Molecular Studies Reveal Frequent Misidentification of *Aspergillus fumigatus* by Morphotyping

S. Arunmozhi Balajee,1 David Nickle,2 Janos Varga,3† and Kieren A. Marr1,2,4*

Program of Infectious Diseases, Fred Hutchinson Cancer Research Center,1 and Departments of Microbiology2 and Medicine,4 University of Washington, Seattle, Washington, and Department of Microbiology, University of Szeged, Hungary3

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*Aspergillus fumigatus* has been understood to be the most common cause of invasive aspergillosis (IA) in all epidemiological surveys. However, recent studies have uncovered a large degree of genetic heterogeneity between isolates morphologically identified as *A. fumigatus*, leading to the description of a new species, *Aspergillus lentulus*. Here, we examined the genetic diversity of clinical isolates identified as *A. fumigatus* using restriction enzyme polymorphism analysis and sequence-based identification. Analysis of 50 clinical isolates from geographically diverse locations recorded the presence of at least three distinct species: *A. lentulus*, *Aspergillus udagawae*, and *A. fumigatus*. In vitro, *A. lentulus* isolates demonstrated decreased susceptibility to antifungal drugs currently used for IA, including amphotericin B, voriconazole, and caspofungin; *A. udagawae* isolates demonstrated decreased in vitro susceptibility to amphotericin B. Results of the present study demonstrate that current phenotypic methods to identify fungi do not differentiate between genetically distinct species in the *A. fumigatus* group. Differential antifungal susceptibilities of these species may account for some of the reported poor outcomes of therapy in clinical studies.

Invasive aspergillosis (IA) is a frequent complication of cytotoxic therapy. Despite availability of new antifungal drugs, some patients with IA fail to respond to antifungal therapy, resulting in death (8). *Aspergillus fumigatus*, the major etiological agent of IA, is identified largely by its macroscopic and microscopic features and, despite small variations in phenotypic agent of IA, is identified largely by its macroscopic and microscopic features and, despite small variations in phenotype, has been largely regarded as a single species. However, results of recent molecular studies demonstrate that several phenotypically identified *A. fumigatus* isolates may be genetically distinct (2, 9, 11, 15). For example, sequence analysis of the alkaline protease gene of four Australian clinical *A. fumigatus* isolates revealed substantial variation (7 to 11%) from *A. fumigatus* nucleotide sequence; further phylogenetic analysis demonstrated that these isolates fell in clusters distinct from *A. fumigatus* (11). The study by Hong et al. (9) described two new species in the *A. fumigatus* group using DNA sequence analysis; still other studies used the internal transcribed spacer region, benA, and intergenic sequence data (14, 16) to study the population structure of *A. fumigatus*.

Apart from sequence analysis, other molecular epidemiological studies have also hinted at the extensive genotypic variability of *A. fumigatus* isolates recovered from both patients and the environment (4, 5, 6, 20), and it has been demonstrated that *A. fumigatus* may exist in nature as two genetically different subgroups, one group that thrives predominantly in the air and another group well adapted to survival in water; both groups of *A. fumigatus* cause IA in humans (20). Thus, it is becoming apparent that morphological identification of *A. fumigatus* underestimates the difference between members, and molecular studies may more precisely define differences between species within the *A. fumigatus* group.

We recently identified and described one new species, *Aspergillus lentulus* within the section *Fumigati* as a cause of IA in hematopoietic stem cell transplant patients in our center in Seattle (2). Previously, it was noted that *A. lentulus* isolates demonstrate relatively low in vitro susceptibility to multiple antifungal drugs, including amphotericin B (AMB), itraconazole (ITZ), voriconazole (VRZ), and caspofungin (CAS). Discovery of the new species from our center prompted us to screen other *A. fumigatus* clinical culture collections in the United States using a two-step screening process that incorporates restriction fragment length polymorphism (RFLP) analysis followed by multilocus sequence typing (MLST). Results of this study reveal the widespread representation of *A. lentulus* as clinical isolates in diverse geographic locations and the existence of a previously described soil saprophyte, *Aspergillus udagawae*, as a cause of human disease.

**MATERIALS AND METHODS**

*Isolates and media.* Previous studies demonstrated that all *A. lentulus* isolates were slow sporulating (2). In this study 33 poorly sporulating *A. fumigatus* clinical isolates that were collected from different centers were screened for *A. lentulus*. This included 10 isolates from the culture collection of the U.S. Centers for Disease Control (CDC; provided by David Warnock), 12 isolates from the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, Texas (UT; provided by Michael Rinaldi), 3 isolates from Unité de Mycologie Moléculaire, Institut Pasteur, France (provided by Eric Dannaoui), and 8 isolates from the Fred Hutchinson Cancer Research Center (FHCRC; Seattle, WA). In addition, 17 isolates that were identified as *A. fumigatus* and were not poor sporulators were also included (13 isolates from the CDC and 4 isolates from the FHCRC culture collection). Reference *Neosartorya udagawae* (including both mating types) were provided by Janos Varga (University of Szeged, Hungary). *A. fumigatus* clinical reference isolates AC293 and B5323 were obtained from David Denning (University of Manchester, United Kingdom) and June Kwon-Chung (National Institutes of Health, Maryland), respectively.

Media used included RPMI 1640 medium with l-glutamine but without bicarbonate, buffered with 0.165 morpholinepropanesulfonic acid to pH 7.0 (Sigma
Chemical Co., St. Louis, MO); potato dextrose agar (PDA; Becton Dickinson, Sparks, MD), malt extract agar (MEA; Becton Dickinson, Sparks, MD), Sabouraud dextrose broth (Becton Dickinson, Sparks, MD), and Aspergillus minimal medium (MM; [2]). All fungal isolates were revived from frozen stocks on PDA. The antifungal agents AMB (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT) and VRZ (Pfizer Pharmaceuticals, New York, NY) were dissolved in dimethyl sulfoxide, and CAS (Merck and Co. Inc., Rathway, NJ) was dissolved in distilled water. Further dilutions were made in RPMI medium, as outlined by the Clinical and Laboratory Standards Institute (formerly NCCLS) (13).

Screening by RFLP. A method that exploits a restriction enzyme site polymorphism in the rod1 gene region was developed to rapidly discriminate between A. fumigatus and potential A. lentulus isolates. Specifically, a 487-bp region of rod1 was PCR amplified using the primers F (5’-GGGGCAATGCAAGGAAGACC-3’) and R (5’-AGGGCAATGCAAGGAAGACC-3’) (7). Due to a change of C to T at position 209 in A. lentulus, a StyI restriction site is lost; digestion of the 487-bp product yields two fragments (209 bp and 278 bp) from A. fumigatus only. Genomic DNA was prepared by mycelial disruption using alternate freeze-thaw cycles in liquid nitrogen and digestion with a DNeasy tissue kit (no. 69306; QIAGEN, Hilden, Germany). The 487-bp region of rod1 was PCR amplified, and the resultant amplicons were purified with a QIAquick PCR Purification Kit as described previously (2). Twenty-five microliters of the ampleron was incubated at 37°C for 2 hr in 5 µl of 10× buffer, 2 µl of the restriction enzyme StyI, and 50 µl of sterile water, and the reaction mixture was heat inactivated at 65°C for 20 min. Products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and visualized.

All isolates (n = 16) identified as non-A. fumigatus (lacking digestion) were characterized by sequencing regions of the β-tubulin (benA) and rodA genes using primers described previously (2, 7). A. fumigatus isolates AF293 and B5233 were included as references. In addition, several fungi that were identified as A. fumigatus using the StyI restriction digestion method were randomly selected for MLST to validate the RFLP screening and for phylogenetic analysis. These included the following isolates: CDC40, CDC25, FH221, FH99, FH102, FH1, FH6, and FH219 (CDC and FHRC [FH] culture collections); and P1237, P1119, and P1112 (collection of E. Dannaoui).

For sequencing, the PCR amplicons were purified with a QIAquick PCR purification kit, and 100 ng of amplicon was mixed with 4 µl of BigDye (PE-Applied Biosystems) and 10 pmol of primer (same as the respective PCR primers). Ten microliters of the reaction mixture was run in a PCR system 9700 thermocycler (PE-Applied Biosystems) with an initial denaturation step at 95°C for 5 min followed by 30 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Products were directly sequenced on a Perkin-Elmer ABI model 373 DNA sequencer in accordance with the protocols supplied by the manufacturer. The resultant nucleotide sequences were edited with Sequencer. Each set of gene sequences was aligned with CLUSTALW and then manually adjusted, when needed, using MacClade version 4.08.

Phylogenetic analyses. All of the sequences generated from the benA and rodA genes were subjected to phylogenetic analysis, as described previously (2). Aspergillus clavatus was used as an outgroup (4), and sequences of other closely related isolates in the Aspergillus section Fumigati were obtained from GenBank, when available. Phylogenetic trees were estimated using maximum-likelihood methods (ML) in PAUP* as described previously (2). Genetic distance between isolates (where appropriate) were estimated in PAUP* with Model of Evolution as determined by the ML method. We used an HKY + F + I model of evolution to correct for multiple hits. Tree searches were started with Neighbor-Joining tree, and the tree space was explored using the SPR (subtree pruning and regrafting) branch-swapping algorithm to arrive at the ML estimate of the phylogeny. Bootstrap values were generated from 1,000 pseudoreplicates. Statistical analyses were performed using the software package JMP, version 3.1 (JMP Statistical Discovery Software, Cary, N.C.).

Morphological examination. All non-A. fumigatus isolates and five A. fumigatus isolates, including AF293 (A. fumigatus Fresenius) and B5233, were cultured under a range of conditions to characterize growth differences between the species. In brief, 10 µl of the conidial suspension (10⁶ conidia/ml) was placed in the center of MM agar plates and incubated at 45°C, 48°C, and 50°C for 3 days. The presence or absence of growth at the end of a 3-day incubation period was recorded. Colony morphology, sporulation, and microscopic characteristics on MEA, PDA, and MM were examined (2).

For mating assays, clinical isolates were crossed with respective tester strains of either mating type A or a on MEA and incubated at 25°C for 7 to 21 days. Isolates were examined for the presence of fruiting bodies.

Susceptibilities of the 16 non-A. fumigatus isolates to CAS, VRZ, ITZ, and AMB were determined by previously published methods (3). Briefly, conidia were filtered through two layers of gauze and visually counted with a hemocytometer to ensure that there was no hyphal contamination. As per the current CLSI recommendations, MICs of ITZ, VRZ, and AMB were defined as the lowest concentrations of the respective drug that resulted in 100% growth reduction compared to growth in the drug-free control (11). For CAS, the minimal effective concentration was defined as the minimum concentration of drug that produced morphological alterations, such as abnormal hyphal growth with highly branched tips, swollen germ tubes, and distended balloon-like hyphe under a light microscope (9). Susceptibility was determined in three different experiments.

RESULTS

Fifty fungi, all previously identified as A. fumigatus by morphology, were screened by the PCR-RFLP method to identify A. lentulus isolates. Initial screening revealed 16 isolates predicted to be non-A. fumigatus and 34 isolates predicted to be A. fumigatus. The benA and rodA genes were sequenced. Ampli- cons from isolates predicted to be A. fumigatus in PCR-RFLP screening were 99 to 100% homologous to reference A. fim- nigatus AF293 and B5233 strains. Details of the isolates and GenBank accession numbers are presented in Table 1.

Recovery of A. lentulus from other culture collections. Eight of the sixteen “non-A. fumigatus” isolates were found to be A. lentulus by benA and rodA sequence homology to the reference A. lentulus isolate FH5 (2). Specifically, the benA sequences of the isolates FH265, FH278, CDC59, and UT2411 were 100% homologous; CDC61, CDC65, and UT3351 were 99% homologous; and UT1323 was 98% homologous to the A. lentulus gene of A. lentulus FH5. The rodA sequences of the isolates UT2411 and UT3351 were 96% and 97% homologous to the rodA gene of A. lentulus FH5, while the rodA sequences of the remaining six isolates were 98% homologous to the rodA gene of FH5. All eight isolates fell into a cluster with the previously described A. lentulus isolates (FH4, FH5, FH7, and FH220) in ML trees generated from benA and rodA sequences (Fig. 1 and 2). A. lentulus isolates were recovered from patients at hospitals in diverse geographical locations (Table 2).

All of the A. lentulus isolates were slowly sporulating on all media tested. Microscopically, most of the newly identified A. lentulus isolates appeared very similar to A. fumigatus AF293, having subclavate conidial heads giving rise to uniseriate flask-shaped vesicles bearing blue-green ornamented conidia. However, there was a growth difference between the two species with incubation at high temperatures. Specifically, none of the A. lentulus isolates grew at 50°C, while all of the A. fumigatus isolates tested grew at this high temperature. Four of the eight A. lentulus isolates did not grow at 48°C, while the remaining four grew extremely poorly. A. fumigatus isolates grew profusely at 48°C. All the A. lentulus isolates grew well at 45°C.

Recovery and characterization of A. udagawae isolates. Eight of the 16 isolates that were non-A. fumigatus by PCR-RFLP were not identified as A. lentulus by MLST. The benA and rodA sequences of these isolates were 98% homologous to the benA and 99% homologous to rodA genes of N. udagawae. The ML trees generated from sequences of the benA and rodA regions demonstrated grouping with N. udagawae reference strains, with high bootstrap support (Fig. 1 and 2). Since the topology of the ML trees also appeared to associate the eight clinical isolates with closely related Neosartorya aureola, ML trees and genetic distances were calculated from the rodA and benA sequences from the eight clinical isolates with respect to N. aureola, Aspergillus viridinutans, and N. udagawae. Results of
this analysis revealed that isolates CDC22, CDC57, CDC58, FH103, FH104, FH105, FH106, and UT1516 clustered with N. udagawae with a bootstrap support of >90% in both rodA and benA regions, supporting the likelihood of shared descent (Fig. 3a and b). Also, the genetic distance of all the eight clinical isolates to N. udagawae were significantly smaller than that to N. aureola when calculated using both benA and rodA regions (data not shown).

Among isolates identified as N. udagawae by MLST, isolates FH103, FH104, FH105, and FH106 were isolated from the same patient (Table 1). These isolates were originally identified by phenotype as Neosartorya fischeri by the clinical microbiology laboratory at the University of Washington medical center. Isolates CDC57 and CDC58 were identified as A. fumigatus "albino" type, and isolate CDC22 was identified as A. fumigatus by the CDC. Isolate UT1516 was identified as A. fumigatus by the Fungus Testing Laboratory of the University of Texas Health Science Center at San Antonio. With the

### TABLE 1. Isolates used in the study

| Isolate | Gene | Accession no. | Patient location | Anatomic site | Year |
|---------|------|---------------|-----------------|---------------|------|
| FH265   | benA | DQ058685      | Seattle         | BAL           | 2004 |
| FH265   | rodA | DQ058369      |                 |               |      |
| FH278   | benA | DQ058386      | Seattle         | BAL           | 2005 |
| FH278   | rodA | DQ058370      |                 |               |      |
| CDC59   | benA | DQ011685      | New York        | Lung          | 1999 |
| CDC59   | rodA | DQ011687      |                 |               |      |
| CDC61   | benA | DQ011687      | California      | Sputum        | 2001 |
| CDC61   | rodA | DQ011688      |                 |               |      |
| CDC65   | benA | DQ058387      | California      | Lung          | 2001 |
| CDC65   | rodA | DQ058371      |                 |               |      |
| UT3351  | benA | DQ058388      | California      | Sputum        | 2003 |
| UT3351  | rodA | DQ058372      |                 |               |      |
| UT1323  | benA | DQ058389      | Texas           | BAL           | 2004 |
| UT1323  | rodA | DQ058373      |                 |               |      |
| CDC58   | benA | DQ058391      | NK              | NK            | 1995 |
| CDC58   | rodA | DQ058375      |                 |               |      |
| CDC37   | benA | DQ058392      | NK              | NK            | 1995 |
| CDC37   | rodA | DQ058376      |                 |               |      |
| CDC22   | benA | DQ058393      | NK              | Sputum        | 2001 |
| CDC22   | rodA | DQ058377      |                 |               |      |
| UT1561  | benA | DQ058394      | NK              | NK            | 2004 |
| UT1561  | rodA | DQ058378      |                 |               |      |
| FH103   | benA | DQ058395      | Seattle         | BAL           | 2002 |
| FH103   | rodA | DQ058379      |                 |               |      |
| FH104   | benA | DQ058396      | Seattle         | BAL           | 2002 |
| FH104   | rodA | DQ058380      |                 |               |      |
| FH105   | benA | DQ058397      | Seattle         | BAL           | 2002 |
| FH105   | rodA | DQ058381      |                 |               |      |
| FH106   | benA | DQ058398      | Seattle         | Lung          | 2002 |
| FH106   | rodA | DQ058382      |                 |               |      |
| FH1     | benA | DQ438503      | Seattle         | Lung          | 1998 |
| FH1     | rodA | DQ439735      |                 |               |      |
| FH6     | benA | DQ438506      | Seattle         | Kidney        | 1995 |
| FH6     | rodA | DQ439738      |                 |               |      |
| FH99    | benA | DQ438534      | Seattle         | Nose          | 2003 |
| FH99    | rodA | DQ439768      |                 |               |      |
| CDC40   | benA | DQ438520      | Washington, DC  | BAL           | 2001 |
| CDC40   | rodA | DQ439752      |                 |               |      |
| FH219   | benA | DQ438543      | Seattle         | Lung          | 1995 |
| FH219   | rodA | DQ439774      |                 |               |      |
| FH221   | benA | DQ438545      | Seattle         | Kidney        | 1998 |
| FH221   | rodA | DQ439777      |                 |               |      |
| P1112   | benA | DQ438540      | France          | Sputum        | 1997 |
| P1112   | rodA | DQ439772      |                 |               |      |
| P1119   | benA | DQ438541      | France          | Sputum        | 1997 |
| P1119   | rodA | DQ439773      |                 |               |      |
| P1237   | benA | DQ438542      | France          | BAL           | 1997 |
| P1237   | rodA | DQ439774      |                 |               |      |

N. udagawae (A) benA DQ058399 NA NA NA
N. udagawae (A) rodA DQ058383 NA NA NA
N. udagawae (a) benA DQ058400 NA NA NA
N. udagawae (a) rodA DQ058384 NA NA NA

* NK, not known; NA, not applicable; BAL, bronchoalveolar lavage.
exception of CDC22, which was abundantly sporulating, the remaining seven were poorly sporulating on all laboratory media tested. Microscopically, most of the isolates closely resembled *A. fumigatus*, having subclavate vesicles bearing uniseriate phialides. Conidia were about 2 to 3 μm in diameter and subglobose, appearing dull green to grayish green. Two of the isolates, CDC22 and UT1516, had nodding heads reminiscent of the conidial heads of *A. viridinutans*.

Since *N. udagawae* has a known sexual state and is heterothallic, mating assays were performed with reference isolates.

FIG. 1. ML tree of partial nucleotide sequence of *rodA* region of the *Aspergillus* isolates revealing three distinct clades: clade 1, *A. fumigatus*; clade 2, *A. lentulus*; and clade 3, *A. udagawae*. Sequences of isolates from section *Fumigati* were derived from the GenBank database and are denoted by an asterisk, and sequences of isolates derived from a previous study are shown in black (2). Shown in gray are sequences derived from this study. Bootstrap values generated from 1,000 pseudoreplicates are shown.
Repeated attempts to induce mating in these isolates failed; hence, all eight isolates were classified as *A. udagawae*, the anamorphic state of *N. udagawae*. None of the eight *A. udagawae* isolates grew at 48°C and 50°C, and they grew poorly at 45°C.

**Antifungal susceptibilities.** In vitro, *A. lentulus* isolates exhibit relatively decreased but variable susceptibilities to all antifungals (Table 2). *A. udagawae* isolates demonstrate particularly decreased susceptibilities to AMB and relatively low susceptibilities to VRZ. Susceptibilities of the *A. fumigatus* reference isolate Af293 are shown in Table 2.

**DISCUSSION**

Classification and identification of filamentous fungi, unlike bacteria, rely mainly on morphological criteria, but limitations of phenotypic typing of fungal pathogens are being increasingly
recognized. Multilocus phylogenies of diverse fungi have repeatedly revealed species within one previously accepted morphospecies (10, 17). Some of the new species revealed by MLST have demonstrated important virulence differences between the species; for example, while the North American class 1 clade of *Histoplasma capsulatum* typically causes disease in immunosuppressed individuals, the North American class 2 clade can cause disease in healthy hosts (18). The present study demonstrates that multiple genetically distinct species are misidentified as *A. fumigatus* by morphological typing methods. Preliminary data suggest that differential antifungal susceptibilities among misidentified species may contribute to poor clinical outcomes with standard diagnostic and therapeutic approaches.

Our previous study using MLST showed that four phenotype-identically identified *A. fumigatus* isolates recovered from patients with invasive aspergillosis represent a new species, *A. lentulus*, distinct from *A. fumigatus* and other members of the *Aspergillus* section *Fumigati*. Herein, we report that this organism has a widespread distribution as a clinical pathogen. Presence of *A. lentulus* isolates in Korea, The Netherlands, and Australia (9, 11) and recent recovery of three more *A. lentulus* isolates in Japan (T. Yaghuchi, Chiba University, personal communication) emphasize the widespread occurrence of *A. lentulus* as clinical isolates worldwide.

Results of our study also identified several isolates of *A. udagawae* (teleomorph, *N. udagawae*) among phenotype-identically identified *A. fumigatus* isolates. Most members of the genus *Neosartorya* are ubiquitous fungi, having been isolated from soil, house dust, and food. *N. udagawae* Horie, Miyaji, and Nishimura strains were first isolated from Brazilian soil; since the initial identification and description in *N. udagawae* 1995, there have been no reports of infections caused by *N. udagawae* or its anamorph *A. udagawae*, and neither has been recovered from clinical samples. Since none of the clinical isolates were able to mate with the tester strains of *N. udagawae*, these isolates are classified as *A. udagawae* (anamorphic state). The apparent loss of sexuality in these fungi may be explained in part to the pathogenic lifestyle of the clinical isolates and/or repeated subculturing on laboratory medium. Similarly, we recently observed that several clinical isolates of *Neosartorya pseudofischeri* could not be induced to produce ascoma in the laboratory (1). Fruiting body formation appears to be an unstable marker for the confirmatory identification of the clinical *Neosartorya*.

Microscopic morphology of two *A. udagawae* isolates revealed “noding” conidial heads that were previously described to be a characteristic feature of another member of the *A. fumigatus* group, *A. viridinutans*. Explicit assignment of a phenotype such as a “noding” head to identify *A. viridinutans* appears unwarranted, especially given the recent observation of variation among *A. viridinutans* isolates (19). It is notable that these two species, along with *N. aureola*, appear to be closely related (Fig. 1 and 2). However, detailed analysis of the eight clinical isolates revealed that these isolates are more likely to be *N. udagawae* and not *N. aureola*, considering both phylogenetic (Fig. 3a and b) and genetic distance studies. It is likely that, given the large evolutionary distances in the data set that included all the isolates (Fig. 1 and 2), the small genetic distances between *N. udagawae* and *N. aureola* were not immediately notable.

With the exception of one isolate (CDC22), all of the *A. udagawae* and *A. lentulus* isolates were slow sporulating. At the same time, five *A. fumigatus* isolates (FH102, FH99, P1112, P1119, and P1237) had a slow sporulation phenotype. Hence, the slow sporulation phenotype is not exclusive to *A. lentulus* and *A. udagawae*, but it may serve to indicate a high likelihood that the isolate is not *A. fumigatus*. It is not clear why there is so much genetic variability among slowly sporulating isolates. The phenotype might depict a difference in native environment or even represent an adaptive change within the host.

Isolates of *N. udagawae* demonstrated relatively high AMB MICs. We recently described isolates of another *Neosartorya* species, *N. pseudofischeri*, that were misidentified as *A. fumigatus* by phenotype and for which the VRZ MICs were high (1). Susceptibility differences of both these *Neosartorya* species are yet to be corroborated in animal studies. Clinically meaningful differences may suggest that further steps are necessary to identify these organisms in order to guide antifungal treatment. One can speculate that some degree of treatment failure of IA may be associated with infection caused by unrecognized non-*A. fumigatus* isolates.

Despite the effective application of MLST for fungal identification systems, these methods are not yet available in the routine clinical mycology laboratory. Development of other rapid, economical, and user-friendly identification systems is important since morphological criteria alone do not differentiate *Aspergillus* species. One such method that could be used to rapidly screen for *A. lentulus* is the PCR-RFLP method that we have described herein. Sequence analysis of the *benA* and *rodA* genes of several random isolates after PCR-RFLP revealed that this method accurately differentiated the non-*A. fumigatus* from the *A. fumigatus* isolates. Another method that

### Table 2. Antifungal susceptibilities of *A. lentulus* and *A. udagawae* isolates

| Isolate   | AMB | ITZ | VRZ | CAS |
|-----------|-----|-----|-----|-----|
| *A. lentulus* |     |     |     |     |
| FH265     | 1   | 0.5 | 1   | 2   |
| FH278     | 1   | 1   | 1   | 2   |
| CDC59     | 1   | 0.5 | 2   | 2   |
| CDC61     | 2   | 0.5 | 2   | 16  |
| CDC65     | 1   | 1   | 2   | 16  |
| UT3351    | 2   | 1   | 2   | 2   |
| UT1322    | 2   | 1   | 2   | 2   |
| UT2411    | 2   | 1   | 1   | 2   |

| *A. udagawae* |     |     |     |     |
|---------------|-----|-----|-----|-----|
| CDC58        | 4   | 0.25 | 1   | 0.015 |
| CDC57        | 0.5 | 0.125 | 1   | 0.03  |
| CDC22        | 4   | 0.25 | 1   | 0.015 |
| UT1561       | 2   | 0.125 | 0.25 | 0.03  |
| FH103*       | 2   | 0.5  | 1   | 0.06  |
| FH104*       | 2   | 0.5  | 1   | 0.015 |
| FH105*       | 2   | 0.5  | 1   | 0.015 |
| FH106*       | 2   | 0.5  | 1   | 0.03  |
| A1293        | 0.5 | 0.25 | 0.25 | 0.125 |

* a MICs are given for AMB, ITZ, VRZ; for CAS, the minimum effective concentration is given.

b Isolates recovered from a single patient.
could be conveniently used in a microbiology laboratory to differentiate these three species would be to exploit the temperature-related growth differences. *A. fumigatus* was the only species that reproducibly grew at 50°C. Larger studies will be needed to ensure that these growth differences can be used as stable phenotypic markers for species differentiation.

Correct species demarcation is important from a taxonomy viewpoint, but differences in susceptibility profiles may also indicate clinical relevance. Since the current study sampled isolates preselected to be slowly sporulating, we cannot estimate the prevalence of different species as causative agents of IA. Studies are under way to screen a large, diverse bank of *A. fumigatus* isolates for the presence of these species using a MLST scheme that includes a larger panel of genes. Judicious integration of molecular speciation methods with available classical phenotyping could yield more accurate identification of *Aspergillus* species and potentially more appropriate tailoring of antifungal therapies.

**FIG. 3.** Analysis of a subclade of isolates that did not type as *A. fumigatus* or *A. lentulus* by the RFLP method. ML tree of *rodA* (a) and *benA* (b) nucleotide sequences of the subset. Values above the node indicate bootstrap values, generated from 1,000 pseudoreplicates.
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