

Genetic Relatedness versus Biological Compatibility between *Aspergillus fumigatus* and Related Species

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Aspergillus section *Fumigati* contains 12 clinically relevant species. Among these *Aspergillus* species, *A. fumigatus* is the most frequent agent of invasive aspergillosis, followed by *A. lentulus* and *A. viridinutans*. Genealogical concordance and mating experiments were performed to examine the relationship between phylogenetic distance and mating success in these three heterothallic species. Analyses of 19 isolates from section *Fumigati* revealed the presence of three previously unrecognized species within the broadly circumscribed species *A. viridinutans*. A single mating type was found in the new species *Aspergillus pseudofelis* and *Aspergillus pseudoviridinutans*, but in *Aspergillus parafelis*, both mating types were present. Reciprocal interspecific pairings of all species in the study showed that the only successful crosses occurred with the *MAT1-2* isolates of both *A. parafelis* and *A. pseudofelis*. The *MAT1-2* isolate of *A. parafelis* was fertile when paired with the *MAT1-1* isolates of *A. fumigatus*, *A. viridinutans*, *A. felis*, *A. pseudoviridinutans*, and *A. wyomingensis* but was not fertile with the *MAT1-1* isolate of *A. lentulus*. The *MAT1-2* isolates of *A. pseudofelis* were fertile when paired with the *MAT1-1* isolate of *A. felis* but not with any of the other species. The general infertility in the interspecies crossings suggests that genetically unrelated species are also biologically incompatible, with the *MAT1-2* isolates of *A. parafelis* and *A. pseudofelis* being the exception. Our findings underscore the importance of genealogical concordance analysis for species circumscription, as well as for accurate species identification, since misidentification of morphologically similar pathogens with differences in innate drug resistance may be of grave consequences for disease management.

Invasive aspergillosis (IA) is one of the major mold infections, and it results in high fatality rates among severely immunocompromised patients, especially those with profound neutropenia (1). *Aspergillus fumigatus*, the major etiologic agent of IA, can be readily distinguished from other, less common agents, such as *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger*, and *Aspergillus nidulans*, by the characteristic morphology of its conidial structures (2). *Aspergillus fumigatus* has been reported to be a variable species (3, 4). Variations are commonly observed in colony growth, the robustness of the production of conidia, conidial surface markings, the presence or absence of septation in phialides, and maximum growth temperatures (4–6). Many strains showing such phenotypical variations were considered atypical *A. fumigatus* because they showed conidiogenous structure morphology indistinguishable from that typical of *A. fumigatus* (4). Using multilocus sequence data, several genetically diverse clades that qualify as phylogenetic species (4, 7, 8) were found among the atypical *A. fumigatus* strains, and they have been described as separate species. Examples of such species are *Aspergillus lentulus* and *Aspergillus felis*.

Recently, heterothallic sexual cycles were discovered in *A. lentulus* (9) and *A. felis* (10). Although the sexual state of *Aspergillus viridinutans* has yet to be found, the presence of the *MAT1-1* (11) mating type homolog in the type strain NRRL 4365 suggests heterothallic sexuality. In an outbreeding system, it is possible to compare the phylogenetic distance with biological relatedness through interspecific crosses. Such comparisons between biological species recognition (BSR) and phylogenetic species recognition (PSR) have been done with *Neurospora* species (12, 13). Mat-

ing between numerous *Neurospora* strains revealed several reproductively isolated biological species. Using genealogical concordance of four independent nuclear loci to identify phylogenetic species in these strains, congruence was observed between the results of the two species identification systems, BSR and PSR. Increased genetic distance between parents was associated with decreased sexual reproduction. These results suggest that genealogical concordance PSR can be reliably used to recognize species in fungi that are not candidates for BSR (12).

The need for correct identification of the etiologic agents of IA that resemble *A. fumigatus*, such as *A. lentulus*, *A. viridinutans*, and *A. felis*, is underscored by the fact that these species tend to be refractory to antifungal treatment and produce more chronic infection than *A. fumigatus* (10, 14, 15). Since they are all heterothallic species, we attempted to determine whether the phylogenetic and reproductive methods concurred in species identification and whether reproductive success between different species reflected their genetic distance. While comparing the phylogenetic rela-

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TABLE 1 *Aspergillus* isolates, mating types, and origins^a

Species	Designation	ARS accession no.	Mating type	Source	Origin
<i>A. viridinutans</i>	NRRL 4365 ^T	NRRL 4365 ^T	<i>MAT1-1</i>	Rabbit dung	Australia
<i>A. felis</i>	CBS 130245 ^T	ND	<i>MAT1-2</i>	Cat	Australia
<i>A. felis</i>	CBS 130246	ND	<i>MAT1-1</i>	Cat	Australia
<i>A. fumigatus</i>	AFB62	ND	<i>MAT1-1</i>	Human	USA
<i>A. fumigatus</i>	AFB623	ND	<i>MAT1-1</i>	Human	USA
<i>A. fumigatus</i>	AFIR928	ND	<i>MAT1-2</i>	Environmental	Ireland
<i>A. wyomingensis</i>	CCF 4417 ^T	ND	<i>MAT1-1^b</i>	Environmental	USA
<i>A. wyomingensis</i>	CCF 4416	ND	<i>MAT1-2^b</i>	Environmental	USA
<i>A. lentulus</i>	NRRL 35552 ^T	NRRL 35552 ^T	<i>MAT1-2</i>	Human	USA
<i>A. lentulus</i>	CM-4428	ND	<i>MAT1-1</i>	Human, skin	Spain
<i>A. lentulus</i>	CM-4843	ND	<i>MAT1-1</i>	Human, sputum,	Spain
<i>A. lentulus</i>	CM-5027	ND	<i>MAT1-2</i>	Human, sputum	Spain
<i>A. lentulus</i>	CM-5563	ND	<i>MAT1-1</i>	Human, BAS	Spain
<i>A. parafelis</i> (former <i>A. viridinutans/A. felis</i>)	CM-3147 ^T	NRRL 62900	<i>MAT1-2</i>	Human, OPE	Spain
<i>A. parafelis</i> (former <i>A. viridinutans/A. felis</i>)	CM-5623	NRRL 62901	<i>MAT1-1</i>	Human, lungs	Portugal
<i>A. pseudofelis</i> (former <i>A. viridinutans</i>)	CM-6087 ^T	NRRL 62903	<i>MAT1-2</i>	Human, sputum	Spain
<i>A. pseudofelis</i> (former <i>A. viridinutans/A. felis</i>)	CM-4518	NRRL 62902	<i>MAT1-2</i>	Human, nail	Spain
<i>A. pseudoviridinutans</i> (former <i>A. viridinutans</i>)	NIHAV1 ^T	NRRL 62904	<i>MAT1-1</i>	Human, lymph node	USA
<i>A. pseudoviridinutans</i> (former <i>A. viridinutans</i>)	NRRL 6106	NRRL 6106	<i>MAT1-1</i>	Unknown	Unknown

^a Superscript T, type strain; ARS, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA; ND, not deposited in ARS; BAS, bronchoalveolar secretion; OPE, oropharyngeal exudate.

^b Mating type reported in Nováková et al. (11).

tionship between strains previously identified as *A. lentulus*, *A. viridinutans*, and *A. felis* and a reference strain of *A. fumigatus* (16), it was observed that the species designated *A. viridinutans* contained several cryptic species. These species are described here, and the MIC values for common antifungals and virulence testing in model systems for the new species are reported.

MATERIALS AND METHODS

Isolates. The isolates used in this study are listed in Table 1. The type strains *A. lentulus* NRRL 35552 and *A. viridinutans* NRRL 4365 and NRRL 6106 were obtained from the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA. *Aspergillus wyomingensis* isolates CCF 4417 and CCF 4416 were kindly provided by V. Hubka of Charles University, Prague, Czech Republic. Isolates were cultured at 37°C on *Aspergillus* minimal medium agar (MMA) (17), malt extract agar (MEA), Czapek's solution agar (CZA), or oatmeal agar (OA) (18).

Phylogenetic analysis. DNA isolation, sequencing, and phylogenetic analysis were carried out as described elsewhere (19). Briefly, sequences from each locus were aligned using Clustalw, the best fit model was determined using ModelTest, phylogeny was calculated using likelihood, and clade credibility was determined from 500 bootstrap samples, all with routines contained in Mega5.2. Routines were also run in Mega to determine whether these loci were suitable for determining evolutionary distance as a function of the molecular clock (20). The partition homogeneity test in PAUP* was used to determine the suitability of combining data (21). Highly credible clades were compared between five loci, encoding beta-tubulin (*BT2*), calmodulin (*CF*), minichromosome maintenance factor (*Mcm7*), the second-largest subunit of RNA polymerase II (*RPB2*), and a pre-rRNA processing protein (*Tsr1*). Majority rule was used to determine the species according to genealogical concordance analysis (12, 22). Loci were amplified using primers *Mcm7-709for* and *Mcm7-1348rev* for *Mcm7*, *fRPB2-5F* and *fRPB2-7cR* for *RPB2*, *Tsr1-1453for* and *Tsr1-2308rev* for *Tsr1*, *BT2a* and *BT2b* for *BT2*, and *cf-1L* and *cf-4* for *CF*, as described elsewhere (23–26).

Mating type. Mating type was determined by sequencing amplicons generated with primers AVN-9F (CTAGGCCACTTGATATCGAATG) and AVN-12R (TTGCTCAAGTAACAACAACAGAAG). The template was genomic DNA isolated as described elsewhere (19).

Growth. To determine growth, strains were inoculated onto either MEA or CZA plates and incubated at 37, 42, and 50°C for 2 to 7 days.

Drug resistance. Antifungal MICs were determined according to the Clinical and Laboratory Standards Institute reference method for broth dilution (M38-A2) (27). The drugs tested were voriconazole (Pfizer), itraconazole (Sigma, St. Louis, MO), and amphotericin B (Sigma, St. Louis, MO).

Virulence. *Galleria mellonella* larvae in the final larval stage (Vanderhorst Wholesale, Inc., St. Marys, OH) were inoculated on the same day of their arrival. Larvae (15 per strain) were inoculated with 5 µl of conidial suspension (2×10^7 conidia/ml) as described elsewhere (28, 29) and incubated at 37°C. Mortality was scored based on color change (from pale beige to dark brown or black) and unresponsiveness to tactile stimulus. Two strains of mice were also used for virulence studies, BALB/c mice (Division of Cancer Treatment, National Cancer Institute, NIH, USA) immunosuppressed with hydrocortisone acetate (Sigma, St. Louis, MO) and gp91*phox*-deficient mice (B6.129S6-*Cybbtm1Din/J*) (The Jackson Laboratory, USA). Hydrocortisone acetate treatment was carried out as described elsewhere (18). BALB/c and gp91*phox*-deficient mice were inoculated with 30 µl of conidial suspension containing 3×10^7 and 3×10^5 conidia/ml, respectively. All mice were inoculated via pharyngeal aspiration (30). Groups of 6 and 10 mice per strain were used for gp91*phox*-deficient and BALB/c mice, respectively. Survival was monitored for 15 days, and survival curves were compared using the log-rank test. Studies were performed under a protocol approved by the Institutional Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases at the U.S. National Institutes of Health (NIH).

Mating. Strain crosses were carried out as described elsewhere (18). Briefly, strains of the opposite mating type were inoculated pairwise onto OA plates, which were then sealed with Parafilm and incubated at 30°C. The mating plates were monitored weekly for the formation of cleistothecia and viable ascospores. Ascospore viability assays were carried out as described previously (18). Briefly, ascospores were released from cleistothecia and suspended in water at concentrations adjusted to 1×10^3 ascospores/ml by hemocytometer counts. Aliquots of 100 µl were incubated at 70°C for 30 min to eliminate contaminating conidia and then inoculated onto MEA plates. The plates were incubated at 37°C for 48 h. The number of growing colonies was scored as the number of viable

ascospores. Single colonies were then transferred to MMA slants, incubated at 37°C for 7 days, and stored at 26°C for further analysis. To confirm that the heat treatment at 70°C eliminated all contaminating conidia from the parental strains, the following control was prepared: conidia from the parental strains were harvested from the mating plate, suspended in water, heat treated as described above, plated on MEA, and incubated at 37°C for 2 to 3 days. No growth was observed in any of the controls, indicating efficient killing of the parental conidia.

Hygromycin resistance. Conidia were inoculated onto MEA with or without hygromycin (Sigma, St. Louis, MO) (200 µg/ml) and incubated at 37°C for 6 days. Colonies growing on MEA supplemented with hygromycin were scored as resistant to the drug.

SEM. Conidia and cleistothecia were suspended in 6% aqueous glutaraldehyde solution (Electron Microscopy Sciences, Hatfield, PA) before processing for scanning electron microscopy (SEM). Samples were fixed for 2 h and then allowed to settle on silicon chips for 1 h. Subsequent postfixation with 1% OsO₄ was performed with microwave irradiation (Pelco 3451 microwave processor) in cycles of 2 min on, 2 min off, and 2 min on at 250 W under a vacuum of 15 in. of Hg (Ted Pella, Redding, CA). Specimens were dehydrated in a graded ethanol series for 1 min under vacuum. Samples were then critical point dried (CPD) in a Bal-Tec CPD 030 drier (Balzer, Bal-Tec AG, Balzers, Liechtenstein). Cells were then coated with 75 Å of iridium in an ion beam sputter (IBS) system (South Bay Technology, Inc., San Clemente, CA) and imaged on a Hitachi SU-8000 SEM (Hitachi, Pleasanton, CA).

Accession numbers. The sequences of the genes encoding the mating-type proteins of the *MAT* locus are deposited in GenBank under accession numbers **KJ858505** (*A. parafelis* CM-3147), **KJ858506** (*A. parafelis* CM-5623), **KJ858507** (*A. pseudofelis* CM-6087), **KJ858508** (*A. pseudofelis* CM-4518), **KJ858509** (*A. pseudoviridinutans* NIHAV1), **KJ858510** (*A. pseudoviridinutans* NRRL 6106), and **KJ858511** (*A. viridinutans* NRRL4365). Sequences of the protein coding loci used in the phylogenetic analysis are deposited in GenBank under the following accession numbers: **EF661252**, **EF669805**, **EF669808**, **EF669825**, **KJ572796**, and **KJ914684** to **KJ914697** (*BT2*); **EF661266**, **EF669874**, **EF669877**, **EF669895**, **KJ572797**, and **KJ914698** to **KJ914711** (*CF*); **KJ572799** and **KJ914712** to **KJ914728** (*Mcm7*); **EF661238**, **EF669734**, **EF669738**, **EF669756**, **KJ572800**, and **KJ914729** to **KJ914742** (*RPB2*); and **KJ572801** and **KJ914743** to **KJ914760** (*Tsr1*). The scientific names and descriptions of the novel species were deposited in MycoBank under accession numbers MB 808634, MB 808636, and MB 808637.

RESULTS

Phylogenetic analysis. A total of 19 strains were employed for mating, construction of phylogenetic trees, and phenotypic analysis (Table 1). Of those, nine clinical strains (one isolated in the United States, one in Portugal, and seven in Spain) were originally identified as *A. lentulus* or *A. viridinutans* (15, 31–33; also M. Cuenca-Estrella and E. Mellado, unpublished data). Since three of these strains were recently reclassified as *A. felis* (10), a phylogenetic reexamination was carried out to confirm species identity. Strains CM-4428, CM-4843, CM-5027, CM-5563, CM-3147, CM-5623, CM-4518, CM-6087, and NIHAV1 were chosen for the phylogenetic analysis. The trees were constructed on the basis of genealogical concordance analysis using the sequences of *BT2*, *CF*, *RPB2*, *Mcm7*, and *Tsr1*. Genealogical concordance analysis based on gene sequences from all five loci showed that CM-4428, CM-4843, CM-5027, and CM-5563 form a monophyletic cluster with the *A. lentulus* type strain, confirming their original identification (Fig. 1). The other strains (formerly identified as *A. viridinutans* or *A. felis*) formed three clusters that separated from the type strains of *A. viridinutans* and *A. felis* based on the sequences of *BT2* and *CF* (Fig. 1B and C, arrows). The partition homogeneity test showed that the loci could be combined, and the tree based on the

combined sequences of all five loci strongly supported the presence of three groups distinct from *A. viridinutans* or *A. felis*, i.e., strains CM-3147 and CM-5623 in *Aspergillus parafelis*, strains CM-4518 and CM-6087 in *Aspergillus pseudofelis*, and strains NIHAV1 and NRRL 6106 in *Aspergillus pseudoviridinutans* (Fig. 1A), which could be identified as novel phylogenetic species.

Species descriptions. *Aspergillus parafelis* (Sugui, S. W. Peterson, et Kwon-Chung) sp. nov., *Aspergillus pseudofelis* (Sugui, S. W. Peterson, et Kwon-Chung) sp. nov., and *Aspergillus pseudoviridinutans* (Sugui, S. W. Peterson, et Kwon-Chung) sp. nov. were submitted to MycoBank under accession numbers MB 808634, MB 808636, and MB 808637, respectively.

A. parafelis. (i) **Holotype.** The holotype of *A. parafelis* is NRRL 62900 (= CM-3147), a culture permanently preserved in a metabolically inactive state as a freeze-dried preparation. The culture was isolated in 2004 from a human oropharyngeal exudate specimen at the National Center for Microbiology, Spain.

(ii) **Etymology.** The name is taken from the Greek root “para,” or “beside,” to indicate that the species is beside *A. felis* phylogenetically.

(iii) **Description.** Colonies attained a diameter of 5.5 to 6.5 cm on MEA, as well as on CZA, in 5 days at 37°C (Fig. 2A and B). On MEA, colonies are white with dense aerial hyphae and scanty conidiation. The colony reverse is yellow. Conidiophores are hyaline, bearing conidial heads consisting of flask-shaped vesicles (10 to 20 µm in diameter) with uniseriate phialides producing long chains of conidia (Fig. 2C). Some conidial heads are slightly nodding, while the majority of conidial heads are straight (Fig. 2C and D). Conidia are subglobose (1.5 to 1.7 by 1.8 to 2.2 µm) and green in color, with delicate surface roughness created by shallow grooves (Fig. 2E). Cleistothecia are globose, white, 400 to 800 µm in diameter, and contain numerous 8-spored asci. Ascospores are lenticular, 3.5 to 4.5 by 4 to 4.75 µm, and have two equatorial crests and echinulate convex surfaces (Fig. 2F and G).

A. pseudofelis. (i) **Holotype.** The holotype of *A. pseudofelis* is NRRL 62903 (= CM-6087), a culture permanently preserved in a metabolically inactive state as a freeze-dried preparation. The culture was isolated in 2010 from a human sputum sample at the National Center for Microbiology, Spain.

(ii) **Etymology.** The name is taken from the Greek root “pseudo,” or “false,” indicating that the species is not *A. felis*.

(iii) **Description.** Colonies attained a diameter of 7.5 to 8 cm on MEA or 5 to 5.5 cm on CZA in 5 days at 37°C (Fig. 3A and B). On MEA, colonies are whitish, with dense aerial hyphae and poor sporulation. The colony reverse is yellow. Conidiophores are hyaline, straight or slightly sinuous, and bear columnar conidial heads composed of flask-shaped vesicles (12 to 16 µm diameter) with uniseriate phialides producing long conidial chains (Fig. 3C and D). Conidia are green and subglobose (1.7 to 1.9 by 1.9 by 2.2 µm), with delicate surface roughness and shallow grooves (Fig. 3E). All the strains included in this study were of one mating type, *MAT1-2*, and thus, cleistothecium formation was not observed.

A. pseudoviridinutans. (i) **Holotype.** The holotype of *A. pseudoviridinutans* is NRRL 62904 (= NIHAV1), a culture permanently preserved in a metabolically inactive state as a freeze-dried preparation. The culture was isolated in 2004 from a human mediastinal lymph node specimen at the U.S. National Institutes of Health.

(ii) **Etymology.** The name is taken from the Greek root

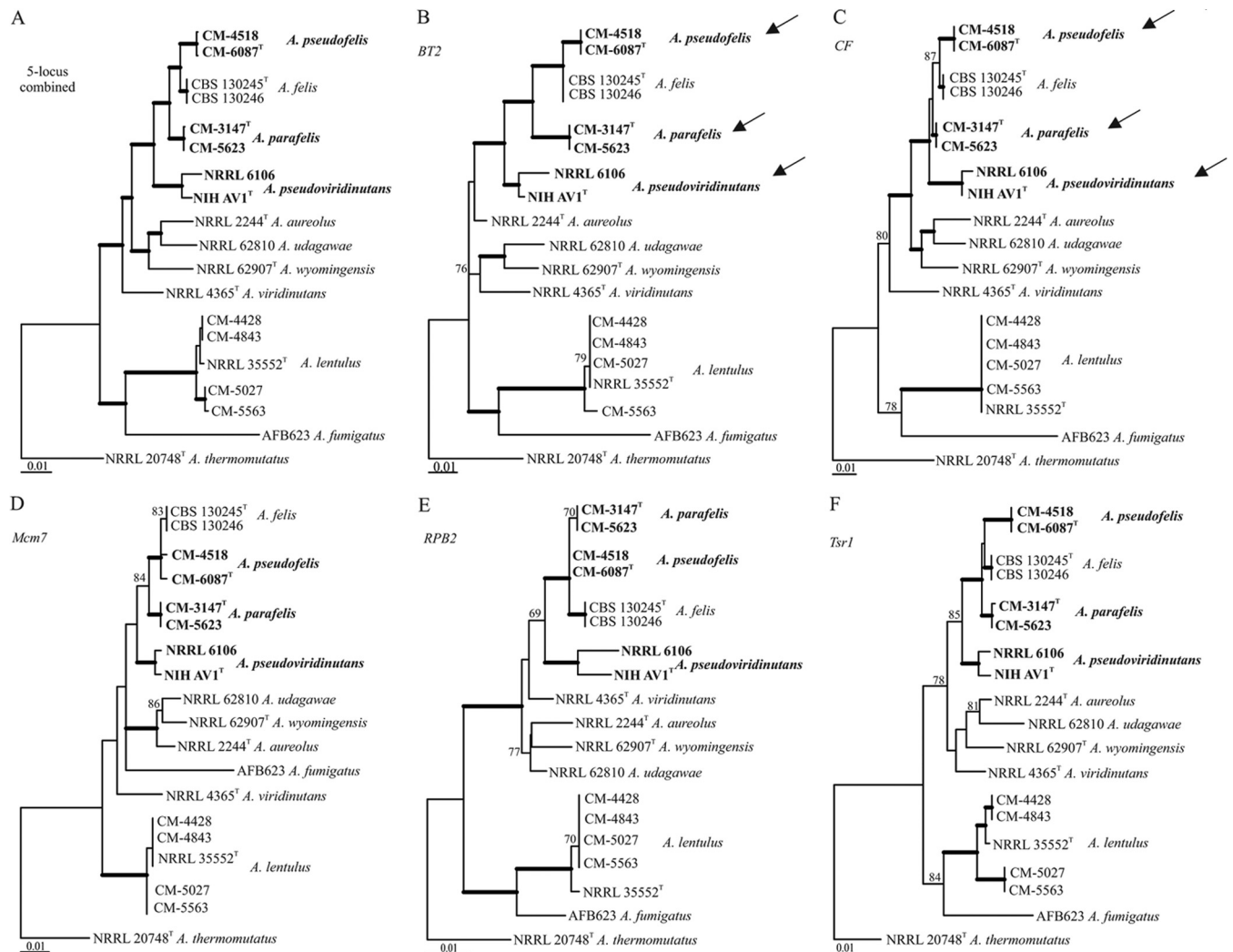


FIG 1 Maximum-likelihood trees based on DNA sequences from the five loci combined and each locus separately. (A) Combined data from five loci, *BT2*, *CF*, *Mcm7*, *RPB2*, and *Tsr1*; (B) beta-tubulin gene (*BT2*); (C) calmodulin gene (*CF*); (D) minichromosome maintenance factor 7 gene (*Mcm7*); (E) gene encoding the second-largest subunit of RNA polymerase II (*RPB2*); (F) preribosomal protein gene (*Tsr1*). Branches with thick lines have 90 to 100% bootstrap support. Bootstrap support of 69 to 89% is indicated by numerals. The validity of the new species is based on the congruence of a majority of statistically supported nodes in the single-locus trees (12, 13).

“pseudo,” or “false,” indicating that the species is not *A. viridinutans*.

(iii) Description. Colonies attained a diameter of 9 to 9.5 cm on MEA or 6 to 6.5 cm on CZA in 5 days at 37°C (Fig. 4A and B). On MEA, colonies are white with dense aerial hyphae and scant conidiation. The colony reverse becomes yellow with age. Conidiophores are hyaline and straight or slightly wavy. The conidial heads are columnar and consist of round, flask-shaped vesicles (7.5 to 10 μm diameter) with uniseriate phialides that produce conidial chains (Fig. 4C and D). Conidia are green and globose (1.75 to 2 μm), with a rough surface texture and uneven waviness (Fig. 4E). The two strains belonging to this species are of the *MAT1-1* mating type, and thus, sexual reproduction could not be evaluated.

Phenotypical analysis. Phenotypical analyses were carried out to evaluate the biotypes of the novel strains. *Aspergillus fumigatus* strain B-5233 and the type strains of *A. lentulus*, *A. felis*,

and *A. viridinutans* were included in the assays for purposes of comparison.

Conidial morphology. SEM revealed that the conidial topographies of *A. parafelis* and *A. pseudofelis* were distinct from those of *A. felis* and *A. viridinutans*. The surface texture of *A. felis* conidia resembled a walnut shell, with smooth waviness and deep grooves that were widely spaced (Fig. 5A), whereas the conidial surface in *A. viridinutans* was less wavy and had shallower grooves (Fig. 5C). The surface textures of *A. parafelis* and *A. pseudofelis* conidia were similar to each other, with jagged waviness and narrowly spaced grooves arranged in irregular reticulation (Fig. 5B and D). The surface roughness observed in *A. pseudoviridinutans* conidia resembled that of *A. fumigatus* conidia except that in *A. fumigatus* conidia, the texture was due to prominent spikes (Fig. 5G, arrowheads), whereas in *A. pseudoviridinutans* conidia, the surface roughness resulted from an accentuated topographic unevenness (Fig. 5H). The conidia of *A. parafelis* appeared to be linked to each

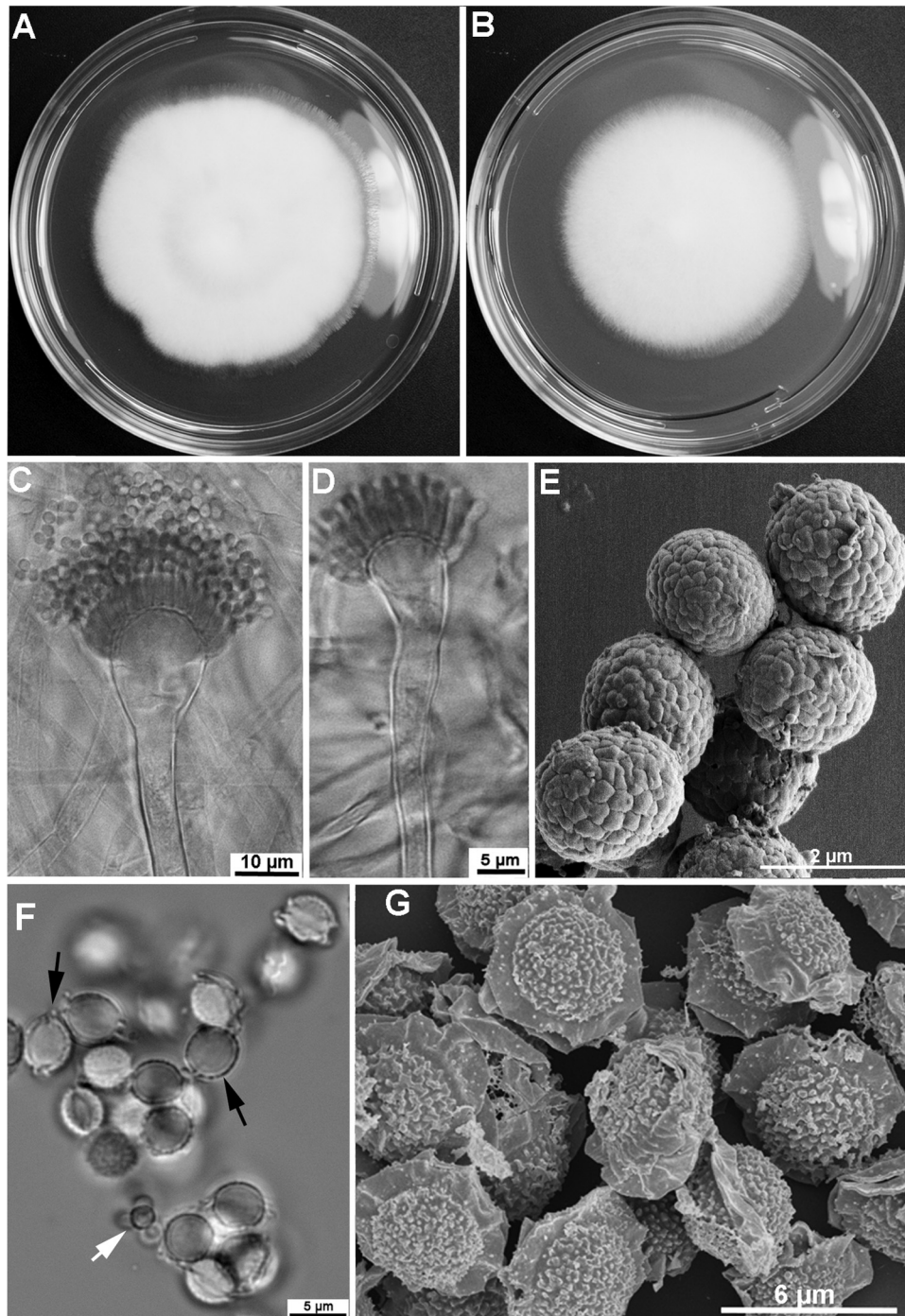


FIG 2 *Aspergillus parafelis* CM-3147^T (T, type strain). (A, B) Colonies grown on malt extract agar (MEA) (A) and Czapek's solution agar (CZA) (B) for 5 days at 37°C; (C, D) variation in conidiophores: straight (C) and slightly nodding (D); (E) SEM of conidia; (F, G) ascospores viewed by light microscopy (F) and SEM (G). (F) White arrow points to conidia; black arrows point to ascospores.

other by a snap-and-lock type of mechanism, with a connecting pin and a docking orifice located in opposite poles of each conidium (Fig. 5E and F, arrows). The conidia of *A. felis*, *A. pseudofelis*, *A. pseudoviridinutans*, *A. viridinutans*, and *A. fumigatus* show appendages, often seen bipolarly, which appear to function as bridges connecting the conidia (Fig. 5A, C, D, G, and H, arrows). Whether the connection between conidia is species specific is un-

clear. Further examination is required to analyze the connectivity in detail.

Growth. At 37°C, *A. parafelis*, *A. pseudofelis*, and *A. pseudoviridinutans* showed an intermediate growth rate between those of *A. fumigatus* (fast) and *A. viridinutans* (slow) (Fig. 6). Of note, the growth of the two *A. pseudofelis* strains, CM-6087 and CM-4518, was distinct. The colonies of CM-6087 attained a larger diameter

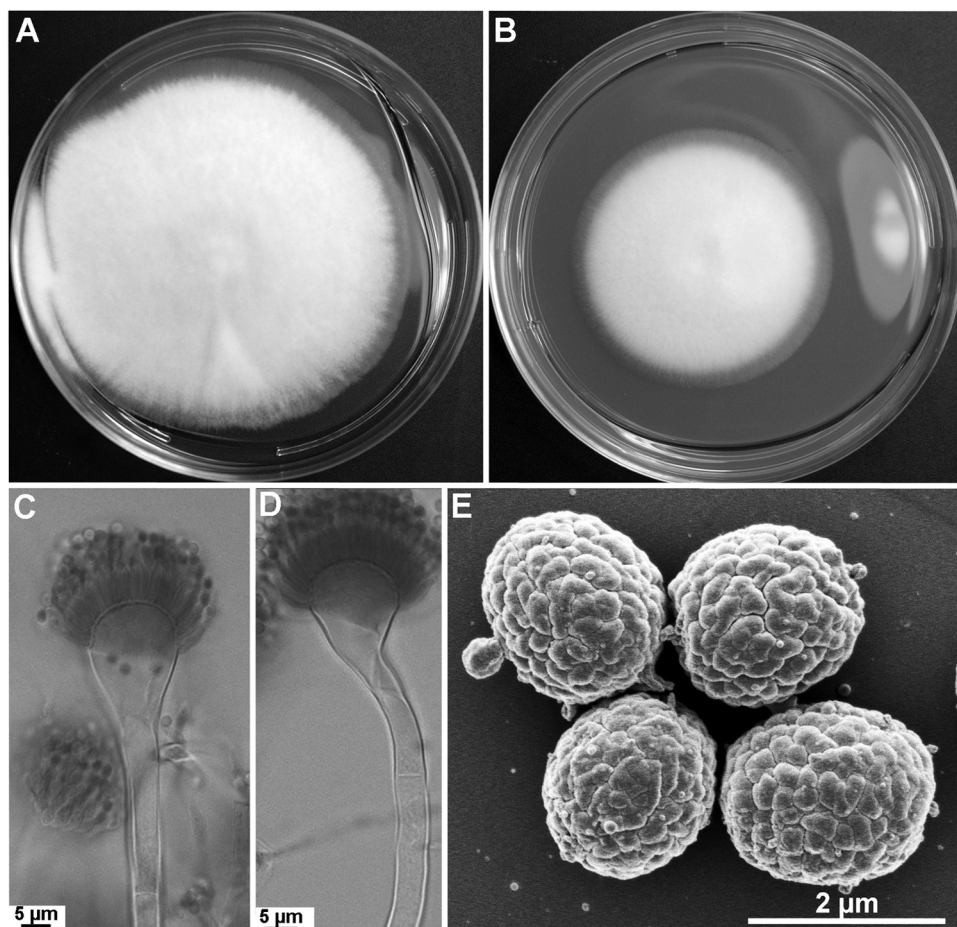


FIG 3 *Aspergillus pseudofelis* CM-6087^T. (A, B) Colonies grown on malt extract agar (MEA) (A) and Czapek's solution agar (CZA) (B) for 5 days at 37°C; (C, D) variation in conidiophores: straight (C) and slightly nodding (D); (E) SEM of conidia.

than those of CM-4518 but sporulated poorly, whereas the colonies of CM-4518 produced abundant conidia. However, the conidia produced by these strains exhibited identical surface topographies as observed by SEM (data not shown). At 42°C, all the strains showed reduced growth but the growth pattern was similar to that observed at 37°C. At 50°C, only *A. fumigatus* was able to grow.

Drug resistance. Antifungal susceptibilities were assessed by determining the MIC values (27) for amphotericin B, itraconazole, and voriconazole (Table 2). Among the three drugs, susceptibility to amphotericin B varied the least, with the *A. viridinutans* type strain being the most susceptible species. With voriconazole, the highest MIC values were observed in *A. felis*, *A. parafelis*, *A. pseudofelis*, and *A. pseudoviridinutans* NIHAV1. *Aspergillus lentulus* showed an intermediate susceptibility, while *A. fumigatus* and *A. viridinutans* were the most susceptible to voriconazole. The greatest variation of MIC values was observed with itraconazole. *Aspergillus fumigatus*, *A. lentulus*, *A. viridinutans*, and *A. pseudoviridinutans* NRRL 6106 showed MICs of ≤ 2 µg/ml, while the other species showed MICs of ≥ 8 µg/ml. Of note, *A. parafelis* and *A. pseudofelis* strains were able to grow at 16 µg/ml, the highest concentration of the drug tested, suggesting significant resistance to itraconazole.

Virulence. Two models were used for the virulence assays. In

the first model, larvae of *G. mellonella* were used as the host. In this model, *A. fumigatus* was significantly more virulent than the *A. lentulus* and *A. viridinutans* type strains (Fig. 7A). Strains of *Aspergillus pseudofelis* and the *A. felis* type strain showed significantly higher virulence than *A. fumigatus* B-5233 ($P \leq 0.0001$) (Fig. 7B and data not shown), while *A. parafelis* and *A. pseudoviridinutans* showed virulence similar to that of *A. fumigatus* B-5233 (data not shown). The survival curves also showed that the two isolates representing *A. parafelis* possessed similar virulence. The same concordance in virulence was found in the isolates of *A. pseudofelis* and *A. pseudoviridinutans*.

Virulence comparisons were then performed in two murine hosts, BALB/c mice immunosuppressed with hydrocortisone and mice with chronic granulomatous disease (CGD). Strains CM-3147 (*A. parafelis*), CM-6087 (*A. pseudofelis*), and NIHAV1 (*A. pseudoviridinutans*), along with the type strains of *A. viridinutans*, *A. lentulus*, and *A. felis*, were chosen for the virulence assays. In immunosuppressed BALB/c mice, *A. parafelis*, *A. pseudofelis*, and *A. felis* showed slightly higher virulence than *A. fumigatus* ($P \leq 0.04$) (Fig. 7D and data not shown), whereas *A. pseudoviridinutans* and *A. lentulus* displayed virulence similar or lower to that of *A. fumigatus* (Fig. 7C and D). The *Aspergillus viridinutans* type strain was the least virulent of all the strains and was the only strain that

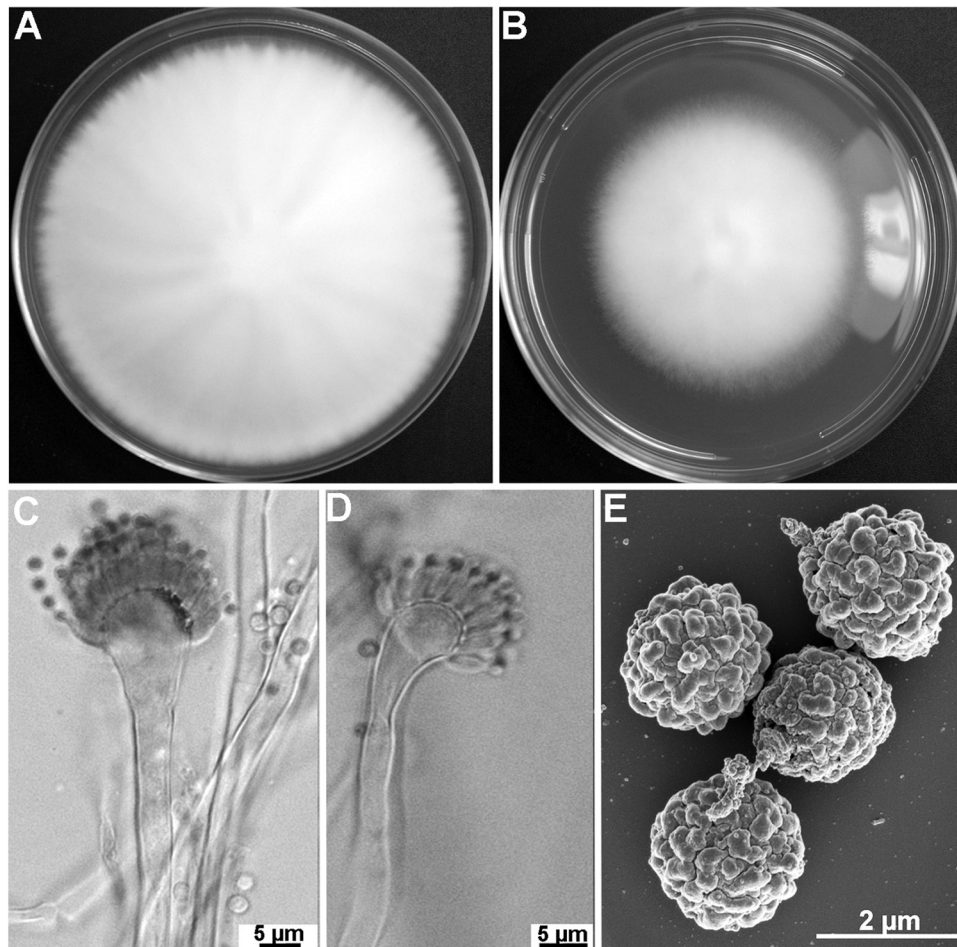


FIG 4 *Aspergillus pseudoviridinutans* NIHAV1^T. (A, B) Colonies grown on malt extract agar (MEA) (A) and Czapek's solution agar (CZA) (B) for 5 days at 37°C; (C, D) variation in conidiophores: straight (C) and slightly nodding (D); (E) SEM of conidia.

showed significantly reduced virulence compared to that of *A. fumigatus* ($P = 0.0001$) (Fig. 7C).

In the CGD model, while *A. fumigatus* caused 100% fatality in 11 days, none of the other species tested caused death in the same period of time. Figure 7E shows a representative survival curve of CGD mice inoculated with either *A. fumigatus* or *A. parafelis* CM-3147. The results of the virulence assays in hydrocortisone acetate-treated mice suggested that, similar to *A. fumigatus*, the novel species are capable of causing fatal infection in severely immunosuppressed hosts. However, dissimilar to *A. fumigatus*, the novel species are less virulent in the host that is deficient in the production of reactive oxygen species. Whether the novel species are isolated more often from patients with any particular type of immunodeficiency is unknown.

Mating. Sexual crosses included *A. fumigatus*, *A. lentulus*, *A. viridinutans*, *A. felis*, *A. wyomingensis*, *A. parafelis*, *A. pseudofelis*, and *A. pseudoviridinutans* (Table 3). Intraspecific mating was observed between several *A. lentulus* strains of the opposite mating type, confirming the functional heterothallism of this species (9). Analysis of 8-week-old crosses between CM-5027 and CM-5563, the most fertile *A. lentulus* isolates, revealed abundant cleistothecia in the junction zones of the two opposite mating type strains. The ascospore viability was 8% on average, and equal ratios of

MAT1 to *MAT2* mating types were observed among the progeny. *A. parafelis* isolates CM-3147 (*MAT1-2*) and CM-5623 (*MAT1-1*) also completed their sexual cycle in 8 weeks. Isolates of the other species used in this study (*A. viridinutans*, *A. pseudofelis*, and *A. pseudoviridinutans*) were either all *MAT1-1* or all *MAT1-2* mating type, and thus, their intraspecific fertility could not be assessed.

Two cases of unanticipated fertility were observed among the interspecific crosses (Table 3). *Aspergillus parafelis* isolate CM-3147 (*MAT1-2*) mated successfully with *MAT1-1* isolates of *A. fumigatus*, *A. pseudoviridinutans*, *A. viridinutans*, *A. felis*, and *A. wyomingensis*. The *MAT1-1* isolate of *A. parafelis* CM-5623, however, did not mate successfully in any interspecific crosses. In contrast to *A. parafelis* CM-3147, the *A. pseudofelis* *MAT1-2* isolates (CM-4518 and CM-6087) were only fertile when crossed with *A. felis* CBS130246 (*MAT1-1*). In general, interspecific crosses produced cleistothecia with viable ascospores in 4 to 8 weeks. The average viability of the hybrid ascospores harvested from 8-week-old crosses was less than 1%, significantly lower than that from conspecific pairings of *A. parafelis* (4%), *A. lentulus* (8%), and *A. fumigatus* (40%) (18).

Interspecies genetic recombination. Progeny from the cross between *A. parafelis* and *A. fumigatus* were chosen for recombinational analysis of the genetic markers. The mutant AFB623 (18),

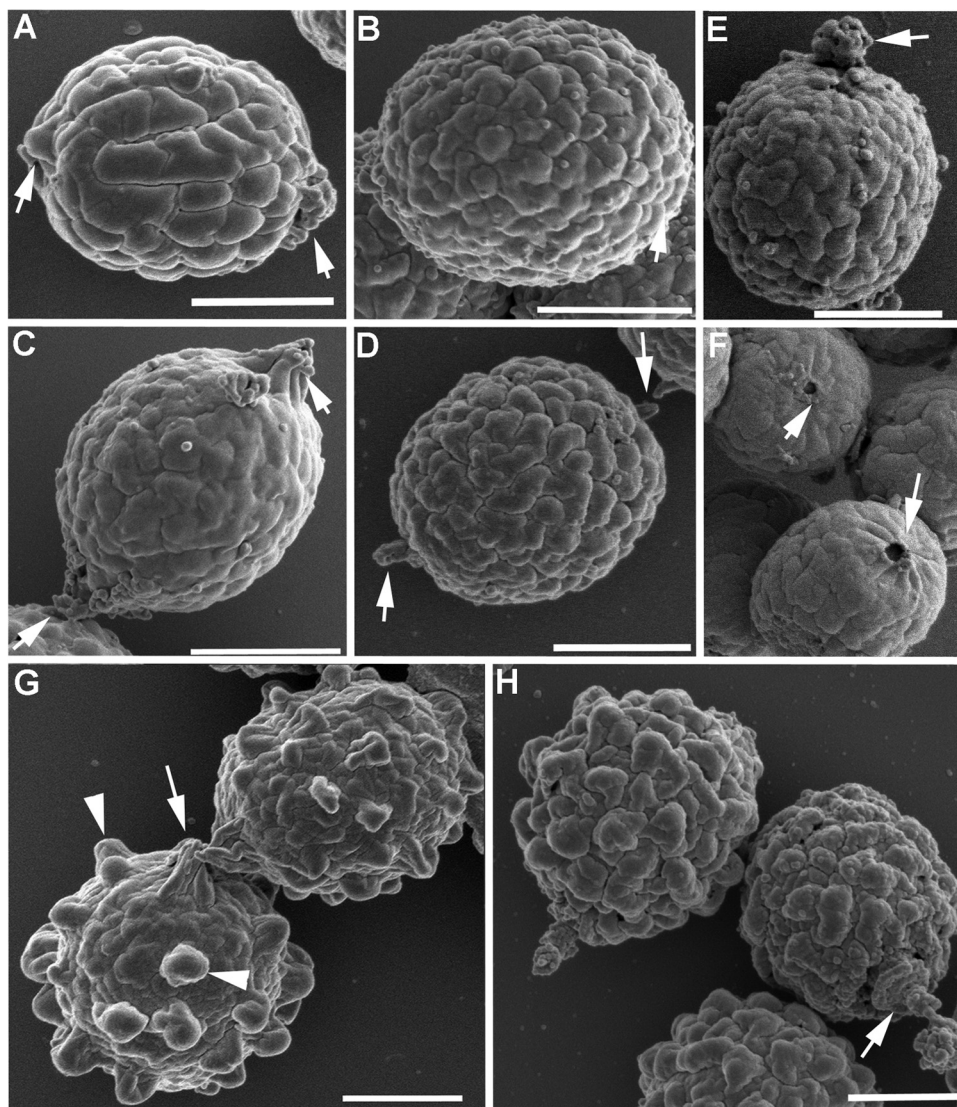


FIG 5 SEM of conidia. (A) *Aspergillus felis*; (B, E, F) *A. parafelis*; (C) *A. viridinutans*; (D) *A. pseudofelis*; (G) *A. fumigatus*; (H) *A. pseudoviridinutans*. Arrowheads point to spikes (G), and arrows point to appendage-like structures (A, C, D, E, G, H) or round orifices (F) located on the conidial surface. Bars = 1 μm .

derived from *A. fumigatus* supermater AFB62 (18), was used as a mating partner to facilitate genetic recombination analysis (Fig. 8). This mutant, a *MAT1-1* mating type strain, produces brown conidia due to deletion of the *abr2* gene (AFUA_2g17530) (18) and harbors a hygromycin resistance cassette used for the deletion of the gene. The genetic markers analyzed for recombination included conidial color, hygromycin resistance, thermotolerance at 50°C, and mating type. For recombinational analysis, eight offspring from an 8-week-old mating plate were randomly chosen at an early growth stage, before conidiation. The selected progeny included parental ditypes and recombinants (Table 4). Progeny P2 was brown, hygromycin resistant, thermotolerant to 50°C, and of the *MAT1-1* mating type, all from the AFB623 parent. Progeny P3 and P8 were green, susceptible to hygromycin, unable to grow at 50°C, and of the *MAT1-2* mating type, all from the *A. parafelis* parent. Progeny P1 displayed a mixed phenotype with brown conidia, hygromycin resistance, *MAT1-2* mating type, and inability to grow at 50°C. Progeny P4, P5, P6, and P7 also showed a

mixed phenotype. They produced green conidia that were hygromycin resistant and thermotolerant. However, the conidia harbored *MAT1-1* and *MAT1-2* mating types. Since their conidial sizes were slightly larger than that of either parent, these may be diploids or aneuploids, which could have resulted from chromosome missegregation during meiosis due to the genetic diversity between the parental strains. Among these progeny, only P5 and P7 were fertile; they produced a complete sexual cycle with parent *A. fumigatus* AFB623 but not with parent *A. parafelis* CM-3147. Interestingly, only P5 underwent self-mating with production of cleistothecia and viable ascospores. The fertility of P1, P2, P3, and P8 was also tested by backcrosses with parental strains. The *MAT1-2* progeny, P1, P3, and P8, were crossed with *A. fumigatus* AFB623, whereas the *MAT1-1* progeny, P2, was crossed with *A. parafelis* CM-3147. At 6 weeks, all four crosses showed cleistothecia with viable ascospores at the junction zone.

Ultrastructure comparisons. Scanning electron microscopy revealed that the ascospores produced by *A. parafelis* are lenticu-

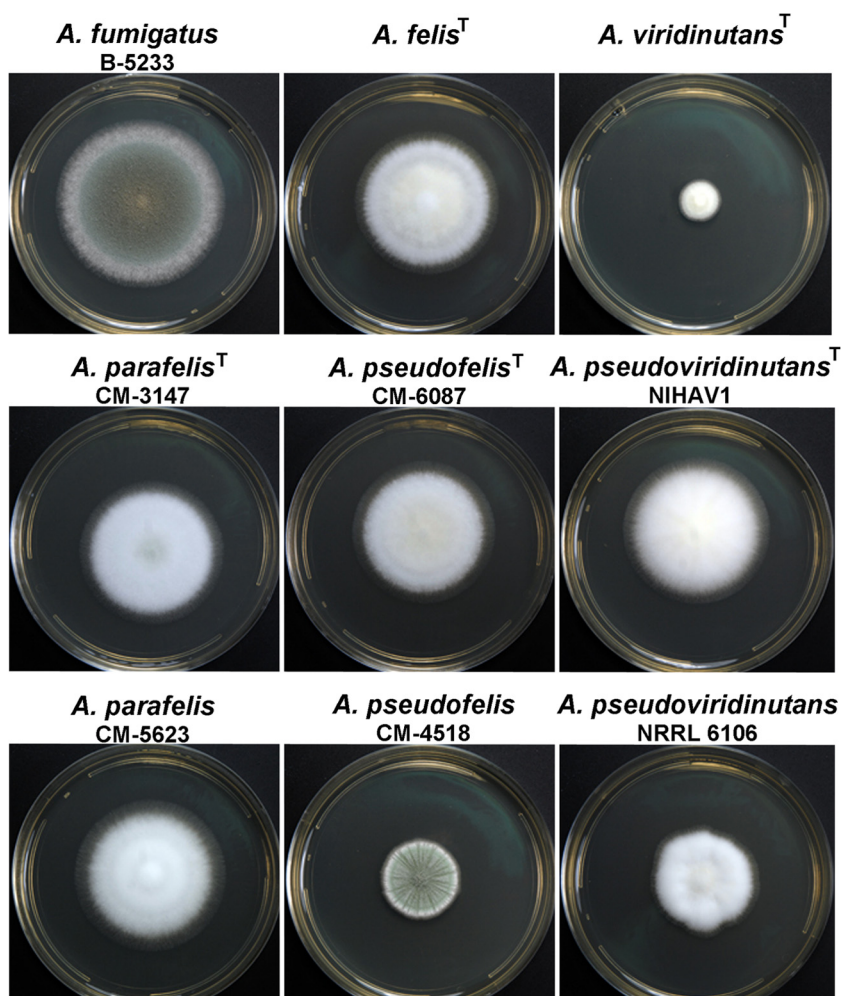


FIG 6 Colonies grown on malt extract agar (MEA). Plates were inoculated and then incubated at 37°C for 3 days.

lar, with two prominent equatorial crests and echinulate convex surfaces (Fig. 9B and E). The groove between the crests was ornamented with blunt toothlike projections arranged in double rows (Fig. 9E, arrow). Ascospores from *A. fumigatus* are also lenticular with two equatorial crests, but their convex surface is ornamented with unorganized tissue flaps and the groove between the crests is

smoother and without the toothlike projections (Fig. 9A and D, arrow). The surface markings on the hybrid ascospores produced by *A. parafelis* CM-3147 and *A. fumigatus* AFB623 resembled those of *A. parafelis* more than those of *A. fumigatus*, except that the toothlike projections between the equatorial crests were not as homogeneous in size and shape as those of *A. parafelis* (Fig. 9C and F, arrow). The hybrid ascospores produced by mating of *A. parafelis* CM-3147 with *A. viridinutans*, *A. felis*, or *A. pseudoviridinutans* (Fig. 9G to L) or by mating of *A. pseudofelis* with *A. felis* showed the same general morphology: a lenticular spore body with two equatorial crests, toothlike projections in the groove between the crests, and echinulate convex surfaces. Figure 9J shows the toothlike structures aligned on the outer edge of one of the ascospore hemispheres (arrow). A similar view is shown in Fig. 9L.

TABLE 2 MICs of amphotericin B, itraconazole, and voriconazole

Strain	MIC ($\mu\text{g/ml}$) of:		
	Amphotericin B	Itraconazole	Voriconazole
<i>A. fumigatus</i> B-5233	1	2	2
<i>A. lentulus</i> ^T	1	1	4
<i>A. viridinutans</i> ^T	0.25	1	1
<i>A. felis</i> ^T	1	8	8
<i>A. parafelis</i> CM-3147	2	>16	8
<i>A. parafelis</i> CM-4518	2	>16	8
<i>A. pseudofelis</i> CM-5623	2	>16	8
<i>A. pseudofelis</i> CM-6087	2	>16	8
<i>A. pseudoviridinutans</i> NIHAV1	2	>16	8
<i>A. pseudoviridinutans</i> NRRL 6106	2	2	2

DISCUSSION

The identification of *Aspergillus* species is a multifaceted process that involves morphological, physiological, and phylogenetic parameters. Until DNA sequencing became readily available, several clinical strains were misidentified as *A. fumigatus* based solely on the morphological resemblance of their conidiogenous structures to those of *A. fumigatus*. The use of unlinked sequences from

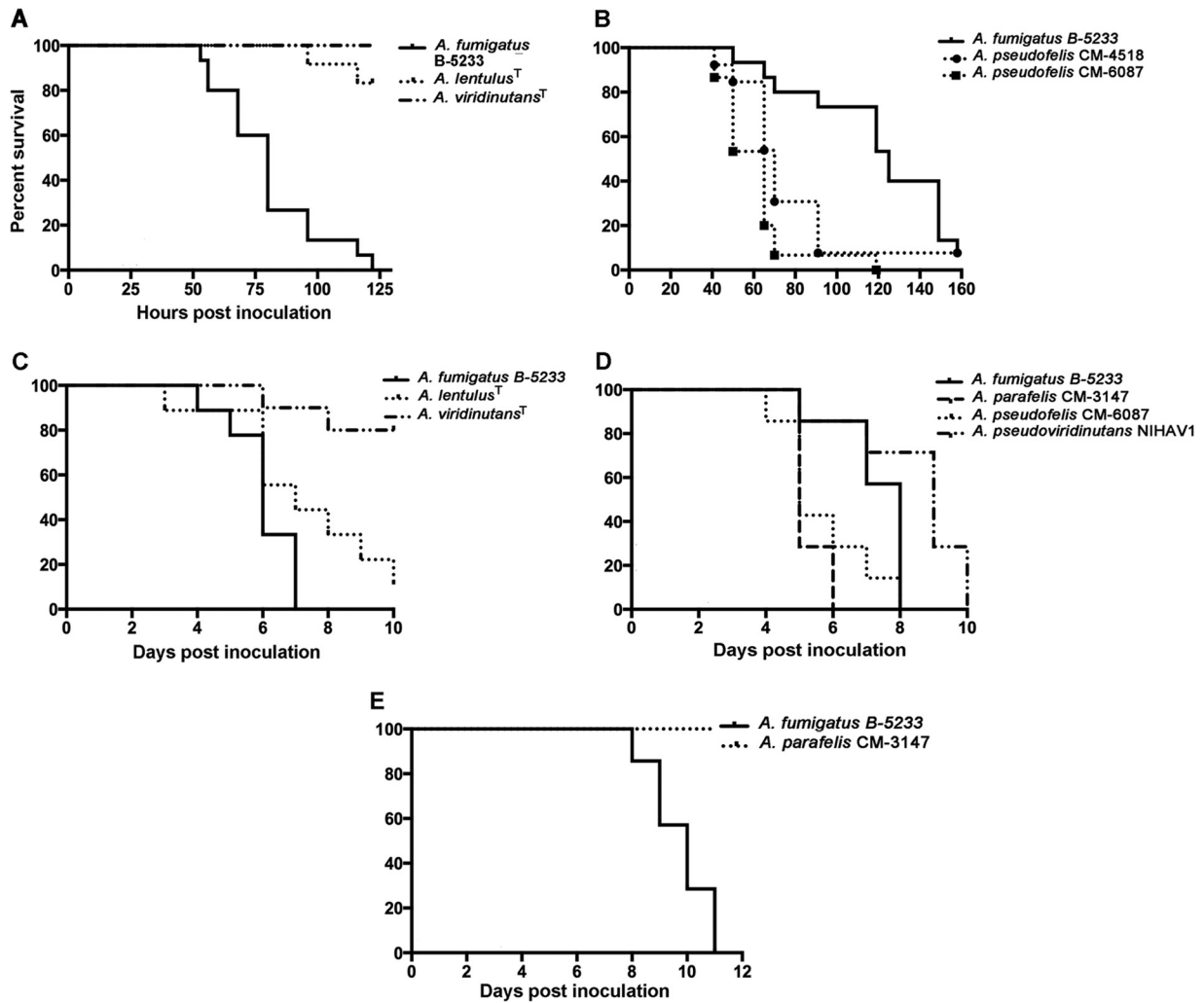


FIG 7 Virulence of *Aspergillus* species in larvae and murine models. (A, B) Larvae of *G. mellonella*; (C, D) BALB/c mice immunosuppressed with hydrocortisone; (E) CGD mice. Hosts were inoculated with *A. fumigatus* (B-5233), *A. parafelis*, *A. pseudofelis*, *A. pseudoviridinutans*, and type strains of *A. lentulus* and *A. viridinutans*. *G. mellonella* larvae were inoculated with 2×10^7 conidia/ml and incubated at 37°C. Mice were inoculated via postpharyngeal aspiration with 3×10^7 (BALB/c) or 3×10^5 (CGD) conidia/ml.

polymorphic loci was instrumental in distinguishing the isolates that resembled *A. fumigatus* from the true *A. fumigatus* (6). Since then, there has been a steady increase in the number of species in section *Fumigati*, which includes *A. fumigatus* (Table 5). The concordance of multiple-gene genealogies has been used previously to

examine the phylogenetic relationship in other species. For instance, phylogeny based on three unlinked loci confirmed the prediction that the *Neurospora discreta* complex included several separate species, which correlated with the morphological variability and reproductive behavior in that complex (13).

TABLE 3 Intra- and interspecific mating among sister species of section *Fumigati*

MATI-2 strain	Fertility of MATI-1 strain							
	<i>A. fumigatus</i> AFB623	<i>A. lentulus</i> CM-5563	<i>A. felis</i> CBS130246	<i>A. parafelis</i> CM-5623	<i>A. viridinutans</i> NRRL 35552 ^T	<i>A. pseudoviridinutans</i>		<i>A. wyomingensis</i> CCF 4417
						NIHAV1	NRRL6106	
<i>A. fumigatus</i> AFIR928	+	–	–	–	–	–	–	–
<i>A. lentulus</i> CM-5027	–	+	–	–	–	–	–	–
<i>A. felis</i> CBS 130245 ^T	–	–	+	–	–	–	–	–
<i>A. parafelis</i> CM-3147	+	–	+	+	+	+	+	+
<i>A. pseudofelis</i> CM-6087	–	–	+	–	–	–	–	–
<i>A. pseudofelis</i> CM-4518	–	–	+	–	–	–	–	–
<i>A. wyomingensis</i> CCF 4416	–	–	–	–	–	–	–	+

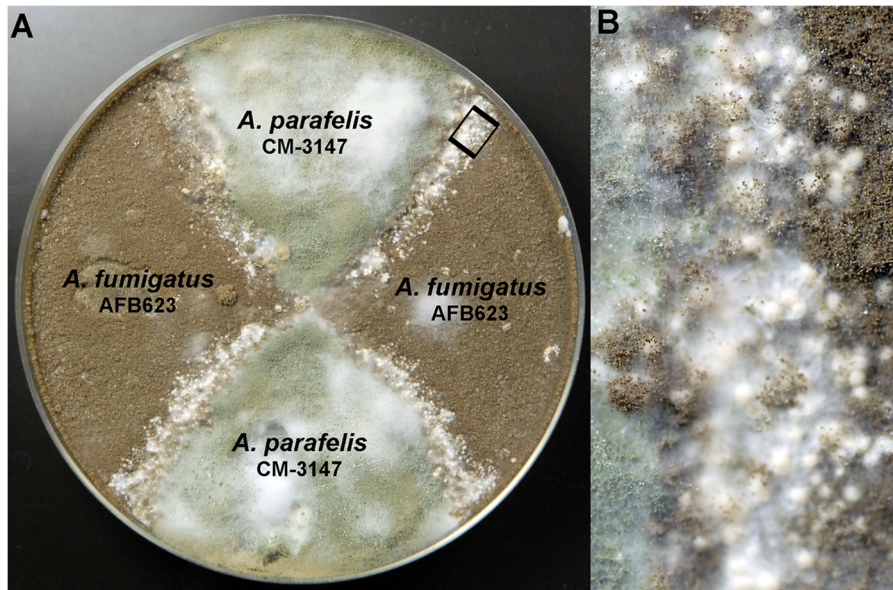


FIG 8 Mating between *A. parafelis* CM-3147 and *A. fumigatus* AFB623. (A) An 8-week-old cross with an abundant production of cleistothecia; (B) magnification of inset in panel A showing cleistothecia produced in the junction zone of *A. parafelis* (green conidia) and *A. fumigatus* AFB623 (brown conidia).

In this study, the genealogical analysis carried out with five loci provided evidence that the five strains previously identified as *A. viridinutans* contained novel phylogenetic species. Three of these strains (CM-3147, CM-5623, and CM-4518) (Table 1) were recently reclassified as *A. felis* (10) based on the DNA sequence of either *BT2* or *CF*. Barrs et al. reported that among the 24 strains identified as *A. felis*, CM-3147, CM-5623, and CM-4518 belonged to a small group (five strains) isolated from humans (10). Further phylogenetic analysis performed in this study, using the sequences of *RPB2*, *Mcm7*, and *Tsr1* in addition to those of *BT2* or *CF*, demonstrated that in at least three of the five loci, these three strains clustered in separate monophyletic clades from *A. felis*. Thus, although these strains are biologically very close to *A. felis*, they should be considered different species. The phylogenetic analysis

based on all five loci established the identity of a third novel species, *Aspergillus pseudoviridinutans*, formed by strains NIHAV1 and NRRL 6106. The morphological, physiological, and genetic variability within the *A. viridinutans* complex has been an issue since the late 1990s (34, 35). Phylogenetic analyses based on the sequences of *BT2*, an alkaline protease gene (*Alp*), the hydrophobin gene, and *CF* showed multiple clades among strains identified as *A. viridinutans* (4, 36). Recently, a revision based on phylogenetic analysis using the *BT2* or *CF* sequences, as well as the biotypical characteristics, recognized the existence of five species within this complex, *Aspergillus aureolus*, *Aspergillus udagawae*, *A. felis*, *A. viridinutans*, and *A. wyomingensis* (11).

Phenotypic and morphological analysis corroborated the identification of the novel species identified in this study. Growth, thermotolerance, and drug resistance were similar among the three novel species but dissimilar to those of *A. viridinutans* and *A. fumigatus*. Furthermore, virulence assays in *G. mellonella* larvae and mice showed that *A. viridinutans* was markedly less virulent than the three novel species. The *Aspergillus viridinutans* type strain was isolated from rabbit dung (37), whereas the strains used in this study were mostly isolated from human patients, which may explain the differences in virulence. SEM revealed that (i) the surface topographies of *A. parafelis* and *A. pseudofelis* conidia were similar to each other but different from that of *A. felis* and (ii) the conidial morphology of *A. pseudoviridinutans* was markedly different from those of *A. viridinutans*, *A. parafelis*, and *A. pseudofelis*; both findings support the phylogenetic analysis (Fig. 5).

No differences in the biotypes of *A. parafelis* and *A. pseudofelis* were found within the parameters tested. However, a comparison of the *MAT1-2* locus of the two *A. pseudofelis* strains, CM-6087 and CM-4518, with that of three other aspergilli (*A. parafelis* CM-3147, *A. felis* CBS130245, and *A. fumigatus* AF293) showed 100% identity between the two strains of *A. pseudofelis* and 10 single-nucleotide polymorphisms (SNPs) unique to the *A. pseudofelis* isolates. One SNP was found in the open reading frame of one of

TABLE 4 Interspecies genetic recombination

Parental strain or progeny	Mating type(s)	Conidial color	Growth at 50°C	Hygromycin resistance
Parents				
<i>A. fumigatus</i> AFB623	<i>MAT1-1</i>	Brown	Yes	Yes
<i>A. parafelis</i> CM-3147	<i>MAT1-2</i>	Green	No	No
Progeny				
P1	<i>MAT1-2</i>	Brown	No	Yes
P2	<i>MAT1-1</i>	Brown	Yes	Yes
P3	<i>MAT1-2</i>	Green	No	No
P4	<i>MAT1-1</i> and <i>MAT1-2</i>	Green	Yes	Yes
P5	<i>MAT1-1</i> and <i>MAT1-2</i>	Green	Yes	Yes
P6	<i>MAT1-1</i> and <i>MAT1-2</i>	Green	Yes	Yes
P7	<i>MAT1-1</i> and <i>MAT1-2</i>	Green	Yes	Yes
P8	<i>MAT1-2</i>	Green	No	No

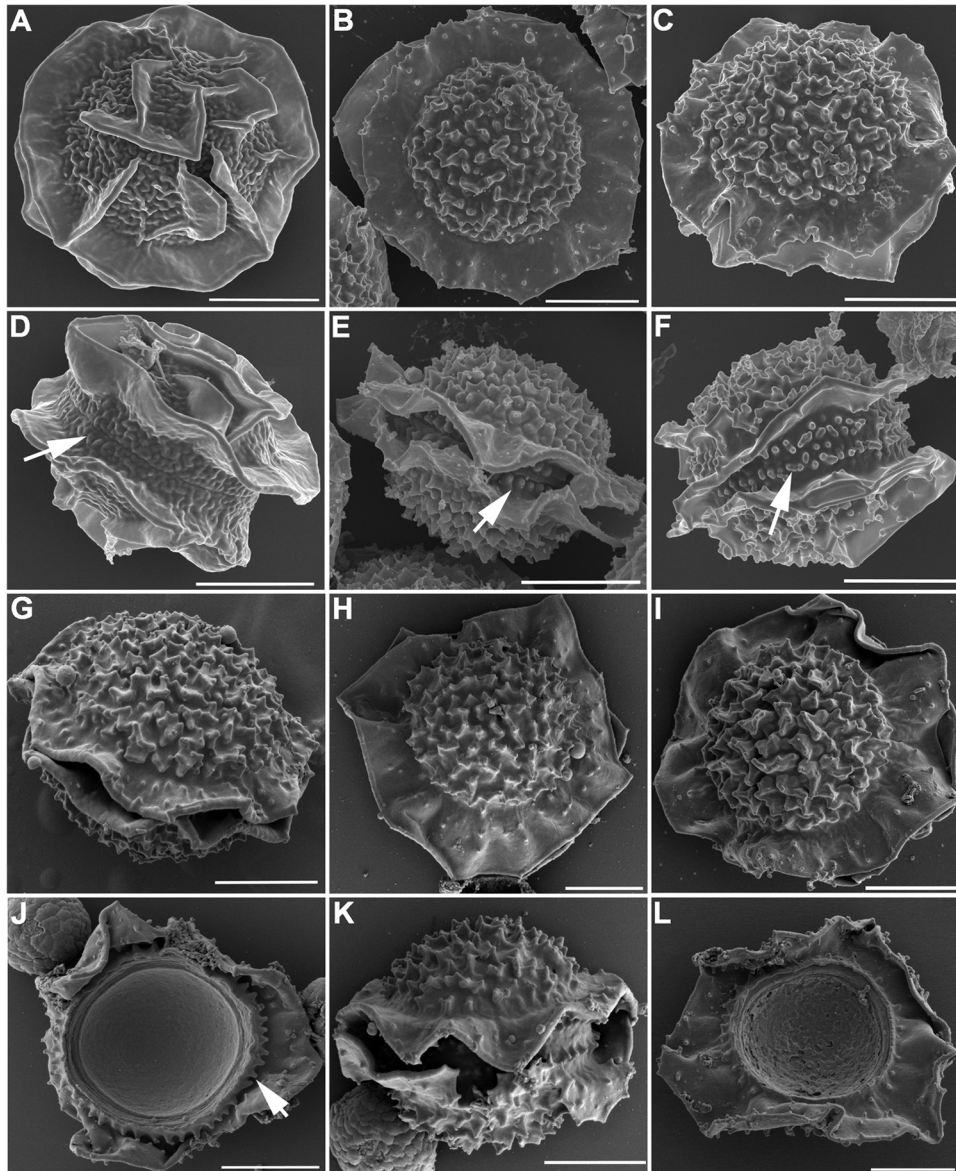


FIG 9 SEM of ascospores. (A, D) *A. fumigatus* AFIR928 \times AFB623; (B, E) *A. parafelis* CM-3147 \times CM-5623; (C, F) *A. parafelis* CM-3147 \times *A. fumigatus* AFB623; (G, J) *A. parafelis* CM-3147 \times *A. viridinutans* type strain; (H, K) *A. parafelis* CM-3147 \times *A. felis* CBS 130246; (L, L) *A. parafelis* CM-3147 \times *A. pseudoviridinutans* NIHAV1. Bars = 2 μ m.

the mating proteins of the *MAT* locus, and five SNPs were present in the other mating protein. While the sequence of the *MAT* locus is not traditionally used for species identification, the unique SNPs of *A. pseudofelis* reinforce the hypothesis that they are separate species.

The phylogenetic distinction between members of section *Fumigati* is well established. However, their biological relatedness remains unclear. Crossbreeding between species was used as a tool to study the correlation between phylogenetic distance and biological reproductive success (or isolation). Mating between *A. fumigatus* and the related species included in this study revealed that reproductive compatibility varied among phylogenetically close sibling species. *A. fumigatus* and *A. lentulus*, though phylogenetically closer to each other than to the other sibling species, were reproductively isolated, but *A. pseudofelis* and *A. felis* were both

phylogenetically close and reproductively compatible. In addition, mating between the *MAT1-2* isolate of *A. parafelis* and the *MAT1-1* isolates of *A. fumigatus*, *A. viridinutans*, *A. felis*, *A. pseudoviridinutans*, and *A. wyomingensis* (Fig. 8) suggests that phylogenetic divergence might have preceded the reproductive isolation in some of the related species. Disagreement between mating success and genealogical concordance is not an unprecedented phenomenon; a lack of correlation was also observed in the ancestral nature of the mating type genes in the *Fusarium graminearum* complex (38, 39). The finding of promiscuous mating seen in some *A. parafelis* and *A. pseudofelis* isolates demonstrates a mechanism for interspecific (horizontal) gene flow that could result in the variable phylogenies found at the different loci used in this study and the rejection of a molecular clock in the sequences as a consequence. This is apparently a rare occurrence, as to the best of

TABLE 5 List of *Aspergillus* species included in section *Fumigati*

Species ^a	Reference strain
<i>A. brevipes</i> (37)	CBS 118.53 ^T
<i>A. caatingaensis</i> (44)	IFM 61335 ^T
<i>A. duricaulis</i> (37)	CBS 481.65 ^T
<i>A. felis</i> * (10)	CBS 130245 ^T
<i>A. fumigatiaffinis</i> * (37)	IBT12703 ^T
<i>A. fumigatus</i> * (37)	CBS 133.61 ^T
<i>A. fumisynnematus</i> * (37)	IFM 42277 ^T
<i>A. huiyanae</i> (45)	IFM57847 ^T
<i>A. lentulus</i> * (37)	NRRL 35552 ^T
<i>A. marvanovae</i> (46)	CCM8003 ^T
<i>A. novofumigatus</i> * (37)	IBT 16806 ^T
<i>A. parafelis</i> * (this study)	CM-3147 ^T
<i>A. pernambucoensis</i> (44)	IFM 61342 ^T
<i>A. pseudofelis</i> * (this study)	CM-6087 ^T
<i>A. pseudoviridinutans</i> * (this study)	NIHAV1 ^T
<i>A. siamensis</i> (47)	KUFC 6349
<i>A. turcosus</i> (37)	KACC 4209 ^T
<i>A. unilateralis</i> (37)	CBS 126.56 ^T
<i>A. viridinutans</i> * (37)	NRRL 4365 ^T
<i>A. waksmanii</i> (46)	NRRL 179 ^T
<i>A. wyomingensis</i> (11)	CCF 4417 ^T
<i>N. assulata</i> (37)	KACC 4169 ^T
<i>N. aurata</i> (37)	CBS 466.65 ^T
<i>N. aureola</i> (37)	CBS 105.55 ^T
<i>N. australensis</i> (37)	CBS 112.55 ^T
<i>N. coreana</i> (37)	KACC 41659 ^T
<i>N. denticulata</i> (37)	CBS 652.73 ^T
<i>N. fennelliae</i> (37)	CBS 598.74 ^T
<i>N. ferencczii</i> (37)	CBS 121594 ^T
<i>N. fischeri</i> * (37)	CBS 544.65 ^T
<i>N. galapagensis</i> (37)	CBS 117522 ^T
<i>N. glabra</i> (37)	CBS 111.55 ^T
<i>N. hiratsukae</i> * (37)	CBS 294.93 ^T
<i>N. indohii</i> (48)	CBM-FA-934 ^T
<i>N. laciniosa</i> * (37)	KACC 41657 ^T
<i>N. multiplicata</i> (37)	CBS 646.95 ^T
<i>N. nishimurae</i> (37)	CBS 116047
<i>N. papuensis</i> (37)	CBS 841.96 ^T
<i>N. pseudofischeri</i> * (37)	NRRL 20748 ^T
<i>N. quadricincta</i> (37)	CBS 135.52 ^T
<i>N. shendaweei</i> (49)	IFM 5761 ^T
<i>N. spathulata</i> (37)	CBS 408.89 ^T
<i>N. spinosa</i> (37)	CBS 483.65 ^T
<i>N. stramenia</i> (37)	CBS 498.65 ^T
<i>N. sublevispora</i> (50)	IFM 53598 ^T
<i>N. takakii</i> (51)	CBM-FA-884 ^T
<i>N. tatenoi</i> (37)	CBS 407.93 ^T
<i>N. tsunodae</i> (49)	IFM57609 ^T
<i>N. tsurutae</i> (48)	CBM-FA-93 ^T
<i>N. udagawae</i> * (37)	CBS 114217 ^T
<i>N. warcupii</i> (37)	NRRL 35723 ^T

^a The novel species described in this study are highlighted in boldface. Human pathogens are marked with an asterisk (52).

our knowledge, no other promiscuous maters were described in section *Fumigati*. However, there is no reason to assume that such interspecies mating does not occur from time to time. In fact, interspecies mating has been shown to occur in *Aspergillus* section *Flavi* (40). In mating between *Aspergillus flavus* and *Aspergillus minisclerotigenes*, which were designated as two distinct species according to beta-tubulin and calmodulin sequences (41), not

only were they able to undergo complete sexual cycles but their progeny were fertile when backcrossed with the parental strain of the opposite mating type (40).

Phenotype analysis of hybrid ascospores produced by mating of *A. fumigatus* with *A. parafelis* suggested that genetic recombination similar to that found in intraspecific mating occurred in half of the progeny. The presence of both mating loci in the other half of the progeny suggested the occurrence of chromosomal missegregation resulting in diploids or aneuploids. Such events occur frequently in matings between genetically diverse strains, e.g., in mating between the two varieties (var. *neoformans* and var. *grubii*) of *Cryptococcus neoformans* or between *C. neoformans* and its sister species *C. gattii* (42). The rate of aneuploid and diploid clone production was higher among the progeny isolated from mating between *C. neoformans* and *C. gattii*. The genetic diversity that between the two *Cryptococcus* species is considerably higher than that between the two varieties within *C. neoformans* (43).

The findings in this study indicate that mating between species may not be reliable to determine species boundaries among the members of section *Fumigati*, since *A. parafelis* was able to mate with several of its sister species. In cases such as section *Fumigati*, majority rule concordance analysis should be used to recognize species boundaries. This study also reinforces the need of multiple loci for a higher resolution of the phylogenetic species boundary and accurate species identification. In cases where analysis of multiple loci is not feasible, either due to time constraints or availability of resources, the sequences of the beta-tubulin (*BT2*) and calmodulin (*CF*) genes, the two most commonly found sequences in public databases, can be used for the initial identification of the *Aspergillus* species. It is essential, however, to confirm the identity of the species with a third locus. Erroneous identification of clinical isolates might result in grave consequences for patients. For instance, the three novel species described here showed considerably higher resistance to antifungal drugs than did the type strain of *A. viridinutans*; thus, disease caused by these species is likely to be refractory to routine antifungal therapies and might require more aggressive therapy than is commonly used for aspergillosis due to *A. fumigatus*.

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