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Aspergillus fuscicans (Aspergillaceae, Eurotiales), a new species in section *Usti* from Argentinean semi-arid soil

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Abstract

Aspergillus fuscicans, a new species within *Aspergillus* section *Usti* from Argentinean semi-arid soil is introduced. Molecular, morphological and physiological studies were conducted, based on sequence analysis of partial β-tubulin and calmodulin sequence data. *Aspergillus fuscicans* formed a distinct, well-defined clade related to *A. calidoustus* and *A. pseudodeflectus*. In addition, *A. fuscicans* was able to grow and sporulate at 37 ºC, and had a negative Ehrlich reaction. Morphological and physiological features could be used to differentiate the new species from its phylogenetically related taxa.

Introduction

In 1965, Raper & Fennell proposed the "*Aspergillus ustus* species group" which included *A. ustu*s (Bainier) Thom & Church (1926: 152) along with four other species. This species group was characterized by radiate to loosely columnar conidial heads in shades of olive gray to red gray and the production of Hülle cells scattered throughout the colony in irregular masses that are not associated with pigmented mycelium.

The *Aspergillus ustus* group was provided with a recognized nomenclatural designation as *Aspergillus* section *Usti* (Gams *et al*. 1985). Several authors have revised the taxonomy of the group (Kozakiewicz 1989, Klich 1993, Peterson 2000). Through the application of polyphasic approaches, the number of species described for the section *Usti* has recently increased. Houbraken *et al*. (2007) have published a monograph including *A. insuetus* (Bainier) Thom & Church (1929) (revived) and *A. keveii* Varga, Frisvad & Samson (2007) as taxonomic novelties. Peterson (2008) examined the relationships of the genus *Aspergillus* using phylogenetic analysis and assigned 15 species to this section. Varga *et al*. (2008) studied a large set of *A. ustus* isolates from clinical and environmental sources and described *A. calidoustus* Varga, Houbraken & Samson (2008) as a new species. Samson *et al*. (2011) erected *Aspergillus carlsbadensis* Frisvad, Varga & Samson, *A. californicus* Frisvad, Varga & Samson, *A. germanicus* Frisvad, Varga & Samson, *A. pseudoustus* Frisvad, Varga & Samson and *A. turkensis* Varga, Frisvad & Samson. In addition, they proposed a new combination *Aspergillus monodii* (Locquin-Linard) Varga, Frisvad & Samson. Through a study of microfungal communities in Spanish caves, *A. baeticus* A. Nováková & Hubka (2012) and *A. thesauricus* Hubka & A. Nováková (2012) were described (Nováková *et al*. 2012). Jurjevic & Peterson (2016) added *A. collinsii* Ž. Jurjević & S. W. Peterson (2016) and *A. asper* Ž. Jurjević & S.W. Peterson (2016) to the section.

A thermoresistant soil–borne fungi survey from Argentina has been carried out since 2009, this study has given several new *Aspergillus* species. The present contribution deals with isolates belonging to *Aspergillus* section *Usti*. These isolates could not be confidently placed in previously described species. The objective of this work was to present and describe a new *Aspergillus* sect. *Usti* species based on partial sequences of calmodulin and β–tubulin genes and morphological and physiological analyses.

Materials & Methods

Isolation

The strains were isolated from a semi–arid soil sample from Catamarca, Argentina. Five g of soil were transferred to 100 mL of melted (45–50 ºC) Malt Extract Agar Oxoid (MEA, CM0059) prepared with the addition of 50 ppm of chloramphenicol, and heated at 75 ºC for 30 min. The mixture was incubated at 30 ºC for up to 30 d (Samson *et al*. 2000). The strains are deposited at BAFC collection (herbarium acronyms are according to Thiers, 2017).

Morphological studies

For standard descriptions, spore suspensions in semi–solid agar were inoculated at three points on MEA, Oatmeal Agar (OA), Czapek Yeast Agar (CYA), Czapek Agar (CZ), Blakeslee Malt Extract Agar (BMEA) and Creatine Sucrose Agar (CREA) according to formulae provided by Samson *et al*. (2000). All media were poured into 9 cm plastic Petri dishes. Plates were inoculated in a three-point pattern using a micropipette and inoculum size of 1 µl per spot. Incubation was carried out in the dark, at 25 ºC during 7 d for all media plates (Samson *et al*. 2014); cultures on CYA were also incubated at 37 ºC, and cultures on MEA were also incubated at 40 and 42 ºC. Colony diameters were measured using a ruler. For color standards and color nomenclature Ridgway´s table (1912) was utilized to describe colony colors. Stern (2004) was used to prepare the Latin diagnosis.

To detect the presence of indol metabolites Ehrlich reaction was use by filter paper method (Lund 1995; Samson *et al*. 2011).

For micro–morphological observations mounts were made in lactic acid (85 % w/w) from MEA colonies, a drop of alcohol 70% v/v was added to remove air bubbles and excess of conidia. Preparations were observed through a Zeiss Axioscop microscope equipped with a drawing tube.

DNA extraction, PCR amplifications and sequencing

After 5 d of growth on PDB (Potato Dextrose Broth) at 25 ºC in darkness, the mycelium was harvested, dried and transferred to 1.5 mL microcentrifuge tubes for DNA extraction. The DNeasy Qiagen kit was used for DNA extraction following manufacturers´ instructions. The DNA obtained was quantified by electrophoresis on 1% agarose gels ran at 120 mV for 15–20 min. DNA was visualized by fluorescence with ethidium bromide. Parts of the calmodulin (*CaM*) and β–tubulin (*BenA*) was amplified and sequenced. The amplification reactions were performed with the pair primers CMD5 and CMD6 (Hong *et al*., 2005), Bt2a and Bt2b (Glass & Donaldson 1995).

All the PCR reactions were performed with a Mastercycler gradient thermocycler (Eppendorff, USA). The PCR reaction for *CaM* was performed as follows: in 20 µl of total volume of 1x Master Mix New England Biolabs, 1.25 mM $MgCl_2$, 50–100 ng/µl genomic DNA. The amplification program was a first denaturation step at 94 ºC for 1 min, 30 cycles at 94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min with a final elongation step at 72 ºC for 3 min. The PCR reaction for *BenA* was performed as follows: in 50 µl of total volume 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.2 mM dNTPs, 0.2 µM primers, 3 mM $MgCl_2$, 1 U Taq polymerase (Invitrogen life technologies, Brazil), 50–100 ng/µL genomic DNA. The amplification program was a first step with 5 cycles: denaturation step at 94 ºC for 1 min, 68 ºC for 90 s, 72 ºC for 2 min; 25 cycles at 94 ºC for 1 min, 64 ºC for 90 s, 72 ºC for 2 min with a final elongation step at 72 ºC for 10 min. The resulting products were purified with Wizard SV Gel and PCR Clean–Up System (Promega). Sequencing was conducted under Big Dye TM Terminator v 3.1 (Applied Biosystems) based on Sanger´s method. The reacted products were purified using ethanol precipitation and analyzed with a Genetic Analyzer 3130xl at Unidad de Genómica, Instituto de Biotecnología (UGB, IB–INTA, Argentina).

Molecular phylogenetic analyses

The sequences were selected following Samson *et al*. (2011) with addition of new species from Nováková *et al*. (2012). Alignments were made using the Clustal W algorithm in Bioedit v. 7.0.5.3 (Hall 1999). Alignments and phylogenetic analyses for *CaM* and *BenA* are deposited in TreeBASE under accession number 10690.

Heuristic searches were conducted using TNT ver. 1.1 (Goloboff *et al*. 2008). During the search we used equal weights and no additive characters, and gaps were treated as missing data. Before searches, all uninformative characters were deactivated. The searches were done using Multiple TBR + TBR with 10000 hold and 1000 replicates. Bootstrap values were calculated from 1000 replicates. All the characters were considered with the same weight. Parsimony-based analyses of the sequence data were performed for the two genes separately. Neighbour Joining analysis using the MEGA 6.1 software (Tamura *et al*. 2013) was performed and with 500 bootstrap replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura & Nei 1993) and are in the units of the number of base substitutions per site.

Results: Taxonomic treatment

Aspergillus fuscicans S. M. Romero, A. I. Romero, Barrera, V.A & Comerio **sp. nov.**
MB 823159
(Fig. 1.A–H)

Type:—ARGENTINA. Catamarca: from Corral Quemado to Papachacra (27°07,545'S, 66°56,598'W, 2152 m elev), 10 January 2009, *S.M. Romero* ET1611 (Holotype BAFC 52653 dried culture). *CaM* and *BenA* gene sequences deposited at GenBank, accession numbers KY853415 and KY853416). BAFCcult 4564, culture *ex type*.

Etymology: *fuscus –i*, adjective, greyish brown to blackish, very dark blackish brown; *–icans*, suffix, indicates process of becoming or resemblance sometimes so close as to be almost identical; *fusc–* + *–icans* = *fuscicans*; *fuscicans*: which becomes very dark blackish brown.

Diagnosis: Habitu morphologiaque A. usto et affinibus speciebus similis; ad A. calidoustum accedens, sed ab ea specie conidiis modice majoribus ac cellulis obtengentibus non modo irregulariter elongatis sed etiam oblongis oviformibusque recedens.

Description: Colonies on **CYA**, 25 ºC, 7 d, 29–35 mm, sulcate to plicate, greenish grey to dark grey (Buffy Olive, R. Pl. XXX; Fuscous to Chaetura Drab, R. Pl. XLVI) presenting narrow white margins, sporulation good to abundant, yellow pigment diffusing into the agar, exudate droplets scarce with yellowish to orange brown color; reverse yellow with brownish centers. After two incubation weeks, colonies becoming darker (Chaetura Drab to Fuscous–Black, R. Pl. XLVI) and reverse brownish yellow. At 37 ºC, 7 d, 25–34 mm diam., plicate, grey (Drab to Heir Brown, R. Pl. XLVI) presenting wide white to beige margins, good sporulation, occasionally yellow pigment diffusing into the agar, exudate droplets absent; reverse brownish). On **CZ**, 25 ºC, 7 d, 23–24 mm diam., sulcate, yellowish white to grey (near Heir Brown, R. Pl. XLVI), sporulation scarce, or abundant presenting narrow white margins, diffusible pigment absent, clear exudate droplets abundant at colony centers; reverse pale to yellow). On **MEA**, 25 ºC, 7 d, 37–50 mm diam., velutinous to rather floccose, grey (near Deep Grayish Olive, R. Pl. XLVI) with wide white margins, very good sporulation, diffusible pigment absent, exudate droplets abundant and clear; reverse brownish to brownish orange. The colonies become very dark blackish brown to black in two weeks of incubation (see detail in Fig. 1.C). At 40 ºC, 7 d, 4–5 mm diam. At 42 ºC no growth. On **BMEA**, 25 °C, 7 d, 48–59 mm diam., velutinous, sometimes with an overlying white mycelium, grey (Deep Grayish Olive, R. PL. XLVI), very good sporulation describing a subtle annular pattern, wide white margins, diffusible pigment absent, exudate absent; reverse yellow to greenish. On **OA**, 25°C, 7 d, 40–50 mm diam., plane, very dark grey, almost black (Deep Greyish Olive to Chaetura Black, R. Pl. XLVI), very good sporulation, sometimes with an overlying white mycelium, soluble pigment light greenish yellow, abundant brownish exudate droplets; reverse yellow green to green (Citron Yellow to yellowish Citrine, R. Pl. XVI; Lettuce Green, R. Pl. V). On **CREA**, 25 ºC, 7 d, 14–21 mm diam., loose colorless mycelium, weak sporulation, neither acid nor base production). On **YES**, 25 ºC, 7 d, 40–42 mm diam., sulcate, rather floccose, sporulation medium to good, grey, (Light Grayish Olive to Deep Grayish Olive, R. Pl. XLVI), yellow pigment diffusing into the agar; reverse greyish yellow to vivid orange.

Teleomorph not observed. Conidial heads radiate. Conidiophores pigmented, brown but diminutive hyaline conidiophores when produced from aerial hyphae, 50–180 × 4–6 µm. Vesicles globose to pyriform, 10–16 µm diam., biseriate. Metulae 4–6 × 3 µm. Phialides 5–6 µm long. Conidia globose, coarsely roughened to echinulate, and even forming bars, 3.5–5 µm diam. Hülle cells hyaline, sparsely produced (a little more abundant in BMEA), irregularly elongated to oblong to ovoid, decreasing in number through successive subcultures.

Additional isolate examined: ARGENTINA, Catamarca, from Corral Quemado to Papachacra (27°07,545'S, 66°56,598'W, 2152 m elev), 25 August 2011, *S.M. Romero* ET4611, BAFCcult 4565.

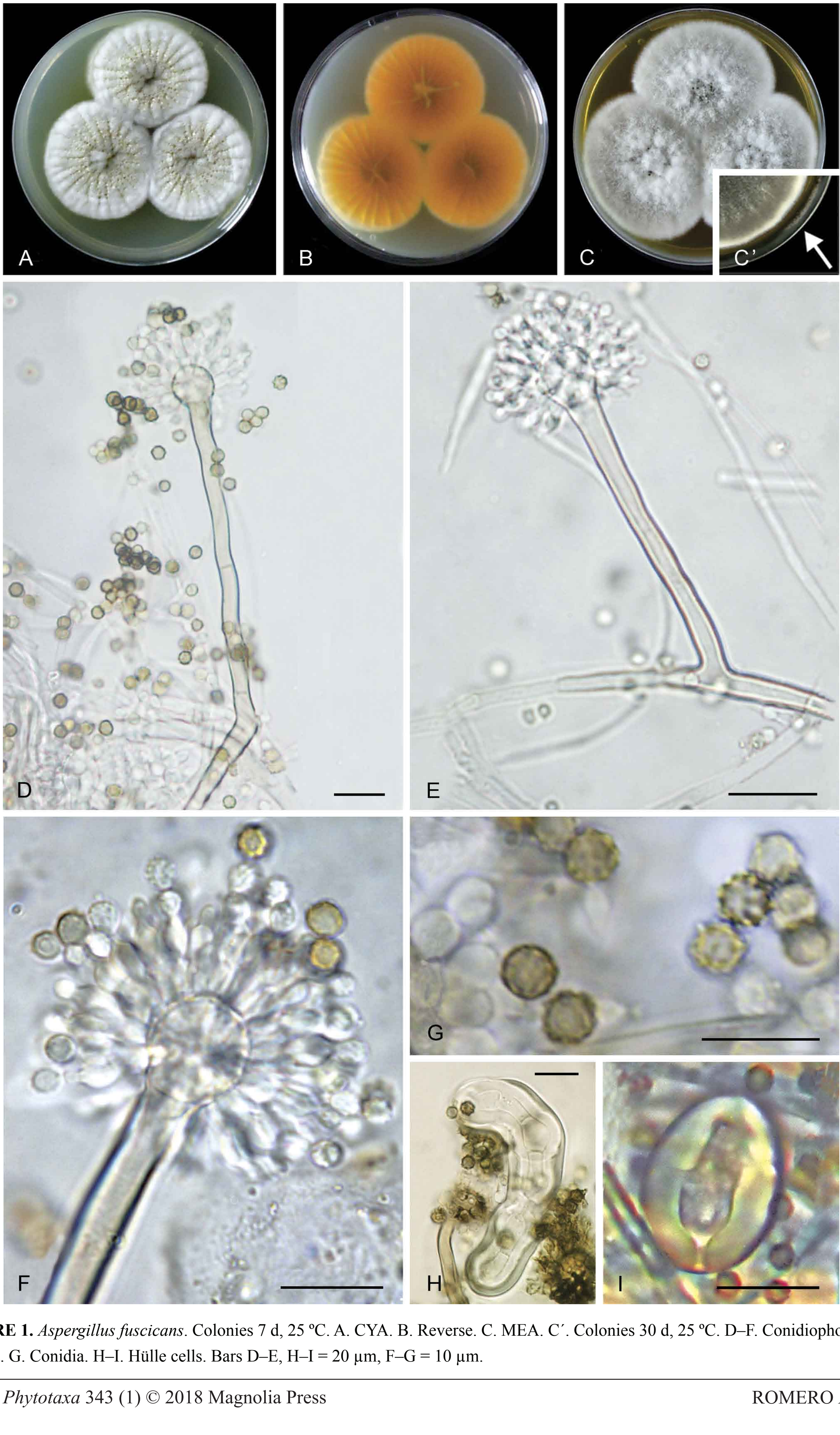

FIGURE 1. *Aspergillus fuscicans*. Colonies 7 d, 25 ºC. A. CYA. B. Reverse. C. MEA. C´. Colonies 30 d, 25 ºC. D–F. Conidiophores and conidia. G. Conidia. H–I. Hülle cells. Bars D–E, H–I = 20 µm, F–G = 10 µm.

Molecular phylogenetic analyses: The *CaM* data set included 28 taxa and 472 characters and 28 and 449 for *BenA*. The *CaM* MP analysis yielded 6 optimal trees and a consensus strict tree of 833 steps with a consistency index CI = 0.51 and a retention index RI = 0.68 from 220 informative characters. The *BenA* MP analysis yielded six optimal trees and a consensus strict tree of 585 steps with a CI = 0.53, RI = 0.71 from 170 informative characters. The MP strict consensus trees based on the *BenA* and *CaM* dataset are shown in Fig 2, together with the accession numbers of the query sequences and the 27 reference sequences, obtained from GenBank and *Aspergillus versicolor* as outgroup. The NJ analysis resulted in a phylogram, which had a similar topology as that of the MP analysis; the NJ bootstrap values are shown in the MP cladogram (Figs. 2 and 3). In the *BenA* MP tree (Fig. 2), *A. fuscicans* grouped with *A. pseudodeflectus* (MP = 100 % bs; NJ = 90 %) and these two were related with *A. calidoustus* (MP = 100 % bs; NJ = 90 %). In the *CaM* phylogenetic analysis (Fig. 3), the three species grouped together in a polytomy (MP = 100 % bs; NJ = 98 %).

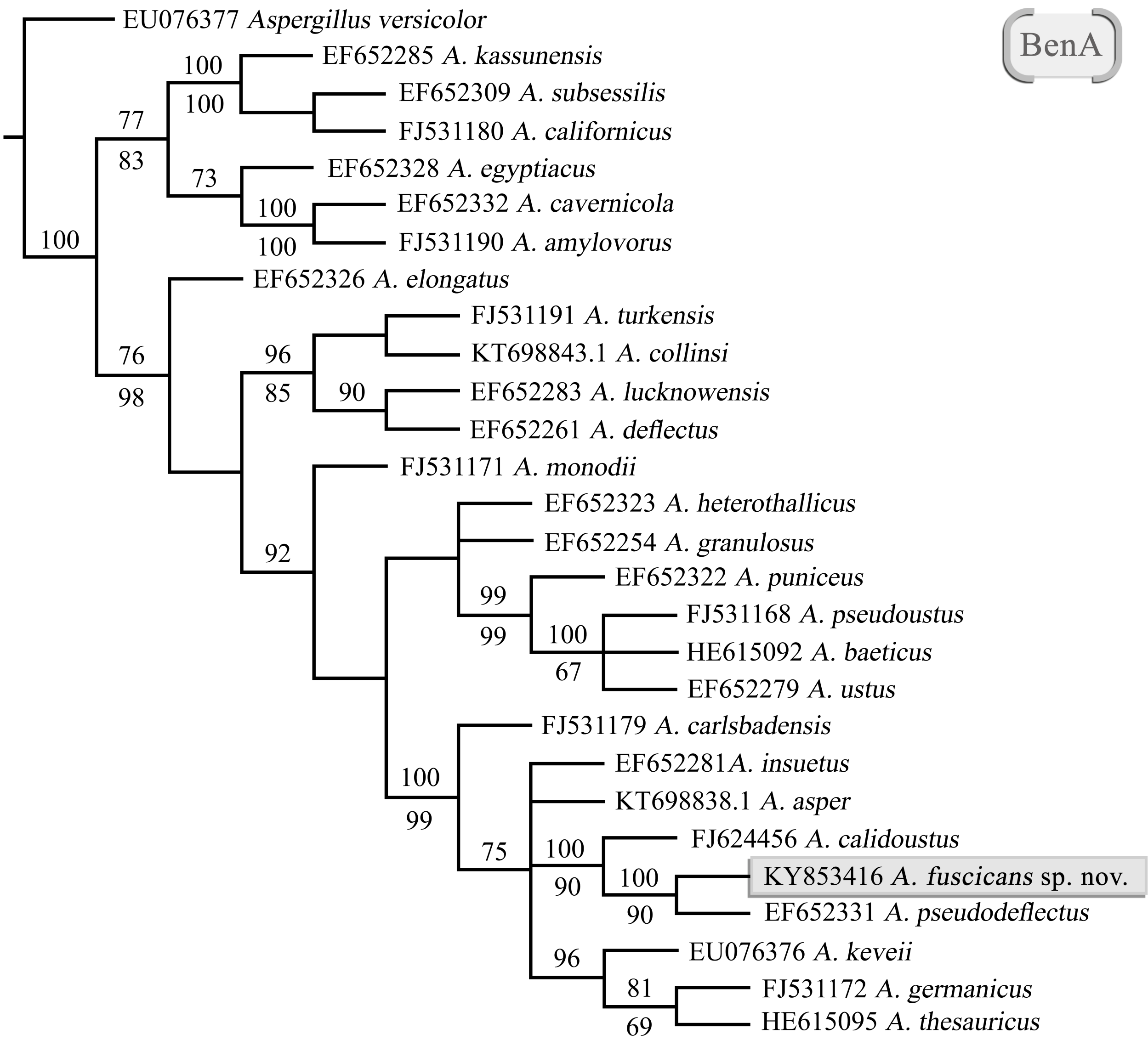

FIGURE 2. Strict consensus phylogenetic cladogram constructed with maximum parsimony analysis with *BenA* sequences. MP and NJ bootstrap values >50% are shown above and below branches, respectively. Terminal nodes given as GenBank accession number and species name.

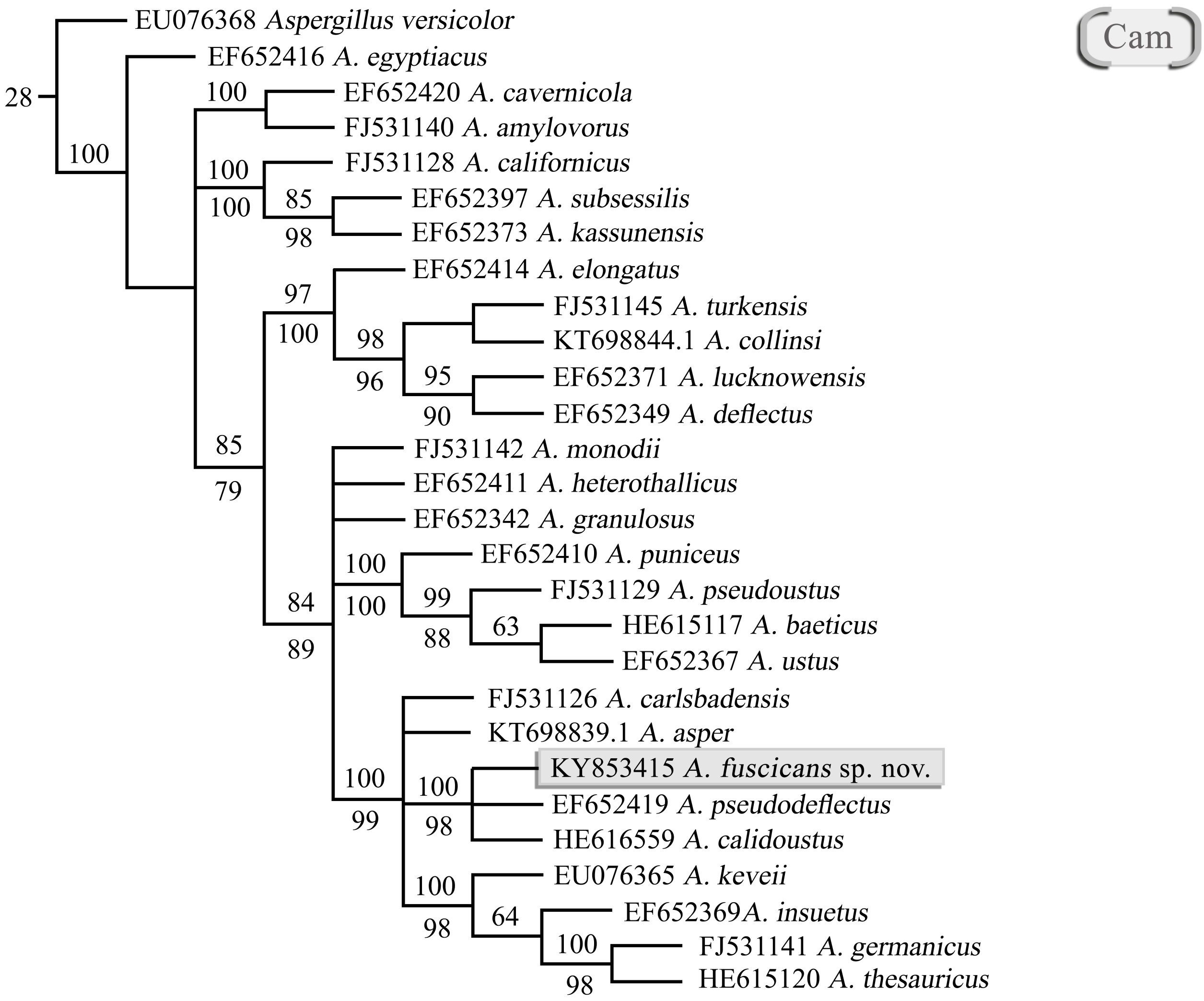

FIGURE 3. Strict consensus phylogenetic cladogram constructed with maximum parsimony analysis with *CaM* sequences. MP and NJ bootstrap values >50% are shown above and below branches, respectively. Terminal nodes given as GenBank accession number and species name.

Discussion

Aspergillus ustus is a common filamentous fungus found in foods, soil and indoor air environments (Samson *et al*. 2011). *Aspergillus calidoustus*, *A. fuscicans*, *A. pseudodeflectus* Samson & Mouch. (1975: 345) and *A. ustus* are closely related species. Nevertheless, *A. fuscicans* has enough differences from the previously known species of the *Aspergillus* section *Usti* to be proposed as new. *Aspergillus fuscicans* strains were able to grow at 37 ºC, whereas *A. ustus* does not. On the other hand, *A. fuscicans*, as *A. calidoustus* and *A. pseudodeflectus* have good growth at 37 ºC. According to Houbraken *et al*. (2007), the Ehrlich test constitutes a useful character to separate species within this section *Usti*. *Aspergillus fuscicans* had a negative Ehrlich reaction and this character can be used to distinguish this species from *A. calidoustus*, a species having a positive reaction with Ehrlich reagents (see Table 1). In addition, the conidium size of *A. fuscicans* differs from *A. calidoustus* and *A. pseudodeflectus* (Table 1). The heavy sporulation on MEA and the production of Hülle cells, both absent features in *A. pseudodeflectus*, support *A. fuscicans* as a new biological entity. Table 1 presents some more features for differentiation of *A. fuscicans* from its closest relatives.

TABLE 1. Morphological and physiological features of *A. fuscicans* and allied species of section *Usti*.

Species	Diam (mm)		Ehrlich Test	CREA	MEA colour	Conidia (µm)
	CYA37	YES				
A. calidoustus	20–35	36–41	Violet	Weak to moderate growth, hyaline mycelium	Brownish grey	2.7–3.5
A. fuscicans	**29–35**	**40–42**	**Negative**	**Weak growth, hyaline mycelium**	**Grey**	**3.5–5**
A. pseudodeflectus	15–20	20–30	Negative	Weak to moderate growth, hyaline mycelium	No sporulation	3.5–5
A. ustus	–	43–49	Negative	Good growth, faint yellow mycelium	Hair brown	3.2–4.5

The position of BAFCcult 4564 was the same in both analyses (MP and NJ) for both genetic markers (*CaM* and *BenA*). The strain always grouped, with statistical support, close to *A. calidoustus* and *A. pseudodeflectus*. The relation between these two species agrees with Houbraken *et al*. (2007), Varga *et al*. (2008), Samson *et al*. (2011) and Nováková *et al*. (2012). The phylogenetic results agree with the morphological and physiological characters reinforcing the proposal of the new species.

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