

Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*

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Aspergillus fumigatus is a saprotrophic fungus whose spores are ubiquitous in the atmosphere¹. It is also an opportunistic human pathogen in immunocompromised individuals, causing potentially lethal invasive infections^{2,3}, and is associated with severe asthma and sinusitis⁴. The species is only known to reproduce by asexual means⁵, but there has been accumulating evidence for recombination and gene flow from population genetic studies^{5–8}, genome analysis^{9,10}, the presence of mating-type genes^{8,10} and expression of sex-related genes⁸ in the fungus. Here we show that *A. fumigatus* possesses a fully functional sexual reproductive cycle that leads to the production of cleistothecia and ascospores, and the teleomorph *Neosartorya fumigata* is described. The species has a heterothallic breeding system; isolates of complementary mating types are required for sex to occur. We demonstrate increased genotypic variation resulting from recombination between mating type and DNA fingerprint markers in ascospore progeny from an Irish environmental subpopulation. The ability of *A. fumigatus* to engage in sexual reproduction is highly significant in understanding the biology and evolution of the species. The presence of a sexual cycle provides an invaluable tool for classical genetic analyses and will facilitate research into the genetic basis of pathogenicity and fungicide resistance in *A. fumigatus*, with the aim of improving methods for the control of aspergillosis. These results also yield insights into the potential for sexual reproduction in other supposedly 'asexual' fungi.

A. fumigatus is found worldwide in soils and on organic debris, where it is important in nutrient recycling^{1,2}. The species has long been considered asexual, with dispersal achieved by the abundant production of haploid conidia⁵. Inhaled conidia are normally eliminated by the innate immune response. However, *A. fumigatus* has become the most prevalent airborne fungal pathogen as a result of its ability to cause infections in immunocompromised hosts, with a human mortality rate of at least 50% (refs 2, 3, 11). Airborne conidia are also allergens, associated with asthma, allergic sinusitis and bronchoalveolitis^{2,4}.

The importance of *A. fumigatus* to human health has led to genome sequencing and investigations into the population biology of the species^{7,9,12}. Studies have revealed that, despite its supposedly asexual status, *A. fumigatus* has many characteristics of a sexual species. Genome screening detected 215 genes implicated in sexual development¹⁰, including a high-mobility group (HMG)-domain gene located at a 'mating-type' (*MAT*) locus typical of sexually reproducing heterothallic (obligately outcrossing) euascomycete fungi¹³. Such HMG genes are believed to be ancestral sex determinants in fungi¹⁴. For sex to occur in heterothallic fungi, it is necessary for isolates of opposite *MAT1-1* and *MAT1-2* mating types to be present¹⁵; by convention these contain either a *MAT1-1* alpha-domain or *MAT1-2* HMG-domain gene, respectively, at the *MAT* locus¹³. These *MAT*

genes encode transcription factors that are master regulators of sexual reproduction, required for normal sexual development in *Aspergillus* and other species^{13,16}. A related study⁸ identified isolates of *A. fumigatus* containing the complementary alpha-domain *MAT* gene, and showed expression of *MAT1-1* and *MAT1-2* and of genes encoding sex pheromones and pheromone receptors. In addition, a nearly 1:1 ratio of *MAT1-1*:*MAT1-2* isolates was found among a worldwide screening of 290 isolates, which is consistent with latent sexuality⁸. Gene recombination was also detected within a subset of isolates⁸, in agreement with other studies of *A. fumigatus* showing evidence of recombination and no detectable population structure, albeit with predominantly clonal reproduction^{6,7,12,17}. The observed recombination was attributed to past meiotic events, a so far undetected 'cryptic' sexual state or parasexual gene flow^{5,6,8}.

Previous population studies of *A. fumigatus* involved analysis of global collections or limited regional sample sets^{7,8,12,17}. In this study we screened for mating type⁸ in an Irish environmental population of *A. fumigatus*, composed of 91 isolates collected from five locations in Dublin during 2005 in the course of aerial sampling work¹⁸ (Supplementary Table 1). Isolates were also fingerprinted by randomly amplified polymorphic DNA (RAPD)-PCR to detect clonality and assess genetic relatedness (see Methods). *MAT1-1* or *MAT1-2* specific bands amplified from all except one isolate (Supplementary Fig. 1). Eighty-eight of the isolates had unique RAPD profiles, showing genetic variability within the Dublin population. A ratio of 49.4% *MAT1-1* to 50.6% *MAT1-2* was evident in the clone-corrected data set, with no significant difference in mating-type distribution at any of the collection sites ($P > 0.05$; Supplementary Table 2) and no evidence for clustering of isolates according to collection site or mating type (Fig. 1). Indeed, both mating types were found in samples collected within minutes of each other, showing that *MAT1-1* and *MAT1-2* isolates are in close proximity in nature. Results are in agreement with previous studies that found *MAT* genotypes in nearly equal proportions^{8,12}, although one survey reported a predominance of *MAT1-2* (ref. 17).

Species identity of the Dublin isolates was verified by molecular analysis, given possible misidentification of *A. fumigatus sensu stricto* by morphotyping^{19,20}. A subset of 12 representative isolates (6 *MAT1-1*, 6 *MAT1-2*) was chosen (Supplementary Table 1), and regions of their β -tubulin (*benA*) and carboxypeptidase-5 (*cyp*) genes were sequenced (S. A. Balajee, D. Nickle, L. Razai, S. F. Hurst & K. A. Marr, personal communication; see Methods). All isolates had identical sequences to those of known *A. fumigatus* depositions (for example, GenBank AY685162 (*benA*) and DQ438742 (*cyp*)) except for AfIR931, which had only one nucleotide difference in the *cyp* amplicon.

We then set up crosses with the 12 confirmed *A. fumigatus sensu stricto* isolates in all possible combinations of opposite mating types ($n = 36$) on a range of growth media at different temperatures to

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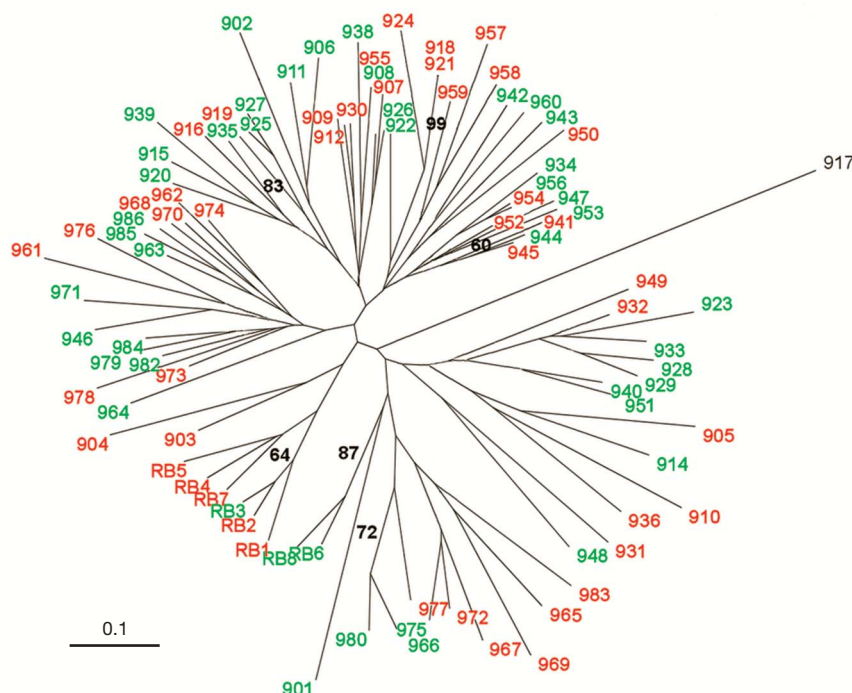


Figure 1 | Genetic diversity of 91 *A. fumigatus* isolates from Dublin, Ireland. Radial dendrogram derived from RAPD-PCR data pooled from primers UBC90, R151, R108 and RC08. The tree was constructed by neighbour-joining analysis with branch lengths drawn to reflect genetic distance derived from Jaccard's coefficient of RAPD band matching (scale bar: 0.1 = 10%)

determine whether it was possible to induce sexual reproduction *in vitro* (see Methods). After 6 months of incubation, mature cleistothecia (spherical fruiting bodies characteristic of sex in the aspergilli²¹) were found on pairings grown on Parafilm-sealed Oatmeal agar²² plates at 30 °C in the dark (Fig. 2a–d and Table 1). The light-yellow cleistothecia (typically 150–500 µm in diameter) formed singly or in small clusters of two to five, mainly along the junction (barrage zone) where hyphae of the parental isolates came into contact, and to a smaller extent within mycelium on either side of the barrage zone. The related heterothallic species *Neosartorya spathulata* produces cleistothecia scattered or grouped in clusters²³, whereas *N. fennelliae* generally produces copious numbers of cleistothecia along lines of mycelial contact²⁴. There was marked variation in the numbers of cleistothecia produced between different pairings (Table 1). It is not yet known whether this variation is typical of the species. Cleistothecia failed to develop when other media and incubation temperatures were used, or in pairings between isolates of the same mating type. When cleistothecia were squashed, numerous yellowish-white to greenish-white lenticular ascospores (4–5 µm in diameter) with two equatorial crests were observed, together with occasional intact asci (Fig. 2e–g). The ridged ornamentation of the ascospores is similar to that in the homothallic species *N. assulata*²⁰. The teleomorph (sexual stage) of *A. fumigatus* was assigned to the genus *Neosartorya* on the basis of phylogenetic relatedness and morphology of the cleistothecia and ascospores^{12,20}, and named *Neosartorya fumigata* O'Gorman, Fuller & Dyer *sp. nov.* (see Supplementary Notes for Latin diagnosis with English description).

Ascospores of *N. fumigata* germinated on 2% malt extract agar (MEA) at 28 °C within 15 h and also germinated after exposure to 70 °C for 90 min (Fig. 2h), heat resistance of ascospores being typical of *Neosartorya* species²⁰. Ascospore-derived cultures gave rise to characteristic *A. fumigatus* colonies bearing abundant asexual conidia under routine growth conditions.

Finally, 15 ascospore progeny from each of three different crosses were assessed for meiotic recombination by examining the segregation

genetic difference). Numbers at nodes indicate the percentage bootstrap support (based on 1,000 resamplings) for which the cluster is supported (only values of 60% or more are shown, for clarity). *MAT1-1* and *MAT1-2* genotypes are shown in red and green, respectively.

of five genetic markers (four RAPD bands and the mating-type genotype) (Fig. 3). Between 53% and 67% of progeny showed unique genotypes, with only 7–13% of progeny identical to one of the parents (on the basis of the markers examined), providing clear evidence of recombination during the sexual cycle (Supplementary Tables 3–5). The segregation of mating type was consistent with a heterothallic breeding system. Fisher's exact test confirmed that genetic markers were equally distributed between the progeny in each cross, verifying the null hypothesis of 1:1 Mendelian segregation as a result of independent assortment.

A. fumigatus was first described 145 years ago²⁰, but its teleomorph has remained undiscovered until now. Indeed, previous efforts failed to induce sexual reproduction despite considerable effort²⁵ (G. S. May, N. Osheroov and J. Kwon-Chung, personal communication). There are several possible explanations of why the sexual cycle has not been reported before. Environmental parameters rarely encountered in nature might be required to trigger sex¹⁵, or cleistothelial production may be confined to substrata that have not been systematically monitored. Sexuality has now been demonstrated in the once presumed asexual pathogen *Candida albicans*, with mating suggested to occur only in particular host niches²⁶. Similarly, sexual reproduction has been detected in previously 'asexual' phytopathogenic *Tapesia* and *Mycosphaerella* species on certain host substrates^{27,28}. Alternatively the species as a whole may be experiencing a 'slow decline' in fertility⁵ — possibly linked to variation in *MAT* gene expression^{5,25}. Thus, the Dublin isolates might represent a rare subset of fertile isolates, although there is evidence that *A. fumigatus* comprises one global, recombining population^{7,12} (see Supplementary Discussion).

The discovery of a sexual cycle in *A. fumigatus* provides insights into the biology and evolution of the species. It helps explain the presence of diverse genotypes despite predominantly clonal reproduction^{6,7,17}, conservation of sex-related genes^{10,29}, aspects of genome evolution²⁹ and defence against repetitive elements². In addition, ascospores might aid survival in adverse environmental conditions.

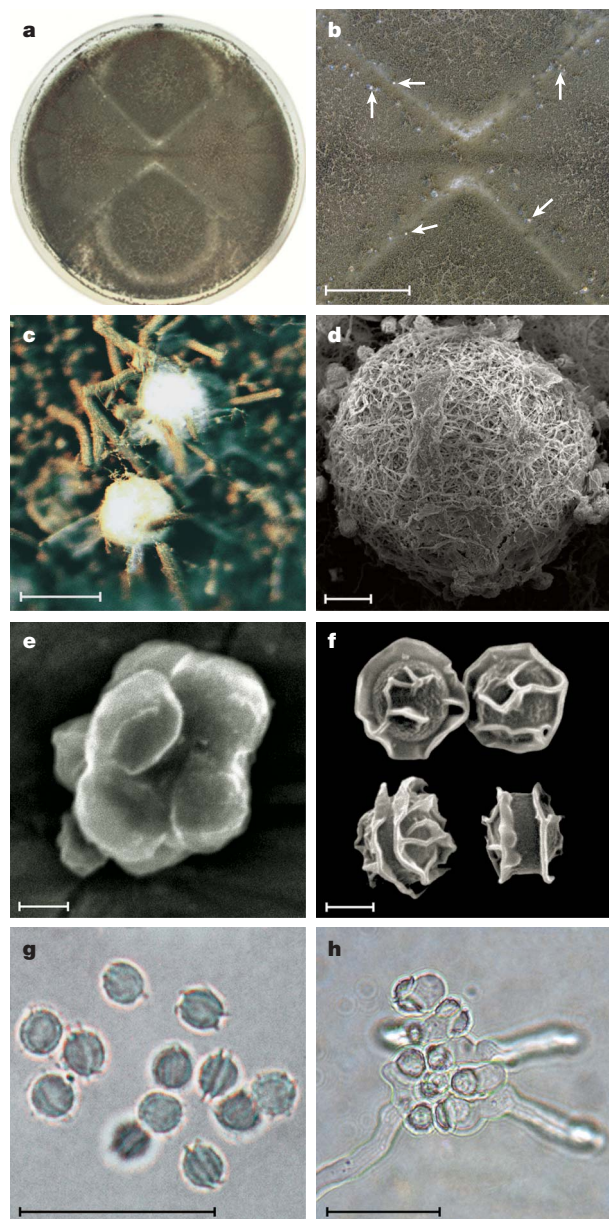


Figure 2 | *Neosartorya fumigata* sp. nov. **a**, Paired culture of AfRB2 $\times$ AfIR956 incubated for 6 months at 30 °C on Oatmeal agar medium in a 9-cm diameter Petri dish. **b**, Cleistothecia (arrows) along junctions of intersecting colonies of opposite mating type. Scale bar, 1 cm. **c**, Cleistothecia among chains of conidia. Scale bar, 400 μ m. **d**, Scanning electron micrograph of a cleistothecium, showing the peridium of interwoven hyphae. Scale bar, 100 μ m. **e**, **f**, Scanning electron micrograph of an eight-spored ascus (**e**) and ascospores (**f**). Scale bars, 2 μ m. **g**, **h**, Ascospores ungerminated (**g**) and germinating after exposure to 70 °C for 60 min (**h**). Scale bars, 20 μ m.

Table 1 | Numbers of cleistothecia produced by *A. fumigatus* crosses

Cross		Number of cleistothecia					
		MAT1-1					
		AfIR909	AfIR931	AfIR954	AfIR957	AfIR974	AfRB2
MAT1-2	AfIR901	+	+	+	—	+++++	+
	AfIR915	+	+	—	+	+++	++
	AfIR928	++	>	+++	++	+++++	>
	AfIR956	+	++	+	+	++	++
	AfIR964	++	+	+	++++	>	++
	AfRB3	+	+	+	++	+++	+++++

Ratings indicate the mean number of cleistothecia produced from two replicate crosses on Oatmeal agar medium in 9-cm diameter Petri dishes after incubation in the dark for 6 months at 30 °C: —, none; +, 1–19; ++, 20–39; +++, 40–59; +++++, 60–79; ++++++, 80–100; >, more than 100 cleistothecia.

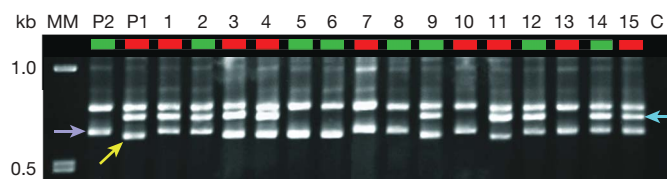


Figure 3 | Segregation patterns of molecular markers. Representative RAPD-PCR data from *N. fumigata* parental isolates (P1 and P2) and 15 ascospore progeny from the cross AfIR974 $\times$ AfIR964 using primer OPW-10. MM, molecular mass marker; P2, AfIR964; P1, AfIR974; lanes 1–15, progeny 974-964-1, 974-964-3 to 974-964-16; C, water control. Coloured arrows indicate bands designated OPW-10-1 (purple), OPW-10-2 (yellow) and OPW-10-3 (blue) (Supplementary Table 3). Lane headings are shown in red and green to indicate MAT1-1 and MAT1-2 genotypes, respectively. kb, kilobase.

The discovery also has significant medical implications. Sexual reproduction can result in progeny with increased virulence or resistance to antifungal agents, and can confound diagnostic tests based on the assumption of clonality^{5,30}. However, the sexual cycle also offers a valuable tool with which to determine the genetic basis of traits of interest. Pathogenicity in *A. fumigatus* seems multifactorial in nature^{2,11}, with genome analysis revealing potential targets⁹. Classical genetic analyses may help in elucidating the contribution of particular components, thereby guiding disease control strategies.

Results are also of general significance, given that almost one-fifth of all fungi have no known sexual stage. This includes many *Aspergillus*, *Penicillium*, *Coccidioides* and *Malassezia* species of great economic and medical importance. Some of these species have apparently functional MAT and other sex-related genes^{10,21} (Supplementary Discussion). Recombination in asexual populations is often attributed to parasexual reproduction. However, parasexuality is limited to isolates of the same vegetative compatibility grouping¹⁵. According to the *A. fumigatus* model, cryptic sexuality is a more likely source of recombination, and isolation of fresh cultures combined with concerted laboratory crossing efforts of compatible mating types may lead to a sexual revolution for many of these other supposed ‘asexuals’.

METHODS SUMMARY

Aspergillus fumigatus isolates were crossed in pairwise MAT1-1 and MAT1-2 combinations in duplicate on Oatmeal agar medium²² (Pinhead Oatmeal; Odlum Group) and were incubated inverted at 15, 30 or 45 °C in the dark. Crosses were also established on 2% MEA (Oxoid), Czapek Dox agar and *Aspergillus* complete medium⁸ at 30 °C in the dark. Spore suspensions of each isolate (5×10^5 conidia ml⁻¹) were prepared from seven-day-old cultures. Two 1- μ l aliquots of each spore suspension were separately inoculated onto the agar surface about 4 cm apart and perpendicular to aliquots of conidia of the opposite mating type. This configuration created four interaction/barrage zones as colonies grew (see Fig. 2a, b). Plates were sealed with one layer of Parafilm. Crosses were examined for cleistothecia periodically over 6 months with Olympus SZH10 Stereo and BX45 light microscopes. Cleistothecia were removed with a sterile needle tip and cleaned of adhering conidia by gentle rolling across a drop of water under sterile conditions. Individual cleistothecia were added to 100 μ l of 0.05% Tween 80 and ruptured by squashing with a needle tip. The resulting macerate (of peridium, asci, ascospores and contaminating conidia) was transferred to 1.9 ml of 0.05% Tween 80 and vortex-mixed for 1 min to disrupt the asci. The suspension was heated at 70 °C for 60 min, which prevented the germination of conidia. Aliquots (100 μ l) of the suspension were spread plated on 2% MEA (9-cm plates) and incubated overnight at 28 °C. Single-ascospore cultures were established on 2% MEA by transferring individual germinating ascospores with a LaRue lens cutter attached to an Olympus BX45 microscope.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions C.O’G., H.T.F. and P.S.D. designed the experiments. C.O’G. performed most of the experiments. C.O’G. and P.S.D. analysed the results and wrote the manuscript. All authors contributed to editing the manuscript.

Author Information DNA sequences have been deposited in GenBank under accession numbers EU541353 and EU541354 (carboxypeptidase-5) and EU541355 (β-tubulin). The holotype of *Neosartorya fumigata* has been deposited in the Herbarium of the Royal Botanic Gardens, Kew, under accession number K(M)159484. The assignment *Neosartorya fumigata* O’Gorman, Fuller & Dyer sp. nov. has been deposited in MycoBank under accession number MB 512563. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to C.O’G. (celine.ogorman@ucd.ie) or P.S.D. (paul.dyer@nottingham.ac.uk).

METHODS

Maintenance and crossing of isolates. Single-spore cultures of each isolate were routinely maintained on MEA slopes at 4 °C in the dark. Crosses were set up on Oatmeal agar medium²², Czapek Dox agar³¹ and *Aspergillus* complete medium⁸. **Light microscopy and scanning electron microscopy.** For micro-morphological examination of *N. fumigata* sexual reproductive structures, mounts were made in clear lactophenol from cultures grown on Oatmeal agar medium²² and colours were assigned using Methuen colour names and numbers³². Dimensions of cleistothecia and ascospores ($n=20$) were measured with the software package Olympus DP-Soft, version 5.0, linked to an Olympus BX-45 light microscope and an Olympus C-5060 Wide Zoom digital camera. For scoring purposes, cleistothecia were defined as spherical white to yellow structures more than 150 µm in diameter. Scanning electron microscopy was performed with a Jeol JSM-5410LV scanning electron microscope. For preparation for scanning electron microscopy, mature cleistothecia were transferred to aluminium stubs with the use of double-sided adhesive tape. A small drop of 0.05% Tween 80 was added and the cleistothecia were crushed. The suspensions were air-dried and sputter-coated with gold.

DNA extraction. Cultures were grown in 2% liquid malt extract for 5 days at 28 °C, washed with a 0.1 M phosphate buffer (pH 7.0) and ground into a fine powder under liquid nitrogen. Genomic DNA was extracted with a DNeasy Plant Mini Kit (Qiagen) in accordance with the manufacturer's instructions.

Mating-type PCR assay. The mating-type genotype of each isolate was determined with a multiplex-PCR assay as previously described⁸, with reaction volumes of 25 µl containing 50–100 ng of DNA. Segregation of mating-type genes in ascospore progeny has previously been used to infer the breeding systems of other filamentous ascomycete species, especially lichen-forming fungi^{33,34}.

Statistical analyses. The null hypothesis was for a 1:1 ratio of the two mating types, as is characteristic for a randomly mating population of haploid organisms³⁵. The hypothesis was tested with χ^2 and contingency χ^2 tests, with $P < 0.05$ considered significant. Fisher's exact test was used instead of the χ^2 test when sample sizes were small, because it is more accurate when expected frequencies are less than five³⁶.

RAPD-PCR protocol for clone-correction and genetic relatedness. RAPD-PCR was performed with cycling conditions described previously³⁷. Four primers were used to fingerprint the 91 *A. fumigatus* isolates: RC08 (5'-GGATGTCGAA-3')³⁸, R108 (5'-GTATTGCCCT-3')³⁹, R151 (5'-GCTGTAGTGT-3')³⁹ and UBC90 (5'-GGGGGTAGG-3')⁴⁰. PCR amplifications were performed in 25-µl reaction volumes containing 0.1–1.0 ng of DNA. Each amplification reaction contained 2.5 µl of 10×DyNAzyme buffer, 0.75 U of DyNAzyme II Polymerase (2 U µl⁻¹; Finnzymes OY, Flowgen Laboratories), 0.15 µl of 25 mM dNTP mix (ABgene), 50 µmol of a single ten-base-pair primer and ultra-pure water (Sigma). Amplicons were resolved in 1.5% agarose gels (data not shown). Clear, reproducible RAPD-PCR amplicons from each primer were scored as 0 (amplicon absent) or 1 (amplicon present) in Microsoft Excel. The data from all primers were pooled for each isolate, and a pairwise similarity matrix was calculated with Jaccard's coefficient⁴¹ with the program FREETREE⁴² v. 0.9.1.50. A bootstrapped dendrogram with 1,000 replications was produced with NJ Plot. It is noted that the most divergent isolate detected by this analysis, Afir917, was also the only isolate that failed to amplify a MAT product in the multiplex mating-type assay, suggesting sequence divergence in the MAT region and that this isolate was not *A. fumigatus sensu stricto*.

RAPD-PCR for genotypic markers. The 12 parental isolates (indicated in Supplementary Table 1) were screened with 37 ten-base RAPD primers (Operon Biotechnologies Kits OP-A, -J, -W and -X) for the amplification of bands suitable for genotypic markers. Five primers (A-05, J-01, W-10, W-19 and X-05) that amplified clear, reproducible bands unique to one of the parental isolates were selected. RAPD-PCR reactions were performed as described above. Segregation of RAPD-PCR markers has previously been used to determine sexual breeding systems in several other ascomycete fungi^{33,43–45}.

Gene sequencing. The genes encoding β -tubulin and carboxypeptidase-5 were amplified with the primer sets benA (forward, 5'-AATTGGTGCCGCT-TTCTGG-3'; reverse, 5'-AGTTGTCGGGACGGAATAG-3') and cyp (forward, 5'-GAACATTAGCCCCAGTTGAG-3'; reverse primer, 5'-CACTTCTCTTG-CACGTAGTC-3'), respectively (S. A. Balajee, D. Nickle, L. Razai, S. F. Hurst & K. A. Marr, personal communication). The amplified DNA fragments were purified with a QIAquick PCR purification kit (Qiagen). DNA sequencing of the forward strand of each fragment was performed at the Biopolymer Synthesis and Analysis Unit, University of Nottingham. The resulting sequences were aligned in CLUSTALW⁴⁶ using the program BioEdit⁴⁷.

Measurement of ascospore germination. Using a fine-tipped needle, an intact mature cleistothecium was removed from a 12-month-old culture of an *N. fumigata* Afir956 × AfirB2 cross. An ascospore suspension was prepared and heat-treated. Ascospore concentration (about 4×10^4 spores ml⁻¹) was determined with a haemocytometer. Defined areas on 2% MEA plates were each spread inoculated with 50 µl of the heat-treated ascospore suspension. Plates were sealed with one layer of Parafilm and incubated at 28 °C in darkness overnight. Coverslips were then placed on the agar surfaces over the defined areas, and levels of ascospore germination were determined. The percentage germination of heat-treated (70 °C, 60 min) ascospores was $50.5 \pm 3.4\%$ ($n=600$; mean $\pm$ s.e.m.) after 15 h at 28 °C.

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