

## CRYPTIC SPECIATION IN THE COSMOPOLITAN AND CLONAL HUMAN PATHOGENIC FUNGUS *ASPERGILLUS FUMIGATUS*

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**Abstract.**—Microbes and other organisms smaller than one to a few millimeters in size are hypothesized to have global populations, in contrast to the geographically restricted ranges of larger organisms. However, fungi, which routinely have reproductive propagules no larger than 10 micrometers, challenge the generality of this hypothesis because recent studies have shown that globally distributed morphological species embrace two or more geographically restricted phylogenetic species. We used the concordance of gene genealogies to recognize phylogenetic species in the globally distributed opportunistic human pathogenic fungus, *Aspergillus fumigatus*. Based on DNA sequence data of five loci for each of 63 individuals collected from five continents, we have delineated two phylogenetic species in this single morphological species. Unlike all other fungi examined to date, both genetically isolated groups showed a global distribution with no evidence of a correlation between genotype and geographic location. Sexual reproduction has never been observed in *A. fumigatus*, but when the same data were used to explore the association of alleles at the five loci for one of the phylogenetic species, evidence was found to support recombination. The discovery of a cryptic species is medically relevant because different species are likely to differ in virulence or drug resistance. The discovery of a globally distributed *A. fumigatus* species clade highlights the need for ecological studies of the fungus to either document global dispersal or propose alternative mechanisms by which it persists as single, global phylogenetic population.

**Key words.**—Darwinian medicine, genealogical species concept, global microbial distribution, medical mycology, microbial biogeography, microbial reproduction.

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Claims that species of some organisms smaller than 10 mm and most species smaller than 1 mm have global distributions (Finlay 2002) may seem startling to evolutionary biologists familiar with the remarkable diversity of endemic animals and plants. Modern advocates of the hypothesis that small eukaryotes and nearly all prokaryotes lack endemism base their claims on studies of morphological species of ciliates (Fenchel and Finlay 2004). An alternative explanation for conflicting estimates of species ranges for large and small organisms is that morphology is a poor means of recognizing microbial species.

In the fungi, a group of small organisms whose reproductive propagules routinely measure two orders of magnitude smaller than 1 mm, several morphological species that were thought to have global distributions have been shown to harbor two or more phylogenetic species, each with geographically restricted distributions (e.g., Koufopanou et al. 1997; Geiser et al. 1998a; Burt et al. 2000; Kasuga et al. 2003). Additionally, in organisms an order of magnitude smaller than fungi, prokaryotic Archaea and Bacteria, multilocus genotyping has shown that genetically isolated clades have geographically restricted distributions (Papke et al. 2003, Whitaker et al. 2003) and that there is a positive correlation between genetic and geographic distances (Whitaker et al.

2003). Therefore, it might seem reasonable to argue that small organisms with global distributions are an artifact of morphological species recognition.

*Aspergillus fumigatus* is a cosmopolitan and ubiquitous ascomycete fungus, one of “the most common microorganisms present in compost and other organic material undergoing decomposition” (Raper and Fennell 1965). According to morphological criteria, the species *A. fumigatus*, with propagules 2  $\mu\text{m}$  in diameter, is globally distributed. Optimal growth is at 37°C, but the fungus can tolerate temperatures of 45°C or higher. The fungus makes unusually small mitospores, about 2  $\mu\text{m}$  in diameter. The ability to grow at a human body temperature, the ability to disperse spores deep into the human lung, and perhaps other (as yet undiscovered) attributes enable *A. fumigatus* to cause an opportunistic infection in hosts with compromised immune systems (Denning et al. 2002). In this species, phenotypically indistinguishable individuals can be recovered throughout the world, either from the environment or from infected human hosts. To examine the hypothesis that *A. fumigatus* is a globally distributed fungus, we applied phylogenetic species recognition by exploring the congruence of gene genealogies (Baum and Shaw 1995) for five regions of nuclear DNA sequenced for 63 individuals collected from patients, hospitals, and other environments on five continents. We discovered that *A. fumigatus* encompasses two genetically isolated, phylogenetic species. More than 90% of the individuals of our study belonged to one of the two species, and for that species there is no evidence of endemism or any correlation between ge-

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notype and geographic origin. *A. fumigatus* is the first example of a fungus with a global distribution.

*Aspergillus fumigatus* can reproduce clonally but sexual reproduction never has been observed in this fungus, putting it in the company of almost 20% of described fungi. However, evidence for recombination has been found in other morphologically asexual fungi (Taylor et al. 1999a), raising the possibility that *A. fumigatus* also has this capacity. To analyze reproductive mode, we analyzed the same DNA sequence data using tests for association among alleles. There is evidence for recombination in one of the phylogenetic species of *A. fumigatus* using two of these tests.

#### Species Recognition

Any description of a species empirically tests a species concept; common criteria for delineating species include phenotype, reproductive isolation or genetic divergence. Traditionally, mycologists have used phenotype for morphological species recognition (MSR) (Harrington and Rizzo 1999; Petersen and Hughes 1999), but morphological species may contain more than one reproductively isolated (Perkins et al. 1976; Turner et al. 2001) or genetically isolated (Taylor et al. 2000) group. Although reproductive isolation is a universal standard for species recognition (Coyne and Orr 2004), and although biological species recognition (BSR) as implemented by mating tests is successful for some fungal species (e.g., Dettman et al. 2003a), it has not been systematically applied throughout the fungal kingdom for a variety of reasons. A fungus may be unculturable (e.g., *Pneumocystis carini* (Cushion et al. 1991)) or asexual (one-fifth of described fungi including, e.g., *Coccidioides immitis* (Taylor et al. 1999a)). Sex may be difficult to induce in the laboratory (e.g., *Candida albicans* (Miller and Johnson 2002; Soll et al. 2003)). Sexual fungi may possess an unbalanced distribution of mating types (e.g., *Histoplasma capsulatum* (Kwon-Chung et al. 1974, 1984) and *Cryptococcus neoformans* (Lengeler et al. 2000)). In contrast to both MSR and BSR, phylogenetic species recognition (PSR) using nucleic acid variation can be used to describe species of phenotypically uniform, apparently asexual and even uncultivable fungal lineages (Taylor et al. 2000). Bacteria, Archaea, and most fungi are haploid and lack multigene families, which makes them ideal candidates for PSR under the Geneological Concordance Phylogenetic Species Concept (GSC) (Baum and Shaw 1995; Shaw 1998). To quote Hudson and Coyne (2002, p. 1557), a species recognized by GSC is a "basal, exclusive group of organisms, whose members are all more closely related to each other than they are to any organisms outside the group, and that contains no exclusive group within it." In all cases in which the PSC has been used to describe species of global isolates of a morphological fungal species, cryptic species have been recognized, for example within *Schizophyllum commune* (James et al. 1999), *Sclerotinia sclerotiorum* (Carbone and Kohn 2001a) and outbreeding *Neurospora* species (Dettman et al. 2003b) (and see also Demanche et al. 2001; Fisher et al. 2002; Franzot et al. 1999; Kasuga et al. 2003; Koufopanou et al. 1997; Sullivan et al. 1995; Xu et al. 2000). BSR and PSR have been compared for species of *Neurospora* and the biological species recognized by success in mating tests are

nearly the same as the phylogenetic species described by the concordance of multiple gene genealogies (Dettman et al. 2003a); the two species concepts are effectively congruent.

PSR has been used in another morphological species of *Aspergillus*, *A. flavus*, to describe cryptic species (Geiser et al. 1998a) and previous research on Northern hemisphere isolates of *A. fumigatus* used genetic fingerprint data to show that individuals could be divided into several distinct clades, suggestive of cryptic speciation (Debeaupuis et al. 1997). However, because fingerprint data can be problematic due to unavoidable assumptions regarding the homology of fragments of a similar size, in this study of *A. fumigatus* we used DNA sequence variation of five polymorphic loci to develop multilocus sequence genotypes. Multilocus sequence genotypes have proven useful for population genetic studies of fungi (Taylor and Fisher 2003) and other microbes, including bacteria (Maiden et al. 1998).

In this study a clade was considered to be an independent evolutionary lineage, and recognized as a phylogenetic species, if it was present in the majority of single locus genealogies. Although phylogenetic species recognition by genealogical concordance may provide a conservative measure of numbers of species (Young 1998; Hudson and Coyne 2002), knowledge of well-supported clades, by whatever name, will provide the necessary foundation for all subsequent comparative biology.

#### Biogeography

Where phylogenetic species of fungi have been recognized by genetic isolation, often a correlation between genotype and geographic range has been discovered. For example, the basidiomycete *Schizophyllum commune*, originally thought to be one cosmopolitan species (Raper et al. 1958), is in fact made up of three species, each with a unique geographic distribution (James et al. 1999). Other examples of cryptic species with distinct ranges include the ascomycetes *Histoplasma capsulatum* (Kasuga et al. 2003), *Coccidioides immitis* (Koufopanou et al. 1997; Fisher et al. 2001), *Candida albicans*, (Forche et al. 1999; Schonian et al. 2000), *Sclerotinia sclerotiorum* (Carbone and Kohn 2001a,b), and *Neurospora* spp. (Dettman et al. 2003a,b). Even in the genus *Aspergillus*, species with restricted geographic ranges have been found; For example, one of the phylogenetic species of *A. flavus* predominated in equatorial regions and the southern hemisphere (Tran-Dinh 2003). In contrast to these studies, prior research on *A. fumigatus* found no correlation between membership in any of several clades and geography (Debeaupuis et al. 1997). However, sampling by Debeaupuis et al. (1997) was restricted to individuals from the Northern Hemisphere (Europe and the United States), and we aimed to test their conclusions by assembling our own collection of individuals from Africa, Latin America, and Asia, as well as Europe and North America.

#### Understanding Asexuality

*Aspergillus fumigatus* is among the approximately one-fifth of species in the fungal kingdom that appear to be exclusively clonal, based on reproductive morphology (Hawksworth et al. 1995). Like the others, *A. fumigatus* has close sexual rel-

atives, that is, *Neosartorya fischerii* (which is self fertile) and other *Neosartorya* species that are obligately outbreeding (Geiser et al. 1998b). Given that truly asexual organisms are considered to be very rare (Judson and Normark 1996), it would be astonishing to find that one-fifth of fungal species lack any ability to fuse and recombine. The same DNA sequence data used to implement PSR by concordance of gene genealogies has been used to provide evidence that apparently asexual species, including other *Aspergillus* species, do recombine in nature (Burt et al. 1996; Gräser et al. 1996; Geiser et al. 1998a; Fisher et al. 2000; Anderson et al. 2001). To search for evidence of recombination in *A. fumigatus* we used the sequence data of the five polymorphic loci to test for an association among alleles.

## MATERIALS AND METHODS

### *Fungal Strains*

Isolates of *A. fumigatus* were gathered from Africa (9), Europe (20), North America (12), South America (6), and Asia (13), and we also included three isolates of unknown origin, plus three isolates of the closest known relative of *A. fumigatus* (Geiser et al. 1998b), *N. fischeri* (Table 1).

Isolates were named as follows: either Af or Nf to stand for *Aspergillus fumigatus* or *Neosartorya fischeri*, a letter to indicate the source of the isolate (environmental, hospital, or patient), a number to indicate replicate number (0 indicates the isolate is a unique combination of source and country; 1, 2, or 3 indicate an isolate is the first, second, or third culture of that combination of source and country), and a letter abbreviation to mark the country or state in which the isolate was collected. Isolates were classified as environmental, hospital, or from a patient according to whether *A. fumigatus* was cultured from an environmental source other than a hospital (for example soil) the environment of a hospital, or directly from a patient infected with *A. fumigatus*. The nomenclature contrasts to the common terminology of the medical literature, in which "environmental" includes the environment of a hospital, here termed "hospital."

### *Cultures*

Mycelia were grown and agitated in 100 ml of potato dextrose broth at 37°C for five days. Mycelia were collected via filtration, frozen in liquid nitrogen, and lyophilized.

### *DNA Extraction, Amplification, and Sequencing*

DNA was extracted using a modified CTAB extraction protocol (Platt 1999). Approximately 200 mg of lyophilized tissue were placed in a 2 ml microcentrifuge tube with two-to-three 3-mm glass beads. Each tube was agitated for 30 sec on a Beadbeater (Biospec Products, Inc., Bartlesville, OK). One ml of CTAB extraction buffer (Doyle and Doyle 1987) was added to the pulverized sample and the tube incubated at 60°C for 45 m. The samples were chloroform extracted with 750 µl of chloroform:isoamyl alcohol (24:1). The DNA was further purified using Quiagen's DNeasy kit (Quiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

Target loci were identified by using microsatellite repeat

motifs to search within the partial genome of *A. fumigatus* and locate putatively variable regions. Five loci were developed and named 10654, 12858, 13578, 15187, and 18548. Each of the five loci encompasses a microsatellite repeat and surrounding nucleotides. Oligonucleotide primer sequences for each locus are as follows: 10654F TTCACAAAGGCAACTCTCAT T and 10654R GACCTCTACAGACTCGTGATA; 12858F C TAGTTCGCTTGATGTGCATA and 12858R TCTCATTCTT CCATTCTATTC; 13578F ATGGTTGACAGAGCAATACT A and 13578R TTACATCCAGGCAGGCAAGCA; 15187F G CTACAGCTACAGCTACAGCA and 15187R ATGAATAA AAGGGATAGCAAT; 18548F ATCCTGAGCCCGTAGAGA A and 18548R AAAGAGTCGTATCATCATAGT. Gene fragments were amplified from genomic DNA using a 25 µl or 50 µl polymerase chain reaction with either an MJ Research PTC-100 programmable Thermal Controller (MJ Research, Inc., Watertown, MA) or an Eppendorf Mastercycler (Eppendorf Scientific, Inc., Westbury, NY). Each reaction included 10 mmol/L Tris-HCL pH 8.3, 50 mmol/L KCL, 2.5 mmol/L MgCl<sub>2</sub>, 0.1 mg/ml gelatin, 20 µmol/L dNTPs, 0.4 µmol/L of each primer (Operon Technologies, Inc., Alameda, CA), and 0.05 µ/µl of AmpliTaq DNA polymerase (Applied Biosystems, Inc., Foster City, CA). Cycling profiles included an initial denaturation at 94°C for 2 min; 34 cycles of 94°C for 20 sec, an annealing temperature of 60°C for 30 sec, and 72°C for 1 min; a 5 min extension at 72°C; and a final soak at 4°C. Initially, an annealing temperature of 60°C was used for primers 10654, 15187, and 18548, while an annealing temperature of 55°C was used for primers 12858 and 13578. The annealing temperature was reduced to as low as 53°C for two isolates that were difficult to amplify (Af\_e\_2\_Col and Af\_e\_0\_IRAN). Products were electrophoresed on 1.5% agarose gels to verify the amplification of a single fragment of the appropriate length. Amplified products were purified with the Qiaquick PCR Purification Kit (Qiagen, Inc.) and nucleotide sequences were determined using the cyclic reaction termination sequencing method, using florescently labeled dideoxyribonucleotide triphosphates. DNA samples were sequenced completely in both directions with an ABI Prism 373 DNA Sequencer, ABI Prism 377 DNA Sequencer, or a DNA Prism 3100 DNA Sequencer; using either BigDye Terminator Cycle Sequencing Kits Version I or Version II (Applied Biosystems, Inc.). Sequences were visually inspected with Sequencing Analysis and Sequence Navigator (vers. 3.4.1 and 1.0.1, respectively; Applied Biosystems Inc., Foster City, CA). The sequences of each locus were aligned using Clustal X (vers. 1.81, CSC Scientific Computing Ltd., Otaniemi, Finland) and alignments adjusted by eye. Sequences were exported as a NEXUS file for subsequent analyses. Sequences of each of the five loci of two strains (Af\_e\_2\_JAP and Af\_p\_0\_II) are deposited in the NCBI nucleotide database (GenBank accession numbers AY551465-AY551474) and alignments are available from TreeBASE ([www.treebase.org](http://www.treebase.org); submission number SN1785).

### *Compatibility and Phylogenetic Analyses*

The internal consistency of each locus was evaluated using the program SITES (May 2002, available from J. Hey at <http://lifesci.rutgers.edu/~heylab>). An internally inconsistent lo-

TABLE 1. Isolates of *Aspergillus fumigatus* and *Neurospora fischeri*.

Name	Culture collection	Sent As:	Taxon	Sequence data					Origin	Source
				10654	18548	15187	13578	12858		
Af.e.0DM	5	Cam 931140-2	<i>A. fumigatus</i>	x	x	x	x	x	Denmark	cow
Af.e.0.INDIA	1	NRRL 5587	<i>A. fumigatus</i>	x	x	x	x	x	India	soil
Af.e.0.IRAN	4	MRC 889	<i>A. fumigatus</i>	x			x	x	Iran	pistachio nuts
Af.e.0.Md	7	SRRC 323	<i>A. fumigatus</i>	x	x	x	x		Maryland	chicken
Af.e.0.THAI	9	IAM 2004	<i>A. fumigatus</i>	x	x	x	x	x	Thailand	rice
Af.e.1.AUST	8	FRR 5073	<i>A. fumigatus</i>	x	x	x	x		Australia	air
Af.e.1.COL	3	46851	<i>A. fumigatus</i>	x	x	x	x	x	Colombia	environmental
Af.e.1.INDO	8	FRR 4617	<i>A. fumigatus</i>	x	x	x	x	x	Indonesia	environmental
Af.e.1.JAP	9	IAM 3006	<i>A. fumigatus</i>	x	x	x	x	x	Japan	soil
Af.e.1.MOZ	4	MRC 726	<i>A. fumigatus</i>	x	x	x	x	x	Mozambique	peanuts
Af.e.1.SA	4	MRC 1189	<i>A. fumigatus</i>	x	x	x	x	x	South Africa	rice
Af.e.2.COL	3	46915	<i>A. fumigatus</i>	x	x	x	x	x	Colombia	environmental
Af.e.2.INDO	8	FRR 623	<i>A. fumigatus</i>	x	x	x	x	x	Indonesia	environmental
Af.e.2.JAP	9	IAM 12766	<i>A. fumigatus</i>	x	x	x	x	x	Japan	soil
Af.e.2.MOZ	4	MRC 909	<i>A. fumigatus</i>	x	x		x	x	Mozambique	fish
Af.e.2.SA	4	MRC 2010	<i>A. fumigatus</i>	x	x	x	x	x	South Africa	sorghum
Af.e.3.COL	3	46922	<i>A. fumigatus</i>	x	x	x	x	x	Colombia	environmental
Af.e.3.INDO	8	FRR 4269	<i>A. fumigatus</i>	x	x	x	x	x	Indonesia	environmental
Af.e.3.JAP	9	IAM 12765	<i>A. fumigatus</i>	x	x	x	x	x	Japan	soil
Af.e.3.SA	4	MRC 2168	<i>A. fumigatus</i>	x	x	x	x	x	South Africa	cacao
Af.e.4.COL	3	46893	<i>A. fumigatus</i>	x	x	x	x	x	Colombia	environmental
Af.e.4.INDO	8	FRR 581	<i>A. fumigatus</i>	x	x	x	x	x	Indonesia	environmental
Af.e.4.SA	4	MRC 2424	<i>A. fumigatus</i>	x	x	x	x	x	South Africa	sorghum
Af.e.5.INDO	8	FRR 582	<i>A. fumigatus</i>	x	x	x	x	x	Indonesia	environmental
Af.e.5.SA	4	MRC 2024	<i>A. fumigatus</i>	x	x	x	x	x	South Africa	sorghum
Af.e.6.SA	4	MRC 10831	<i>A. fumigatus</i>	x	x	x	x	x	South Africa	snails
Af.e.x.x	9	IAM 2511	<i>A. fumigatus</i>	x	x				unknown	soil
Af.h.0.CAN	10	CDC E3	<i>A. fumigatus</i>	x	x	x	x	x	Montreal	hospital
Af.h.1.FR	5	3640-20	<i>A. fumigatus</i>	x	x	x	x	x	France	hospital
Af.h.2.FR	5	HD243	<i>A. fumigatus</i>	x	x	x	x	x	France	hospital
Af.h.3.FR	5	HD334	<i>A. fumigatus</i>	x	x	x	x	x	France	hospital
Af.h.4.FR	5	HD46	<i>A. fumigatus</i>	x	x	x	x	x	France	hospital
Af.h.5.FR	5	HD47	<i>A. fumigatus</i>	x	x	x	x		France	hospital
Af.h.6.FR	5	HD70	<i>A. fumigatus</i>	x	x	x	x	x	France	hospital
Af.h.7.FR	5	HD88	<i>A. fumigatus</i>	x					France	hospital
Af.p.0.II	1	NRRL 5109	<i>A. fumigatus</i>	x	x	x	x	x	Illinois	patient
Af.p.0.INDIA	6	F/5443	<i>A. fumigatus</i>	x	x	x	x	x	India	patient
Af.p.0.Sd	2	FH 97-11439	<i>A. fumigatus</i>	x			x		South Dakota	patient
Af.p.1.AUST	6	AF262	<i>A. fumigatus</i>	x	x	x	x	x	Australia	patient
Af.p.1.Ca	6	AF72	<i>A. fumigatus</i>	x	x	x	x	x	California	patient
Af.x.0.AUST	6	AF240	<i>A. fumigatus</i>	x	x	x	x	x	Australia	unknown
Af.p.1.Fa	2	FH 98-6747	<i>A. fumigatus</i>	x	x	x	x		Florida	patient
Af.p.1.FR	5	FdeF 9406118	<i>A. fumigatus</i>	x	x	x	x	x	France	patient
Af.p.1.GER	5	S217	<i>A. fumigatus</i>	x	x	x	x	x	Germany	patient
Af.p.1.NETH	5	M317	<i>A. fumigatus</i>	x	x	x	x	x	Netherlands	patient
Af.p.1.Wa	2	FH 97-16552	<i>A. fumigatus</i>		x	x	x	x	Washington	patient
Af.p.2.AUST	6	AF270	<i>A. fumigatus</i>	x	x	x	x	x	Australia	patient
Af.p.2.Ca	6	AF91	<i>A. fumigatus</i>	x	x	x	x	x	California	patient
Af.p.1.COL	3	29263	<i>A. fumigatus</i>	x	x	x	x	x	Colombia	patient
Af.p.2.FR	5	FdeF 9406132	<i>A. fumigatus</i>	x	x	x	x		France	patient
Af.p.2.GER	5	Sh17829	<i>A. fumigatus</i>	x	x	x	x	x	Germany	patient
Af.p.2.NETH	5	M408	<i>A. fumigatus</i>	x	x	x	x	x	Netherlands	patient
Af.p.2.Wa	2	FH 97-2826	<i>A. fumigatus</i>	x	x	x	x	x	Washington	patient
Af.p.2.COL	3	46334	<i>A. fumigatus</i>	x	x	x	x	x	Colombia	patient
Af.p.3.FR	5	FdeF 9406144	<i>A. fumigatus</i>	x	x	x	x	x	France	patient
Af.p.3.GER	5	Sh18199	<i>A. fumigatus</i>	x	x	x	x	x	Germany	patient
Af.p.3.NETH	5	M562	<i>A. fumigatus</i>	x	x	x	x	x	Netherlands	patient
Af.p.3.Wa	2	FH 98-1380	<i>A. fumigatus</i>	x	x	x	x	x	Washington	patient
Af.p.4.GER	5	Sh19010	<i>A. fumigatus</i>	x	x	x	x	x	Germany	patient
Af.p.4.Wa	2	FH 97-18249	<i>A. fumigatus</i>	x	x	x	x	x	Washington	patient
Af.p.5.GER	5	Sh21673	<i>A. fumigatus</i>	x	x	x	x	x	Germany	patient
Af.p.x.x	9	IAM 2034	<i>A. fumigatus</i>	x	x	x	x	x	unknown	patient
Af.x.x.x	9	IAM 2046	<i>A. fumigatus</i>	x	x	x	x	x	unknown	unknown
Nf.e.0.Az	1	NRRL A-7617	<i>N. fischeri</i>		x				Arizona	soil
Nf.e.0.GER	7	SRRC 317	<i>N. fischeri</i>		x				Germany	canned apples
Nf.e.0.MOZ	4	MRC 723	<i>N. fischeri</i>	x	x		x		Mozambique	cassava

TABLE 2. Culture collection addresses.

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1. NRRL (Northern Regional Research Laboratory), currently the Agricultural Research Service Culture Collection, United States Department of Agriculture, Peoria, IL, U.S.A.
  2. Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.
  3. Dr. Juan McEwen, Corporacion Para Investigaciones Biologicas, Medellin, Colombia.
  4. Medical Research Council, Tygerberg, South Africa.
  5. Dr. Jean-Paul Latgé, Laboratoire d'*Aspergillus*, Institut Pasteur, Paris, France.
  6. Dr. Caroline B. Moore, Department of Microbiology, Hope Hospital, Salford, U.K.
  7. SRRC (Southern Regional Research Center), United States Department of Agriculture, New Orleans, LA, U.S.A.
  8. Dr. Ailsa Hocking, Food Science Australia, CSIRO, North Ryde, NSW, Australia.
  9. IAM Culture Collection, The University of Tokyo, Japan.
  10. Dr. Viviane Loo, McGill University Health Centre, Montreal, Quebec, Canada.
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cus might be subject to either significant levels of internal recombination or high levels of homoplasy.

We used three tree-building methods (neighbor-joining (NJ), maximum-parsimony (MP), and Bayesian analysis) to determine if any significant taxonomic or geographic structure exists within *A. fumigatus*. For NJ and MP trees we used PAUP 4.0b10 (Swofford 1998). For MP analysis, starting trees were obtained via stepwise addition with simple addition sequence. Tree bisection-reconnection was used for branch-swapping, and branches were collapsed if the maximum branch length was zero. A MP phylogeny was generated for each of the five loci, and the combined dataset. The analysis of the combined dataset excluded fungal isolates for which only one or two loci were sequenced. Bootstrap analysis using 100 heuristic replicates was used to determine support for the clades of each locus. Parsimony analyses included insertion/deletions (indels) that were consistently and unambiguously alignable across all taxa, and microsatellite length variants; each indel was treated as a single evolutionary event by recoding a single site within the indel or at the end of the microsatellite repeat as a multistate character. Remaining gaps were treated as missing data.

Finally, a Bayesian phylogenetic analysis was used to determine the probability of taxonomic structure given the data at each individual locus, as well as the combined data from all five loci using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). In the Bayesian analysis, indels and microsatellite repeats were excluded from the data. First, the model of nucleotide substitution that best fit the data of each locus was determined by adding parameters until the likelihood of an initial MP tree did not improve significantly, according to a likelihood ratio test, using ModelTest 3.06 (Posada and Crandall 1998). In the analysis of the combined loci, parameter estimates of each locus were unlinked, allowing independent substitution models for each locus. Three replicate analyses were used to calculate the posterior probabilities, as estimated from uniform priors, for the clades of each locus and the combined loci. Each replicate included four simultaneous and incrementally heated Markov chains; each replicate used default heating values. Markov chains were initiated from a random tree and were run for 1,000,000 generations. Samples were taken every 100th generation. Log-likelihood values were plotted against generation number to ensure that the likelihood stabilized (and to determine "burn-in"; all samples taken prior to burn-in were discarded. The clade posterior probabilities and overall topology of each replicate were com-

pared to verify that each consensus tree converged on a similar phylogeny.

#### *A. fumigatus* Reproductive Mode

The congruence or incongruence of individual gene genealogies was used to infer asexuality or sexuality. If a species is sexual, each locus is expected to have a unique evolutionary history; the data are not combinable (Kroken and Taylor 2001; Taylor et al. 1999b). Analyses of reproductive mode excluded isolates of the "occultum" group (see Results), and also excluded isolates from which only one or two loci were sequenced. First, an incongruence length difference/partition homogeneity test (ILD/PHT) (Farris et al. 1995; Huelsenbeck et al. 1996) was used to assess whether or not the data of individual loci might be combined. This test compares the sum of the MP tree lengths for the observed loci to the distribution of sums of tree lengths of 100 datasets in which the parsimony-informative sites have been shuffled between loci. If the loci are congruent, the shuffling will have little or no effect, and the sum of the tree lengths of the original dataset will be similar to the sums of the tree lengths of the artificial datasets. In an asexual species loci are effectively linked and congruent; shuffling will have little effect. But in a species with recombination loci are incongruent and the sum of the tree lengths of the original dataset will be less than the sums of the tree lengths of the shuffled datasets. The ILD/PHT has a high probability of a type I error; it is likely to reject the null hypothesis when that hypothesis should not be rejected. For this reason we chose a conservative threshold for significance (Cunningham 1997). We used a score of  $P < 0.0001$  to reject the null hypothesis of clonality, or congruence between loci.

A permutation tail probability test was used to compare the observed parsimony tree length to a distribution of tree lengths based on 100 resampled datasets. Extra steps suggest a homoplasious distribution of character states (typical of species capable of recombination, Burt et al. 1996; Kroken and Taylor 2001), and if artificially recombined datasets are longer than the observed tree, clonality is suggested. A score of  $P < 0.05$  or less was used to reject the null hypothesis of recombination (Burt et al. 1996).

Finally, the Index of Association ( $I_A$ ) (Maynard-Smith et al. 1993) of these data was compared to the  $I_A$ s of artificially recombined datasets (Burt et al. 1996; Taylor et al. 1999a). The  $I_A$  measures associations between alleles at different loci,

TABLE 3. The characters of each locus.

Locus	Sample no.		Total	Characters			Base frequencies			
	<i>Aspergillus fumigatus</i>	<i>Neurospora fischeri</i> <sup>1</sup>		Constant	Parsimony uninformative	Parsimony informative	A	C	G	T
15187	58	0	567	554	5	8	23.3	25.1	19.7	31.8
13578	60	1	233	231	0	2	13.9	20.6	8.7	56.8
12858	55	0	383	375	3	5	31.0	23.8	16.5	28.7
18548	60	3	364	359	2	3	31.0	16.9	22.6	29.5
10654	61	1	500	490	1	9	25.5	22.5	22.4	30.0

<sup>1</sup> *N. fischeri* excluded from analyses of individual loci; isolate Nf.e.0.MOZ included in analysis of combined loci.

and is greater if alleles are linked (an  $I_A$  of 1 indicates complete linkage between alleles at different loci). For this analysis the most balanced polymorphism of each locus was chosen for inclusion in a dataset which included only one character from each locus. The dataset was exported to the program MultiLocus 1.2 (available from A. Burt at [http://www.bio.ic.ac.uk/evolve/software/multilocus/multilocus\\_mac.sea.hqx](http://www.bio.ic.ac.uk/evolve/software/multilocus/multilocus_mac.sea.hqx)). A null hypothesis of recombination may be rejected if the  $I_A$  of the observed dataset is significantly greater (is closer to 1) than the  $I_A$ s of artificially recombined datasets (the  $I_A$ s of the artificial datasets will be close to 0).

RESULTS

*Compatibility analyses of individual loci.*—No evidence of homoplasy was found in four internally consistent loci (15187, 13578, 12858, and 18548). The fifth locus (10654) showed evidence of homoplasy. Homoplasy might be caused by frequent mutation or internal recombination. An inspection of the sequence data suggests that frequent mutation is more likely than internal recombination. The inconsistency was localized to variable positions 198, 266, 311, and 441. The two most common sequence types (GCTC, 28 of 61 sequences, and GCTA, 20 of 61 sequences) would need only a single mutation to give rise to two of the other sequence types (GCTC to GTTC, nine of 61 sequences, and GCTA to

ACTA, one of 61 sequences). However, a fifth sequence type (ACCC, three of 61 sequences) is more difficult to explain. It might have involved a double mutation from one of the very common sequence types (GCTC to ACCC). None of the variants would be easily generated by internal recombination.

*Phylogenetic analyses of individual loci.*—The five loci included a total of 27 parsimony informative characters (Table 3). An heuristic search of locus 15187 found two MP trees of 14 steps each (CI = 1.000, RI = 1.000) (Fig. 1A); of locus 13578 found one MP tree of two steps (CI = 1.000, RI = 1.000) (Fig. 1B); of locus 12858 found one MP tree of eight steps (CI = 1.000, RI = 1.000) (Fig. 1C); of locus 18548 found two MP trees of six steps each (CI = 1.000, RI = 1.000) (Fig. 1D); of locus 10654 found 32 MP trees of 13 steps each (CI = 0.8462, RI = 0.9688) (Fig. 1E).

One clade was well supported by the data from four of five individual loci (Figs. 1A–D, Table 4); it included five isolates of *A. fumigatus* collected from patients, hospitals, or the environment on four continents. One of these cultures (Af.p.0.II) was originally sent to us as *A. fumigatus* var. *ellipticus*. For convenience, we refer to the clade as the “occultum” group from this point forward. In the fifth locus, 10654, four of the five members of the occultum group form a clade that also includes two nonoccultum individuals; the fifth member of the occultum group is in the “fumigatus” clade (Fig. 1E).

Topologies derived from NJ and Bayesian analyses were very similar or identical to those obtained using MP. Only the “occultum” clade was consistently well supported by Bayesian posterior probabilities, and it was supported by four of the five loci (Figs. 1A–D).

*Phylogenetic analysis of combined loci.*—An heuristic search of the combined loci (and including an isolate of *N. fischeri*) found 20,000 MP trees of 74 steps each (CI = 0.7703, RI = 0.8851). The “occultum” group emerged as a well supported clade (Fig. 2).

The phylogeny included one additional well supported clade, which groups Af.e.0.INDIA, Af.e.0.THAI, and Af.e.3.INDO. In contrast to the “occultum” clade, these individuals were only supported as a monophyletic clade by one of the individual loci. For likelihood-based analysis, combining data from all five loci required that separate substitution models be simultaneously considered for each partition of the data. With this approach the variation in substitution patterns was included in a total-evidence analysis of phylogenetic structure in *A. fumigatus*. As with the parsimony analysis, one strongly supported branch separated the

TABLE 4. Characters providing support for an *Aspergillus fumigatus* variety *ellipticus* clade.

Code	Locus	Position	Change	CI
A	15187 <sup>1</sup>	86	Transition	1.000
B		286	Transversion	1.000
C		337	Transition	1.000
D	13578 <sup>2</sup>	44	Transversion	1.000
E	12858 <sup>2</sup>	134	Indel	1.000
F		159	Transition	1.000
G		263	Transversion	1.000
H		274	Transition	1.000
I	18548 <sup>1</sup>	266	Transition	1.000
J	10654 <sup>3</sup>	262	Microsatellite Length	0.667
K		443	Polymorphism Transversion	0.500
L		267	Transition	1.000
M		419	Indel	1.000
N		8	Transversion	1.000
O		169	Transversion	1.000

<sup>1</sup> Description of first of three equally parsimonious trees.

<sup>2</sup> Analysis gives a single tree.

<sup>3</sup> Description of first of 89 equally parsimonious trees.

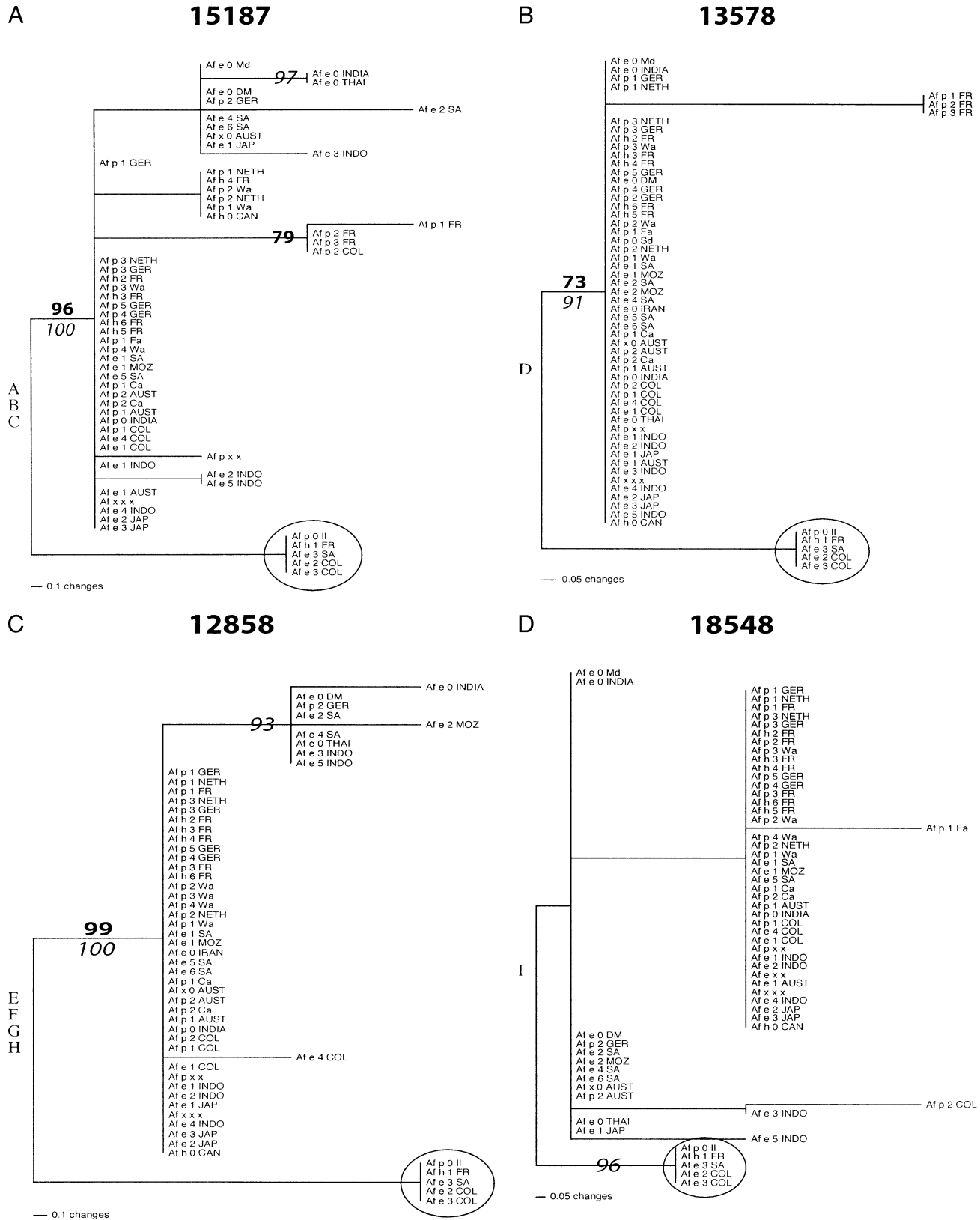


FIG. 1. Gene genealogies of five individual loci made by MP analysis. Parsimony bootstrap support above 70 is bold. Bayesian posterior probability above 90 is italicized. Phylogenies are unrooted; to facilitate comparisons between loci, they are displayed using the *Aspergillus fumigatus* “occultum” clade as an outgroup. Indices of consistency are given for lettered characters, see Table 4. (A). Locus **15187**: one of three equally parsimonious phylogenies. (B). Locus **13578**: a single most parsimonious phylogeny. (C). Locus **12858**: a single most parsimonious phylogeny. (D). Locus **18548**: one of three equally parsimonious phylogenies.

E

10654

Biogeography

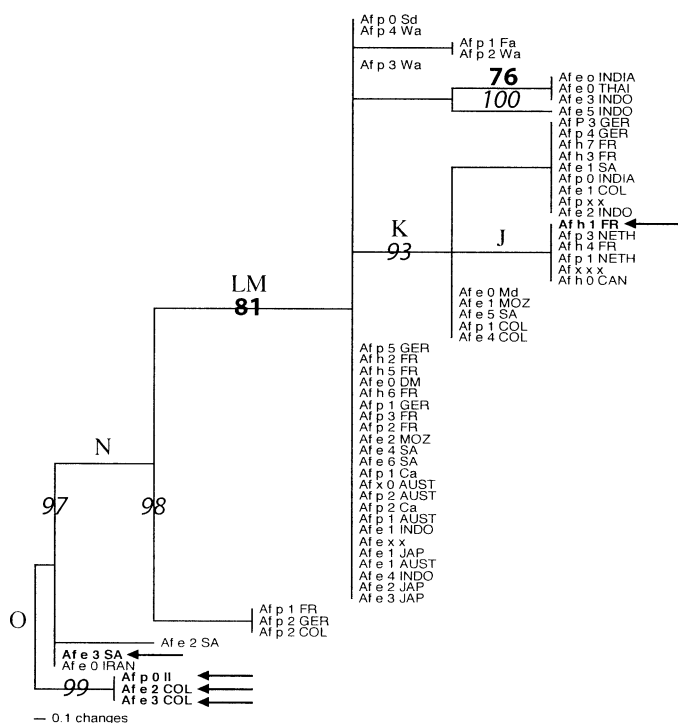


FIG. 1. Continued. (E). Locus 10654: one of 89 equally parsimonious phylogenies using the Af.p.0-II, Af.e.2-COL, and Af.e.3-COL clade as the outgroup.

“occultum” clade from the remaining *A. fumigatus* individuals (Fig. 2). Additional smaller clades were also supported. The first is the clade which groups Af.e.0-INDIA, Af.e.0-THAI, and Af.e.3 INDO. The second clade includes Af.p.2-GER and Af.e.2-SA. This group is not supported as a monophyletic clade by any single locus. The third clade combines Af.p.1-FR, Af.p.2-FR, Af.p.3-FR, and Af.p.2-COL. Once again, the group is not supported as a monophyletic clade by any of the single loci.

**Rooting the *A. fumigatus* phylogeny.**—Polymorphisms in beta tubulin, hydrophobin, and mitochondrial cytochrome b genes indicate that *N. fischeri* is the closest known relative to *A. fumigatus* and *A. fumigatus* var. *ellipticus* (Geiser et al. 1998b; Varga et al. 2000; Wang et al. 2000). In this study only three of the five loci could be amplified from *N. fischeri*. In two loci (10654 and 13578), *N. fischeri* was allied with *A. fumigatus* “fumigatus,” but in the third locus (18548) it grouped apart from both *A. fumigatus* “fumigatus” and *A. fumigatus* “occultum” (data not shown). In the combined analysis, *N. fischeri* is allied with the large *A. fumigatus* “fumigatus” clade. The nearest apparent outgroup of these fungi appears to be too distant to provide a well-supported root for species phylogenies, as is also true for other ascomycetes including *Neurospora* (Dettman et al. 2003b), *Coccidioides* (Koufopanou et al. 2001), and *Histoplasma* (Kasuga et al. 1999, 2003).

These data also were used to explore the biogeography of both the *A. fumigatus* “fumigatus” and *A. fumigatus* “occultum” clades. No structure is apparent from a visual inspection of the data (Figs. 1 and 2). The “fumigatus” clade typically groups isolates from different countries and continents, see for example a clade combining isolates from Denmark, Germany, South Africa, Mozambique, India, Thailand, and Indonesia (Fig. 1C), or the combination of Af.p.2-GER from Europe and Af.e.2-SA from Africa (Fig. 2). Isolates from France do appear to group together for two loci (Figs. 1A, B); however, other isolates from France do not group with this clade and in one case (Fig. 1A) an isolate from Colombia is also included in the clade. Similarly, the “occultum” clade includes isolates from North and South America, Europe, and Africa.

#### Sexuality within the *A. fumigatus* “fumigatus” Clade

To determine if the population of *A. fumigatus* is clonal or possesses a genetic structure more typical of sexual species, we used the same nucleotide sequence data described above, but excluded individuals of the “occultum” clade. Even minimal recombination will disrupt associations between alleles at different loci. We used three tests to identify multilocus linkage disequilibrium: the incongruence length difference/partition homogeneity test (ILD/PHT), the parsimony tree length permutation test (PTLPT), and an index of association ( $I_A$ ) test.

The ILD/PHT was done either without locus 10654 (because hypermutability could not be excluded as the source of homoplasy of this locus), or with just the first or second half of the locus (as neither half is homoplasious by itself). When every individual was included in a single analysis the null hypothesis of clonality was rejected in each of the three cases, but not at a conservative threshold for significance (54 individuals,  $P = 0.02$  in each test). When individuals with identical genotypes were pruned so that each genotype was represented by only a single individual, the null hypothesis of clonality was also rejected, but with more confidence (23 individuals; excluding 10654,  $P = 0.017$ ; including the first part of 10654,  $P = 0.002$ ; including the second half,  $P = 0.0065$ ). Yet none of the analyses met our very conservative criterion for significance ( $P < 0.0001$ ). We are aware that the ILD/PHT test has been reported to give evidence of conflicts among datasets when none exists (Yoder et al. 2001), and therefore consider these analyses only in combination with the following tests.

The PTLPT test of associations among alleles at the five loci gave a tree length for the observed data set of 38 steps, significantly ( $P < 0.01$ ) below the range of tree lengths for datasets resampled to mimic recombination (60–73 steps). The null hypothesis of recombination could be rejected even when the data were clone corrected so that each genotype was represented by only a single individual ( $P < 0.01$ , 44–52 steps after clone correction).

The  $I_A$  used only the single polymorphic nucleotide position with the most balanced allele frequency of each locus. The nucleotide positions and the frequency of the rare allele for each biallelic position follow: locus 10654 (position 443,



## Combined Loci

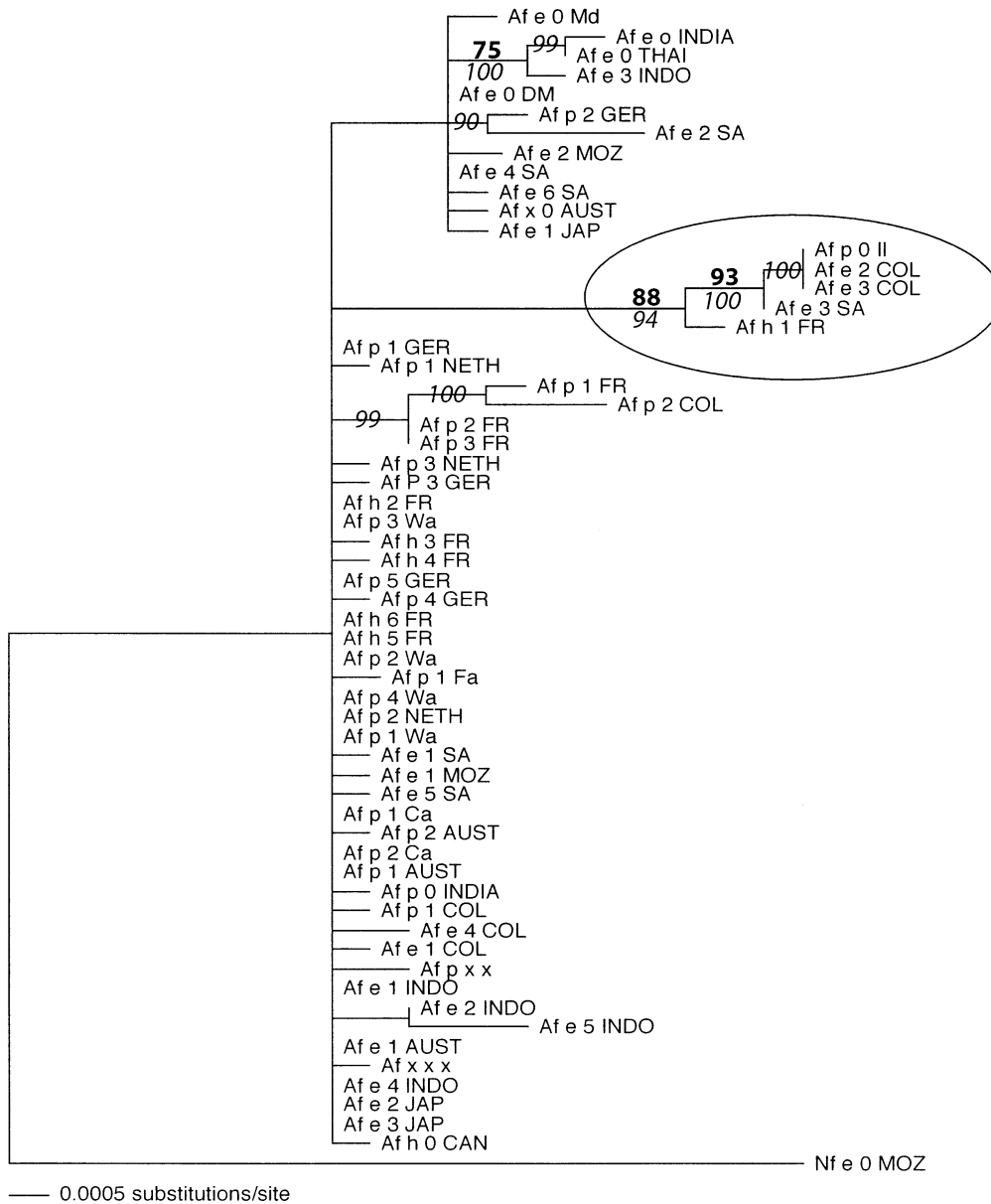


FIG. 2. Bayesian analysis of five loci combined with separate substitution models for each partition. Parsimony bootstrap support above 70 is bold. Bayesian posterior probability above 90 is italicized. The phylogeny is rooted using *Neurospora fischeri* as an outgroup.

frequency 0.35), locus 18584 (position 764, frequency 0.26), locus 15187 (position 961, frequency 0.18), locus 13578 (position 1619, frequency 0.35), and locus 12808 (position 1847, frequency 0.09). In a subset of these analyses data were clone corrected so that individuals with identical genotypes at the five most balanced nucleotide positions were excluded; each multilocus genotype was represented by only one individual. An  $I_A$  test of all 54 individuals rejected the null hypothesis of recombination ( $P < 0.001$ ), but an  $I_A$  test after clone correction could not do so ( $P = 0.202$ ). Taken as a whole these analyses are consistent with the observation that *A. fumigatus* reproduces clonally, by abundant production of

conidia, but our results also suggest that *A. fumigatus* “*fumigatus*” can recombine. However recombination has not fully disrupted the associations between alleles caused by clonal reproduction, and the signal of those associations is apparent even when the data are clone corrected.

## DISCUSSION

Phylogenetic species recognition based on the concordance of gene genealogies was used to recognize two, genetically isolated species in *A. fumigatus*. The species are informally named *A. fumigatus* “*fumigatus*” and *A. fumigatus* “*occul-*

tum.” *Aspergillus fumigatus* “fumigatus” included 58 of 63 individuals and in this species the data are consistent with both clonal reproduction and recombination. There is no correlation between genotype and geographic location in either species, making this study the first genetic documentation of a fungal species with a global distribution and no evidence of endemism.

#### *Cryptic Species*

A clade was recognized by PSR-GSC as an independent evolutionary lineage if it appeared as a well-supported branch in the majority of single locus genealogies. This majority-rule criterion acknowledges that coalescence for every genealogy, as proposed by Baum and Shaw (1995), is an unrealistic requirement (Harrison 1998, Hudson and Coyne 2002). In contrast to Dettman et al. (2003b), we did not recognize a clade as a species if it appeared in only one genealogy, even if that branch was not contradicted in additional genealogies. Our approach is conservative and seems appropriate to the description of novel species of a human pathogen; this approach is the one described by Shaw (1998).

Using these criteria we recognize two genealogical species within the morphological species *A. fumigatus*: *A. fumigatus* “fumigatus,” with more than 90% of the individuals representing five continents, and *A. fumigatus* “occultum” (*L.*, hidden or secret), with individuals representing five genotypes from Illinois, Columbia, France, and South Africa (including an isolate received as *A. fumigatus* var. *ellipticus*). Each of the clades is well supported by a distance analysis of resampled datasets and by Bayesian probabilities for four of the five loci, and well supported by parsimony analysis of resampled datasets in three of the five loci. Neither clade is found in the homoplasious fifth locus. Although no information is available about the effective population size of *A. fumigatus* or the substitution rates at the five loci studied here, the temporal separation of two strains that are reciprocally monophyletic at four of five nuclear loci is likely to be quite large (Hudson and Coyne 2002). In a phylogeny using all five loci, the branch separating the genealogical species *A. fumigatus* “fumigatus” from “occultum” is well supported and *A. fumigatus* “fumigatus” is not basal (Baum and Shaw 1995).

Additional clades are delineated within the phylogeny of the combined loci that are not found in a majority of individual genealogies. Further work with greater numbers of isolates or loci may show that these lineages deserve the rank of species, however, our conservatism in not naming these lineages as species is supported by the recent comparison of phylogenetic and biological species recognition in *Neurospora*, where internal clades represented by few individuals and supported by a minority of gene genealogies often were found nested within species subsequently defined using both phylogenetic and biological species concepts (Dettman et al. 2003a).

Currently the “fumigatus” and “occultum” clades are supported only by the criterion of genetic isolation. However, future morphological or ecological studies may differentiate the two species. One of the isolates of the occultum clade was sent to us as an *A. fumigatus* var. *ellipticus*. This variety

was originally described as having ellipsoidal and smooth-walled conidia (Raper and Fennell 1965). Our measurements of 100 spores each from 10 individuals, five of the “fumigatus” clade and all five of the “occultum” clade, failed to find consistent differences in morphology (D. Baker, unpubl. data). A previous recommendation to elevate *A. fumigatus* var. *ellipticus* to the species *A. neoellipticus* based on mitospore ornamentation (Kozakiewicz 1989) was not followed by Frisvad and Samson (Frisvad and Samson 1990) for similar reasons; only one individual of *A. fumigatus* var. *ellipticus* was shown to have the unusual ornamentation and the spore morphology of *A. fumigatus* sensu stricto were found to be extremely variable. Moreover, Frisvad and Samson (1990) reported that individuals labeled as *A. fumigatus* var. *fumigatus* or *A. fumigatus* var. *ellipticus* created identical mycotoxins and other secondary compounds. In other ascomycetes, phylogenetic clades deemed worthy of species recognition show consistent differences in morphology and mycotoxin production, for example, *Fusarium* (O’Donnell 2000; Ward et al. 2002), *Stachybotrys* (Cruse et al. 2002; Andersen et al. 2003), and even *A. flavus* (Geiser et al. 2000). Now that two *A. fumigatus* clades can be recognized it will be possible to study many additional aspects of phenotype, including the ability to cause disease and preferred microenvironment.

Our data support the hypothesis that any isolate of either the “fumigatus” or the “occultum” clade is a potential pathogen, an idea originally formulated from European and North American collections of *A. fumigatus* (Debeaupuis et al. 1997, Bart-Delabesse et al. 1998, see also Rosehart et al. 2002). These taxa are accidental human pathogens and are not transmitted from host to host; infection provides no apparent benefit to the fungi. However, the lack of a correlation between disease and environmental, geographic or genetic origin does not mean that individuals are equally virulent or resistant to antifungal pharmaceuticals (Perea and Patterson 2000; Dannaoui et al. 2001; Kontoyiannis and Lewis 2002).

Phylogenetic species recognition using concordance of multiple nuclear genes enjoyed widespread use with microbes earlier than with animals because often it is the only means of recognizing microbial species. Microbes possess relatively few phenotypic characters, making nucleic acid characters attractive, and microbial genomes are typically haploid with few gene families, making it easier to find useful nuclear genes. However, in animals, too, the use of multiple nuclear markers as well as mitochondrial markers now is routine as demonstrated by recent studies of *Drosophila* (Ballard et al. 2002; Kopp and Barmina 2005) and Hemiptera (Abe et al. 2005), which show that nuclear and mitochondrial genes may support conflicting results. Nor is the use of PSR-GSC restricted to small animals, for example, a recent study of right whales found cryptic species, and in this case nuclear and mitochondrial genes were in agreement (Gaines et al. 2005).

#### *Sexuality*

*Aspergillus fumigatus* “fumigatus” clearly is able to reproduce clonally and appears to recombine, as well. Evidence of recombination by the  $I_A$  test was found only when genotypes represented by more than one individual were pared to a single representative, that is, clone corrected. Clone cor-

rection was irrelevant in two other tests of association. ILD/PHT analysis rejected a null hypothesis of clonality even without clone correction, but not at a conservative threshold for significance. In contrast, PTLPT analyses rejected a null hypothesis of recombination even when data were clone corrected. Previous analyses of isoenzyme and sequence specific DNA primer (SSDP) data also found limited evidence for recombination (Varga 2003).

Genes for both mating types have been found in *A. fumigatus* (Pöggeler 2002; Dyer et al. 2003) and in the future additional loci specific to mating and meiosis may be identified. The emerging question is not whether *A. fumigatus* can recombine, but how it recombines and the relative contribution of its clonal and sexual reproductive modes to its evolutionary history and ecology.

Unfortunately, evidence for recombination rarely translates into knowledge of how or when recombination last occurred. For example, in the human pathogenic fungus *Candida albicans*, populations from France (Pujol et al. 1993), North Carolina (Gräser et al. 1996), Angola and Madagascar (Forche et al. 1999; Schonian et al. 2000) provide evidence for both clonality and recombination. In addition, mating type-like loci are found (Hull and Johnson 1999), and *C. albicans* has been experimentally mated both inside a mammalian host (Hull et al. 2000) and in vitro (Magee and Magee 2000). Evidence against recombination in nature is an excess or paucity of heterozygotes at some loci in natural isolates of this diploid yeast, although the same study found apparently recombinant haplotypes in the same isolates (Tavanti et al. 2004). A comparison of nuclear and mitochondrial polymorphisms suggests that ancestral recombination may have given way to a current, exclusively clonal, reproductive mode (Anderson et al. 2001).

As mentioned in the introduction, morphological evidence for sexual reproduction is lacking for one-fifth of fungi. However, where population genetic methods have been used to test for association of alleles in morphologically asexual fungi, evidence has been found for recombination. For example, the human pathogens *Coccidioides posadasii* (Burt et al. 1996) and *C. immitis* (Fisher et al. 2000), the toxigenic fungus *A. flavus* (Geiser et al. 1998b), and the lichenized fungus *Letharia* ‘‘lupina’’ (Kroken and Taylor 2001).

Similar issues are faced by evolutionists working with animals. Complete lack of sexual reproduction is claimed for only one animal clade, bdelloid rotifers (Welch and Meselson 2000), but other animals have been proposed to be asexual. For example, recombination has been demonstrated in a putatively ancient asexual aphid lineage, but the data cannot distinguish between mitotic and meiotic recombination (Normark 1999). Even rare bouts of recombination are likely to influence the persistence of otherwise asexual lineages, as has been discussed for the parasitoid wasp genus *Lysiphlebus* (Belshaw et al. 1999). Although much of the recent empirical work on the evolution of sex has focused on animals (Law and Crespi 2002, Simon et al. 2002, Van Doninck 2002, Delmotte et al. 2003, Vorburger et al. 2003), fungi also provide excellent models for experimental studies of the value of sex (Goddard et al. 2005).

### Biogeography

Our inability to discover any correlation between genotype and geography in a global collection of *A. fumigatus* individuals makes this fungus the first to be shown by population genetic evidence to have a global population structure with no endemism. Our result is consistent with earlier studies of more limited scope, for example, no correlation was found between genotype and geography for European and North American individuals (Debeauvais et al. 1997), nor for isolates from French and Italian patients (Bertout et al. 2001), nor for environmental and clinical isolates from Canada (Rosehart et al. 2002). Neither has any correlation between genotype and geography been found in another, study using a different collection of *A. fumigatus* individuals and different genetic markers (Rydholm et al. 2003). All other fungi postulated to have global, hemispherical, or even continental populations have proved to have more narrowly endemic populations when population genetic data were collected (e.g., Koufopanou et al. 1997; Geiser et al. 1998a; Forche et al. 1999; James et al. 1999; Schonian et al. 2000; Carbone and Kohn 2001a,b; Fisher et al. 2001; Kasuga et al. 2003; Tran-Dinh 2003). Studies of animal species with large geographic ranges have also elucidated endemic populations. For example, analyses of multiple nuclear loci in the cosmopolitan and human-associated fly, *Drosophila ananassae*, show that this species embraces central populations in Southeast Asia, from which peripheral populations emerged in parallel with human migration (Das et al. 2004). Two studies of insects show that dispersal and subsequent geographic isolation can lead to population differentiation in surprisingly short times, even in the absence of severe population bottlenecks. Parasitoid wasps imported to North America from Europe for biological control of pea aphids fewer than 50 years ago are now differentiated from European populations (Hufbauer et al. 2004), and Caribbean island populations of *Drosophila* established just under 100,000 years ago show genetic isolation expected of distinct species, but still share significant ancestral polymorphism (Wilder and Hollocher 2003). The short historical and geological times over which populations can become differentiated makes it even more remarkable that *A. fumigatus* maintains a global population.

Our finding for *A. fumigatus* would seem to support both the historical view of fungi as cosmopolitan, and the persisting view that microbes (including bacteria and protists) possess broad, panmictic distributions (Finlay 2002; Fenchel and Finlay 2004). However, *A. fumigatus* is exceptional in this respect, and the studies of many other fungi showing endemism referenced above, as well as studies of prokaryotes (Whitaker et al. 2003; Papke and Ward 2004) refute these views. How does *A. fumigatus* maintain a global population when all other fungi examined to date are unable to do so?

The lack of a correlation between geographic and genetic distance must result from unrestricted gene flow over distance and time (Templeton 1998) caused by the unlimited dispersal of gametes or other propagules, that is, the classical explanation for the cosmopolitan distribution of microbes (Finlay 2002; Fenchel and Finlay 2004; Papke and Ward 2004). The spores of *A. fumigatus* are unusually small for a fungus (about 2–3  $\mu\text{m}$  in diameter) (Latge 1999) and perhaps this feature

facilitates global dispersal. However, the larger spores of other fungi also can travel long distances. For example, the relatively hefty spores of the plant pathogenic rust fungus *Puccinia graminis* fsp. *tritici* (urediniospores 26–40  $\mu\text{m}$   $\times$  16–22  $\mu\text{m}$ ; [Cummins 1971]) can travel from Southern Africa to Australia (Watson and De Sousa 1983), and the powdery mildew *Blumeria graminis* f.sp. *hordei* (conidia 24–35  $\mu\text{m}$   $\times$  12–16  $\mu\text{m}$ ; (Braun 1995)) can travel across the North Sea from Europe to the British Isles (Brown et al. 1991). In human pathogenic fungi of the genus *Coccidioides*, wind storms are known to spread spores from one population into the geographic ranges of other populations and species (Pappagianis 1988). How then do endemic species and populations of *Coccidioides* persist under these conditions? A likely explanation is that populations of the natural hosts of the fungi, small mammals, are strongly associated with geography and that the fungal populations are well adapted to their local hosts, that is, spores from one population impinge on the other, but they cannot displace the locally adapted individuals to become established in the new area.

What makes *A. fumigatus* unusual is not its ability to disperse spores, but its apparent lack of local adaptation. This may be due to the fact that *A. fumigatus* is not a parasite, but instead is isolated from decaying plant debris and soil and is a ubiquitous and cosmopolitan component of the mycota of compost. Hypotheses to explain the global population structure of *A. fumigatus* need to be challenged by ecological studies of the fungus to confirm or propose alternative mechanisms by which *A. fumigatus* “fumigatus” crosses barriers to dispersal. Experiments might also explore local adaptation in both the “fumigatus” and “occultum” clades. *Aspergillus* species are readily isolated from nature and straightforward to culture in the laboratory; this fungus is an excellent model for studies of microbial biodiversity and biogeography.

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