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Aspergillus citrinoterreus, a New Species of Section *Terrei* Isolated from Samples of Patients with Nonhematological Predisposing Conditions

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The use of molecular identification techniques has revealed an increasing number of new species within *Aspergillus* section *Terrei*. We phenotyped a set of 26 clinical isolates that showed genetic differences from *Aspergillus terreus sensu stricto* by analyzing sequences from PCR-amplified β-tubulin and calmodulin genes and the internal transcribed spacer region. Since the isolates were phylogenetically and morphologically different from all of the members of *Aspergillus* section *Terrei*, they are described here as a new species, *Aspergillus citrinoterreus*, so named because it produces a diffusible yellowish pigment in agar. *A. citrinoterreus* isolates were significantly more susceptible to itraconazole, voriconazole, and posaconazole than *A. terreus sensu stricto* isolates were; in contrast, the amphotericin B MICs for both species were high. *A. citrinoterreus* was found in clinical samples from patients with proven or probable invasive aspergillosis and colonized patients, none of whom had hematological malignancies as predisposing conditions. However, they did have other underlying conditions such as chronic obstructive pulmonary disease, cirrhosis, and cancer or had received a solid organ transplants and presented not only with invasive pulmonary aspergillosis but also with mediastinitis. *A. citrinoterreus* isolates were detected for the first time in 2002. In all cases of invasive aspergillosis, *A. citrinoterreus* was found to be a copathogen, mostly with *A. fumigatus*.

Invasive aspergillosis affects patients with hematological and nonhematological conditions such as chronic obstructive pulmonary disease (COPD) (1–3). Most cases of invasive aspergillosis are caused by *Aspergillus fumigatus* and *A. flavus*. *A. terreus* is the third most common cause of invasive aspergillosis and a particularly prevalent microorganism in some geographic areas (4–8).

Molecular tools can provide an accurate picture of the epidemiology of invasive aspergillosis and have revealed the presence of cryptic *Aspergillus* species frequently missed by conventional techniques (9–11). The number of newly described species within the section *Terrei* has grown during the last few years and includes *A. alabamensis*, *A. allahabadii*, *A. ambiguous*, *A. aureoterreus*, *A. carneus*, *A. floccosus*, *A. hortai*, *A. microcysticus*, *A. neoafricanus*, *A. neoindicus*, *A. niveus*, *A. pseudoterreus*, and *A. terreus sensu stricto* (12). To date, most of these species, with the exception of *A. terreus sensu stricto*, have not been reported to cause invasive aspergillosis in humans.

We previously used molecular techniques to identify a set of *Aspergillus* section *Terrei* isolates collected from clinical samples from patients admitted to a general teaching hospital (13). Most of the isolates were identified as *A. terreus sensu stricto*, but a clade of isolates showed some remarkable genetic differences. The isolates comprising the clade have been phenotypically characterized and are reported here as representatives of a proposed new species within *Aspergillus* section *Terrei*, namely, *Aspergillus citrinoterreus*.

MATERIALS AND METHODS

Hospital description. This study was carried out at Hospital Gregorio Marañón, a large tertiary-care hospital serving a population of approximately 715,000 inhabitants of Madrid, Spain. The institution cares for all types of patients at risk of acquiring aspergillosis, including solid organ and bone marrow transplant recipients and patients with hematological malignancies, HIV infection, and COPD.

Study population and fungal isolates. We studied 26 *A. terreus sensu lato* isolates from the lower respiratory tract ($n = 21$), wounds ($n = 4$), and an abscess ($n = 1$) from 18 patients with proven ($n = 1$) or probable ($n = 5$) invasive aspergillosis or *Aspergillus* colonization ($n = 12$) admitted to Hospital Gregorio Marañón (Madrid, Spain) (Table 1). The isolates, which differed genetically from *A. terreus sensu stricto*, comprised 18 strains collected from 2002 to 2010 and studied in our previous investigation (13) and 8 additional strains collected from 2010 to 2012. Patients were classified according to the revised criteria of the EORTC (14); patients with COPD fulfilled Bulpa's criteria (15). We included 10 clinical isolates of *A. terreus sensu stricto* as controls.

DNA extraction, amplification, and sequencing. Genomic DNA was extracted from conidial suspensions of the isolates with the QIAamp DNA minikit (Qiagen, Heidelberg, Germany). The internal transcribed spacer (ITS) region and fragments of the β-tubulin (*Tub*) and calmodulin (*Cal*) genes were amplified and sequenced as previously described (13). Ampli-

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TABLE 1 Clinical sources and years of isolation of *A. citrinoterreus* isolates

A. citrinoterreus isolate	Clinical source	Yr of isolation
GM 228	Sputum	2006
GM 464	Bronchial secretion	2007
GM 541	Sputum	2007
GM 673	Sputum	2007
GM 676	Sputum	2007
GM 837	Abscess	2007
GM 1381	Wound	2008
GM 1426	Wound	2008
GM 1479	Wound	2008
GM 1504	Wound	2008
GM 1532	Sputum	2008
GM 1597	Sputum	2008
GM 1777	Sputum	2008
GM 2006	Sputum	2009
GM 2025	Sputum	2009
GM 2137	Sputum	2009
GM 2568	Sputum	2010
GM 2597	Sputum	2010
GM 3921	Bronchial secretion	2011
GM 3934	Bronchial secretion	2011
GM 3939	Bronchial secretion	2011
GM 3959	Bronchial secretion	2011
GM 3967	Bronchial secretion	2011
GM 4016	Bronchial secretion	2011
GM 4511	Bronchial secretion	2002
GM 4611	Sputum	2012

cons were purified with illustra GFX PCR DNA and Gel Band Purification kits (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, United Kingdom).

Phylogenetic analyses. Sequences from each locus were aligned by MEGA version 5.05 (16) and ClustalW (17) and manually improved when necessary. For patients with multiple samples from which the same fungus was isolated, only the isolate found in the first sample ($n = 18$) was included in the phylogenetic analyses. Neighbor-joining analyses using the Kimura two-parameter model were applied to each data partition individually in order to compare and check the stability of the individual phylogenies. Since no incongruence was found, the three loci were combined into a single data set. Phylogenetic reconstructions of the combined data set were made on the basis of maximum-likelihood (ML) analysis with Mega 5.05 and Bayesian inference (BI) analysis with MrBayes version 3.1.2 (18). For ML analysis, nearest-neighbor interchange was used as a heuristic method, gaps were treated as partial deletions with a 95% site coverage cutoff, and the robustness of the branches was estimated with a 1,000-generation ML bootstrapped data set (bootstrap values), for which a value ≥70% was considered significant. For BI analysis, two parallel runs of four incrementally heated Markov chains were performed for 800,000 generations with a sample frequency of 1,000 generations. The 50% majority rule consensus tree and Bayesian posterior predictive values were calculated after removing the first 25% of the samples; Bayesian posterior predictive values of ≥0.95 were considered significant. The best-fit model for each data partition (GTR+G for ITS and SYM+G for *Tub* and *Cal*) was estimated with MrModelTest version 2.3 (19). In addition, 65 sequences representing 19 type and reference strains were retrieved from GenBank and included in the phylogenetic analyses (Table 2).

Morphological and physiological study. Colony features and growth rates were determined for all of the isolates grown on creatine agar (CREA), Czapek yeast autolysate agar (CYA), malt extract agar (MEA), or oatmeal agar (OA) after 7 days of incubation at 25, 37, 40, or 45°C in darkness (12). The color notations used in the descriptions are those of Kornerup and Wanscher (20). Morphological observations and measurements were recorded for all of the isolates grown on MEA after 7 days at 25°C mounted in 85% lactic acid. Photographs of the microscopic structures were made with a Zeiss Axio Imager M1 light microscope with Nomarski differential interference contrast and phase-contrast optics (Zeiss, Oberkochen, Germany) and recorded with a DeltaPix Infinity X digital camera.

Antifungal susceptibility testing. Susceptibilities to the antifungals itraconazole (ITC), voriconazole (VRC), posaconazole (PSC), and amphotericin B (AMB) were determined by the CLSI M38-A2 procedure (21). The final concentrations of the antifungal agents tested ranged from 0.003 to 8 μg/ml. The antifungal susceptibilities of the 26 isolates were compared with those of 72 clinical isolates of *A. terreus sensu stricto* from our collection. Pairwise comparisons of both species were performed by using the Mann-Whitney test to calculate differences in antifungal susceptibility.

Accession numbers. Information about *Aspergillus citrinoterreus* has been submitted to MycoBank and assigned accession number MB810584. Newly determined sequences for clinical strains of *A. citrinoterreus* and *A. terreus sensu stricto* were submitted to GenBank under accession numbers KP175260 to KP175266, KP175270 to KP175278, KP175284 to KP175295, and LN680657 to LN680712 (Table 2).

RESULTS

Phylogenetic analysis of the combined data set (Fig. 1) included 1,423 bp (ITS, 485 bp; *Tub*, 395 bp; *Cal*, 543 bp). The ingroup consisted of 49 strains from 14 taxa, and *Aspergillus neoniveus* CBS 261.73 and *Aspergillus flavipes* CBS 260.73 were used as the outgroups. *Aspergillus* section *Terrei* was well delimited (bootstrap support value of 84, Bayesian posterior predictive value of 1.00) and consisted of 14 well-supported lineages, 13 of which corresponded to species currently accepted as members of this section. The 18 clinical strains and 4 additional reference strains previously assigned to *A. terreus sensu stricto* (NRRL 260, NRRL 1913, UOA/HCPF 9927, and UOA/HCPF 10158-2) were grouped in a fully supported clade (bootstrap support value of 100, Bayesian posterior predictive value of 1.00) that was a sister to *A. terreus sensu stricto* (97.7% sequence similarity). Since the former set of isolates was phylogenetically and morphologically different from all of the members of this section, they are described below as a new species, *Aspergillus citrinoterreus*. This clade was also consistently formed with a high level of statistical support in the individual *Tub* and *Cal* phylogenies (data not shown). In contrast, the analysis of the single ITS region showed insufficient resolution for many of the species of *Aspergillus* section *Terrei*, including the new species *A. citrinoterreus*.

Taxonomy. *Aspergillus citrinoterreus* Guinea, Sandoval-Denis, Escribano, Bouza & Guarro, sp. nov. (Fig. 2).

Etymology. So named because it produces a diffusible yellowish pigment.

Diagnosis. The new species is closely related to *A. terreus sensu stricto*, from which it differs by producing acid on CREA and forming light-colored colonies with abundant intense diffusible yellow pigment, septate stipes, globose to subglobose yellowish conidia, and smaller obovoid accessory conidia.

On CYA, colonies were velvety to dusty with a slightly floccose center, flat or slightly folded, at first white and then becoming brownish orange to grayish brown (5C3 to 5D3), reaching 33 to 35 mm in diameter at 7 days. Colonies in the reverse were grayish yellow to brownish orange (4B6 to 5C5) with abundant diffusible pastel yellow to light yellow pigment (3A4 to 4A4). On MEA, colonies were velvety

TABLE 2 GenBank accession numbers of clinical strains of *A. citrinoterreus* and *A. terreus sensu stricto* and reference sequences used in this study

Species and strain[a]	GenBank accession no.[b]		
	ITS	*Tub*	*Cal*
A. alabamensis CBS 125693^T		EU147769	EU147583
A. allahabadii CBS 164.63^T	AY822638	EF669531	EF669559
A. ambiguus CBS 117.58^T	EF669606	EF669534	EF669564
A. aureoterreus CBS 503.65^T	EF669580	EF669524	EF669538
A. carneus CBS 494.65^T	EF669611	EF669529	FJ531220
A. citrinoterreus			
GM 228^T	**KP175260**	**LN680657**	**LN680685**
GM 464	**KP175261**	**LN680658**	**LN680686**
GM 541	**KP175262**	**LN680659**	**LN680687**
GM 673	**KP175263**	**LN680660**	**LN680688**
GM 676	**KP175264**	**LN680661**	**LN680689**
GM 837	**KP175265**	**LN680662**	**LN680690**
GM 1381	**KP175266**	**LN680663**	**LN680691**
GM 1532	**KP175270**	**LN680664**	**LN680692**
GM 1597	**KP175271**	**LN680665**	**LN680693**
GM 1777	**KP175272**	**LN680666**	**LN680694**
GM 2006	**KP175273**	**LN680667**	**LN680695**
GM 2025	**KP175274**	**LN680668**	**LN680696**
GM 2137	**KP175275**	**LN680669**	**LN680697**
GM 2568	**KP175276**	**LN680670**	**LN680698**
GM 2597	**KP175277**	**LN680671**	**LN680699**
GM 3921	**KP175278**	**LN680672**	**LN680700**
GM 4511	**KP175284**	**LN680673**	**LN680701**
GM 4611	**KP175285**	**LN680674**	**LN680702**
NRRL 260	EF669587	EF669521	EF669545
NRRL 1913	EF669579	EF669518	EF669537
UOA/HCPF 9927	FJ878636	GQ376134	JF927631
UOS/HCPF 10158-2	FJ878638	GQ376135	JF927633
A. flavipes CBS 260.73^T		EU014084	
A. floccosus CBS 116.37^T	FJ531205	FJ491714	FJ531219
A. hortai CBS 124230^T	FJ531192	FJ491706	FJ531242
A. microcysticus CBS 120.58^T	EF669607	EF669515	EF669565
A. neoafricanus CBS 130.55^T	EF669585	EU147719	EF669543
A. neoindicus CBS 444.75^T	EF669616	EF669532	EF669574
A. neoniveus CBS 261.73^T	FJ531198	EU014098	EF669570
A. niveus CBS 115.27^T	EF669616	EF669528	EF669573
A. pseudoterreus CBS 123890^T	EF669598	EF669523	EF669556
A. terreus			
CBS 17A1		EU147714	EU147528
CBS 117.37	FJ531206	FJ491704	FJ531223
CBS 594.65	AY822634	EU147709	EU147523
CBS 601.65^T	EF669586	EU147708	EF669544
GM 1738	**KP175286**	**LN680675**	**LN680703**
GM 1832	**KP175287**	**LN680676**	**LN680704**
GM 1839	**KP175288**	**LN680677**	**LN680705**
GM 1876	**KP175289**	**LN680678**	**LN680706**
GM 2001	**KP175290**	**LN680679**	**LN680707**
GM 2036	**KP175291**	**LN680680**	**LN680708**
GM 2042	**KP175292**	**LN680681**	**LN680709**
GM 2088	**KP175293**	**LN680682**	**LN680710**
GM 2186	**KP175294**	**LN680683**	**LN680711**
GM 2263	**KP175295**	**LN680684**	**LN680712**
UOA/HCPF 3706	GQ461901	GQ376128	JF927627

[a] A superscript T indicates a type strain.
[b] The accession numbers of sequences newly generated in this study are in bold.

to felty with a floccose center and flat with a white, dusty, and regular margin. They were greenish orange (5B3 to 5B6) in color, reaching 23 to 25 mm in diameter at 7 days. The reverse side of colonies was light yellow (4A4) with abundant yellow diffusible pigment. On OA, colonies were sandy to dusty, with abundant submerged mycelium, flat, and pastel yellow to light yellow (3A4 to 4A4). Colonies in the reverse were pastel yellow with abundant pastel yellow (2A4 to 3A4) diffusible pigment. On CREA, colonies were felty to fluffy, flat, greenish orange (5B3), reaching 35 to 40 mm in diameter at 7 days, with slight acid production at 25 and 37°C, except for two strains (GM 3967 and GM 4016) that produced large amounts of acids, leading to complete agar acidification and a color change after 3 days of incubation at 25 and 37°C. Conidiophores were hyaline and short (100 to 500 μm), widening from 2 to 5 μm at the base to 4 to 8 μm at the apex. They were also septate, smooth, and thick walled, gradually swelling to a globose to subglobose vesicle measuring 12 to 22 μm in diameter. Vesicles were typically biseriate at maturity, although some were monoseriate in young cultures. Metulae were cylindrical (5.5 to 9 by 1.5 to 3 μm), smooth, and thin walled, covering two-thirds of the vesicle surface. Conidiogenous cells were cylindrical with an apical constriction (5 to 9 by 1 to 2.5 μm), smooth, and thin walled. Conidia were globose to subglobose (2 to 3 by 1.5 to 3 μm), light yellow, smooth, thin walled, and arranged in compact columns. Accessory conidia were obovoid to ellipsoidal (3.5 to 4.5 by 3.5 to 4 μm), smooth, and thin walled and formed directly on hyphae or from short stalks (0.5 to 1 by 1 to 1.5 μm).

Holotype. Spain, from human sputum, 2006, T. Peláez (CBS H-22005; ex-type cultures CBS 138921 = GM 228).

A. citrinoterreus isolates were significantly more susceptible to ITC, VRC, and PSC than isolates of *A. terreus sensu stricto* were. In contrast, AMB MICs were high for both species (Table 3). *A. citrinoterreus* was found in clinical samples from patients with proven or probable invasive aspergillosis or from patients with colonization, none of whom had hematological malignancies as an underlying condition. Characteristics of the patients with invasive aspergillosis are shown in Table 4. Patients had predisposing conditions such as COPD, cirrhosis, and cancer or had received solid organ transplants and presented with invasive pulmonary aspergillosis and extrarespiratory involvement such as

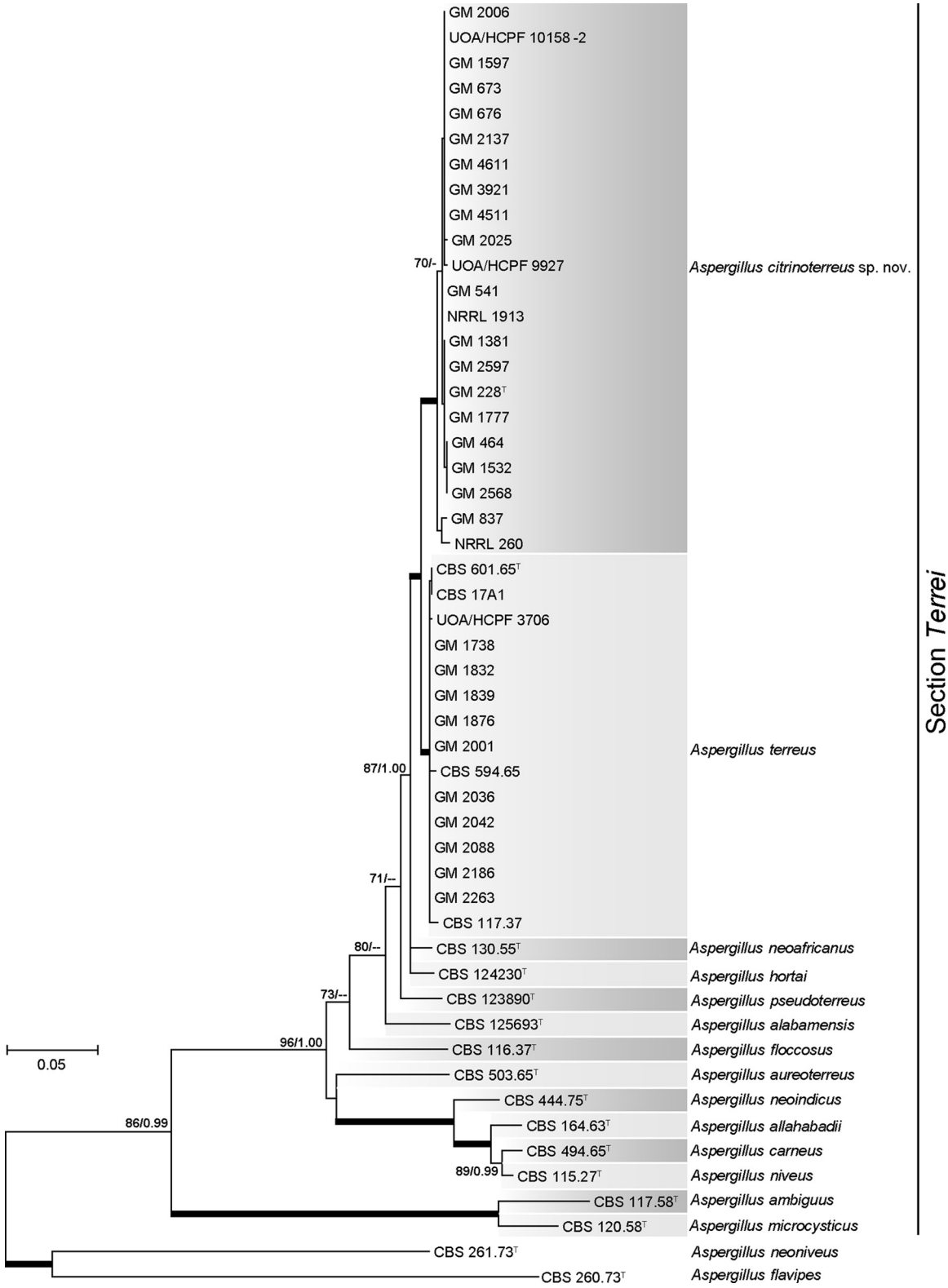

FIG 1 ML tree inferred from combined ITS, *Tub*, and *Cal* sequences of *Aspergillus* isolates from section *Terrei*. Branch lengths are proportional to phylogenetic distances. ML bootstrap support values of ≥70% and Bayesian posterior predictive values of ≥0.95 are shown above the branches. Strongly supported branches (bootstrap support value of 100, Bayesian posterior predictive value of 1.00) are in bold. Sequences of *Aspergillus neoniveus* and *Aspergillus flavipes* were used to root the tree. A superscript T indicates a type strain. CBS, culture collection of the CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands; GM, clinical strains stored at Hospital Gregorio Marañón; UOA/HCPF, University of Athens/Hellenic Collection of Pathogenic Fungi.

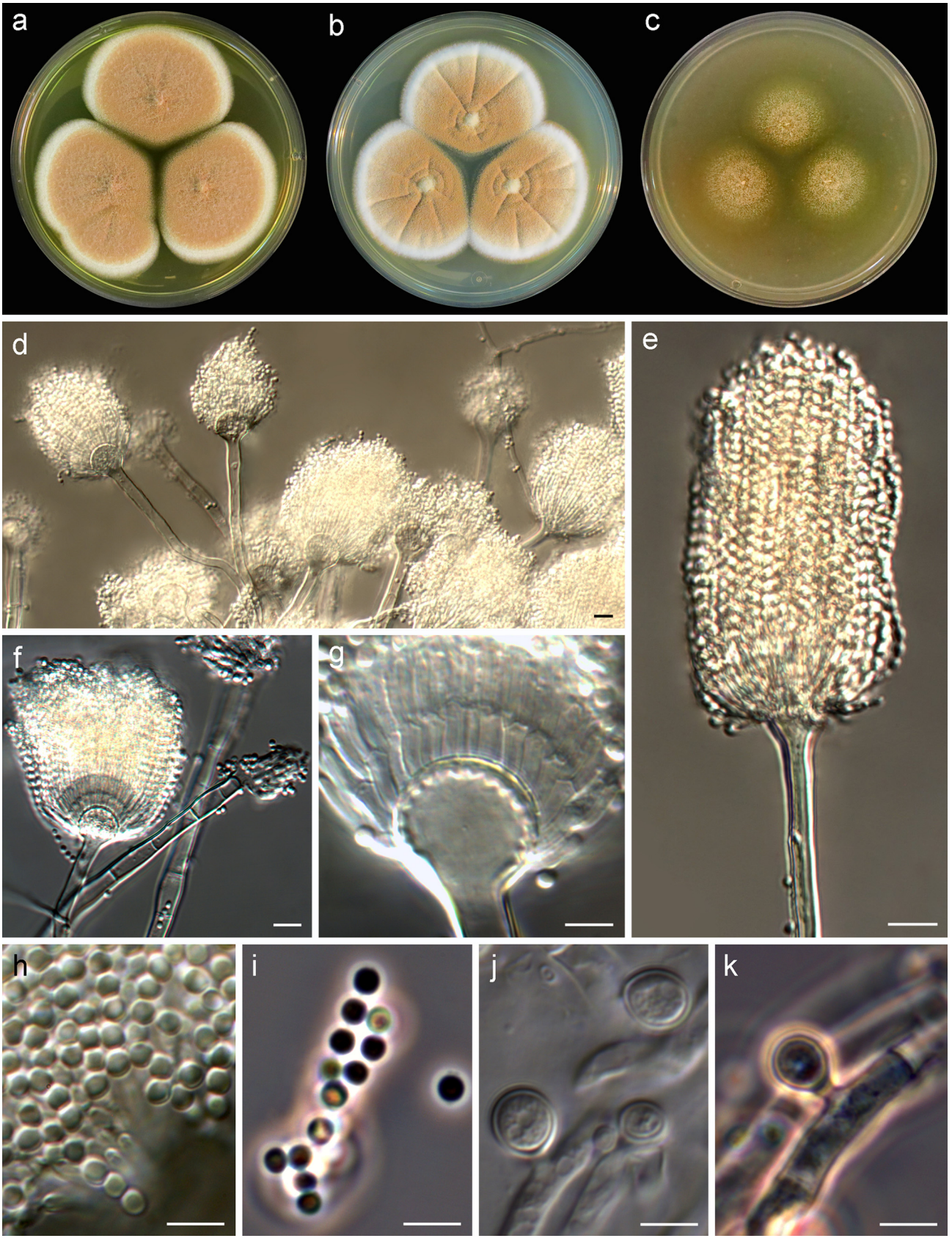

FIG 2 Images of *Aspergillus citrinoterreus* sp. nov. (CBS 138921). Panels: a to c, colonies on MEA, CYA, and CMD, respectively, after 7 days at 25°C; d to f, conidiophores; g, vesicle, metulae, and phialides; h and i, conidia; j and k, accessory conidia. Scale bars: d to f, 10 μm; g to k, 5 μm.

TABLE 3 Antifungal susceptibilities of 26 *A. citrinoterreus* and 72 *A. terreus sensu stricto* isolates to ITC, VRC, PSC, and AMB

Antifungal agent and species	Geometric mean MIC in μg/ml	MIC_{90} (range) in μg/ml	*P* value
AMB			
A. citrinoterreus	11	≥8 (4 to ≥8)	0.432
A. terreus sensu stricto	12.1	≥8 (2 to ≥8)	
ITC			
A. citrinoterreus	0.67	1 (0.25 to 2)	<0.001
A. terreus sensu stricto	1.06	2 (0.25 to 2)	
VRC			
A. citrinoterreus	0.51	1 (0.125 to 1)	<0.001
A. terreus sensu stricto	1.13	2 (0.125 to 2)	
PSC			
A. citrinoterreus	0.46	1 (0.125 to 1)	<0.001
A. terreus sensu stricto	0.85	1 (0.25 to 1)	

mediastinitis. Isolates of *A. citrinoterreus* were found for the first time in 2002. In all cases, *A. citrinoterreus* was found to be a copathogen, mostly with *A. fumigatus* (Table 4).

DISCUSSION

We describe *A. citrinoterreus*, a new species within *Aspergillus* section *Terrei*. *A. citrinoterreus* was found in samples from patients without hematological malignancies and in patients with and without invasive aspergillosis. In addition, it was slightly more susceptible to azoles than *A. terreus sensu stricto* is.

In recent years, with the aid of phylogenetic analyses, several new species belonging to *Aspergillus* section *Terrei* have been described (12, 22). Samson et al. (12) demonstrated that numerous taxa of section *Terrei*, which in the past were reduced to varietal status among *A. terreus* on the basis of phenotypic criteria, corresponded to distinct but closely related phylogenetic species, many of which were almost indistinguishable by morphological criteria. Similarly, although subtle, the differences in morphological, physiological, and antifungal-susceptibility features between *A. citrinoterreus* and its closest relative, *A. terreus*, are consistent with the results of the individual and combined three-gene phylogenetic analyses that allowed us to propose *A. citrinoterreus* as a new species. In 1934, Blochwitz described *Aspergillus boedijni*, a species that, like *A. citrinoterreus*, differed from *A. terreus* by forming yellowish conidia and a diffusible pigment (23). However, *A. boedijni* was considered a morphological variety of *A. terreus* (24) and was later considered to be synonymous with *A. terreus* (25). *A. citrinoterreus*, however, can be differentiated from the original description of *A. boedijni* by its larger and hyaline conidiogenous cells, larger conidia, and abundant production of a diffusible yellow pigment. In contrast, *A. boedijni* produces intense yellow conidiogenous cells and a diffusible pigment that is at first yellow but turns light brown to reddish with age. Although there is no type or authentic strain available of *A. boedijni*, a reference strain considered by Thom and Raper in 1945 to belong to the latter species (CBS 594.65) (24) clustered within *A. terreus sensu stricto*, which confirmed it to be distinct from the new species *A. citrinoterreus*.

Arabatzis and Velegraki (26) recently described the sexual morph of *A. terreus* on the basis of mating crosses and multilocus sequence analyses based on the same genetic loci applied in our study. Their phylogenetic analyses revealed a topology similar to that observed in our results, with several internal branches. However, the findings were not statistically significant. Interestingly, according to our phylogenetic analysis, most of the fertile strains in that study showed 99% sequence similarity to *A. citrinoterreus*, while only a single strain grouped within *A. terreus sensu stricto*. Accordingly, there is evidence that the teleomorph described corresponds to the sexual state of *A. citrinoterreus*. The use of mating crosses to infer species limits can be misleading because of the fertility of closely related phylogenetic species, indicating that phylogenetic divergence can precede reproductive isolation (27, 28).

A. citrinoterreus was found in samples from patients with and without invasive aspergillosis. In the six patients with invasive aspergillosis, *A. citrinoterreus* was found to be a copathogen, mainly with *A. fumigatus*. Therefore, it is difficult to assess the clinical significance of this new species. Further studies to clarify the association between invasive aspergillosis and *A. citrinoterreus* should be carried out. Of particular interest is the fact that we isolated *A. citrinoterreus* only from patients with nonhematological predisposing conditions, such as COPD; this group of patients has received greater attention during the last few years (3).

The AMB MICs for isolates of *Aspergillus* section *Terrei* are commonly high, and the outcome of patients treated with VRC is better than that of patients treated with a polyene (7, 29, 30). The Infectious Diseases Society of America guidelines recommend the use of VRC instead AMB for the treatment of infections caused by *A. terreus* (31). The low number of patients included in our series and the fact that *A. citrinoterreus* was a copathogen make it difficult to draw conclusions about the appropriate antifungal treat-

TABLE 4 Clinical characteristics of the six patients with invasive aspergillosis from whom *A. citrinoterreus* was isolated

Patient	Yr of episode	Ward of admission	Underlying condition(s)	Region affected	IA[a] diagnosis	Antifungal treatment	Outcome	Coinfecting species
153	2002	Geriatrics	COPD	Pulmonary	Probable	Liposomal AMB	Poor	*A. fumigatus*
3317	2007	Pneumology	COPD	Pulmonary	Probable	Liposomal AMB	Poor	*A. fumigatus*
3503	2008	Major heart surgery unit	Surgery, COPD, heart transplantation	Mediastinal	Proven	VRC	Favorable	*A. fumigatus*, *A. calidoustus*
3797	2010	Oncology	COPD, solid cancer	Pulmonary	Probable	VRC	Poor	*A. fumigatus*
3890	2011	Intensive care unit	Corticosteroids	Pulmonary	Probable	VRC	Poor	*A. fumigatus*, *A. flavus*
3986	2012	Digestive medicine	Liver transplantation	Pulmonary	Probable	VRC followed by anidulafungin	Favorable	*A. lentulus*

[a] IA, invasive aspergillosis.

ment for patients infected with this species. In addition, the observed lower azole MICs than for *A. terreus sensu stricto* should be studied in the future.

In conclusion, we describe *A. citrinoterreus*, a new species within *Aspergillus* section *Terrei* that was found in samples from patients with nonhematological predisposing conditions. Further studies are required to determine the potential pathogenic role of this new species, which is easily misidentified as the well-known pathogen *A. terreus*.

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This study does not present any conflicts of interest for us.

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