EUKARYOTIC CELL, Apr. 2008, p. 630–638 Vol. 7, No. 4
1535-9778/08/$08.00+0 doi:10.1128/EC.00425-07

Aspergillus calidoustus sp. nov., Causative Agent of Human Infections Previously Assigned to *Aspergillus ustus*▽

János Varga,[1,2] Jos Houbraken,[1] Henrich A. L. Van Der Lee,[3] Paul E. Verweij,[3] and Robert A. Samson[1]*

CBS Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands[1]; Department of Microbiology, Faculty of Science and Informatics, University of Szeged, H-6701 Szeged, P.O. Box 533, Hungary[2]; and Department of Medical Microbiology, Radboud University Nijmegen Medical Center, Nijmegen University Center for Infectious Diseases, P.O. Box 9101 6500 HB, Nijmegen, The Netherlands[3]

Received 20 November 2007/Accepted 7 February 2008

***Aspergillus ustus* is a relatively rare human pathogen causing invasive infections in immunocompromised hosts. In this study isolates originating from clinical and other sources have been examined using molecular, morphological, and physiological approaches to clarify their species assignment. Phylogenetic analysis of partial β-tubulin, calmodulin, actin, and intergenic transcribed spacer sequences indicated that none of the clinical isolates recognized previously as *A. ustus* belongs to this species. All but two of these isolates formed a well-defined clade related to *A. pseudodeflectus* based on sequence analysis of protein-coding regions. Morphological and physiological examination of these isolates indicated that they are able to grow above 37°C, in contrast with *A. ustus* isolates, and give a positive Ehrlich reaction, in contrast with related species including *A. granulosus*, *A. ustus*, and *A. pseudodeflectus*. These isolates are proposed as a new species, *A. calidoustus*. Antifungal susceptibility testing showed that this species has decreased susceptibilities to several antifungal drugs. The triazoles are inactive in vitro, including the new azole posaconazole.**

Aspergillus ustus is a common filamentous fungus found in food, soil, and indoor air environments worldwide (27). At the same time, this species is considered a relatively rare human pathogen that can cause invasive infection in immunocompromised hosts. To date, only 22 cases of invasive aspergillosis have been reported to be caused by *A. ustus* (1, 13, 20, 21, 36, 37, 39; also O. V. Vasilenko, N. V. Bezmelnitsyn, S. A. Keselman, E. M. Shulutko, and A. V. Pivnik, unpublished data). Almost half of these cases were classified as primarily cutaneous aspergillosis, so the skin was suggested as the primary site of infection (17, 30). Invasive aspergillosis caused by *A. ustus* is associated with high mortality rates, primarily due to the reduced susceptibilities of *A. ustus* isolates to various antifungal drugs (32). More recently, *A. ustus* was implicated in a cluster of eye infections (29).

A. ustus belongs to the *Aspergillus* section *Usti* together with *A. granulosus*, *A. puniceus*, and *A. pseudodeflectus* (4, 12, 22); it is characterized by radiate to loosely columnar conidial heads in shades of olive gray to red gray and produces Hülle cells scattered throughout the colony in irregular masses that are not associated with pigmented mycelium (23). However, *A. ustus* is morphologically highly variable; Raper and Fennell (23) stated that "not a single strain can be cited as wholly representative of the species as described." *A. ustus* isolates may vary in their colony color from mud brown to slate gray, with colony reverse colors from uncolored through yellow to dark brown (14, 23). Molecular data, including randomly amplified polymorphic DNA analysis (20, 24) and sequence analysis of parts of the rRNA gene cluster (8, 10, 22), also indicate that this species is highly variable. These molecular and chemical data indicate that more species may be present in the broad description of *A. ustus* provided by Raper and Fennell (23).

We examined a large set of *A. ustus* isolates from clinical and environmental sources to clarify the taxonomic status of the species. The methods used included sequence analysis of the intergenic transcribed spacer (ITS) region (including ITS regions 1 and 2 and the 5.8 S rRNA gene of the rRNA gene cluster) and parts of the β-tubulin, calmodulin, and actin genes; macro- and micromorphological analysis; and antifungal susceptibility testing of the isolates.

MATERIALS AND METHODS

Morphological examinations. The strains examined are listed in Table 1. Both clinical and environmental strains were grown as three-point inoculations on Czapek yeast extract agar (CYA), Czapek agar, malt extract agar (MEA), creatine sucrose agar (CREA), and yeast extract sucrose agar (YES) at 25, 30, and 37°C for 7 days (composition of media is according to reference 27). All isolates were examined for production of cyclopiazonic acid and other alkaloids that react with Ehrlich reagent by using a filter paper method described previously (16). For micromorphological examinations light microscopy (Zeiss Axioskop 2 Plus; Carl Zeiss B.V., Sliedrecht, The Netherlands) was been employed.

Isolation and analysis of nucleic acids. The cultures used for the molecular studies were grown on malt peptone broth using 10% (vol/vol) malt extract (Oxoid, Basingstoke, United Kingdom), 0.1% (wt/vol) Bacto peptone (Becton Dickinson, Le-Pont-de-Claix, France), and 2 ml of medium in 15-ml tubes. The cultures were incubated at 25°C for 7 days. DNA was extracted from the cells using a Masterpure yeast DNA purification kit (Epicenter Biotechnology, Madison, WI) according to the instructions of the manufacturer. The ITS region was amplified using primers ITS1 and ITS4 as described previously (38). Amplification of part of the β-tubulin gene was performed using the primers Bt2a and Bt2b (7). Amplifications of the partial calmodulin and actin genes were set up as

* Corresponding author. Mailing address: CBS Fungal Biodiversity Centre, P.O. Box 85167, 3508 AD Utrecht, The Netherlands. Phone: 31 30 2122600. Fax: 31 30 2512097. E-mail: r.samson@cbs.knaw.nl.

▽ Published ahead of print on 15 February 2008.

TABLE 1. Isolates in *Aspergillus* section *Usti* examined in this study

Species	Strain no(s).	Source and/or origin
A. calidoustus	CBS 112452	Indoor air, Germany
A. calidoustus	CBS 113228	ATCC 38849, IBT 13091
A. calidoustus	CBS 114380	Wooden construction material, Finland
A. calidoustus[T]	CBS 121601, 677	Bronchoalveolar lavage fluid, proven invasive aspergillosis; Nijmegen, The Netherlands[a]
A. calidoustus	CBS 121602, 678	Bronchial secretion, proven invasive aspergillosis; Nijmegen, The Netherlands[a]
A. calidoustus	CBS 121589, 682	Autopsy lung tissue sample, proven invasive aspergillosis; Nijmegen, The Netherlands[a]
A. calidoustus	CBS 121603, 741	Elevator shaft in hospital, Nijmegen, The Netherlands
A. calidoustus	CBS 121604, 924	Patient room, Nijmegen, The Netherlands
A. calidoustus	CBS 121605, 943	Laboratory, Nijmegen, The Netherlands
A. calidoustus	CBS 121606, 2725	Sputum, Nijmegen, The Netherlands
A. calidoustus	CBS 121607, 3297	Feces, Nijmegen, The Netherlands
A. calidoustus	CBS 121608, 6989	Bronchoalveolar lavage, Nijmegen, The Netherlands
A. calidoustus	7843	Pasteur Institute, Paris, France
A. calidoustus	8623	Oslo, Norway
A. calidoustus	9331	Mouth wash, Nijmegen, The Netherlands
A. calidoustus	9371	Mouth wash, Nijmegen, The Netherlands
A. calidoustus	9420	Bronchial secretion, Nijmegen, The Netherlands
A. calidoustus	9692	Hospital ward, Nijmegen, The Netherlands
A. calidoustus	V02-46	Tongue swab, Nijmegen, The Netherlands
A. calidoustus	V07-21	Bronchial secretion, Nijmegen, The Netherlands
A. calidoustus	V17-43	Bronchial secretion, Nijmegen, The Netherlands
A. calidoustus	V22-60	Skin biopsy, Nijmegen, The Netherlands
A. calidoustus	CBS 121609, 902	Postcataract surgery endophthalmitis
A. calidoustus	907	Postcataract surgery endophthalmitis
A. calidoustus	908	Postcataract surgery endophthalmitis
A. calidoustus	64	Postcataract surgery endophthalmitis
A. calidoustus	67	Postcataract surgery endophthalmitis
A. calidoustus	CBS 121610, 91	Postcataract surgery endophthalmitis
A. calidoustus	351	Osteorickets
A. calidoustus	482	Postcataract surgery endophthalmitis
A. calidoustus	CBS 121611, FH 166	Patient 4[b]
A. calidoustus	CBS 121616, FH 168	Environmental[b]
A. calidoustus	FH 165	Patient 5b[b]
A. calidoustus	CBS 121614, FH 98	Patient 5a[b]
A. calidoustus	CBS 121615, FH 97	Patient 6[b]
A. calidoustus	CBS 121613, FH 94	Patient 2[b]
A. calidoustus	CBS 121612, FH 90	Patient 1[b]
A. calidoustus	FH 91	Patient 1[b]
A. calidoustus	NRRL 26162	Culture contaminant, Peoria, IL
A. calidoustus	NRRL 281	Thom 5634
A. calidoustus	NRRL 277	Thom 5698.754, green rubber
A. granulosus	CBS 588.65[T]	Soil, Fayetteville, AR
A. granulosus	CBS 119.58	Soil, Texas
A. granulosus	IBT 23478	Unknown
A. pseudodeflectus	CBS 596.65	Sugar, Louisiana
A. pseudodeflectus	CBS 756.74[T]	Desert soil, Western Desert, Egypt
A. puniceus	CBS 122.33	A. Blochwitz (1933)
A. puniceus	9377	Mouth wash, Nijmegen, The Netherlands
A. puniceus	V41-02	Feces, Nijmegen, The Netherlands
A. puniceus	NRRL 29173	Indoor air, Saskatoon, Canada
A. puniceus	CBS 495.65[T]	Soil, Zarcero, Costa Rica
A. puniceus	CBS 128.62	Soil, Louisiana
A. ustus	CBS 116057	Antique tapestries, Krakow, Poland
A. ustus	CBS 114901	Carpet, The Netherlands
A. ustus	CBS 261.67[T]	Culture contaminant, United States
A. ustus	CBS 133.55	Textile buried in soil, The Netherlands
A. ustus	CBS 239.90	Biopsies of brain tumor of 10-year-old male, The Netherlands
A. ustus	CBS 113233	IBT 14495, unknown
A. ustus	CBS 113232	IBT 14932, unknown
A. ustus	CBS 121617, NRRL 285	Soil, Iowa
A. ustus	NRRL 280	Bat dung, Cuba
A. ustus	NRRL 1609	Bat dung, Cuba
A. ustus	NRRL 29172	Indoor air, Edmonton, Canada

[a] These samples were taken from the same patient (36).
[b] K. A. Marr.

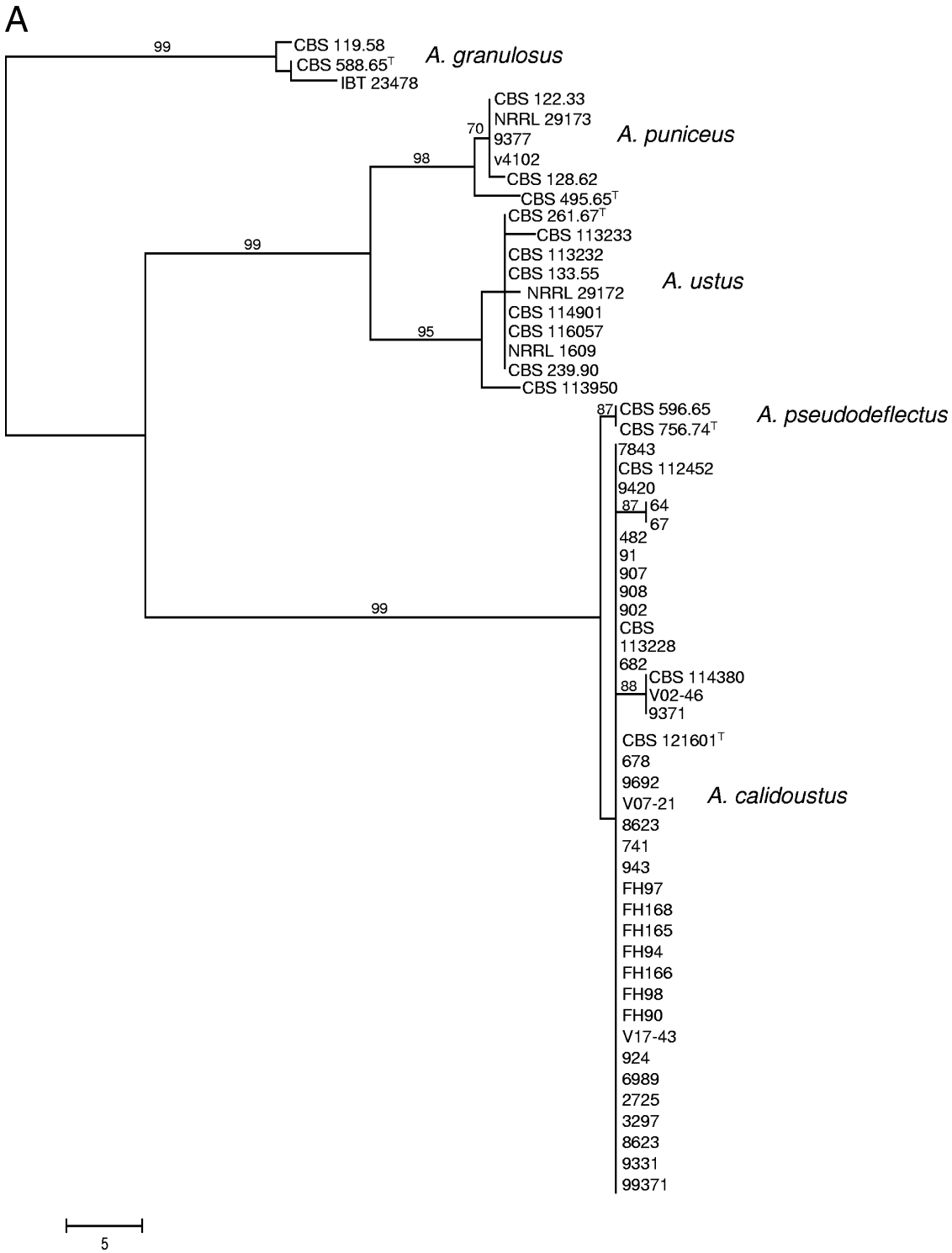

FIG. 1. One of the MP trees based on the β-tubulin (A) and calmodulin (B) sequence data of the examined isolates. Numbers above branches are bootstrap values. Only values above 70% are indicated.

described previously (11). Sequence analysis was performed using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with MT Navigator software (Applied Biosystems, Foster City, CA). All sequencing reaction products were purified by gel filtration through a Sephadex G-50 column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Data analysis. Sequences were aligned using CLUSTAL X (34) and improved manually. The neighbor-joining (NJ) and maximum parsimony (MP) methods were used for the phylogenetic analysis. For NJ analysis (25), the data were first

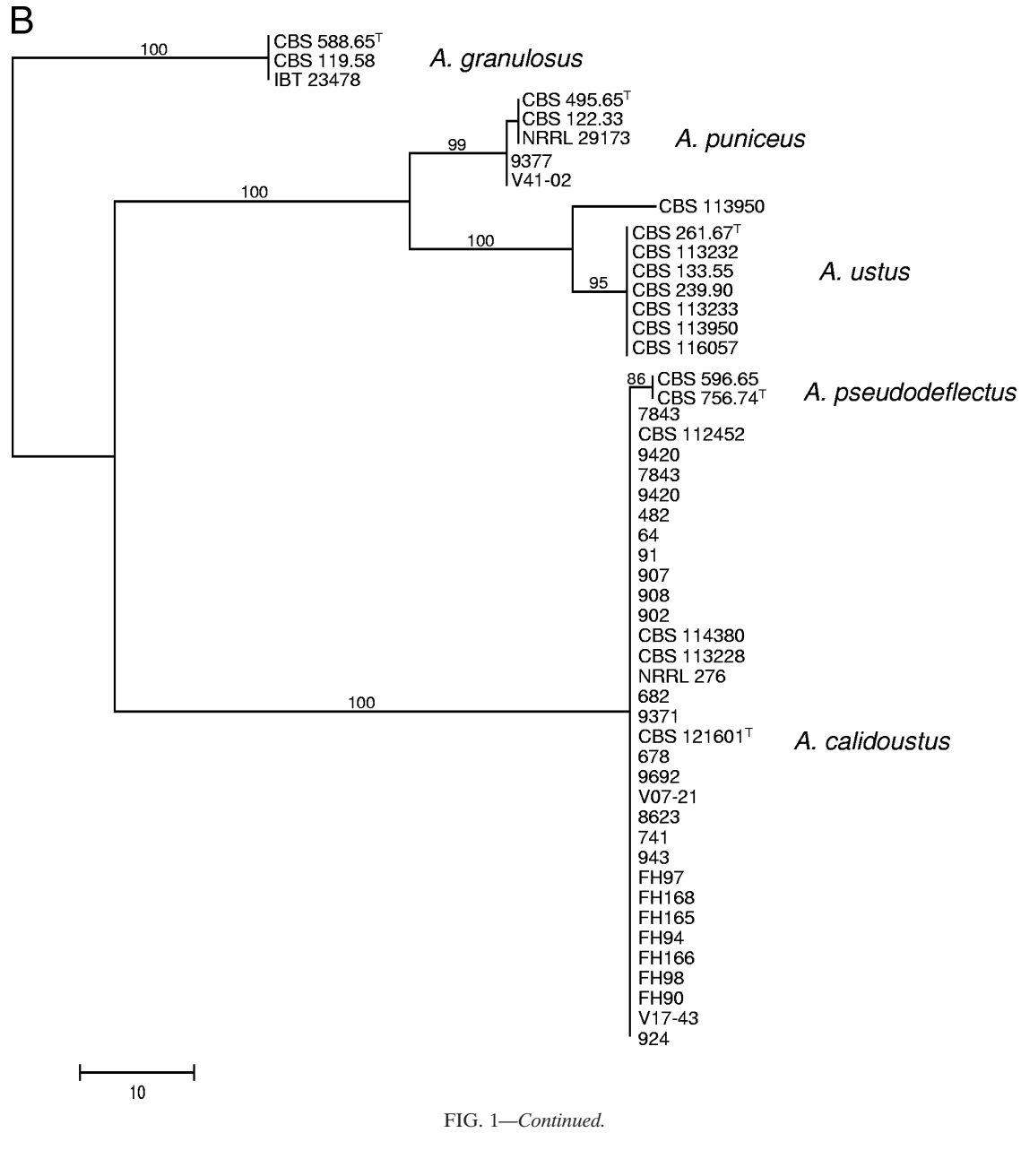

FIG. 1—*Continued.*

analyzed using the Tamura-Nei parameter distance calculation model with gamma-distributed substitution rates (32), which were then used to construct the NJ tree with the MEGA program, version 3.1 (15). To determine the support for each clade, a bootstrap analysis was performed with 1,000 replications. For parsimony analysis, PAUP*, version 4.0, was used (31). Alignment gaps were treated as a fifth character state, and all characters were unordered and of equal weight. MP analysis was performed for all data sets using the heuristic search option with 100 random taxon additions and tree bisection and reconstruction as the branch-swapping algorithm. Branches of zero length were collapsed, and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1,000 bootstrap replications (9). *A. granulosus* isolates were used as outgroups.

Antifungal susceptibility tests. Isolates were revived by subculturing twice on Sabouraud dextrose agar tubes for 5 to 7 days at 35°C. Conidial suspensions were prepared spectrophotometrically and were further diluted in RPMI 1640 medium (with L-glutamine and without bicarbonate) (GIBCO BRL, Life Technologies, Woerden, The Netherlands). Microtiter plates were inoculated to an initial concentration of 1×10^4 to 5×10^4 conidia/ml as recommended by the CLSI for mold testing (18).

The antifungal activities of amphotericin B (Bristol-Meyers-Squibb, Woerden, The Netherlands), flucytosine (or 5-fluorocytosine [5-FC]; Valeant, Zoetermeer, The Netherlands), itraconazole (Janssen Pharmaceutica BV, Tilburg, The Netherlands), voriconazole (Pfizer, Capelle aan de IJssel, The Netherlands), posaconazole (Schering-Plough, Maarssen, The Netherlands), terbinafine (Novartis Pharma, Arnhem, The Netherlands), and caspofungin (MSD, Haarlem, The Netherlands) were determined in vitro using a broth-microdilution method according to CLSI guidelines (M-38A) (18). The concentration range for amphotericin B, terbinafine, itraconazole, voriconazole, and posaconazole was 0.016 to 16 mg/liter; a range of 0.062 to 64 mg/liter was used for 5-FC and caspofungin. MICs were determined after 24 and 48 h of incubation. For amphotericin B the MIC was defined as the lowest concentration that showed no visible growth. For the azoles and 5-FC the MIC was defined as the lowest concentration at which 50% growth inhibition was measured compared with that of the control (19). For caspofungin the minimum effective concentration was determined. All suscepti-

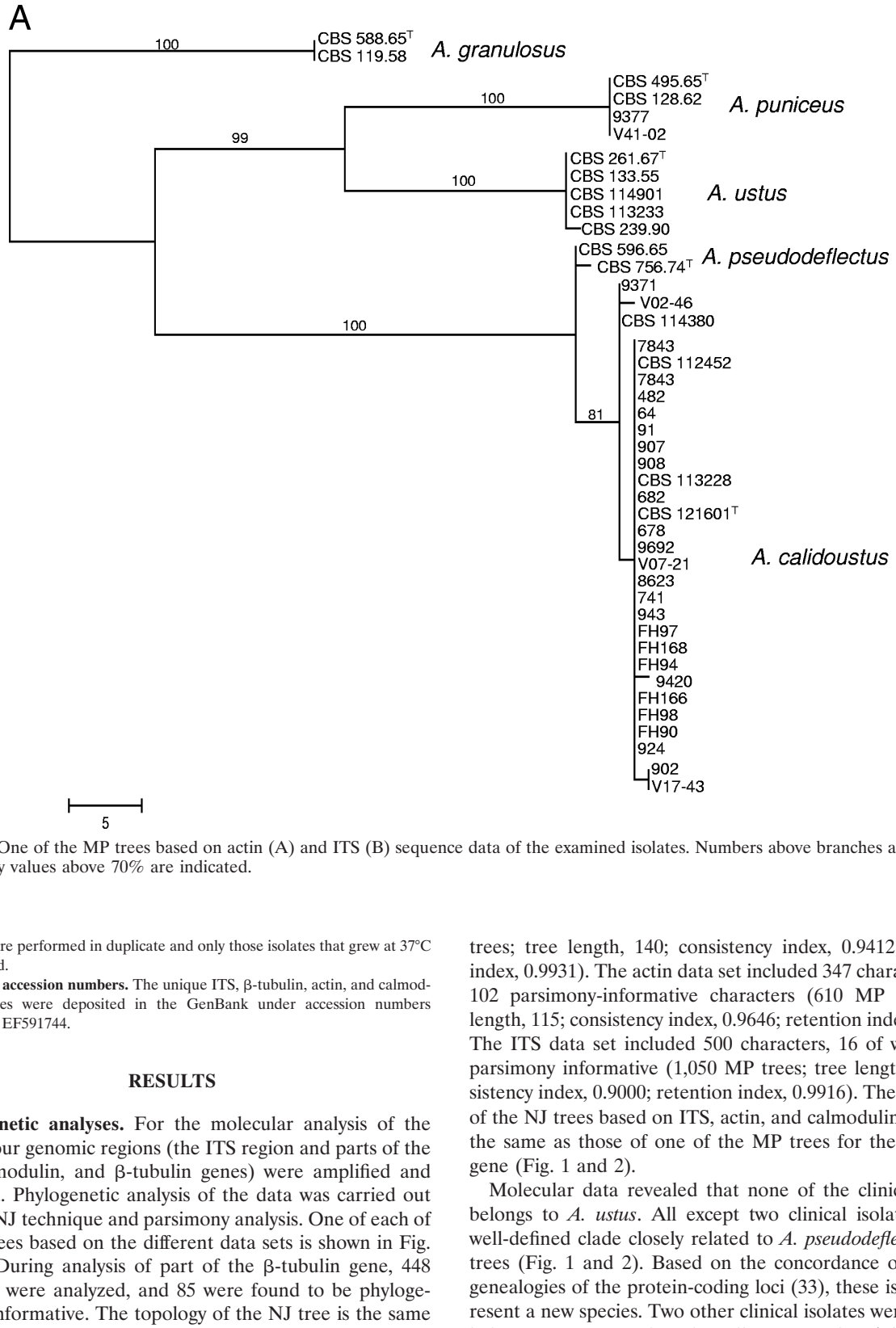

FIG. 2. One of the MP trees based on actin (A) and ITS (B) sequence data of the examined isolates. Numbers above branches are bootstrap values. Only values above 70% are indicated.

bility tests were performed in duplicate and only those isolates that grew at 37°C were included.

Nucleotide accession numbers. The unique ITS, β-tubulin, actin, and calmodulin sequences were deposited in the GenBank under accession numbers EF591703 to EF591744.

RESULTS

Phylogenetic analyses. For the molecular analysis of the isolates, four genomic regions (the ITS region and parts of the actin, calmodulin, and β-tubulin genes) were amplified and sequenced. Phylogenetic analysis of the data was carried out using the NJ technique and parsimony analysis. One of each of the MP trees based on the different data sets is shown in Fig. 1 and 2. During analysis of part of the β-tubulin gene, 448 characters were analyzed, and 85 were found to be phylogenetically informative. The topology of the NJ tree is the same as that of one of the 776 MP trees constructed by the PAUP program (length, 109 steps; consistency index, 0.9314; retention index, 0.9930). The calmodulin data set included 415 characters, with 108 parsimony-informative characters (770 MP trees; tree length, 140; consistency index, 0.9412; retention index, 0.9931). The actin data set included 347 characters, with 102 parsimony-informative characters (610 MP trees; tree length, 115; consistency index, 0.9646; retention index, 0.9929). The ITS data set included 500 characters, 16 of which were parsimony informative (1,050 MP trees; tree length, 25; consistency index, 0.9000; retention index, 0.9916). The topologies of the NJ trees based on ITS, actin, and calmodulin data were the same as those of one of the MP trees for the respective gene (Fig. 1 and 2).

Molecular data revealed that none of the clinical isolates belongs to *A. ustus*. All except two clinical isolates form a well-defined clade closely related to *A. pseudodeflectus* on all trees (Fig. 1 and 2). Based on the concordance of the gene genealogies of the protein-coding loci (33), these isolates represent a new species. Two other clinical isolates were found to belong to *A. puniceus* based on all sequence data (Fig. 1 and 2). Interestingly, some isolates associated with endophthalmitis following cataract surgery formed a subclade based on ITS sequences corresponding to a single conversion of T to C at

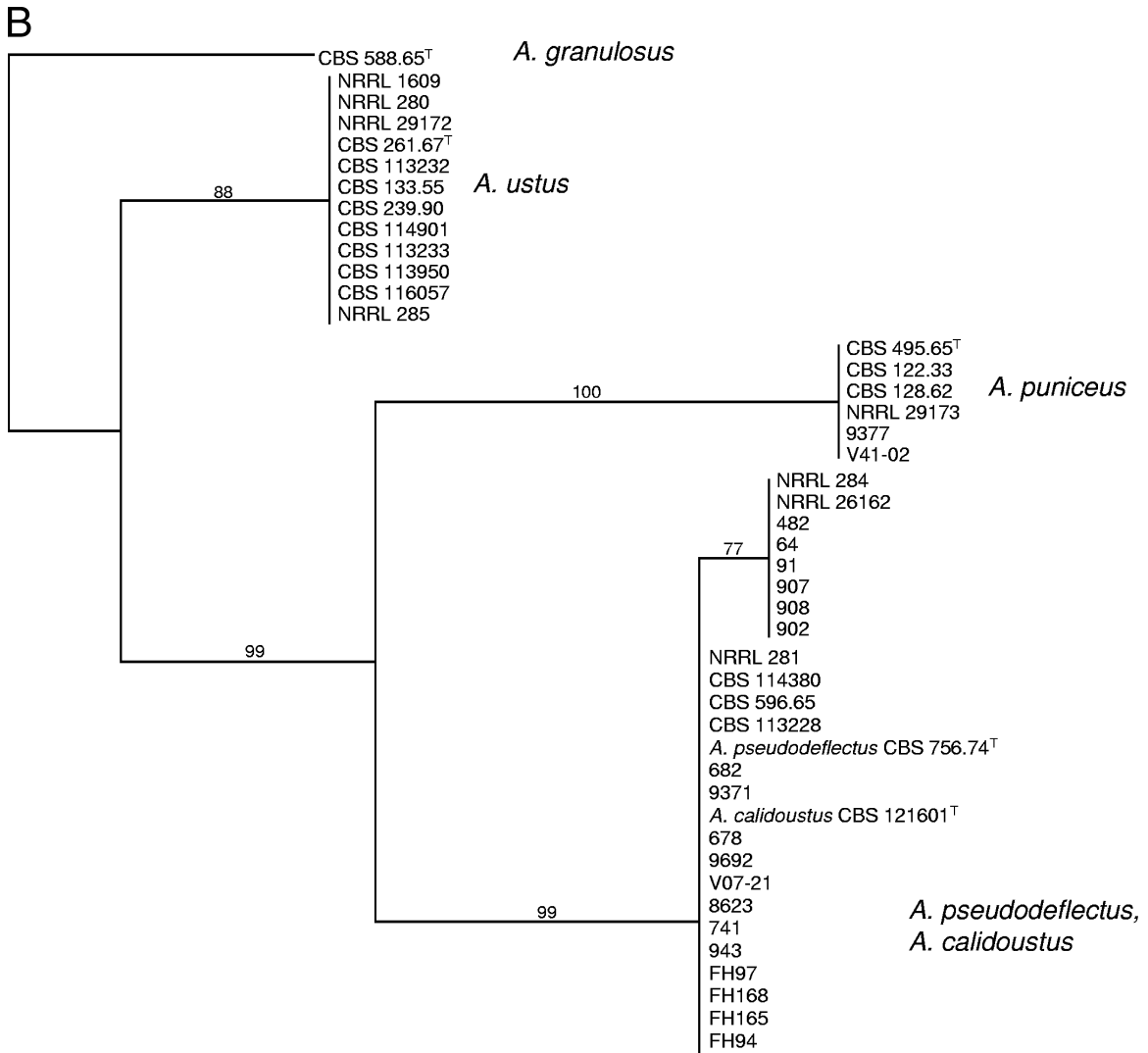

FIG. 2—*Continued.*

position 431 in ITS2 and also two consecutive substitutions (CT to TC) in positions 11 and 12 in their calmodulin genes. Although some of these isolates also produce more curved Hülle cells than observed in other isolates, these features were treated as insufficient to assign them to separate species since their other characteristics were identical to those of the other clinical isolates.

Phenotypic analyses. All clinical isolates were able to grow at temperatures above 37°C, in contrast with *A. ustus* isolates, which could not grow at this temperature (Table 2). Similarly, *A. granulosus* and *A. pseudodeflectus* isolates were also able to grow at 37°C, but *A. puniceus* isolates could not. Regarding Ehrlich reactions, only those isolates that were found to form a distinct clade based on phylogenetic analysis of sequence data gave violet reactions (Table 2).

The clinical isolates grew well on CYA, producing grayish yellow to grayish brown colonies with yellowish brown exudates. Microscopically, their conidial heads were loosely co-

TABLE 2. Morphological characteristics of species of *Aspergillus* section *Usti*

Species	Growth at 37°C	Characterization of Hülle cells	Ehrlich reaction	Mycelium color on CYA
A. ustus	−	Scattered	Negative	Creme to light yellow
A. granulosus	+	Aggregated to colorless clusters	Negative	Inconspicuous
A. puniceus	−	Aggregated to yellowish masses	Negative	Bright yellow
A. pseudodeflectus	++	Absent	Negative	White; poor sporulation
A. calidoustus	++	Scattered	Violet	Grayish yellow to grayish brown

lumnar, their conidia were coarsely roughened to echinulate, and they produced irregularly elongated Hülle cells sparsely in scattered groups (Fig. 3). They were phylogenetically related to *A. pseudodeflectus*, which is also able to grow at 37°C but does not produce Hülle cells but does produce curved brown conidiophores not seen in the clinical isolates (26). It also differs from the clinical isolates in its conidial ornamentation and negative Ehrlich reaction (Table 2).

Antifungal susceptibility tests. The examined isolates were found to have decreased susceptibilities to 5-FC and azoles including itraconazole, voriconazole, and posaconazole. The activity of caspofungin was variable, with some isolates showing low MICs and others showing MICs of 4 mg/liter or greater. Terbinafine appeared to be the most active antifungal agent. No differences were detected among the susceptibilities of isolates from patients or clinical or indoor environments (Table 3).

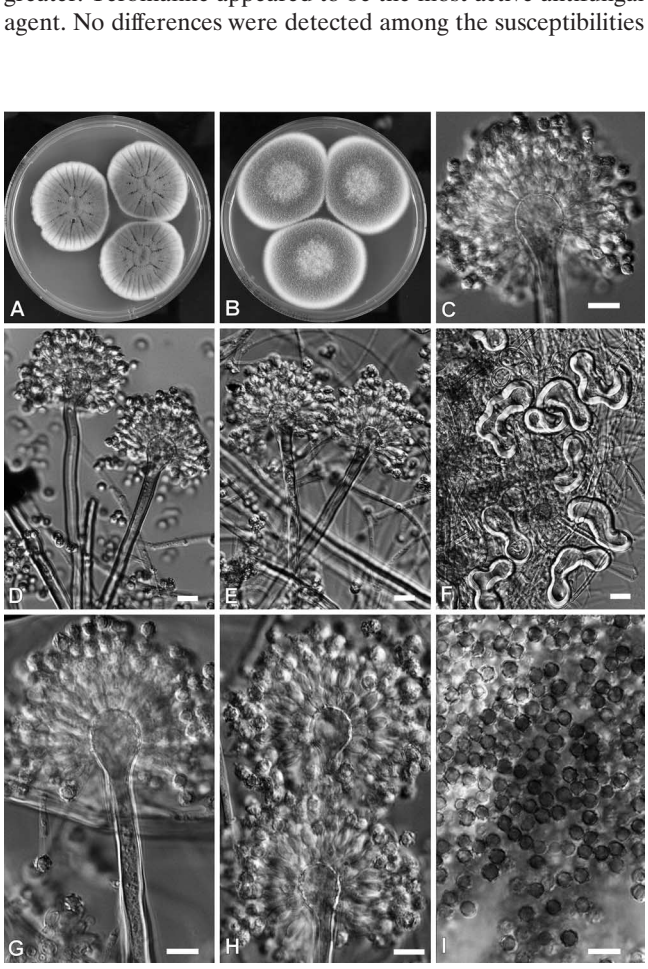

FIG. 3. *A. calidoustus* sp. nov. Colonies were grown on CYA (A) and MEA (B) at 25°C for 7 days. Panels C, D, E, G, and H show conidiophores. (F) Hülle cells. (I) Conidia. Bar, 10 μm.

Based on molecular, morphological, and physiological data, we propose that these (mostly) clinical isolates represent a distinct species in *Aspergillus* section *Usti*, *A. calidoustus* sp. nov. (Fig. 3).

***Aspergillus calidoustus* Varga, Houbraken, and Samson, sp. nov. MB 504846.** Holotype of *A. calidoustus*, here designated CBS 121601^T (677) (dried culture) isolated from bronchoalveolar lavage fluid, proven invasive aspergillosis, Nijmegen, The Netherlands.

Coloniae in 7 dieibus et 25°C in agaro MEA 35–48 mm, in CYA 27–32 mm, in YES 36–41 mm, in CREA 14–22 mm diameter; in 7 dieibus et 37°C in agaro CYA 20–35 mm diameter. Sporulatio in CYA modica vel plus; colonia cinereolutea, brunneogrisea vel cinereobrunnea, hyphis inconspicuis. Exsudatum in guttulis parvis, luteobrunneis; colonia reversa dilute brunnea vel olivaceobrunnea, interdum ad marginem pallide lutea; pigmenta solubilia lutea (brunnea). Capitula conidialia laxe columnaria; stipites curti, (130–)150–300 × 4–7 μm, pariete crasso, laevi, brunneo; vesciculae pyriformes vel late spathulatae, (7–)9–15(–20) μm in lat., biseriatae; metulae (3.9–)4.8–5.8(–6.2) × 2.5–4.5 μm; phialides (5.6–)6.0–6.7(–7.5) × 2.0–3.0 μm; conidia globosa, 2.7–3.5 μm diameter, ornamento exasperato (0.5–0.8 μm in alt.). Cellulae "hülle" sparsae, irregulariter elongatae.

Colonies on MEA 35 to 48 mm, on CYA 27 to 32 mm, on YES 36 to 41 mm, on CREA 14 to 22 mm in diameter after 7 days at 25°C and 20 to 35 mm on CYA after 7 days at 37°C. Moderate to good sporulation on CYA at 25°C, colony color blond/grayish yellow, brownish gray or grayish brown, hyphae

TABLE 3. Antifungal susceptibilities of *A. calidoustus* isolates from different sources

Antifungal agent	MIC (mg/liter [range]) for isolates from:[a]		
	Patients (n = 20)	Clinical environment (n = 4)	Indoor environment (n = 3)
Amphotericin B	1–2	1 (0.5–4)	1 (1–2)
5-FC	8–>64 (2–>64)	2–16	2–32
Itraconazole	>16 (≥16)	>16 (≥16)	>16
Voriconazole	8–16	16 (8–16)	8 (8–16)
Posaconazole	>16	>16	>16
Terbinafine	0.031–0.125	0.016–0.25	0.031–0.0125
Caspofungin	0.25–4 (0.125–4)	2–4 (0.125–8)	0.25–2

[a] MICs are defined as follows: for amphotericin B, the lowest concentration that showed no visible growth; for the azoles and 5-FC, the lowest concentration at which 50% growth inhibition was measured compared with that of the control; for caspofungin, the minimum effective concentration. n, number of isolates examined.

inconspicuous. Exudate present as small yellow brown droplets; reverse light or olive brown, occasionally with (light) yellow edges; yellow (brown) soluble pigments diffusing into the agar. Conidial heads loosely columnar; stipes short 150 to 300 μm (minimum, 130 μm) by 4 to 7 μm, walls thick, smooth, brown; vesicles 9 to 15 μm (range, 7 to 20 μm) wide, pyriform to broadly spathulate, biseriate; metulae covering the upper half to three-fourths of the vesicle, measuring 4.8 to 5.8 μm (range, 3.9 to 6.2 μm) by 2.5 to 4.5 μm; phialides 6.0 to 6.7 μm (range, 5.6 to 7.5 μm) by 2.0 to 3.0 μm; conidia globose 2.7 to 3.5 μm, very rough ornamentation (0.5 to 0.8 μm high), inner and outer wall visible. Hülle cells sparsely produced, irregularly elongated, in scattered groups.

Etymology and distinguishing features. In Latin "calidus" means warm and is used here to refer to the resemblance to *A. ustus* and the ability of the isolates to grow at 37°C. Colonies are characterized by good growth at 37°C, a violet Ehrlich reaction, and coarsely roughened to echinulate conidia.

DISCUSSION

In this study, we examined the phylogenetic placement of 34 *A. ustus* clinical isolates together with environmental isolates and strains from culture collections. Phylogenetic analysis of protein-coding regions of the clinical isolates previously assigned to the *A. ustus* species based on morphological examinations revealed that these isolates represent a new species, *A. calidoustus*. Although the ITS sequences of the clinical isolates were identical to those of *A. pseudodeflectus*, they formed a separate well-defined clade based on β-tubulin, calmodulin, and actin sequence data (Fig. 1 and 2). Previously, the ITS regions have also been found to be of limited value for species delimitation in *Aspergillus* sections *Clavati* (35) and *Nigri* (28). The genetic variability among these isolates was extremely low.

Isolates of *A. calidoustus* can easily be distinguished from *A. ustus* or *A. granulosus* by their ability to grow at or above 37°C and from *A. pseudodeflectus* by using the Ehrlich test, which is used in *Penicillium* systematics for the detection of cyclopiazonic acid and other indole metabolites (3). Based on this reaction, *A. calidoustus* isolates produce such metabolites, while other members of *Aspergillus* section *Usti* do not (12).

A. calidoustus seems to be a relatively widespread species. Apart from the clinical isolates, several collection strains held in the CBS or NRRL collections have also been found to belong to this species (Table 1). In addition, preliminary data indicate that this species is also prevalent in indoor environments (J. Varga and R. A. Samson, unpublished data).

Antifungal susceptibility tests of *A. calidoustus* isolates show that most antifungal drugs have limited or no activity, which is in accordance with previous studies where "*A. ustus*" (*A. calidoustus*) isolates were found to be resistant to amphotericin B, echinocandins, and azole derivatives (5, 6, 21, 36). The class of the triazoles has a prominent role in the management of patients with invasive aspergillosis, and breakthrough infections caused by "*A. ustus*" have been reported in patients treated with voriconazole and itraconazole (21). We found that even the most recently registered compound, posaconazole, which is commonly the drug with the greatest intrinsic activity against *Aspergillus* species, showed no in vitro activity. Posaconazole was recently shown to be very effective in preventing invasive fungal infection and invasive aspergillosis in high-risk patients with acute myeloid leukemia, myelodysplastic syndrome, or graft-versus-host disease (2). However, it is important to realize that *A. calidoustus* might cause breakthrough infection in patients on posaconazole prophylaxis due to the lack of activity of the drug. In another study, terbinafine and the azole derivative UR-9825 were found to be the most active against "*A. ustus*" isolates among the seven antifungal drugs tested (6); however, these drugs are either experimental (UR-9825) or not used for the treatment of invasive aspergillosis due to the lack of clinical evidence of efficacy. Later studies clarified that this isolate belongs to the *A. insuetus* species (12). Further work is in progress to define the diversity and clinical significance of *A. calidoustus* isolates.

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