, and meiosis, in accord with preliminary genome reports [2, 3]. It is predicted that genes specific to sex and recombination will decay and be lost in truly asexual species [16], with evidence available for directional selection for pseudogenes preventing outcrossing [17]. This led to the suggestion of the possibility of mating in *A. fumigatus*, and a model for the evolution of the mating-type locus was proposed by Galagan et al. (personal communication). We now provide experimental evidence for sexuality in *A. fumigatus*, which validates these claims, as follows.

Identification of *MAT1-1* Idiomorph

For sex to occur in heterothallic fungi, it is necessary for isolates of complementary mating type "MAT" to be present [5, 18]. Mating type in filamentous ascomycetes is determined by genes found at a single *MAT* locus, which contains either a *MAT1-1* or *MAT1-2* "idiomorph" highly divergent in sequence [4, 5, 19]. *MAT1-1* idiomorphs contain a characteristic α box

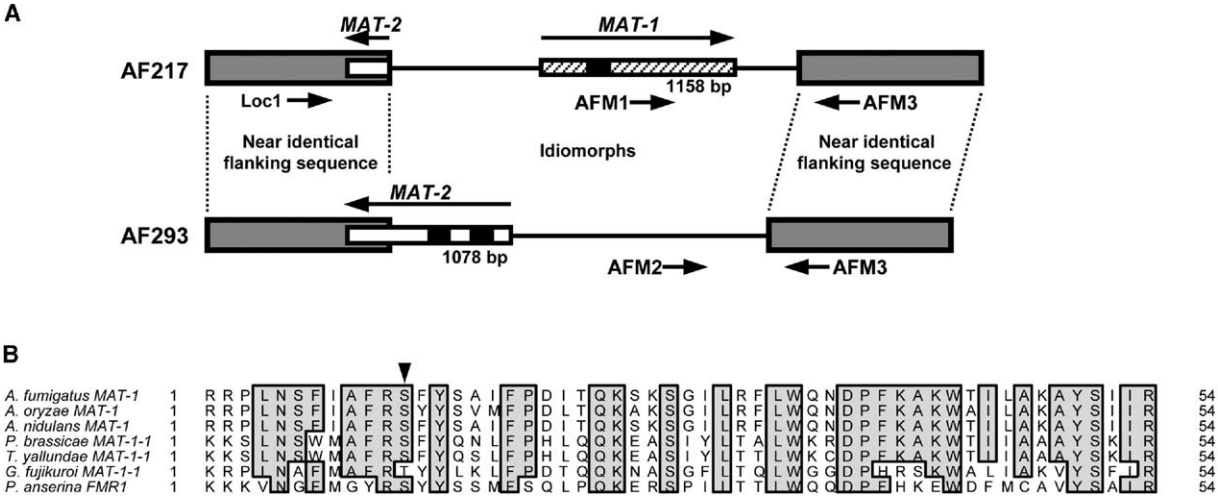

Figure 1. *MAT* Locus of *Aspergillus fumigatus*

(A) A schematic arrangement of the *A. fumigatus* idiomorph region shows the difference in organization between isolates AF217 (*MAT1-1*) and AF293 (*MAT1-2*). Textures of boxes indicate nearly identical flanking regions (gray), *MAT-1* (stippled gray), *MAT-2* (white), and introns (black); lines extending from boxes represent idiomorph sequence. Diagram also shows position of primers (AFM1, AFM2, AFM3, Loc1) used in multiplex mating-type test and for amplification of idiomorph region (direction of arrow indicates 5′ to 3′ sequence).

(B) Multiple alignment of α box region of *A. fumigatus* MAT-1 protein with other ascomycete MAT-1 family proteins. Inverted arrow indicates location of conserved intron.

gene, whereas *MAT1-2* idiomorphs contain a single open reading frame (ORF) encoding a high mobility group (HMG) gene [5,19]. The genome analysis of *A. fumigatus* clinical isolate AF293 revealed the presence of a *MAT* locus with a single *MAT1-2* HMG mating-type gene (Galagan et al. and Nierman et al., personal communication). We therefore investigated whether compatible *MAT1-1* isolates of the *A. fumigatus* could be found in nature, the presence of such mating partners being a prerequisite for sexual reproduction.

A degenerate PCR approach, with primers designed to amplify the partially conserved *MAT1-1* α box region, was utilized in an attempt to detect *A. fumigatus* isolates containing a *MAT1-1* idiomorph. An amplicon of the predicted size (ca. 140 bp) was obtained from five of nine trial isolates. The PCR product from Swedish environmental isolate AF217 (see Table S1 in the Supplemental Data available with this article online) was cloned and sequenced and was found to have closest homology to the *MAT1-1* gene from *A. nidulans* (E = $5e10^{-6}$) [20]. We then chromosome walked to obtain the entire α box gene and surrounding region from AF217. Fragments of 2.5 and 2.0 kb were obtained in the 5′ and 3′ directions, respectively. These were sequenced, and the combined data (deposited as GenBank accession number AY898661) were compared to the genome sequence of AF293 (http://www.tigr.org/tdb/e2k1/afu1/; [21]). This comparison revealed a structural organization typical of the idiomorph-based *MAT* loci present in sexually reproducing heterothallic ascomycete fungi [4, 5] (Figure 1A). Highly conserved flanking regions were found up- and downstream (97% and 98% identical over 635 and 258 nucleotides, respectively) from an idiomorphic region that showed little or no sequence similarity between isolates AF217 and AF293. The idiomorph region of AF217, termed *MAT1-1* under standard nomenclature [19], is 2041 bp in size and contains a single ORF of 1158 bp, interrupted by one putative intron, encoding a deduced protein of 368 amino acids. This ORF, formally termed *MAT1-1-1* (abbreviated to *MAT-1* for convenience [19]), has the hallmarks of a *MAT-1* family gene, for example an α box and an intron at a conserved position (Figure 1B) [5]. Therefore, isolate AF217 was designated to be of mating type *MAT-1* [5, 19]. Meanwhile, the idiomorph region of AF293, termed *MAT1-2* [19], is 2372 bp in size and also contains a single ORF (1078 bp), the previously identified *MAT1-2* HMG gene (here abbreviated to *MAT-2* [19]) ([2, 3]; Galagan et al. and Nierman et al., personal communication). AF293 was therefore designated mating type *MAT-2* [5, 19]. Notably, although the *MAT-2* ORF commences within the idiomorph region, the final 360 bp of the gene were found to lie within the flanking sequence common to both AF217 and AF293 (Figure 1A). Terminal regions of *MAT-1* genes have elsewhere been found to lie within the flanking regions bordering idiomorphs [22], but to our knowledge, this situation has not been observed before for a fungal *MAT-2* gene. The entire *MAT1-1* idiomorph was cloned and sequenced from an independent clinical isolate AF250 from the United Kingdom (Table S1) with primers Loc1 and AFM3 to confirm this observation (Figure 1A). The DNA sequences of the *MAT1-1* region from AF217 and AF250 (GenBank accession number AY898660) were found to be identical. Mating-type genes have elsewhere been shown to be highly conserved within, although not between, ascomycete species [23].

It is noteworthy that the presence of both *MAT1-1* and *MAT1-2* idiomorphs of *A. fumigatus* supports the model proposed by Galagan et al. (personal communi-

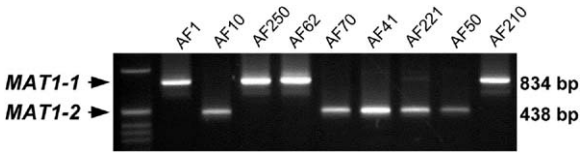

Figure 2. Representative Results from a Multiplex PCR Assay to Determine Idiomorph Genotype (Mating Type) of Isolates of *Aspergillus fumigatus*

The PCR assay involves one common primer and two mating-type-specific primers, with amplicons of either 834 or 438 bp generated by *MAT1-1* or *MAT1-2* isolates, respectively. Lane 1 shows a molecular weight marker, and lanes 2–10 show amplicons from isolates of *A. fumigatus*; code numbers are indicated above each lane.

cation) of evolution of *MAT* loci within the Aspergilli. In particular, the presence of partial *MAT-2* sequence within the idiomorph-flanking region is consistent with the proposal that species such as *A. fumigatus* arose from a homothallic ancestor in which *MAT-1* α and *MAT-2* HMG genes originally were adjacent, but the genes then became separated as a result of a translocating break or aberrant segregation. This might also partially explain why only a single ORF is present in the *MAT1-1* idiomorph of *A. fumigatus*, whereas up to three ORFs have been detected in other ascomycete *MAT1-1* idiomorphs [5, 22]. We have also recently identified a *MAT1-1*/*MAT1-2* idiomorphic organization in *A. oryzae* (M.P., P.S.D., K. Kitamoto and D.B. Archer, unpublished data). However, it remains conceivable that homothallic Aspergilli arose from heterothallic ancestors, as suggested for *Neurospora* species [4], and convincing evidence for such a transition has been provided for *Cochliobolus* species [24]. Elsewhere, different reproductive lifestyles have arisen in yeasts as a result of evolution of the *MAT* locus [25], and alternative models for the evolution of fungal *MAT* regions have been proposed [26].

Mating-Type Diagnostic Test and Worldwide Idiomorph Distribution

We next wished to assess the distribution in nature of isolates of *A. fumigatus* of complementary *MAT1-1* and *MAT1-2* genotype. To achieve this, we devised a multiplex PCR-based mating-type diagnostic test. This utilized two mating-type-specific primers located within the *MAT1-1* or *MAT1-2* idiomorphs (AFM1 and AFM2, respectively), together with a "common" primer (AFM3) in the flanking region bordering both idiomorphs (Figure 1A). This enabled a single PCR amplification to be performed incorporating all three primers, with isolates of the *MAT1-1* or *MAT1-2* genotype predicted to generate amplicons of 834 or 438 bp, respectively. A similar multiplex diagnostic test has been used successfully to determine the mating type of isolates of the cereal eyespot pathogens *Tapesia acuformis* and *T. yallundae* [27], and related PCR tests have been devised for other plant pathogenic fungi, including *Mycosphaerella graminicola*, *Rhynchosporium secalis*, *Pyrenopeziza brassicae*, and *Fusarium* species [28–30] and the human pathogen *Cryptococcus neoformans* [31]. Trial PCRs with the *A. fumigatus* diagnostic confirmed the utility of the test (Figure 2). Furthermore, the ability of the diagnostic to detect heterokaryosis of idiomorphs was assessed by mixing 100 ng of genomic DNA from a *MAT-1* isolate with serial dilutions of genomic DNA from a *MAT-2* isolate and vice versa. Mixed DNAs in a ratio of 1:10 were readily detected with this method (data not shown).

Table 1. Distribution of *MAT1-1* and *MAT1-2* Idiomorphs (Mating Type) among a Worldwide Collection of Clinical and Environmental Isolates of *Aspergillus fumigatus*

Sample Source	Mating-Type Frequency[a]		χ^2[b]
	MAT1-1	*MAT1-2*	
Clinical	40.8 (40)	59.2 (58)	3.31 (1)
(Clone corr.)[c]	40.7 (33)	59.3 (48)	2.78 (1)
Environmental	44.8 (73)	55.2 (90)	1.77 (1) 0.39[d] (1)
(Clone corr.)[c]	44.7 (71)	55.3 (88)	1.82 (1) 0.33[d] (1)
Total	43.3 (113)	56.7 (148)	4.69[e] (1)
(Clone corr.)[c]	43.3 (104)	56.7 (136)	4.27[e] (1)

[a] Numbers in parentheses refer to number of isolates.
[b] Number in parentheses indicates degrees of freedom.
[c] Clone-corrected data.
[d] Contingency χ^2 value.
[e] Significant at p = 0.05.

The test was then applied to DNA extracted from a worldwide collection of 290 isolates comprised of clinical and environmental strains of *A. fumigatus* mainly from Europe and North America, but including isolates from Asia, Africa, Australasia, and South America (Table S1). Discriminatory *MAT1-1* or *MAT1-2* bands were successfully amplified from 86% of isolates, with the alternative primer set (AF51/AF31 and AF52/AF32) producing diagnostic bands for a further 4% of isolates. In no instances were both mating-type bands amplified from a single isolate; that is, there was no indication of heterokaryosis at the *MAT* locus. However, it was not possible to obtain products from the remaining isolates. This was most likely due to degradation of DNA (some extracts had been stored in excess of 10 years) and/or low DNA concentration (as reported for other *MAT* diagnostic tests [28]) because the test yielded results with almost all (>98.5%) freshly extracted DNA samples.

Results revealed the presence of isolates of both mating types in approximately equal proportion, with overall 43.3% *MAT1-1* and 56.7% *MAT1-2* (Table 1). The same figures were obtained for the clone-corrected data set, with 8% of isolates from the same geographical sites found to be possible clonal samplings (Table 1; Table S1). There was no significant difference in ratios between clinical and environmental isolates, consistent with other genetic analyses that have failed to discriminate between these sources [1]. This contrasts with *C. neoformans*, in which a link between mating type and virulence is evident in certain serotypes [31, 32]. There was no evidence for geographical isolation of one mating type from the other. Indeed, isolates of both mating types were found together in the same subpopulations and were even obtained from the same clinical patients (Table S1). Further analysis of mating types within specific geographic regions revealed no significant differences in distribution between *MAT1-1* and *MAT1-2* for all regions except France (Table 2). Here, a clear bias toward *MAT1-2* isolates was evident (despite clone correction), which was responsible for

Table 2. Distribution of *MAT1-1* and *MAT1-2* Idiomorphs (Mating Type) among Isolates of *Aspergillus fumigatus* from Specific Continents and Countries (Clone-Corrected Data)

Continent	Country	Mating-Type Frequency[a]		χ^2[b]	Contingency[b] χ^2
		MAT1-1	*MAT1-2*		
Europe	France	36.8 (25)	63.2 (43)	4.76[c] (1)	
	Germany	65.2 (15)	34.8 (8)	2.13 (1)	
	Scandinavia	47.1 (8)	52.9 (9)	0.06 (1)	
	UK	42.1 (8)	57.9 (11)	0.47 (1)	5.73 (3)
North America	Canada	38.5 (5)	61.5 (8)	0.69 (1)	
	USA	54.3 (19)	45.7 (16)	0.26 (1)	0.95 (1)
South America	Various	35.7 (5)	64.3 (9)	1.14 (1)	
Africa	Various	36.4 (4)	63.6 (7)	0.82 (1)	

[a] Numbers in parentheses refer to number of isolates.
[b] Number in parentheses indicates degrees of freedom.
[c] Significant at p = 0.05.

the overall significant dominance of *MAT1-2* isolates in the worldwide sampling (Table 1). Intriguingly, certain French collections (Loh, Mar, and Mou; Table S1) included *MAT1-1*- and *MAT1-2*-genotype isolates that shared the same RFLP pattern, suggesting that these were very closely related strains (results not shown).

The results of the worldwide survey are highly significant because the detection of a near 1:1 distribution ratio of *MAT-1*:*MAT-2* isolates is consistent with occasional sexual reproduction [27, 28, 33, 34]; asexual populations generally show strong deviation from this ratio [35, 36]. It has been proposed that frequency-dependent selection, a type of balancing selection favoring rare genotypes, will lead to equal frequencies of mating types within sexually reproducing populations [28, 37]. However, another contributory factor might be the possibility that *A. fumigatus* was recently descended from a homothallic ancestor containing both HMG and α box genes, as proposed by Galagan et al. (personal communication). This would lead to the presence of both *MAT-1* and *MAT-2* isolates as a result of separation of the HMG and α box genes in the arising "heterothallic" species without the necessary involvement of any sexual reproduction—although subsequent genetic drift from a 1:1 distribution might be expected in the absence of sex.

Population Genetic Studies

We also performed population genetic analyses to determine whether there was evidence for recombination or clonality in natural populations of *A. fumigatus*. We identified three intergenic regions, *inter1*, *inter2*, and *inter3* (514–950 bp in size), and sequenced these regions from a subset of 106 global isolates, including 36 isolates from five subpopulations (from Friesburg, Germany; Durham, USA; Finland; and Nunavut and NW Ontario, Canada [Table S1]). Arising data were analyzed in two ways. First, isolates of opposite mating type were grouped and evaluated for shared substitutions at the three loci. During any extended period of asexuality, lineages of each mating type would be expected to have a separate evolutionary history with distinct traits apparent, and any substitutions shared between isolates of opposite mating type would have had to accumulate by convergence [38]. However, no clear differences were evident between the *MAT-1* and *MAT-2* pools in the present study. Nine of 24 total polymorphisms in the three regions were found in isolates of opposite mating type. Given the low probability of isolates of both mating types' sharing the same substitutions through convergence [38], these data suggest that *MAT-1* and *MAT-2* isolates of *A. fumigatus* were exchanging substitutions while these regions were evolving—consistent with a history of recombination by sexual or parasexual means. If sexuality was lost, it was lost after the evolutionary-history period represented by these shared substitutions. Second, the index of association (I_A) for three individual polymorphic sites (one drawn from each data set) shared by the most strains (the most balanced polymorphisms) was determined and compared with artificially recombining populations [9, 39, 40]. The I_A of the observed data set was found to be 0.24, whereas the I_A of 1000 artificially recombining data sets ranged from –20 to 0.29, with a mean value of zero. In the data set, the alleles of different loci are not significantly associated, and the null hypothesis of recombination could not be rejected; that is, these results present clear evidence that recombination had occurred within the test samples. Population genetic analysis was also attempted with the LDhat package [41], but results were inconclusive (data not shown).

Expression of Sex-Related Genes

We finally investigated whether putative genes involved in mating processes (newly identified from the genome analysis [Galagan et al. and Nierman et al., personal communication]) were expressed at the RNA level during mycelial growth of *A. fumigatus.* A semiquantitative RT-PCR approach was used to study expression of the mating-type genes (*MAT-1* and *MAT-2*) and genes encoding an α-factor-like pheromone precursor (*ppgA*) and two pheromone receptors (*preA* and *preB*) ([2]; Galagan et al., personal communication).

Significantly, both *MAT-1* and *MAT-2* were expressed under both growth conditions tested in a mating-type-specific manner (Figure 3). The pheromone-receptor genes *preA* and *preB* were also expressed, with no clear differences evident between the *MAT-1* and *MAT-2* isolates. In contrast, the expression level of the α-pheromone precursor gene was higher in the *MAT-1* than the *MAT-2* isolate (Figure 3). This is believed to be the first report of expression of pheromone-precursor and

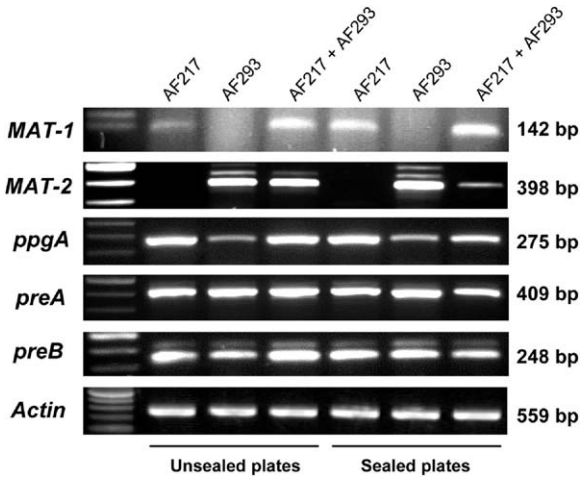

Figure 3. Expression of Genes Involved in Putative Mating Processes in *Aspergillus fumigatus*

RNA transcripts, together with actin as a control gene, were amplified by RT-PCR from cultures grown on solid media for 4 days on plates either "unsealed" or "sealed" with two layers of Whatman film to restrict gas exchange. Cultures were either of *MAT-1* or *MAT-2* genotype (isolates AF217 and AF293, respectively), or they were cocultures of *MAT-1* and *MAT-2*. Lane 1 shows a molecular weight marker, and lanes 2–7 show amplicons from isolates of *A. fumigatus*; code numbers are indicated above each lane.

receptor genes in a supposedly asexual filamentous fungus. A similar expression pattern has been observed in *Neurospora crassa*, a heterothallic ascomycete, in which pheromone-receptor gene expression is constitutive, whereas pheromone-precursor gene expression is mating-type dependent [42, 43]. Mating-type-specific expression of pheromone-precursor genes has also been reported in the heterothallic *Podospora anserina* and *Cryphonectria parasitica* [44]. However, in *N. crassa*, pheromone-precursor expression is stimulated when isolates of both mating types are present [45], whereas no stimulation was observed in the cocultures of *A. fumigatus*. Also, no difference in expression of genes was detected in sealed versus unsealed plates of *A. fumigatus*, conditions known to promote or prevent sexual differentiation in *A. nidulans* [46]. The sizes of the resulting amplicons confirmed the presence, and processing, of introns within *MAT-2*, *preA*, *preB*, and the actin control (Figure 3).

Conclusions

These results, taken together with the discovery of other genes associated with sexual reproduction in the genome (Galagan et al. and Nierman et al., personal communication), lead us to propose that *A. fumigatus* has a recent evolutionary history of sexual recombination and might have an extant sexual cycle that has yet to be observed. The presence of a *MAT* locus with idiomorphs containing complementary mating-type genes, which are expressed, is a characteristic of a heterothallic sexual species. However, mating-type genes have been shown to be present and expressed in other supposedly asexual fungi, so presence of *MAT* genes alone does not confer sexuality [29, 36, 38, 47, 48]. Drawing on newly available genome data (Galagan et al. and Nierman et al., personal communication), we were able to show experimentally that pheromone-precursor and receptor genes are also expressed during mycelial growth in *A. fumigtatus*. These genes are invariably involved in detection of a mating partner [43], again indicating latent sexuality. Meanwhile, the detection of a near 1:1 distribution ratio of *MAT1-1*:*MAT1-2* isolates in our global survey is also consistent with, although not proof of, sexuality [27, 28, 33–35] rather than asexuality [35, 36]. Genetic analysis of three polymorphic loci involved evaluation of shared substitutions and the index of association and provided additional evidence that recombination is occurring, or has occurred in the past, in natural populations—indicative of sexuality [9, 16, 39, 40]. Indeed, evidence for recombination has already been presented for certain populations of *A. fumigatus* [1, 7, 49] and a related asexual species, *A. flavus* [10]. Recombination may also occur by parasexual means, but this phenomenon has yet to be demonstrated in a natural population of any fungus [1]. Further independent support for sexuality in *A. fumigatus* comes from the presence of a defective transposable element, *AFUT*1, within the genome. The propagation of this element must once have relied on sexual reproduction, and it appears to bear traces of directed mutation (RIP), which is specifically induced at meiosis in other fungal taxa [1, 49]. The genome of *A. fumigatus* contains other major classes of eukaryotic transposable elements, suggested to be another characteristic of sexual species (Galagan et al., personal communication; [50]). It is also noteworthy that *A. fumigatus* has close taxonomic relatives with known *Neosartorya* sexual states [1, 51, 52] and that abortive cleistothecia were formed in pairings between certain isolates of *A. fumigatus* and an "A" mating strain of *N. fennelliae* [53].

The potential presence of a sexual cycle is highly significant to understanding the population biology of this opportunistic pathogen. A heterothallic breeding system would sustain variation within populations, allowing the species to evolve and respond to environmental change [16, 33]. It also affects disease management because sexual recombination may confound diagnostic tests detecting clonal lineages in outbreak settings and would allow gene flow of antifungal resistance genes and pathogenicity genes enhancing virulence [40, 33]. Conversely, the sexual cycle could be used to study the genetic basis of such traits, with the aim of devising improved methods of disease control.

Results are also of more broad significance in providing insights into supposed asexuality in nature. It has been argued that, given the benefits of sex, the presence of asexuality may be viewed as "an evolutionary scandal" and that truly asexual species are likely to be very rare [54]. However, a remarkably high number (almost one-fifth) of all fungi are only known to reproduce by sexual means [49]. Data from the present study suggests that many of these species may also have some unexpected sexual potential. Work is now underway to assess whether a complete sexual cycle of *A. fumigatus* can be induced under laboratory conditions. Given that many fungal species are composed of isolates that exhibit differing degrees of sexual fertility [18, 55–57], it

may be necessary to cross numerous *MAT-1* and *MAT-2* isolates to identify any sexual stage. Thus, *A. fumigatus* (as with other supposedly asexual fungi) may yet prove to be neither "sexual" or "asexual," but rather consist of isolates being on a continuum of sexuality from completely sterile to highly fertile, with genetic factors other than those at the *MAT* locus contributing to sexual productivity [18].

Experimental Procedures

Multiplex PCR Mating-Type Diagnostic and Idiomorph Distribution

The mating-type diagnostic utilized a *MAT1-1*-specific primer, AFM1 (5′-CCTTGACGCGATGGGGTGG-3′), and a *MAT1-2*-specific primer, AFM2 (5′-CGCTCCTCATCAGAACAACTCG-3′), together with a "common" primer, AFM3 (5′-CGGAAATCTGATGTCGCCACG-3′) flanking the *MAT* locus (Figure 1A). Reaction volumes (25 μl) contained 100 ng DNA, 100 ng of primer AFM3, 50 ng of both primers AFM1 and AFM2, 200 μM of each dNTP, and 1 U of Red Hot DNA Polymerase (ABgene). Cycle parameters were 5 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C before a final 5 min at 72°C (all at ramp rate 60°C min^{-1}). Where no results were obtained, alternative sets of primers were used that were either *MAT1-1*- (AF52 [5′-GGAGGATGCCGGTCTTGG-3′] and AF32 [5′-TGGAGGCCGTTGAACAGG-3′]) or *MAT1-2*-specific (AF51 [5′-CCTCCCCATCAATGTGACC-3′] and AF31 [5′-CTCGTCTTTCCACT GCTTCC-3′]), respectively. The null hypothesis of a 1:1 ratio of the two mating types, together with overall environmental and clinical totals, was evaluated with χ^2 and contingency χ^2 tests [58]. To avoid bias in estimation of mating-type frequencies owing to repeat sampling of fungal strains of the same genotype, we typed isolates by use of either RFLP, microsatellite, or DNA sequencing methods [59] to allow clone correction of data [28, 29]. Further analysis within specific geographic regions was undertaken where sufficient numbers were available for the clone-corrected data set [58].

Supplemental Data

Detailed Experimental Procedures regarding strain isolation, growth conditions, and DNA and RNA extraction, as well as PCR parameters, chromosome walking, identification of intergenic regions, implementation of I_A test, and selection of polymorphic sites and protocols for RT-PCR are available online at http://www.current-biology.com/cgi/content/full/15/13/1242/DC1/.

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