Aspergillus fumigatus sp. nov., a New Sibling Species of A. fumigatus

S. Arunmozhi Balajee,1 Jennifer L. Grigsby,1 Edward Hanley,1 David Nickle,2 and Kieren A. Marr1,3,*

Program in Infectious Diseases, Fred Hutchinson Cancer Research Center,1 and Department of Microbiology2 and Department of Medicine,3 University of Washington, Seattle, Washington

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In a prior study, we identified seven clinical isolates of an Aspergillus sp. that were slow to sporulate in multiple media and demonstrated decreased in vitro susceptibilities to multiple antifungals, including amphotericin B, itraconazole, voriconazole, and caspofungin. These isolates were initially considered to be variants of Aspergillus fumigatus because of differences in mitochondrial cytochrome b sequences and unique randomly amplified polymorphic DNA PCR patterns (S. A. Balajee, M. Weaver, A. Imhof, J. Grigsby, and K. A. Marr, Antimicrob. Agents Chemother. 48: 1197–1203, 2004). The present study was performed to clarify the taxonomic status of these organisms by phylogenetic analyses based on multilocus sequence typing of five genes (the β-tubulin gene, the rodlet A gene, the salt-responsive gene, the mitochondrial cytochrome b gene, and the internal transcribed spacer regions). Results revealed that four of the seven variant isolates clustered together in a clade very distant from A. fumigatus and distinct from other members of the A. fumigatus group. This new clade, consisting of four members, was monophyletic with strong bootstrap support when the protein-encoding regions were analyzed, indicating a new species status under the phylogenetic species concept. Phenotype studies revealed that the variant isolate has smaller conidial heads with diminutive vesicles compared to A. fumigatus and is not able to survive at 48°C. Our findings suggest the presence of a previously unrecognized, potentially drug-resistant Aspergillus species that we designate A. lentulus.

Aspergillus fumigatus is identified microscopically by characteristic blue-green conidia borne on uniseriate conidiophores with subclavate vesicles. However, A. fumigatus can be morphologically variable and several new species have been defined within this group on the basis of minor phenotypic differences. Species designation based on morphological characteristics is controversial because morphology is largely dependent on growth conditions (8). Predictions based on phenotypic methods of fungal species recognition are now being challenged by phylogenetic methods, whereby comparisons of nucleotide sequences of multiple genes are used to predict species. Recognizing species on the basis of phylogenetic relationships has uncovered “cryptic” species within morphologically indistinguishable organisms such as in Coccidioides immitis and A. flavus (7, 13).

In a recent study, we identified several poorly sporulating variants of A. fumigatus as a cause of invasive infection in hematopoietic stem cell transplant recipients (1). All of these variants were initially identified as A. fumigatus by morphological characteristics, but they had distinct mitochondrial cytochrome b (mtcyt b) gene sequences and randomly amplified polymorphic DNA PCR patterns. These isolates were found to have low in vitro susceptibilities to multiple antifungal drugs, including amphotericin B (MICs, 1 to 2 μg/ml), itraconazole (MICs, 0.5 to 1 μg/ml), voriconazole (MICs, 4 μg/ml), and caspofungin (minimal effective concentrations, 4 to >32 μg/ml). As detailed in the prior report, patients from whom isolates were recovered all had poor clinical outcomes; all died with invasive aspergillosis despite receipt of antifungal therapy. In the present study, we evaluated the phylogenetic relationships of these variant isolates to A. fumigatus and related species by multilocus sequence typing (MLST). Results suggest the presence of a unique, previously unrecognized species that we designate A. lentulus. Morphological and phylogenetic characterization of this new species in the A. fumigatus series is presented.

MATERIALS AND METHODS

Isolates. Isolates FH1, FH4, FH5, FH6, FH7, FH219, FH220, and FH221 were all obtained from clinical specimens of patients hospitalized at the Fred Hutchinson Cancer Research Center, and isolates CDC40 and CDC25 were clinical isolates from the Centers for Disease Control and Prevention culture collection (provided by David Warnock). All were identified morphologically as A. fumigatus by the clinical microbiology laboratory at the University of Washington Medical Center and at the Centers for Disease Control and Prevention. Well-characterized A. fumigatus isolates AF293 and B5233 were provided by David Denning and June Kwon-Chung, respectively. A. fumigatus isolate AF293 was confirmed to be A. fumigatus Fresenius by the National Collection of Pathogenic Fungi (NCPF 7367) at the Mycology Reference Laboratory, Bristol, United Kingdom, and by the Centraal Bureau voor Schimmelcultures (CBS 101355). Baarn, The Netherlands (16). Neurospora fischeri (isolate 4075) and A. fumigatus var. ellipticus (isolate 5109) were provided by Stephen Peterson (U.S. Department of Agriculture).

MLST. For isolation of genomic DNA, 10° conidia were inoculated into Sabouraud dextrose broth incubated while shaking for 3 days at 37°C, and the hyphal mat was washed in phosphate-buffered saline. Cell suspensions were treated with lyticase (10 U/ml; Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C and then incubated in proteinase K for 1 h (10 μg/ml; Sigma) and in 0.5% sodium dodecyl sulfate (Sigma) for 2 h at 60°C. This suspension was subjected to three freeze-thaw cycles in liquid nitrogen, alternating with vortexing for 1 min with 0.2 g of 2-μm-diameter sterile glass beads (Sigma). Genomic DNA was isolated with the DNeasy tissue kit (QIAGEN 69704; Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions.

PCR primers were designed to amplify regions of the genes for rodlet A (rodA) and mtcytb and the internal transcribed spacer (ITS) regions of the rRNA gene of A. fumigatus (Table 1). PCR primers for β-tubulin were designed with the bendA gene sequence from A. flavus (GenBank accession no. M38265) to find the corresponding sequence in AF293 (www.tigr.org/db/v/afu1). The PCR prim-
ers for the salt-responsive gene (egr) were designed on the basis of the previously submitted A. fumigatus sequence (GenBank accession no. AF146225). PCR amplification of benA, rodA, egr, and ITS regions was performed with 2 to 4 μl (100 ng) of DNA as the template in a total reaction volume of 50 μl consisting of PCR buffer (20 μM Tris-HCl [pH 8.4], 50 mM KCl): 0.2 mM each dATP, dGTP, dCTP, and dTTP; 1.2 to 1.6 mM MgSO4; 0.2 pmol of (each) primer; 1 U of Taq polymerase (Invitrogen-BRL, Life Technologies, Carlsbad, Calif.); and 1× PEX enhancer (Invitrogen). For benA, rodA, egr, and ITS, 30 cycles of amplification were performed in a GeneAmp PCR system 9700 thermalcycler (PE- Applied Biosystems) after initial denaturation of DNA at 94°C for 3 min. Each of the thirty cycles consisted of a denaturation step of 94°C for 15 s, an annealing step of 55°C for 30 s, and an extension step of 68°C for 30 s, and the last cycle was followed by a final extension at 68°C for 3 min. Amplification conditions for the mtcy region consisted of an initial denaturation step of 2 min at 98°C, followed by 30 cycles of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C. A final extension of 8 min at 72°C followed the last amplification cycle. Products were visualized on a 1.5% agarose gel. The amplicons were purified with the QIAquick PCR purification kit (catalog no. 28104), and 100 ng of amplicon was mixed with 4 μl of dH2O, 0.5 μl of 10× buffer, 0.5 μl of 25 mM Mg2+, 0.2 μl of dNTP mix, 0.2 μl of each primer, and 0.2 μl of Taq polymerase (Invitrogen). The resultant nucleotide sequences were edited with the Sequencher™ program.

Phylogenetic analysis. Each set of homologous gene sequences was aligned with CLUSTALW (22) and then manually adjusted when needed with MacClade (15). For studying phylogenetic relationships, sequences of other, closely related, isolates in the Aspergillus section Fumigati were obtained from the GenBank database, when available (Table 2). A. clavatus, a member of section Clavati, was chosen as an outgroup as it had been previously shown to be distinct from Aspergillus section Fumigati by both morphological and phylogenetic analyses (6). As the egr sequence of A. clavatus was not available in the GenBank database, the N. fischeri sequence was included as an outgroup in this analysis instead. Likelihood ratio tests (10, 11) were used to derive a maximum-likelihood (ML) model of evolution that statistically fit the data while at the same time making the fewest assumptions about the evolution of the sequences themselves (18). Parameters derived from the best-fit model were then applied to the data sets to obtain ML trees with PAUP* (20). To arrive at an ML tree, we performed 10 random addition replicates with the most exhaustive branch-swapping algorithm, tree bisection-reconnection. Support for nodes was evaluated through bootstrap- ping with the neighbor-joining algorithm under the same ML model revealed by likelihood ratio tests (4). Bootstrap values were generated from 1,000 pseudoreplicates. Combined phylogeny was also performed from all of the five gene regions with ML and maximum-parsimony algorithms. For parsimony analysis of the combined data set, tree space was searched with the robust Branch and Bound algorithm in PAUP*4 to arrive at the most parsimonious tree. The genetic distances from A. lentulus to A. fumigatus and from A. fumigatus to N. fischeri were estimated in PAUP* with Model of Evolution as determined by the ML method. Statistical analyses were performed using the software package JMP (3.1 ed.; JMP Statistical Discovery Software, Cary, N.C.).

Growth studies. Growth characteristics (radial growth and conidiation) of the Aspergillus isolates were evaluated on Czapek dex agar (CZD; Becton Dickinson, Sparks, Md.) and malt extract agar (MEA; Becton Dickinson) as described elsewhere (1). Briefly, for radial growth determination, 10 μl of the conidial suspension (10⁵ conidia/ml) was placed at the center of each agar plate, the plates were incubated at 25 and 37°C, and the diameter of the growing colony was recorded every 24 h for 5 days. For enumeration of spores, 10 μl of each conidial suspension (10⁶ conidia/ml) was plated and incubated at 37°C for 5 days. After colony growth, a plug of agar (1 cm²) was removed and suspended in 1 ml of distilled water and the number of conidia per ml was calculated.

Growth of the variant isolates and A. fumigatus Af293 and B5233 was evaluated at a range of temperatures. In brief, 10 μl of the conidial suspension (10⁵ conidia/ml) was placed in the center of a CZD agar plate containing Aspergillus minimal agar medium (6.0 g of NaNO3, 0.52 g of KCl, 0.52 g of MgSO4·7H2O, 1.52 g of KH2PO4, 1 ml of trace elements [2.2 g of ZnSO4·7H2O, 1.1 g of H3BO3, 0.5 g of MnCl2·4H2O, 0.5 g of FeSO4·7H2O, 0.16 g of CuSO4·5H2O, 5H2O, (NH4)6Mo7O24·4H2O, and 5.0 g of Na2EDTA in 100 ml of distilled water], 10 g of glucose (pH 6.5), 15 g of agar/ liter of distilled H2O). Plates were incubated at 37, 45, or 48°C for 3 days, and the presence or absence of growth at the end of the incubation period was recorded.

Microscopy. Conidial heads of one variant isolate (FH5) and of A. fumigatus isolate Af293 were compared by differential interference contrast (DIC) microscopy and scanning electron microscopy (SEM). For SEM studies, Aspergillus isolates were grown on potato dextrose agar plugs at 37°C for 3 days. The agar plugs were fixed in half-strength Karnovsky’s fixative for 4 h, rinsed twice in 0.1 M cacodylate buffer (1:1). The plugs were then rinsed twice in 0.1 M cacodylate buffer over a period of 10 min and then passed through increasing concentrations of ethanol (35, 70, 95, and 100%) twice for 30-min periods. Pieces were cut from each sample and transferred to a critical-point dryer for a 20-min cycle. Samples were mounted on aluminum stubs and coated with 30-nm gold-palladium alloy. An Amray 1820D scanning electron microscope was operated at an accelerating potential of 25 kV to view the fungal structures.

Nucleotide sequence accession numbers. Sequences of the benA, rodA, egr, mtetyb, and ITS regions of the variant isolates (FH4, FH5, FH7, and FH220) have been submitted to GenBank and assigned the accession numbers listed in Table 2.

RESULTS

Phylogenetic analyses. Three of seven variant isolates (FH1, FH6, and FH219) and isolates FH221, CDC40, and CDC25 had benA, rodA, egr, and ITS region sequences that were identical to those of A. fumigatus Af293 (data not shown); hence, these were classified as A. fumigatus. Four of the seven variant isolates (FH4, FH5, FH7, and FH220) were distinct from A. fumigatus in all of the gene regions analyzed, as described below. The primers for the β-tubulin gene amplified a total of 471 nucleotides, with 284 nucleotides in introns and 187 nucleotides in exons. In variant isolates FH4, FH5, FH7, and FH220, 34 nucleotide changes occurred in the noncoding regions, yielding 91% nucleotide homology with A. fumigatus. There were four nucleotide changes that occurred as silent changes when the sequences were translated. The ML tree topology inferred from benA revealed that N. fischeri and N. neoelipticus clustered together with A. fumigatus; N. neoelipticus was genetically indistinguishable from A. fumigatus. The ML tree topology also showed that the variant isolates were a monophyletic group, having a most recent ancestor in common with

![Table 1. PCR primers used in this study](https://example.com/Table1.jpg)

| Primer | Target | Sequence | Reference |
|--------|--------|----------|-----------|
| ITS 1 | ITS1-5.8S-ITS2 | 5′-TCCTAGGTTGACCTGGG-3′ | 9 |
| ITS 4 | ITS1-5.8S-ITS2 | 5′-CTCCCGCTTTGATG-3′ | 9 |
| E1m | mtcyb | 5′-TGGATAGTCTAATATGAC-3′ | 26 |
| E2m | mtcyb | 5′-AACCTGCGGGCTTTG-3′ | 26 |
| βTub1 | β-Tubulin (fumA) | 5′-TCCGTAGGGACGGAATAG-3′ | This study |
| βTub2 | β-Tubulin (fumA) | 5′-GCTGGCCAATGTTGGGCCA-3′ | 6 |
| rodA1 | Rodlet A (rodA) | 5′-AGGTATTGCTGAAATTAGG-3′ | This study |
| rodA2 | Rodlet A (rodA) | 5′-AGGTACAGGGCAAGG-3′ | 6 |
| srg1 | Salt-responsive gene (egr) | 5′-ACGAGCTTTCTGAAATTAC-3′ | This study |
| srg2 | Salt-responsive gene (egr) | 5′-GGCCCCAATACCTGAAATTGAT-3′ | This study |
| Organism          | Gene      | Accession no. | Reference                  |
|-------------------|-----------|---------------|----------------------------|
| Aspergillus sp. strain FH5 | bend (β-tubulin) | AY738513 | This study                 |
|                   | rod4      | AY738514 | This study                 |
|                   | ITS       | AY738515 | This study                 |
|                   | mtcytb    | AY73516 | This study                 |
|                   | srg       | AY864050 | This study                 |
| Aspergillus sp. strain FH4 | bend (β-tubulin) | AY738517 | This study                 |
|                   | rod4      | AY738519 | This study                 |
|                   | ITS       | AY738518 | This study                 |
|                   | mtcytb    | AY73526 | This study                 |
|                   | srg       | AY864051 | This study                 |
| Aspergillus sp. strain FH7 | bend (β-tubulin) | AY738520 | This study                 |
|                   | rod4      | AY738522 | This study                 |
|                   | ITS       | AY738521 | This study                 |
|                   | mtcytb    | AY73527 | This study                 |
|                   | srg       | AY864052 | This study                 |
| Aspergillus sp. strain FH220 | bend (β-tubulin) | AY738523 | This study                 |
|                   | rod4      | AY738525 | This study                 |
|                   | ITS       | AY738524 | This study                 |
|                   | mtcytb    | AY73528 | This study                 |
|                   | srg       | AY864053 | This study                 |
| N. aureola        | bend4     | AF057319 | 6                          |
| N. stramenia      | bend4     | AF057330 | 6                          |
| N. pseudofischeri | bend4     | AF057325 | 6                          |
| A. viridinutans   | bend4     | AF057317 | 6                          |
| A. unilateralis   | bend4     | AF057316 | 6                          |
| A. duricaulis     | bend4     | AF057313 | 6                          |
| A. brevipes       | bend4     | AF057311 | 6                          |
| A. clavatus       | bend4     | AF057312 | 6                          |
| N. aurata         | bend4     | AF057318 | 6                          |
| N. tatenoi        | bend4     | AF132227 | 24                         |
| N. multiplicata    | bend4     | AF132228 | 24                         |
| N. hiratsukae     | bend4     | AF057324 | 6                          |
| N. glabra         | bend4     | AF132224 | 24                         |
| N. fennelliae a   | bend4     | AF057321 | 6                          |
| N. fennelliae A   | bend4     | AF057320 | 6                          |
| N. spathulata a   | bend4     | AF057326 | 6                          |
| N. spathulata A   | bend4     | AF057327 | 6                          |
| N. primulina      | bend4     | AF132229 | 24                         |
| N. quadricincta   | bend4     | AF057326 | 6                          |
| N. ulagwae a      | bend4     | AF132230 | 24                         |
| N. ulagwae A      | bend4     | AF132226 | 24                         |
| N. botocatensis   | bend4     | AF132225 | 24                         |
| N. paulistensis   | bend4     | AF132231 | 24                         |
| N. spinosa        | bend4     | AF057329 | 6                          |
| A. clavatus       | rod4      | AF057332 | 6                          |
| N. aureola        | rod4      | AF057339 | 6                          |
| N. stramenia      | rod4      | AF057350 | 6                          |
| N. pseudofischeri | rod4      | AF057345 | 6                          |
| A. viridinutans   | rod4      | AF057337 | 6                          |
| A. unilateralis   | rod4      | AF057336 | 6                          |
| A. duricaulis     | rod4      | AF057333 | 6                          |
| A. brevipes       | rod4      | AF057331 | 6                          |
| N. spathulata A   | rod4      | AF057347 | 6                          |
| N. spathulata a   | rod4      | AF057348 | 6                          |
| N. glabra         | rod4      | AF057343 | 6                          |
| N. hiratsukae     | rod4      | AF057344 | 6                          |
| N. aurata         | rod4      | AF057338 | 6                          |
| N. quadricincta   | rod4      | AF057346 | 6                          |
| N. spinosa        | rod4      | AF057349 | 6                          |
| A. brevipes       | mtcytb    | AB025445 | 26                         |
| A. viridinutans   | mtcytb    | AB025444 | 26                         |
| A. unilateralis   | mtcytb    | AB025442 | 26                         |
| A. duricaulis     | mtcytb    | AB025439 | 26                         |
| A. clavatus       | mtcytb    | AB025454 | 26                         |
| N. aureola        | mtcytb    | AB025517 | 26                         |
| N. stramenia      | mtcytb    | AB025521 | 26                         |
| A. fumigatus var. albus | mtcytb | AB025435 | 26                         |
| N. strumenia      | ITS       | AF459733 | S. W. Peterson, unpublished data, 2001 |
| A. clavatus       | ITS       | AY214444 | U. T. Bui, unpublished data, 2003 |
FIG. 1. ML tree of partial nucleotide sequences of genes for β-tubulin (a) and rodlet A (b) from the variant isolates (in bold), A. fumigatus, and isolates assigned to Aspergillus section Fumigati. Asterisks indicate sequences of strains derived from the GenBank database. ML trees were generated with the PAUP* software; bootstrap values generated from 1,000 pseudoreplicates are shown.
the clade that included *A. fumigatus* and *N. fischeri* isolates (Fig. 1).

The *rodA* primers amplified a total of 461 nucleotides, with 116 nucleotides in introns and 345 nucleotides in exons. Variant sequences contained 21 to 23 nucleotide changes in non-coding regions and 10 changes in coding regions, resulting in 91% homology at the nucleotide level. Translated sequences differed from *A. fumigatus* by five amino acids. The ML tree topology revealed that the variant *A. fumigatus* isolates clustered together as a monophyletic group with high bootstrap support, and the topology also showed that this clade had a most recent ancestor in common with the *A. fumigatus-N. fischeri* clade (Fig. 2). Again, *A. neoellipticus* and *N. fischeri* fell into the *A. fumigatus* clade.

The *srg* primers amplified a 450-bp region containing no introns. The sequences of the variant isolates had 38 to 40 nucleotide changes, which resulted in 91% homology at the nucleotide level. Since the *srg* sequences of other members of the section *Fumigati* were not yet available in the GenBank database, our phylogenetic analysis was restricted to the sequences generated in this study. The ML tree topology revealed that the variant isolates were clustered together, distant from the *A. fumigatus* clade, with nodes having high bootstrap support (Fig. 2).

The *mtcytB* primers amplified a 426-bp fragment. Three nucleotide changes and one amino acid change were present in the variant isolates, resulting in 99% homology with *A. fumigatus* at the nucleotide level. Amplification of the ITS1-5.8S-ITS2 regions of the *Aspergillus* isolates, which have been shown to be phylogenetically useful, generated 612-bp PCR products. Alignment of contiguous sequences revealed two nucleotide changes in the
ITS1 region and three nucleotide changes in the ITS2 region. No nucleotide changes were observed in the short 5.8S region. The variant sequence was 98% homologous to the \textit{A. fumigatus} sequence. Neither the gene for mtcytb nor the ITS sequences yielded well-resolved trees because there are few variable sites in these regions, but all of the variant isolates fell in a cluster well separated from \textit{A. fumigatus} and all of the other isolates in the \textit{A. fumigatus} group (Fig. 2 and data not shown).

Combined-evidence phylogeny trees were generated by both the ML and maximum-parsimony methods. Both analyses generated trees with a topology that resembled that of the ML trees from single loci (Fig. 3a and b). The genetic distance of the variant isolates from \textit{A. fumigatus} across the four gene regions studied was as great as or greater than the genetic distance from \textit{A. fumigatus} to \textit{N. fischeri} (Table 3).

**Phenotypic analyses.** Growth of the variant isolates on CZD and MEA was comparable to growth of \textit{A. fumigatus} isolates at 37°C (1). The variant isolates sporulated poorly in both of the media tested, but conidial germination and hyphal elongation

\begin{table}[h]
\centering
\caption{Genetic distances}
\begin{tabular}{lcccc}
\hline

Gene & Avg GD$^a$ & $t$ test & Kruskal-Wallis test \\
\hline
mtCytB & 0.0092 & 0.0085 & 0.0007 & 0.8839 & 0.8767 \\
ITS & 0.00718 & 0.00572 & 0.00146 & 0.075 & 0.0026 \\
rodA & 0.0923 & 0.0121 & 0.0802 & <0.0001 & 0.0004 \\
benA ($\beta$-tubulin) & 0.0915 & 0.043 & 0.0485 & <0.0001 & 0.0004 \\
\hline

\end{tabular}
\end{table}

\footnotetext{a GD, genetic distance.}
rates of the variant isolates were equivalent to those of *A. fumigatus* (data not shown). Unlike *A. fumigatus*, which grows at 45 and 48°C (2), the variant isolates grew poorly at 45°C and did not grow at all at 48°C.

DIC microscopy images of AF293 and FH5 conidial heads revealed that the vesicles of the variant are smaller, with diameters of 15 and 22 µm for FH5 and AF293, respectively (Fig. 4a and b). The flask-shaped phialides of FH5 also appear somewhat shorter than those of *A. fumigatus*. Conidia arise mostly from the top half of the vesicles in the variant isolates. Scanning electron micrographs of variant FH5 and *A. fumigatus* isolate AF293 also revealed that the variant isolate has smaller conidial heads (Fig. 4c and d), but the conidium characteristics (color, size, shape, and ornamentation) of the variants appear similar to those of *A. fumigatus* (Fig. 4e and f).

Given the genotypic and phenotypic uniqueness of these variant isolates, we propose that they be raised to a species. We propose the name *A. lentulus*, to denote the slow-sporulating phenotype of these fungi.

*Aspergillus lentulus* S. A. Balajee and K. A. Marr sp. nov.

Coloniae in MFA factae erant 30 ad 35 mm diametro post 7 dies in 25°C et 50 ad 55 mm post 7 dies in 37°C. Coloniae texta plerunque fluccosae, colore fere albae, inimixiae cum colonis coloris viridis olearium. Isolates saepe tam male sporulant ut colonia subalba colore apparent. Contrarium coloniae colore flavum necum pigmento diffusabili. Coloniae in CZD similes, sed fluccosiores alboresque cum nulla vel parvissima sporula.

**Holotype.** Colonies of FH5 were grown on potato dextrose agar for 5 days and dried; this specimen was deposited in the National Fungus Herbarium (BPI), U.S. Department of Agriculture, Beltsville, Md., and has been assigned BPI accession number 863540. A live culture of variant isolate FH5 has also been submitted to the American Type Culture Collection, Manassas, Va., and has been assigned number MYA-3566.

**Etymology.** The word *lentulus* means somewhat slow in Latin; this name was chosen to reflect the slow-sporulating phenotype of these fungi.

Colonies on MEA attain a diameter of 30 to 35 mm after 7 days at 25°C and 50 to 55 mm after 7 days at 37°C. Colony texture is mostly floccose, and colonies are usually white, interspersed with olive-green colonies. Isolates often sporulate poorly, giving the colony a whitish appearance. In reverse, colonies are yellow in color with no diffusible pigment. Colonies appear similar on CZD but more floccose and white, with no or very little sporulation at both temperatures. Stipes are smooth walled, colorless, and 250 to 300 µm long; vesicles are diminutive, 8 to 10 µm wide, hyaline, subelavate in shape, and fertile over only half of the area. Few short, flask-shaped, uniseriate phialides give rise to conidia 2.5 to 3.0 µm in diameter. Conidia are fewer than six or seven per chain, bluish to olive green in color, and globose, and the surface is rough with ornamentation.

**Discussion.** We previously identified seven poorly sporulating isolates that were initially identified as *A. fumigatus*; on the basis of unique randomly amplified polymorphic DNA PCR patterns and mitochondrial sequences, we reported these isolates to be *A. fumigatus* variants (1). Although the MICs of multiple antifungals for all of the variant isolates were high and the fungi had a slow-sporulating phenotype, MLST results showed that three of the seven variant isolates were identical to *A. fumigatus* and four belong to a distinct species within the *A. fumigatus* group. The in vitro MICs of multiple antifungals for clinical isolates of this new species, designated *A. lentulus*, are high, and the fungi do not grow at 48°C, which is an important phenotype that is helpful in differentiating them from *A. fumigatus*, which is able to grow at this elevated temperature (2).

The five gene genealogies examined indicate genetic isolation between *A. fumigatus* and *A. lentulus*, providing evidence of a distinct species under the phylogenetic species recognition concept (21). The phylogenetic species recognition concept recognizes species boundaries by using genealogical concordance of multiple independent loci (3). *A. lentulus* isolates were present as a monophyletic clade with high bootstrap support in the majority (three of five) of the single-locus genealogies studied, thus fulfilling the genealogical concordance and genealogical nondiscordance criteria put forward by the phylogenetic species recognition concept (3). Combined-evidence phylogeny trees generated by both the ML and maximum-parsimony methods had a topology similar to that of trees generated from single loci, further supporting the genetic uniqueness of *A. lentulus*.

All four isolates of *A. lentulus* appear to be genetically distinct from fungi assigned to *Aspergillus* section *Fumigati*, specifically, *N. aureola*, *N. fischeri*, *A. viridinutans*, and *A. fumigatus*. It was previously shown that *A. fumigatus* forms a strongly supported clade with *N. fischeri* (7). The present study reveals that *A. lentulus* isolates cluster well separated from the *A. fumigatus*-*N. fischeri* clade. Our results confirm the genetic similarity between *A. fumigatus* and *A. neoellipticus*, as shown by other investigators (6, 14, 26). In contrast, *A. lentulus* is a distinct species, consistently clustering independently, with large genetic distances from both *A. lentulus* and *N. fischeri*.

Although the genes for mtcytB and the ITS regions did not have enough phylogenetic signal (i.e., too few mutations) to resolve the evolutionary relationships with strong bootstrap support, these gene sequences did demonstrate that *A. lentulus* is just as divergent from *A. fumigatus* as is *N. fischeri*.

Interestingly, only four of the seven variant isolates that were initially identified were *A. lentulus* by the MLST method. Although the other three variant isolates were similar to *A. lentulus* by limited genetic analysis and phenotype (1), robust phylogenetic analysis revealed that these three isolates were genetically similar to *A. fumigatus*. Thus, the results of our study agree with the conclusions of prior investigations, finding that morphological characteristics have limited utility for inferring phylogenetic relationships of isolates within *Aspergillus* section *Fumigati* (6). Molecular studies increasingly show that the many known single “species” of fungi actually consist of several phylogenetically different species (8). Phylogenetics has revealed cryptic species in numerous fungi, including *Candida*.
albicans, Cryptococcus neoformans, A. flavus, A. niger, and A. bombycis (5, 7, 17, 19). The species designation of morphologically variable A. fumigatus has been controversial thus far; phylogenetic analysis of specific DNA sequences may help define "species" within this group in a more reliable and objective fashion.

Varga and colleagues found that an atypical A. fumigatus isolate (recovered from soil in Western Australia) was highly divergent on the basis of toxin profiles, restriction fragment length polymorphism, and the sequences of the genes for mitochondrial DNA and β-tubulin; because of these differences, these authors concluded that this isolate may represent a new, unrecognized species. A more recent phylogenetic analysis of another isolate (MK245), recovered as a cause of aspergillosis in a transplant patient in New South Wales, Australia, showed that this isolate may in fact be a new species distinct from A. viridimutans and A. fumigatus (11a). Interestingly, the sequence of the gene for β-tubulin of this Aspergillus sp. isolate (MK245; GenBank accession no. AY590128) is 100% homologous to the A. lentulus sequence (this study; accession no. AY738513), suggesting that A. lentulus isolates may have a wide geographic distribution. More study is necessary to confirm the identity of the Australian isolate.

We report the isolation of a previously unrecognized Aspergillus species, A. lentulus, which currently includes four clinical isolates. Since all A. lentulus isolates caused invasive disease and have decreased susceptibilities to multiple antifungal drugs, correct identification and characterization of these fungi may have both epidemiological and clinical significance. Identification to the species level appears to be important for the genus Aspergillus, as members of this genus may demonstrate variable susceptibility to antifungal drugs (e.g., the MICs of amphotericin B for A. terreus are higher) (25). The significance of the decreased in vitro susceptibility of A. lentulus isolates is unknown, but if studies suggest that they demonstrate in vivo resistance to antifungal drugs, it will become yet more important to differentiate A. lentulus from A. fumigatus in clinical samples. Since poorly sporulating "A. fumigatus" isolates are commonly recovered from clinical specimens (12), this may be an issue of reasonable significance. The A. lentulus isolates examined have smaller vesicles than does A. fumigatus, but it is difficult to distinguish these features on the basis of microscopic examinations alone. However, none of the A. lentulus isolates were able to thrive at 48°C and this may be an important phenotype that can be used in clinical microbiology laboratories to distinguish A. lentulus from A. fumigatus. Molecular methods or metabolite profiles may be necessary to definitively identify these isolates to the species level. Work is under way to further define the clinical significance of this new Aspergillus species.

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