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Polyphasic taxonomy of *Aspergillus* section *Usti*

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Abstract: *Aspergillus ustus* is a very common species in foods, soil and indoor environments. Based on chemical, molecular and morphological data, *A. insuetus* is separated from *A. ustus* and revived. *A. insuetus* differs from *A. ustus* in producing drimans and ophiobolin G and H and *not* producing ustic acid and austocystins. The molecular, physiological and morphological data also indicated that another species, *A. keveii* **sp. nov.** is closely related but distinct from *A. insuetus*. *Aspergillus* section *Usti sensu stricto* includes 8 species: *A. ustus, A. puniceus*, *A. granulosus, A. pseudodeflectus, A. calidoustus*, *A. insuetus* and *A. keveii* together with *Emericella heterothallica*.

Taxonomic novelties: *Aspergillus insuetus* revived, *Aspergillus keveii* **sp. nov.**
Key words: actin, *Aspergillus*, β-tubulin, calmodulin, extrolite profiles, ITS, phylogenetics, polyphasic taxonomy.

INTRODUCTION

Aspergillus ustus is a very common filamentous fungus found in foods, soil and indoor air environments (Samson *et al.* 2002). This species is considered as a rare human pathogen that can cause invasive infection in immunocompromised hosts. However, *A. ustus* has been noted increasingly as causes of invasive aspergillosis in tertiary care centres in the US (Malani & Kaufman 2007). Up to date, 22 invasive aspergillosis cases have been reported to be caused by *A. ustus* (Verweij *et al.* 1999; Pavie *et al.* 2005; Panackal *et al.* 2006; Yildiran *et al.* 2006). Several studies indicate that *A. ustus* isolates are resistant to amphotericin B, echinocandins and azole derivatives (Verweij *et al.* 1999; Pavie *et al.* 2005; Gene *et al.* 2001; Garcia-Martos *et al.* 2005). Other species related to *A. ustus* can also cause human or animal infections. *Aspergillus granulosus* was found to cause disseminated infection in a cardiac transplant patient (Fakih *et al.* 1995), while *A. deflectus* has been reported to cause disseminated mycosis in dogs (Robinson *et al.* 2000; Kahler *et al.* 1990; Jang *et al.* 1986).

A. ustus is a variable species. Raper & Fennell (1965) stated that "not a single strain can be cited as wholly representative of the species as described". Indeed, *A. ustus* isolates may vary in their colony colour from mud brown to slate grey, with colony reverse colours from uncoloured through yellow to dark brown (Raper & Fennell 1965; Kozakiewicz 1989). Molecular data also indicate that this species is highly variable; RAPD analysis carried out in various laboratories could be used to detect clustering of the isolates (Rath *et al.* 2002; Panackal *et al.* 2006), and sequence analysis of parts of the ribosomal RNA gene cluster also detected variability within this species (Henry *et al.* 2000; Peterson 2000; Hinrikson *et al.* 2005).

We examined a large set of *A. ustus* isolates and related species originating from environmental and clinical sources to clarify the taxonomic status of the species, and to clarify the taxonomy of *Aspergillus* section *Usti*. The methods used include sequence analysis of the ITS region (intergenic spacer region and the 5.8 S rRNA gene of the rRNA gene cluster), and parts of the β-tubulin, calmodulin and actin genes, analysis of extrolite profiles, and macro- and micromorphological analysis of the isolates.

MATERIALS AND METHODS

Morphological examination. The strains examined are listed in Table 1. Both clinical and environmental strains were grown as 3-point inoculations on Czapek yeast agar (CYA), malt extract agar (MEA), creatine agar (CREA) and yeast extract sucrose agar (YES) at 25 °C, and on CYA at 37 °C for 7 d (medium compositions according to Samson *et al.* 2004). For micro morphological examination light microscopy (Olympus BH2 and Zeiss Axioskop 2 Plus) was employed.

Extrolite analysis. Extrolites were analysed by HPLC using alkylphenone retention indices and diode array UV-VIS detection as described by Frisvad & Thrane (1987), with minor modifications as described by Smedsgaard (1997). Standards of ochratoxin A and B, aflavinine, asperazine, austamide, austdiol, kotanin and other extrolites from the collection at Biocentrum-DTU were used to compare with the extrolites from the species under study.

Isolation and analysis of nucleic acids. The cultures used for the molecular studies were grown on malt peptone (MP) broth using 10 % (v/v) of malt extract (Brix 10) and 0.1 % (w/v) bacto peptone (Difco), 2 mL of medium in 15 mL tubes. The cultures were incubated at 25 °C for 7 d. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. Fragments containing the ITS region were amplified using primers ITS1 and ITS4 as described previously (White *et al.* 1990). Amplification of part of the β-tubulin gene was performed using the primers Bt2a and Bt2b (Glass 1995). Amplifications of the partial calmodulin and actin genes were set up as described previously (Hong *et al.* 2005). Sequence analysis was performed with the Big Dye Terminator

Table 1. Isolates in *Aspergillus* section *Usti* and related species examined in this study.

Species	Strain No.	Source
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^{T}	Bronchoalveolar lavage fluid, proven invasive aspergillosis, Nijmegen, The Netherlands†
A. calidoustus	CBS 121602	Bronchial secretion, proven invasive aspergillosis, Nijmegen, The Netherlands†
A. calidoustus	CBS 121589	Autopsy lung tissue sample, proven invasive aspergillosis, Nijmegen, The Netherlands†
A. calidoustus	CBS 121603	Elevator shaft in hospital, Nijmegen, The Netherlands
A. calidoustus	CBS 121604	Patient room, Nijmegen, The Netherlands
A. calidoustus	CBS 121605	Laboratory, Nijmegen, The Netherlands
A. calidoustus	CBS 121606	Sputum, Nijmegen, The Netherlands
A. calidoustus	CBS 121607	Feces, Nijmegen, The Netherlands
A. calidoustus	CBS 121608	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	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	907	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	908	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	64	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	67	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	CBS 121610	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	351	Osteorickets
A. calidoustus	482	Post-cataract surgery endophthalmitis
A. calidoustus	CBS 121611	Patient 4, Washington, U.S.A.
A. calidoustus	CBS 121616	Environmental, Washington, U.S.A.
A. calidoustus	FH 165	Patient 5b, Washington, U.S.A.
A. calidoustus	CBS 121614	Patient 5a, Washington, U.S.A.
A. calidoustus	CBS 121615	Patient 6, Washington, U.S.A.
A. calidoustus	CBS 121613	Patient 2, Washington, U.S.A.
A. calidoustus	CBS 121612	Patient 1, Washington, U.S.A.
A. calidoustus	FH 91	Patient 1a, Washington, U.S.A.
A. calidoustus	NRRL 26162	Culture contaminant, Peoria, U.S.A.
A. calidoustus	NRRL 281	Thom 5634
A. calidoustus	NRRL 277	Thom 5698.754, Green rubber
A. granulosus	CBS 588.65^{T}	Soil, Fayetteville, Arkansas, U.S.A.
A. granulosus	CBS 119.58	Soil, Texas, U.S.A.
A. granulosus	IBT 23478 = WB 1932 = IMI 017278iii = CBS 588.65	Soil, Fayetteville, Arkansas, U.S.A.
A. insuetus	CBS 107.25^{T}	South Africa
A. insuetus	CBS 119.27	Unknown
A. insuetus	CBS 102278	Subcutaneous infection left forearm and hand of 77-year-old woman
A. keveii	CBS 209.92	Soil, La Palma, Spain
A. keveii	CBS 561.65	Soil, Panama
A. keveii	IBT 10524 = CBS 113227 = NRRL 1254	Soil, Panama