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Multilocus Sequence Typing of the Pathogenic Fungus *Aspergillus fumigatus*▽

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A multilocus sequence typing (MLST) scheme was devised for *Aspergillus fumigatus*. The system involved sequencing seven gene fragments and was applied to a panel of 100 isolates of *A. fumigatus* from diverse sources. Thirty different sequence types were found among the 100 isolates, and 93% of the isolates differed from the other isolates by only one allele sequence, forming a single clonal cluster as indicated by the eBURST algorithm. The discriminatory power of the MLST method was only 0.93. These results strongly indicate that *A. fumigatus* is a species of a relatively recent origin, with low levels of sequence dissimilarity. Typing methods based on variable numbers of tandem repeats offer higher levels of strain discrimination. Mating type data for the 100 isolates showed that 71 isolates were type *MAT1-2* and 29 isolates were *MAT1-1*.

Differentiation between strains of the same microbial species can be accomplished by multilocus sequence typing (MLST). This method compares nucleotide polymorphisms within regions of five to seven genes, traditionally housekeeping genes, which are under selective pressure to retain function. MLST was developed to facilitate studies of epidemiology and population structure in bacterial populations (32). Polymorphisms giving rise to allelic variants are recorded as bar codes of integers which together constitute a strain sequence type (ST). The digital nature of MLST makes it globally portable so that data can be compiled from multiple contributors. Active MLST schemes for several microorganisms are publicly available at http://www.mlst.net/, where sequence data for MLST alleles can be uploaded to ascertain allele genotypes and strain STs. Recently, MLST was used to investigate populations of human pathogenic fungi, including *Candida albicans* (7), *Candida glabrata* (16), *Candida tropicalis* (48), *Candida krusei* (22), *Cryptococcus neoformans* var. *gattii* (20), *Cryptococcus neoformans* var. *grubii* (30), *Histoplasma capsulatum* (23), and *Coccidioides immitis* (25).

Aspergillus fumigatus is a saprotrophic mold fungus commonly found in soil enriched with organic material (49). This fungus produces huge numbers of airborne spores, which are found ubiquitously in the environment, including the air column (15). Despite having its primary niche in organic material in soil, *A. fumigatus* has become a major pathogenic organism in humans, coincident with compromised or suppressed host immunity (6). In the immunocompromised host, inhaled spores can initiate serious invasive aspergillosis, a condition carrying a prognosis of at least 50% mortality even when antifungal drugs are administered (29). Vulnerable individuals cannot be completely protected from airborne spores; even HEPA-filtered air in bone marrow transplant units may contain four *Aspergillus* spores per m^3, which may be brought in by the patient or staff (28). Ascertaining relationships between the epidemiology of aspergillosis and the population structure of *A. fumigatus* is therefore desirable, and attempts have been made using a variety of genotyping methods, although with little success in discerning population structure within the species (52).

A search of the incompletely sequenced *A. fumigatus* genome revealed some of the genetic elements that may permit a sexual cycle (38). The sexual genetic elements in *A. fumigatus* constitute a putative heterothallic system. The original genome-sequencing strain AF293 has the high-mobility-group domain protein at the *MAT* locus (36). A survey of 290 isolates revealed distributions of strains comprising either the high-mobility-group protein (*MAT1-2*) or the complementary alpha box domain protein (*MAT1-1*) at the *MAT* locus at either 57% or 43%, respectively (37). In the present study, we describe the development of a seven-locus MLST scheme for *A. fumigatus* and discuss the findings from MLST analysis of 100 clinical and environmental isolates. In addition, we have determined the distribution of sexual idiomorphs among our collection. The results show a low level of sequence variation between most isolates of *A. fumigatus*, suggesting a relatively recent evolutionary origin for the species.

MATERIALS AND METHODS

Isolates. The 100 isolates of *A. fumigatus* used in this study were sourced from both clinical and environmental settings (Table 1). They were taken from our own stock collection and provided by the kindness of collaborators. Each isolate originated from a separate source except for IHEM20373, which was obtained from a culture collection but which was found, after it had been typed, to be from the same source as J960330. The isolate originally received as TX2684 was found to consist of colonies of opposite mating types, which were typed separately by MLST as TX2684A and TX2684B. For four isolates, no information concerning the origins was known. Among the remaining 96, 46 came from the United Kingdom, 19 from the United States, 17 from Australia, 8 from France, and 2 each from Belgium, Canada, and The Netherlands (Table 1). Fifty-six isolates had been originally cultured since 2000, and 36 were cultured in the 1990s, 1 in the 1980s, and 2 in the 1970s. Eighteen isolates had previously been typed by PCR of genes encoding the intergenic spacer regions of the large and small

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TABLE 1. List of 100 *A. fumigatus* isolates typed by MLST

Strain	Geographical source	Other details[a]	Decade isolated	ST	UPGMA subgroup	Mating type
81/001	United Kingdom		1980	1	B	*MAT1-1*
AM2002/0066	United Kingdom	Sputum; patient with CF	2000	2	A	*MAT1-2*
TX01-2819	United States		2000	2	A	*MAT1-2*
WC04-200434	Australia	Sputum isolate	2000	3	A	*MAT1-2*
J931199	France		1990	3	A	*MAT1-2*
J932614	France		1990	3	A	*MAT1-2*
WC04-200492	Australia	BAL isolate	2000	4	A	*MAT1-2*
WC04-201352	Australia	Sputum isolate	2000	4	A	*MAT1-2*
AF23	United Kingdom	Environment BMT patient; DNA type H (41)	1990	5	A	*MAT1-1*
AF22	United Kingdom	Environment BMT patient; DNA type H (41)	1990	5	A	*MAT1-1*
J940362	Belgium		1990	5	A	*MAT1-1*
AM2004/0051	United Kingdom	BAL isolate	2000	5	A	*MAT1-1*
WC04-201446	Australia	Rhinitis isolate	2000	5	A	*MAT1-1*
TX01-2725	United States		2000	5	A	*MAT1-1*
WC04-202908	Australia	Isolate from mitral valve tissue	2000	5	A	*MAT1-2*
MGR01-2507	United States		2000	5	A	*MAT1-2*
MGR01-2705	United States		2000	5	A	*MAT1-2*
AM2004/0044	United Kingdom	Sputum; patient with CF	2000	5	A	*MAT1-2*
AM2004/0049	United Kingdom	Sputum; patient with CF	2000	5	A	*MAT1-2*
AM2004/0055	United Kingdom	Sputum; patient with CF	2000	5	A	*MAT1-2*
WC04-201208	Australia	Sputum isolate	2000	5	A	*MAT1-2*
WC03-202715	Australia	BAL isolate	2000	5	A	*MAT1-2*
WC02-202431	Australia	BAL isolate	2000	5	A	*MAT1-2*
J980659/3	Belgium	Isolated from a toenail sample	1990	5	A	*MAT1-2*
TX01-2837	United States		2000	5	A	*MAT1-2*
IHEM20452	Canada	Sputum isolate	1990	5	A	*MAT1-2*
TX01-2841	United States		2000	6	B	*MAT1-2*
AM2004/0054	United Kingdom	Isolated from an ear swab	2000	7	A	*MAT1-1*
TX01-2855	United States		2000	7	A	*MAT1-1*
AF04	United Kingdom	Environment BMT patient; DNA type F (41)	1990	7	A	*MAT1-2*
AF11	United Kingdom	Patient 3; nasal swab; DNA type H (41)	1990	7	A	*MAT1-2*
AF16	United Kingdom	Patient 6; pericardial fluid; DNA type H (41)	1990	7	A	*MAT1-2*
AF02	United Kingdom	Environmental isolate; DNA type F (41)	1990	7	A	*MAT1-2*
AF12	United Kingdom	Patient 1; nasal swab; DNA type H (41)	1990	7	A	*MAT1-2*
WC02-202494	Australia		2000	7	A	*MAT1-2*
AF06	United Kingdom	Environmental isolate; DNA type F (41)	1990	7	A	*MAT1-2*
AM2002/0063	United Kingdom	Isolated from aspergilloma	2000	7	A	*MAT1-2*
AM2002/0065	United Kingdom	Sputum, patient with ABPA	2000	7	A	*MAT1-2*
AF05	United Kingdom	Environmental isolate; DNA type F (41)	1990	7	A	*MAT1-2*
AF07	United Kingdom	Environment BMT patient; DNA type F (41)	1990	8		*MAT1-1*
AF70	United Kingdom	Environmental isolate; DNA type A (41)	1990	8		*MAT1-1*
K4437				8		*MAT1-2*
AF19	United Kingdom	Environmental isolate; DNA type H (41)	1990	9	B	*MAT1-1*
AF69	United Kingdom	Environmental isolate; DNA type A (41)	1990	9	B	*MAT1-1*
AM2004/0056	United Kingdom	Sputum isolate	2000	9	B	*MAT1-1*
J941369	France		1990	9	B	*MAT1-1*
AF60	United Kingdom	Environmental isolate; DNA type A (41)	1990	9	B	*MAT1-2*
AM2002/0067	United Kingdom	Patient with IPA	2000	9	B	*MAT1-2*
AM2004/0043	United Kingdom	Sputum isolate	2000	9	B	*MAT1-2*
R3317	United States			9	B	*MAT1-2*
AF20	United Kingdom	Environmental isolate; DNA type H (41)	1990	10	B	*MAT1-1*
74/037	United Kingdom	Laboratory contaminant	1970	11	A	*MAT1-1*
AF71	United Kingdom	Environmental isolate; DNA type A (41)	1990	11	A	*MAT1-2*
AM2004/0048	United Kingdom	Sputum; patient with CF	2000	11	A	*MAT1-2*
WC04-202367	Australia	BAL isolate	2000	11	A	*MAT1-2*
J960158	France		1990	11	A	*MAT1-2*
AF78	United Kingdom	Environmental isolate; DNA type E (41)	1990	12	B	*MAT1-2*
AM2004/0053	United Kingdom	Isolated from a throat swab	2000	12	B	*MAT1-2*
AF21	United Kingdom	Environmental isolate; DNA type H (41)	1990	13	A	*MAT1-1*
73/063	United Kingdom	Isolated from a lymph node; postmortem	1970	14	B	*MAT1-1*
MCR01-2529	United States		2000	14	B	*MAT1-1*
MGR01-2556	United States		2000	14	B	*MAT1-1*
MGR02-40	United States		2000	14	B	*MAT1-2*
AM2004/0052	United Kingdom		2000	14	B	*MAT1-2*
TX01-2684B	United States		2000	14	B	*MAT1-2*
TX01-2711	United States		2000	14	B	*MAT1-2*
J950637	France		1990	14	B	*MAT1-2*

Continued on following page

TABLE 1—*Continued*

Strain	Geographical source	Other details[a]	Decade isolated	ST	UPGMA subgroup	Mating type
AM2002/0062	United Kingdom	Sputum; patient with ABPA	2000	15	A	*MAT1-2*
J960330	United Kingdom	NCPF7099; patient with invasive aspergillosis	1990	16	A	*MAT1-2*
WC02-203746	Australia	Bronchial washing isolate	2000	16	A	*MAT1-2*
TX01-2748	United States		2000	16	A	*MAT1-2*
IHEM20373	United Kingdom	Originally J960330 but obtained from different culture collection	1990	16	A	*MAT1-2*
J970377	Netherlands		1990	17		*MAT1-2*
J970466	Netherlands		1990	18		*MAT1-2*
MGR01-2659	United States		2000	19	B	*MAT1-2*
WC04-201218	Australia	Isolated from mitral valve tissue	2000	19	B	*MAT1-2*
WC02-200231	Australia	Isolated from leg tissue	2000	20	A	*MAT1-1*
J960181	United Kingdom	AF90 (13)	1990	20	A	*MAT1-1*
J960180	United Kingdom	AF91 (13)	1990	20	A	*MAT1-1*
AM2004/0040	United Kingdom	Sputum isolate	2000	20	A	*MAT1-2*
AM2004/0042	United Kingdom	Sputum isolate	2000	20	A	*MAT1-2*
AM2004/0047	United Kingdom	Sputum; patient with CF	2000	20	A	*MAT1-2*
TX01-2898	United States		2000	20	A	*MAT1-2*
AM2004/0045	United Kingdom	Cough swab; patient with CF	2000	21	B	*MAT1-2*
AM2004/0046	United Kingdom		2000	22		*MAT1-2*
AM2004/0057	United Kingdom	Isolated from an ear swab	2000	23	B	*MAT1-2*
WC04-202056	Australia	Isolated from right maxillary tissue	2000	24	A	*MAT1-2*
WC03-202903	Australia		2000	24	A	*MAT1-2*
WC04-201263	Australia		2000	24	A	*MAT1-2*
WC04-201640	Australia	Sputum isolate	2000	24	A	*MAT1-2*
J941185	France		1990	24	A	*MAT1-2*
TX01-2825	United States		2000	25	A	*MAT1-1*
J941218	France		1990	25	A	*MAT1-1*
IHEM20368	France		1990	25	A	*MAT1-1*
IHEM20453	Canada	BAL isolate	1990	26	B	*MAT1-1*
TX01-2656	United States		2000	26	B	*MAT1-2*
AF05-55308			2000	27	B	*MAT1-2*
B42928				28	B	*MAT1-2*
B64620				29	A	*MAT1-2*
TX01-2684A	United States			30	A	*MAT1-1*

[a] ABPA, allergic bronchopulmonary aspergillosis; BAL, bronchoalveolar lavage; BMT, bone marrow transplantation; CF, cystic fibrosis; IPA, invasive pulmonary aspergillosis.

ribosomal subunits (41). The fungi were stored at −80°C as spore suspensions in YPG-glycerol (1:1) (1% yeast extract [Difco, Sparks, MD], 2% Bacto peptone [Difco], 2% glucose [Fisher, Loughborough, United Kingdom], 50% glycerol [Fisher]) and then streaked onto Sabouraud agar and grown at 35°C until conidia formed.

The identity of the isolates as *A. fumigatus* and not a closely related species, such as *Aspergillus lentulus* or *Aspergillus udagawae*, was confirmed by PCR and StyI restriction digestion as described elsewhere (3).

DNA extraction. Mycelia were grown from conidial inocula in 20 ml of liquid YPG with shaking at 200 rpm at 30°C for 3 days. The mycelia were strained to remove the supernatant, and then 0.3-g glass beads (0.45- to 0.52-mm diameters; Sigma, St. Louis, MO), 300 μl extraction buffer (100 mM Tris-HCl, pH 8, 100 mM NaCl, 2% Triton X-100, 1% sodium dodecyl sulfate, 1 mM EDTA), and 300 μl phenol-chloroform (1:1) were added. The mycelia were disrupted by vortexing for 30 min. To the lysate, 200 μl TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) was added, and insoluble debris was removed in a bench-top centrifuge run at full speed for 5 min. The DNA in the aqueous phase was extracted a second time by adding an equal volume of phenol-chloroform (1:1), vortexing it for 30 s, and repeating the centrifugation step. Nucleic acid from the aqueous phase was precipitated with 1 ml ethanol and pelleted as before. The pellet was resuspended in TE containing 250 μg ml^{-1} RNase (Sigma) and incubated for 30 min at 37°C. DNA was precipitated with 1 ml isopropanol and 10 μl 3 M sodium acetate. Following centrifugation for 10 min, DNA pellets were dried and resuspended in 50 μl water.

Typing of sexual idiomorphs. *MAT1-1* and *MAT1-2* sexual idiomorphs were differentiated by PCR as described previously (37). Briefly, a multiplex PCR was performed with reaction volumes of 25 μl containing 1 μl of genomic DNA as the template, 5 μl 5× GoTaq buffer (Promega, Southampton, United Kingdom), 2 mM $MgCl_2$ (Promega), 200 μM deoxynucleoside triphosphate mix (Invitrogen, Paisley, United Kingdom), and 2.5 units GoTaq DNA polymerase (Promega). The reaction mixture contained 0.64 μM of reverse primer AFM3 (5′-CGGAA ATCTGATGTCGCCACG-3′), which is common to both idiomorphs, and 0.32 μM each of forward primer AFM1, which is specific to *MAT-1* (5′-CCTTGAC GCGATGGGGTGG-3′), and forward primer AFM2, which is specific to *MAT-2* (5′-CGCTCCTCATCAGAACAACTCG-3′). PCR was performed with a thermocycler (TC-412; Techne, Cambridge, United Kingdom) programmed as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final elongation step at 72°C for 5 min.

Selection of gene fragments for MLST scheme. A selection of 27 *A. fumigatus* genes from 0.7 to 3.3 kb was sequenced across a set of 12 isolates chosen to maximize the diversities of geographical sources, anatomical properties, and dates of isolation and therefore increase the likelihood of representation of diverse genotypes. Primers for PCR amplification and sequencing of the seven fragments were designed from sequences taken from GenBank (http://www.ncbi.nlm.nih.gov/) and *A. fumigatus* GeneDB (http://www.genedb.org/genedb/asp/). Results from these pilot sequences were used to determine a final panel of seven gene fragments that gave the highest discrimination for MLST analysis (Table 2). In accordance with feasible automated-sequencing run length, fragments of up to 590 bp containing the most discriminatory single-nucleotide polymorphisms (SNPs) were identified.

Sequencing of MLST fragments. Gene fragments were amplified by PCRs in 96-well microdilution plates to amplify gene fragments of <1 kb with the primers listed in Table 2. Reaction volumes of 50 μl contained 2 μl of genomic DNA as a template, 10 μl 5× Green GoTaq buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 100 μM deoxynucleoside triphosphate mix (Invitrogen), 0.2 μM each of forward and reverse primers (Invitrogen), and 5 U GoTaq DNA polymerase (Promega). PCR was performed with a TC-412 thermocycler (Techne, Cambridge, United Kingdom), with a cycle program of 94°C for 5 min; 35 cycles of 94°C for 1 min,

TABLE 2. Gene fragments and primers used for MLST

Gene fragment	Gene product	GenBank accession no.	Primers[b]	Amplicon size (bp)	Sequencing result		Size of sequenced fragment (bp)
					5′	3′	
ANXC4	Annexin	AY598940	F, 5′-GCGAGATAGCAACACTTCAGT-3′ R, 5′-GGATACTGTTGCCCTAGATTTG-3′	729	GGTCGCCA	GGCTTCGT	400
BGT1	Beta-1,3-glucanosyl transferase	AF38596	F, 5′-GATCGGTTGCCAGTCTTTGA-3′ R, 5′-AATGGACGCAGAATGAAACT-3′	811	GTCTATTC	AAACGAGG	500
CAT1	Catalase	XM_743457	F, 5′-AGCTCAACCGTCGTGAT-3′ R, 5′-TGCCATGCCCAGACATA-3′	754	GACGTCCG	ACTCGGCC	590
LIP	Lipase	AfA24A6.090c[a]	F, 5′-CGCCTCACTTCTCCTCA-3′ R, 5′-TGCGAAATGGCTGACG-3′	802	GGTTTGGC	CGCGAAGG	503
MAT1-2	Mating type protein	XM_746497	F, 5′-ACACCCTTCGACTTTCCA-3′ R, 5′-GATCGCATAGTGTGAGTGG-3′	360	CTCCCAAG	CGGGCTGC	135
SODB	Superoxide dismutase	XM_746579	F, 5′-GCTCCAAGAGCTGCTAC-3′ R, 5′-AAGCGCTTCTCCACAGC-3′	673	GATTATGG	TGACGCCT	510
ZRF2	Zinc transporter	AY611772	F, 5′-CTCATCCAAGCTTGTTTCC-3′ R, 5′-GTACCGCATCACCATCAA-3′	639	GCCTTGAT	TTCGTTGC	400

[a] The sequence of *LIP* is not deposited in GenBank but can be found in the *A. fumigatus* GeneDB (http://www.genedb.org/genedb/asp/index.jsp).
[b] F, forward primer; R, reverse primer.

50°C for 1 min, and 68°C for 1 min; and a final elongation step at 68°C for 10 min. Amplified DNA fragments were purified by combining PCR mixtures with 60 μl of 20% polyethylene glycol 8000 (Sigma) and 2.5 M NaCl solution for 30 min at room temperature and then centrifuging 96-well plates at 2,254 × *g* for 60 min. The plates were inverted onto blotting paper to remove the supernatant. The DNA pellets were washed with 150 μl 70% ethanol and centrifuged for 10 min, and the supernatant was removed by blotting. Plates inverted on tissue paper were centrifuged for 1 min at 500 × *g* to remove residual ethanol. DNA was resuspended in 50 μl distilled H_2O for use as template DNA for subsequent sequencing reactions. Reaction volumes of 5 μl were set up in 96-well plates and contained 1 μl of purified gene fragment, 2 μl of 0.67 μM forward primer, 0.25 μl Big Dye (Applied Biosystems, Warrington, United Kingdom), 0.875 μl 5× buffer (Applied Biosystems), and 0.875 μl distilled H_2O. Reaction mixtures were prepared as described above with the reverse primer. Sequencing PCR was performed with 25 cycles of 96°C for 10 s, 52°C for 5 s, and 60°C for 2 min. DNA was purified by addition of 50 μl of a 250:1 solution of ethanol-sodium acetate and incubation at room temperature for 45 min. DNA was pelleted and washed in 96-well plates as described above. Automated DNA sequencing was performed at the Department of Zoology, University of Oxford, United Kingdom. Forward and reverse DNA sequence chromatograms were analyzed with DNASTAR software to identify interstrain SNPs.

Statistical analysis of MLST data. MLST sequence data were analyzed to determine relationships between the 100 strains. Concatenated SNPs for all MLST alleles of each strain were input with MEGA 3.0 software (26) to generate an unweighted-pair group method with arithmetic averages (UPGMA) dendrogram based on *p*-distance with 1,000 bootstrap replications. eBURST version 3 (http://eburst.mlst.net/) was used to ascertain clonal cluster relationships between isolates on the basis of single allele differences, regardless of numbers of SNP differences between alleles.

RESULTS

***A. fumigatus* strain differentiation by MLST.** A total of 27 gene fragments was sequenced for a set of 12 isolates, expected to be genetically diverse, to determine an optimal panel of gene fragments for MLST. Initially, housekeeping genes were considered potential MLST fragment candidates. These are defined as genes for which the ratio of nonsynonymous/synonymous amino acid changes resulting from SNPs is <1. Traditionally, only such genes were used in bacterial schemes when MLST was first devised (32). *A. fumigatus* housekeeping genes showed such low interstrain sequence variability that we eventually included nonhousekeeping genes to obtain a higher frequency of interisolate SNPs. The 20 genes that were considered but not chosen for the final selection were *AREA*, *CALN*, *CSN*, *CPCA*, *CRNA*, *FACC*, *HIS3*, *HSP70*, *LEU2*, *MAN70*, *MANA*, *NIAD*, *NIIA*, *PEP*, *PREA*, *PREB*, *SOK*, *STEA*, *STUA*, and *TRPC*.

Seven MLST fragments that represented the minimum set needed to differentiate the panel of 12 isolates were selected (Table 2). This MLST scheme was used to type 100 *A. fumigatus* isolates, including both clinical and environmental specimens, from our culture collection (Table 1). The polymorphic sites in the MLST fragments for *A. fumigatus* are shown in Table 3. Details of the MLST system can be found at http://pubmlst.org/afumigatus/. Among the 100 strains analyzed, we distinguished 30 STs (Table 1), giving our scheme a discriminatory index of 0.93 (21). ST 5 was the most common (Table 1), with 18 isolates, followed by ST 7 (12 isolates), ST 14 (8

TABLE 3. Numbers of polymorphic sites within MLST fragments and noncoding sequences and ratios of nonsynonymous to synonymous and nontrivial to trivial amino substitutions resulting from nucleotide changes at polymorphic sites

MLST fragment	No. of polymorphisms		Ratio of amino acid substitutions	
	Total	In noncoding sequences	Nonsynonymous to synonymous	Nontrivial to trivial[a]
ANXC4	8	0	3	0.6
BGT1	7	1	2	0.5
CAT1	8	3	0.67	0.25
LIP	8	0	1.67	0.33
MAT1-2	3	0	3	2
SODB	3	0	0	0
ZRF2	4	0	4	1

[a] A trivial substitution is defined as one that does not alter side chain polarity or acidity/basicity; a nontrivial substitution involves a change of one or both properties.

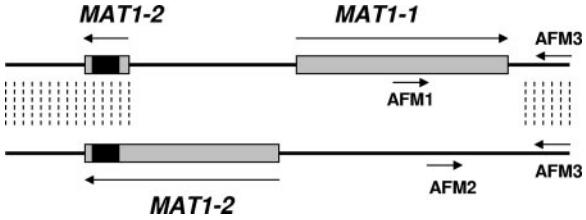

FIG. 1. The *MAT* locus in *A. fumigatus*. Both sexual idiomorphs are shown aligned together as described by Paoletti et al. (37). The upper sequence has only the 3′ portion of the *MAT1-2* ORF and the complete *MAT1-1* ORF. The lower sequence has the complete *MAT1-2* ORF. Dashed lines indicate areas of sequence homology between both idiomorphs. Primers AFM1, AFM2, and AFM3, used for the multiplex PCR to distinguish the sexual idiomorph genotypes, are indicated. The black box indicates the location of MLST fragment *MAT1-2*.

isolates), and ST 20 (7 isolates). For 19 STs, only one or two examples each were found among the isolates tested (Table 1).

Polymorphic sites were found predominantly in coding sequences; however, some were found in introns that were discovered by comparison of our direct sequence data with cDNA sequences in GenBank (Table 3). The *BGT1* fragment used for MLST contained a 71-bp intron without polymorphic sites. The *CAT1* fragment contained two introns of 66 bp and 105 bp, which included, respectively, one and two of the eight SNPs for this gene. One SNP was upstream of the start codon in the *BGT1* fragment (Table 3). MLST can be based on coding and noncoding sequences because the technology relies upon the digital information of genomic DNA sequences and not expressional information. The amino acid substitutions for polymorphisms within the coding sequence were determined. The ratio of nonsynonymous to synonymous amino acid substitutions was determined for each MLST fragment (Table 3) and found to be >1 for five of the seven genes. Therefore, to determine the functionally relevant substitutions, the ratio of substitutions that maintained amino acid side chain polarity and acidity/basicity to those that did not was also calculated. These ratios were <1 for five gene fragments, which we consider acceptably low (Table 3).

Determination of distribution of sexual idiomorphs *MAT1-1* and *MAT1-2*. In addition to applying the MLST scheme to genotype our *A. fumigatus* collection, we determined the mating type for each strain, using a multiplex PCR with primers AFM1, AFM2, and AFM3 (Fig. 1), as described previously (37). The locus-specific amplicon of the sexual idiomorph *MAT1-1* is 834 bp, and that of *MAT1-2* is 438 bp. It should be noted that the *MAT1-2* fragment sequenced in the MLST scheme can be amplified from all *A. fumigatus* isolates because both *MAT* loci carry the 3′ *MAT1-2* coding region, within which lies the 135-bp MLST fragment (Fig. 1). The position of the MLST *MAT1-2* fragment is from nucleotides +1106 to +1240 of *MAT1-2*. Of the 100 *A. fumigatus* strains that we tested, 29% were sexual idiomorph *MAT1-1* and 71% were *MAT1-2*, a ratio of approximately 1:2. There was no association between mating type and ST, since both mating types were represented among the most populous STs.

***A. fumigatus* phylogenetics.** An eBURST analysis of the MLST data, which constructs a tree of STs joined to indicate pairs that differ in only one of the seven alleles sequenced, is shown in Fig. 2. All but 5 of the 30 STs formed a single clonal

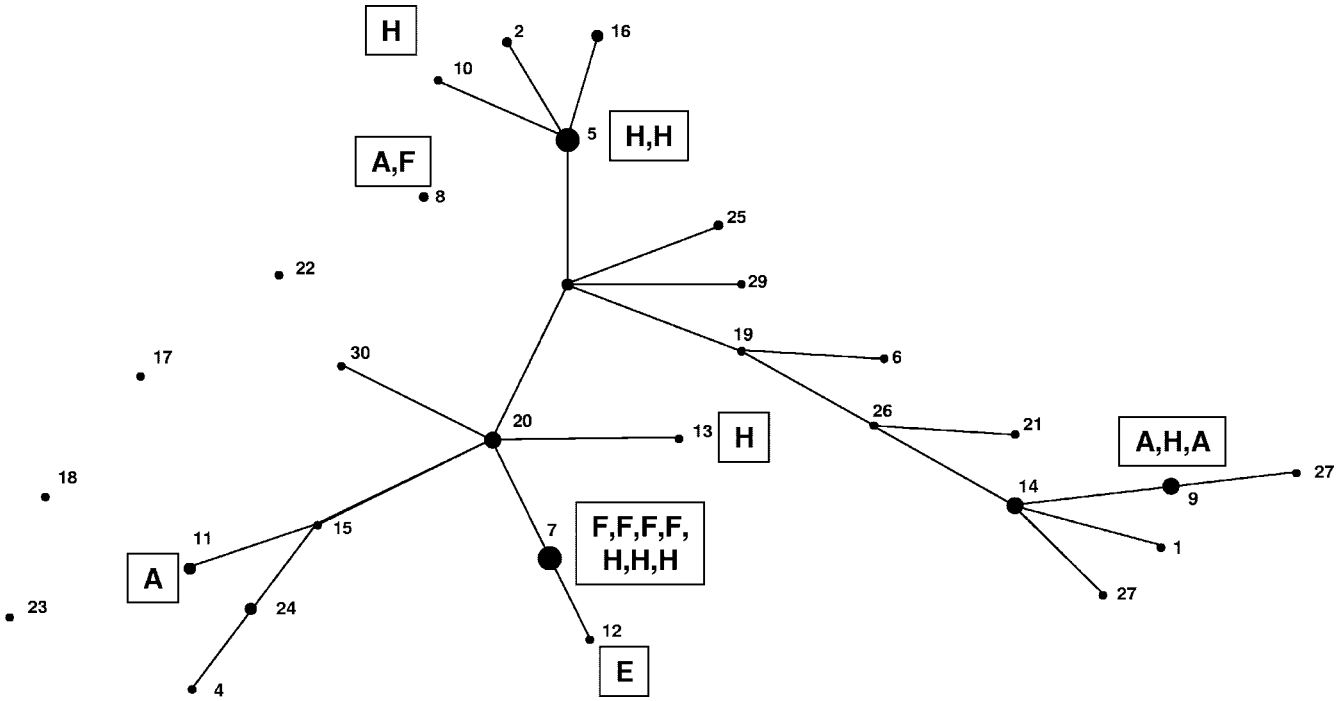

FIG. 2. Clonal cluster and five single STs generated by eBURST analysis of MLST data, representing all 30 STs found from 100 *A. fumigatus* isolates. The putative founder of the complex is ST 20. The diameter of each circle represents the number of members within each ST group. Connecting lines are of arbitrary lengths but indicate that STs linked by a single line are different only at one allele out of seven. The boxed letters indicate genotypes assigned in a previous study (39).

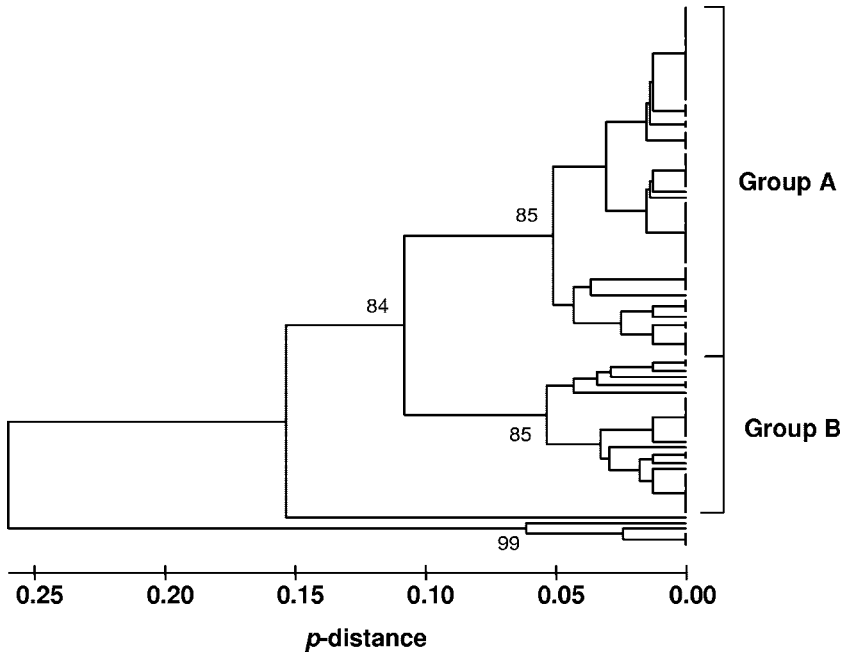

FIG. 3. UPGMA dendrogram for 100 *A. fumigatus* isolates sequenced for MLST. The large cluster, which contained 94% of the isolates, was subdivided into two groups (indicated at the right) based on the high bootstrap values at the cluster nodes (numbers shown on the dendrogram).

cluster of related isolates, with ST 20 as the putative founding isolate, according to eBURST. Figure 2 shows the genotype assignments of the 18 isolates that had been typed in a previous study on the basis of intergenic spacer regions in ribosome-encoding DNA (41). It is obvious that no relation could be found between the positions of STs on the branches of the eBURST clonal cluster and previously determined genotypes.

A UPGMA dendrogram of the MLST data, based on *p*-distance (Fig. 3), separated four of the five isolates that were singletons in the eBURST snapshot (Fig. 2) as distant relatives of the rest. The UPGMA dendrogram divided the isolates along lines similar to those for eBURST, but with ST 23, the fifth eBURST singleton, included with the main cluster in this analysis (Fig. 3). Isolates with STs 8, 17, 18, and 22 remained well distanced from other isolates. The main set of isolates could be differentiated into two subgroups by UPGMA, with strong bootstrap support (Fig. 3). The subgroup assignments of the 100 isolates are listed in Table 1, together with other genotype details. No significant association was found between the subgroups and the geographic origins or mating types of the isolates.

DISCUSSION

The publication of the *A. fumigatus* genome sequence (36) is a driver for molecular genetic studies and has already helped correct taxonomic assignments based on phenotypic traits. Phylogenetic analysis revealed a sibling species of *A. fumigatus*, designated *A. lentulus*, in a study that compared sequences at five loci in slow-sporulating variants with those of Af293 (2), the *A. fumigatus* strain used for whole-genome sequencing (36). Sequence comparison based on part of the β-tubulin gene and 18S rRNA revealed that phenotypically atypical strains previously thought to be *A. fumigatus* may be a separate, more recently evolved species (24). Cryptic speciation within *A. fumigatus* was revealed following a microsatellite analysis of 63 isolates (39). In a recent study, misidentified *A. fumigatus* isolates were reclassified as *A. lentulus* and *A. udagawae* on the basis of restriction fragment length polymorphisms (RFLP) (3). None of our 100 isolates was slow to form conidia, the main phenotypic differentiator for *A. lentulus* and *A. udagawae*, and PCR/StyI testing confirmed that the isolates were *A. fumigatus*. In our experience with MLST analysis of many fungal species, we have never encountered examples in which isolates of another species gave identical PCR products with all the MLST genes when set up for sequencing reactions. This information therefore serves as a double check on species identity.

Comparison of nucleotide sequences of organisms is the most unequivocal method by which strains of any microorganism can be differentiated (50). We therefore devised a seven-gene MLST scheme to genotype *A. fumigatus*. Housekeeping genes possessed very low numbers of polymorphisms in this species and therefore were not useful for designing an MLST scheme. The infrequency with which we found polymorphisms within many genes scattered throughout the genome is a general indicator that *A. fumigatus* has low interstrain variation in its genome, suggesting recent evolution relative to that of other fungal species. MLST is, in effect, the successor to multilocus enzyme electrophoresis (MLEE). MLEE has previously been applied to *A. fumigatus*. Rodriguez et al. (42) found 48 electrophoretic types from 91 isolates tested with 12 polymorphic loci. Bertout et al. (5) differentiated eight electrophoretic types from 50 isolates based on seven loci. These MLEE results therefore support our finding from MLST that the level of interstrain variation for coding regions of genes is low. Sequence-specific DNA primer analysis distinguished 22 geno-

types from 51 isolates, with a discriminatory power of 0.96 (34), and another sequence-specific DNA primer study found 19 genotypes in 81 isolates (46). Once again, typing systems based on sequence differentials failed to show high discriminatory power with *A. fumigatus*.

The total number of polymorphic sites from all seven MLST fragments in our system was 41 (Table 3); therefore, 1.35% of the 3,038 nucleotides from seven MLST fragments have polymorphisms. This "SNP return" is much lower than that for MLST with other pathogenic fungi, such as *C. albicans*, presently with 172 SNPs (6.0%) among the 2,883 bases sequenced; *C. tropicalis*, with 169 SNPs (6.3%) among the 2,677 bases sequenced; and *C. glabrata*, with 122 SNPs (3.7%) among the 3,345 bases sequenced (results from our own current databases for these species). The 1.35% SNP return for *A. fumigatus* is comparable with the polymorphic site rate of 1.6% reported in a study that compared three intergenic sequences in strains of *A. fumigatus* and the closely related taxa *Neosartorya fischeri* and *Neosartorya spinosa*, both of which fall within the subgenus *Fumigati* subgroup *Fumigati* (44). Normally, there is an expectation that intergenic sequences should have greater variability than the coding regions which constitute the majority of our MLST scheme. Rydholm et al. (44) found that the two *Neosartorya* species had greater interstrain variation than *A. fumigatus*. The consistently low typeability of *A. fumigatus* based on DNA sequences of coding and intergenic regions, combined with the observation that 93% of the isolates in our panel fell into a single clonal cluster by eBURST analysis (Fig. 2), reemphasizes the conclusion that *A. fumigatus* must be a recently evolved species and has therefore accumulated fewer mutations.

The fragments of approximately 500 bp used for MLST were chosen primarily because they contained the highest possible numbers of SNPs. They do not represent entire open reading frames (ORFs) and, in the present study, include introns and noncoding regions upstream of ORFs. For *CAT1*, Calera et al. previously described five introns in the ORF, with sizes of 66, 49, 85, 56, and 59 bp (from the 5′ to the 3′ end) (9). Our MLST fragment includes the first two of these introns, but the sizes were 66 and 105 bp, as deduced by comparison of GenBank mRNA data (sequence XM 743457) with our own sequence data. Similarly, for *BGT1*, the first of three introns previously described for this gene (35) was included in our MLST fragment, but comparison of the GenBank mRNA sequence (XM 747418) with our own data indicated an intron of 71 bp instead of the 42 bp originally reported (35).

Earlier approaches to *A. fumigatus* strain differentiation included immunoblot fingerprinting (8), RFLP typing (10), randomly amplified polymorphic DNA typing (31, 33, 40), and DNA fingerprinting by Southern blot analysis and probe hybridization (18). The discriminatory power of each of these approaches was superior to that of our MLST, but in none of the studies did the isolate population yield more than two-thirds of its number as different types. (MLST and its analogous approaches yielded different types amounting to approximately one-third of the number of isolates tested.) By comparison, *C. albicans* MLST generated 351 strain types from a panel of 416 isolates, so 84% of the test population emerged as different types. The discriminatory power of typing by amplified fragment length polymorphisms was not assessed (53), but the data suggest that this approach is at least as effective as RFLP or randomly amplified polymorphic DNA typing. By far, the most successful typing approach for *A. fumigatus* has been microsatellite, or short tandem repeat, analysis. This method has been used successfully by several investigators (4, 14, 19, 43, 51) and has been shown to have a discriminatory power of 0.99 and better (4, 14).

To achieve finer discrimination of *A. fumigatus* strains, some authors have used different methods in combination (1, 42, 45). However, applying multiple tests makes the task of typing *A. fumigatus* more laborious, and some typing methods may be compromised in their accuracy, for example, because interpretation of band sizes is open to error. The advantages of MLST as a typing approach are its portability (sequencing can be done anywhere, with identical results for identical DNA samples) and its archiveability (results can be stored in a central web database). Our study shows that MLST is a practical proposition for typing *A. fumigatus*, but for high-level strain discrimination, microsatellite typing is considerably more effective. This approach, like MLST, also offers portability and archiveability. However, its application to population genetic studies is limited by homoplasy arising from highly mutable sequences.

The *A. fumigatus* genome sequence project has revealed genetic elements for sexual behavior (36–38), opening the possibility that sexual recombination may occur. No observations of mating or meiosis have so far been made for *A. fumigatus*; therefore, this species will remain classified as an asexual haploid fungus for the time being. The *MAT1-2* idiomorph was more commonly found than the *MAT1-1* idiomorph (71% versus 29%) in our panel of isolates. Other studies also found *MAT1-2* to be the dominant mating type: 57% versus 43% of 290 isolates (37) and 55% versus 45% of 102 isolates (44). The predominance of the *MAT1-2* idiomorph may indicate a shift from the 1:1 ratio that is consistent with sexual reproduction. There is some evidence that recombination has occurred in the past for *A. fumigatus* (37); however, other studies (44) have found the same lack of variation between isolates from all parts of the world, indicating a predominantly clonal mode of reproduction. Compared with *Candida albicans*, also considered a largely clonal species, which generates 18 eBURST clonal clusters out of 416 isolates (47), there is very strong evidence for *A. fumigatus* both as a clonally reproducing organism and as a species of a relatively recent origin.

A major application of any microbial strain typing system is to elucidate the epidemiology of infection. The many clinical forms of aspergillosis result from inhalation of *A. fumigatus* from environmental sources, yet research attempting to make positive associations between environmental isolates and isolates infecting patients has not easily made such links, regardless of the typing method used. While some investigators have found examples of indistinguishable *A. fumigatus* isolates in some individual patients and their hospital environments (10, 19, 33), others have reported multiple strain types in the same patient and even the same sample (10, 11, 33). The best evidence for common types in infecting and environmental isolates comes more from statistical clustering of strain types (10, 17, 19, 53) than from unequivocal evidence of an indistinguishable strain type in a clinical sample and a sample, say, from the patient's ward. One study even drew the conclusion of a neg-

ative association between clinical and environmental isolates (43). By contrast, two studies found indistinguishable isolates in several patients, a situation compatible with cross-infection with a common strain type (12, 27); the finding is also more likely to arise in studies based on typing methods with low discriminatory power.

We cross referenced our own data with those from another study, because 18 isolates were genotyped in both studies (41). MLST could further resolve strains that had previously been classified as the same type, with up to seven allelic differences found (41). However, there was no cross-association between types determined by the two methods (Fig. 2). We have no explanation for why the results from ribosomal DNA typing do not match those from MLST.

MLST is a useful tool for differentiating isolates in an unambiguous manner. However, the inherent lack of sequence variability between *A. fumigatus* isolates probably restricts the value of MLST to preliminary screening in situations such as putative endemic outbreaks. High-level strain discrimination for *A. fumigatus* is better served by microsatellite typing.

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