Aspergillus calidoustus sp. nov., Causative Agent of Human Infections Previously Assigned to Aspergillus ustus\V

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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 mised hosts. To date, only 22 cases of invasive aspergillosis pathogen that can cause invasive infection 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.
| 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                | CBS 121601, 677| Bronchoalveolar lavage fluid, proven invasive aspergillosis; Nijmegen, The Netherlands |
| A. calidoustus                | CBS 121602, 678| Bronchial secretion, proven invasive aspergillosis; Nijmegen, The Netherlands        |
| A. calidoustus                | CBS 121589, 682| Autopsy lung tissue sample, proven invasive aspergillosis; Nijmegen, The Netherlands |
| A. calidoustus                | CBS 121603, 741| Patient room, Nijmegen, The Netherlands                                             |
| A. calidoustus                | CBS 121604, 924| Laboratory, Nijmegen, The Netherlands                                               |
| A. calidoustus                | CBS 121605, 943| Sputum, Nijmegen, The Netherlands                                                   |
| A. calidoustus                | CBS 121606, 2725| Feces, Nijmegen, The Netherlands                                                   |
| A. calidoustus                | CBS 121608, 6989| Bronchoalveolar lavage, Nijmegen, The Netherlands                                 |
| A. calidoustus                | 7843          | Pasteur Institute, Paris, France                                                   |
| A. calidoustus                | 8023          | 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            | Patient 16                                                                         |
| A. calidoustus                | 67            | Patient 17                                                                         |
| 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 16                                                                        |
| A. calidoustus                | CBS 121616, FH 168| Environmental                                                                      |
| A. calidoustus                | FH 165        | Patient 5a                                                                         |
| A. calidoustus                | CBS 121614, FH 98| Patient 5b                                                                         |
| A. calidoustus                | CBS 121615, FH 97| Patient 6b                                                                         |
| A. calidoustus                | CBS 121613, FH 94| Patient 2b                                                                         |
| A. calidoustus                | CBS 121612, FH 90| Patient 1b                                                                         |
| A. calidoustus                | FH 91         | Patient 1b                                                                         |
| 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\textsuperscript{T} | Soil, Fayetteville, AR                                           |
| A. granulosus                 | CBS 119.58    | Soil, Texas                                                                         |
| A. granulosus                 | IKT 23478     | Unknown                                                                              |
| A. pseudodeflectus            | CBS 596.55    | Sugar, Louisiana                                                                    |
| A. pseudodeflectus            | CBS 756.74\textsuperscript{T} | Desert soil, Western Desert, Egypt                                                |
| A. puniceus                   | CBS 122.33    | A. Biochwitz (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\textsuperscript{T} | Soil, Zacinto, 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\textsuperscript{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 14392, 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                                                       |

\textsuperscript{a} These samples were taken from the same patient (36).  
\textsuperscript{b} K. A. Marr.
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

![Image of a phylogenetic tree](image.png)

**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.
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 $1 \times 10^4$ to $5 \times 10^4$ conidia/ml as recommended by the CLSI for mold testing (18).

The antifungal activities of amphotericin B (Bristol-Meyers-Squibb, Woerden, The Netherlands), fluconazole (or 5-fluorocytosine [5-FC]; Valeant, Zoetermeer, 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-
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. 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.
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-
luminal, their conidia were coarsely roughened to echinulate, and they produced irregularly elongated Hu¨lle 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 Hu¨lle 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).

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.

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 2. Morphological characteristics of species of Aspergillus section Usti |
|---------------------------------|-----------------|-----------------|-----------------|------------------|
| Species                        | Growth at 37°C | Characterization of Hu¨lle 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           |

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

| Antifungal agent | MIC (mg/liter [range]) for isolates from* |
|------------------|-----------------------------------------|
|                  | 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 |

* 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 dropplets; 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. Hüolle cells sparsely produced, irregularly elongated, in scattered groups.

**Etymological 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. pseudodefectus*, 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. pseudodefectus* 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*” (4) 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 theazole 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. insuustus* species (12). Further work is in progress to define the diversity and clinical significance of *A. calidoustus* isolates.

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