

Aspergillus asper sp. nov. and *Aspergillus collinsii* sp. nov., from *Aspergillus* section *Usti*

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In sampling fungi from the built environment, two isolates that could not confidently be placed in described species were encountered. Phenotypic analysis suggested that they belonged in *Aspergillus* sect. *Usti*. In order to verify the sectional placement and to assure that they were undescribed rather than phenotypically aberrant isolates, DNA was isolated and sequenced at the beta-tubulin, calmodulin, internal transcribed spacer and RNA polymerase II loci and sequences compared with those from other species in the genus *Aspergillus*. At each locus, each new isolate was distant from existing species. Phylogenetic trees calculated from these data and GenBank data for species of the section *Usti* excluded the placement of these isolates in existing species, with statistical support. Because they were excluded from existing taxa, the distinct species *Aspergillus asper* (type strain NRRL 35910^T) and *Aspergillus collinsii* (type strain NRRL 66196^T) in sect. *Usti* are proposed to accommodate these strains.

Introduction

The *Aspergillus ustus* group was first recognized by Thom & Raper (1945) and contained two species. Raper & Fennell (1965) recognized five species in the group. The *Aspergillus ustus* group was provided a recognized nomenclatural designation as *Aspergillus* section *Usti* (Gams *et al.*, 1985). Houbraken *et al.* (2007) added a species to the section. Peterson (2008), using multilocus phylogenetic analysis of DNA sequences, included 15 species in the section while removing two species accepted by Raper & Fennell (1965). Samson *et al.* (2011) accepted Peterson's analysis and added

more novel species to the section. Visagie *et al.* (2014) added another novel species, *Aspergillus porphyreostipitatus* to the section. A notable feature of this section is that about one third of the described species are known only from the type isolate or specimen.

Section *Usti* species are primarily environmental fungi, some cosmopolitan and common, while others are known only from one or a few isolates. *Aspergillus calidouustus* is recognized as an opportunistic human and animal pathogen (Varga *et al.*, 2008; Balajee *et al.*, 2009; Hubka *et al.*, 2012; Panackal *et al.*, 2006) and one species, reported to be *A. ustus*, is algicolous (Liu *et al.*, 2014). Species from sect. *Usti* make a variety of exometabolites (Samson *et al.*, 2011; Liu *et al.*, 2014), but production of the aflatoxin precursor sterigmatocystin that was reported from *A. ustus* (Rabie *et al.*, 1977) could not be verified in subsequent studies (Samson *et al.*, 2011).

In the course of environmental sampling and identification, we obtained isolates of *Aspergillus* section *Usti* that could not be placed in any of the described species. We determined DNA sequences from multiple loci of the isolates and conducted phylogenetic analysis to determine whether these isolates were morphologically abnormal isolates of known species or novel taxa. Descriptions and names for two novel species, *Aspergillus asper* sp. nov. and *Aspergillus collinsii* sp. nov. are provided, based on phenotype and phylogeny of a single isolate in each species.

Abbreviations: BT2, amplified fragment of the beta tubulin gene; CF, amplified fragment of the calmodulin gene; ITS, amplified fragment containing ITS1, 5.8S rDNA and ITS2; RPB2, amplified fragment of the DNA-dependent RNA polymerase II, second largest subunit.

The GenBank/EMBL/DDBJ accession numbers for the BT2, CF, ITS and RPB2 sequences of *Aspergillus asper* are KT698838, KT698839, KT698840 and KT698842, respectively; those for the BT2, CF, ITS and RPB2 sequences of *Aspergillus collinsii* are KT698843, KT698844, KT698845 and RPB2 are KT698848, respectively.

The Mycobank accessions for *Aspergillus asper* and *Aspergillus collinsii* are 814412 and 814413, respectively.

Herbarium accession numbers for *Aspergillus asper* and *Aspergillus collinsii* are BPI 893218 and BPI 893219, respectively. BPI is the United States National Herbarium, USDA, Beltsville, MD, USA.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

Methods

Fungal isolates. Fungal isolates used in this study are listed in Table 1 and are available from the Agricultural Research Service Culture Collection (NRRL), Peoria, IL, USA (<http://nrnl.nrcar.usda.gov>). The CBS Biodiversity Culture Collection, Utrecht, The Netherlands, and the Charles University of Prague culture collection (CCF), Prague, The Czech Republic, also house the type isolates of *A. asper* sp. nov. and *A. collinsii* sp. nov.

Media and examination. Cultures for morphological examination were grown at 25 °C for 7 days in darkness on Czapek yeast autolysate agar (CYA), malt extract agar (MEA; 20 g Difco malt extract, 1 g peptone, 10 g glucose per liter), CYA with 20 % sucrose (CY20S), CYA supplemented with 5 % NaCl (CYAS), dichloran with 18 % (w/v) glycerol agar (DG18), oatmeal agar (OA), potato dextrose agar (PDA), and creatine sucrose agar (CREA) (Health Link; <http://www.healthlinkinc.net>) formulated as specified by Pitt (1980) and Samson *et al.* (2011). Difco malt extract was used. Additional CYA and MEA cultures were incubated at different temperatures (15, 20, 30, 35, 37, 40 and 43 °C) for 7 days. Cultures were grown in duplicate as three-point inoculations in 9 cm diameter Petri dishes. Fungal isolation and microscopy were performed as described by Peterson & Jurjević (2013).

DNA methods. For DNA extraction, cultures were grown in 25 ml malt extract broth (30 g Difco malt extract per liter water) with shaking at 200 r.p.m. and collected by vacuum filtration over filter paper. DNA was extracted from freeze-dried mycelium using the CTAB method (Peterson *et al.*, 2015). Beta tubulin (BT2), calmodulin (CF), internal transcribed spacer region (ITS; containing ITS1, 5.8S rDNA and ITS2) and DNA-dependent RNA polymerase II second largest subunit (RPB2) were amplified from genomic DNA and sequenced using published methods (Peterson & Jurjević, 2013). Phylogenetic analysis of data sets from each locus was carried out with MEGA 6.06 (Tamura *et al.*, 2013) using maximum-likelihood criterion and the model determined using the built-in model selection test routine. All GenBank numbers are provided in Table S1 (available in the online Supplementary Material). Trees were prepared for publication as described previously (Peterson *et al.*, 2015).

Results

Phylogenetic analysis of sequence data from the *RPB2* locus is presented in Fig. 1, and analyses of BT2, CF and ITS loci are presented in Fig. S1 (available in the online Supplementary Material). *A. asper* sp. nov. (Fig. 1, *RPB2*) is in a statistically supported clade containing *Aspergillus thesauricus*, *A. keveii*, *A. pseudodeflectus*, *A. calidoustus* and *A. insuetus*. High bootstrap values (99 %) support the distinction of *A. asper* from its sibling species in the clade. Phylogenetic analysis of the BT2, CF and ITS data also revealed this clade (Fig. S1). The ITS locus is the barcode region and its occasional lack of resolution among closely related species is known (Peterson, 2012). Even so, *A. asper* could be distinguished from other species of sect. *Usti* using the ITS barcode. Fig. 1 shows a 73 % bootstrap value supporting sibling relationship between *A. asper* and the *A. insuetus*, *A. keveii*, *A. thesauricus* clade. Bootstrap values greater than 70 % are viewed as greater than 95 % statistical probability (Hillis & Bull, 1993).

A. collinsii sp. nov. occurs in a 98 % bootstrap supported clade containing *Aspergillus deflectus*, *A. lucknowensis*, *A. turkensis* and *A. elongatus* (Fig. 1), and each of these is strongly

supported (85–100 % bootstrap) as a distinct species by the bootstrap statistic. The single locus trees from the other loci contain no contradictions with the *RPB2* data that are supported by bootstrap data, although *A. elongatus* is, by chance, excluded from the clade in the ITS and BT2 data sets (Fig. S1).

Taxonomy

Description of *Aspergillus asper* Ž. Jurjević & S. W. Peterson sp. nov.

Aspergillus asper (as'per. N.L. masc. adj. *asper* rough, referring to the very rough conidial surface ornamentation).

Description of colonies after 7 days (Fig. 2): CYA at 25 °C, conidial heads radiate, splitting with age, greyish brown, good sporulation, mycelium white to greyish-white, floccose, at margin hyphae subsurface or submerged into medium, no exudate, no soluble pigment, reverse pale-yellow; CYA at 30 °C, abundant sporulation, abundant aggregations of Hülle cells, white to pale yellow, reverse brown; MEA at 25 °C, conidial heads radiate, splitting with age, greyish-brown, poor to good sporulation, mycelium white, floccose, plane, at margin hyphae subsurface or submerged into medium approximately 2–3 mm, no exudate, no soluble pigments, reverse pale brown; MEA at 30 °C, good sporulation, abundant carpet-like aggregation of Hülle cells, white to pale yellow, overgrown with white hyphae; CY20S at 25 °C, conidial heads greyish-brown, poor sporulation, mycelium white, subsurface or submerged into medium, no exudate, no soluble pigments, reverse uncoloured; OA at 25 °C, conidial heads greyish-brown, sporulation poor to good, mycelium white to pale yellowish, floccose, clear exudate sparse, no soluble pigments, reverse pale yellow; PDA at 25 °C, conidial heads greyish-brown, radiate or occasionally short columnar, splitting with age, poor to good sporulation, mycelium white to pale yellow, floccose, moderately deep radial sulca, no exudate or soluble pigments, reverse yellow; DG18 at 25 °C, conidial heads brownish-yellow to grey-green, good sporulation, radiate, mycelium white to pale-yellowish shades, at margin hyphae subsurface or submerged into medium approximately 3–4 mm, floccose, no exudate or soluble pigments, reverse pale yellow; CYAS at 25 °C, conidial heads greyish-brown, sporulation poor to good at centre of colony, radiate, mycelium white, floccose, no exudate or soluble pigments, reverse pale buff; CREA at 25 °C, no sporulation, mycelium white, subsurface or submerged in medium, no acid production.

Stipes smooth, occasionally roughened, near crustaceous, rarely septate, hyaline becoming brownish with age, 10–150×3–4(–5) µm if borne from aerial hyphae, (150–)200–1000(–1150)×(4–)5–9(–10) µm if borne from substrate; vesicles globose to pyriform, (4–)5–10 µm diameter if borne from aerial hyphae, 10–25(–29) µm diameter if borne from substrate, biseriate, metulae cylindrical 3–8(–10)×2.5–4.5(–6) µm, covering 1/2 to 2/3 of vesicle; phialides ampulliform, (4–)5–7(–8)×2.5–3.5(–5) µm; conidia globose to subglobose,

4–5(–7) μm diameter, echinulate walls, abundant Hülle cells irregularly elongate, ovoid, curved to coiled, 10–90 μm long.

Medium-dependent growth characteristics assessed as colony diameter after 7 days of growth at 25 °C on: CYA 38–39 mm, MEA 30–32 mm, CY20S 25–27 mm, CYAS 9–10 mm, DG18

9–10 mm, OA 30–31 mm, PDA 29–30 mm and CREA 30–31 mm. Temperature-dependent growth assessed as colony diameter on CYA and MEA, respectively, after 7 days of growth at: 20 °C 20–21 mm/20–21 mm, 30 °C 50–51 mm/43–45 mm, 35 °C 3–4 mm/no growth, 37 °C no growth/no

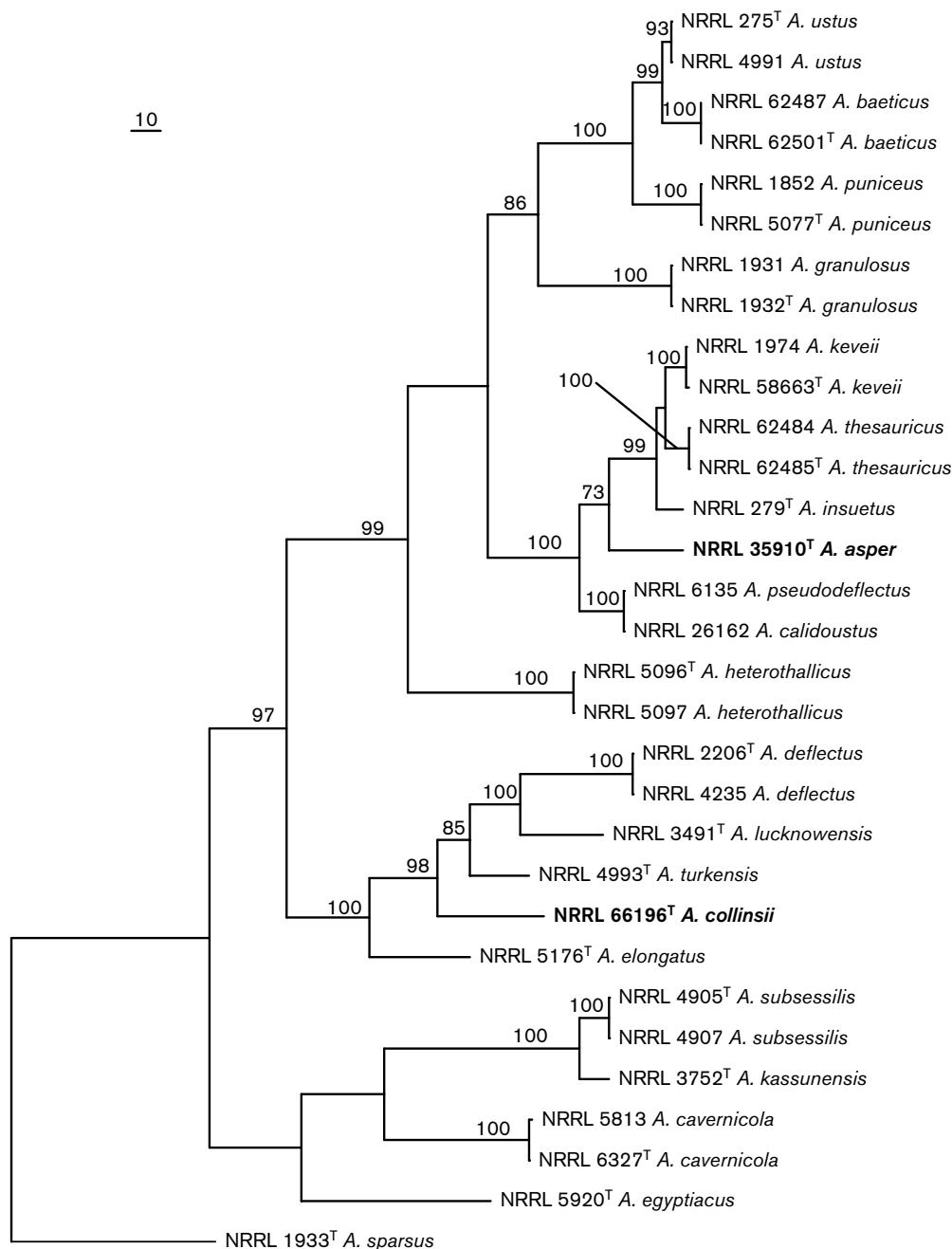


Fig. 1. Phylogenetic analysis of *Aspergillus* section *Usti* based on maximum-likelihood analysis of the RPB2 locus; *Aspergillus sparsus* is the outgroup species. Numbers near nodes are bootstrap percentages based on 1000 bootstrap samplings. *A. collinsii* sp. nov. and *A. asper* sp. nov. are presented in bold text and each is distinguished from other species of section *Usti* with high statistical probability. Intraspecific variability can be assessed from some species that are represented by two isolates. Two distinct species, *Aspergillus pseudodefectus* and *Aspergillus calidoustus*, are not different in this tree, although differences exist at other loci. Bar, 10 substitutions per nucleotide position.

Table 1. Provenance of isolates examined and sequenced in this study

NRRL	Provenance
<i>Aspergillus asper</i> 35910 ^T	USA, Pennsylvania, isol ex air sampler, January 2008, <i>Z. Jurjevic</i> ; =Z-345, culture ex type.
<i>Aspergillus baeticus</i> 62501 ^T	Spain, Gruta de las Maravillas cave, isol ex cave sediment, April 2012, <i>Vit Hubka</i> . culture ex type=CCF 4226
62487	Spain, Gruta de las Maravillas cave, isol ex cave sediment, April 2012, <i>Vit Hubka</i> . culture ex type=CCF 4226
<i>Aspergillus calidoustus</i> 26162	USA, Peoria, isol ex NRRL 3361 as a contaminant, September 1968.
<i>Aspergillus cavernicola</i> 6327 ^T	Romania, isol ex cave soil, Caprei grotto, Defileul Nature Reserve, culture ex type
5813	USSR, isol ex wheat starch, culture ex type of <i>A. amylovorous</i>
<i>Aspergillus collinsii</i> 66196 ^T	USA, isol ex air sample, November 2014, <i>Z. Jurjevic</i> , culture ex type
<i>Aspergillus heterothallicus</i> 5097	Costa Rica, Esparta, isol ex forest soil, <i>J. C. Cavender</i>
5096 ^T	Costa Rica, Esparta, isol ex forest soil, <i>J. C. Cavender</i>
<i>Aspergillus deflectus</i> 2206 ^T	Brazil, Rio de Janeiro, isol ex soil, 1949, <i>A. Cury</i> , isolate ex type
4235	USA, Nebraska, isol ex potting soil, 1955, <i>R. W. Embree</i>
<i>Aspergillus egyptiacus</i> 5920 ^T	Egypt, isol ex sandy soil in olive grove, 1974, culture ex type
<i>Aspergillus elongatus</i> 5176 ^T	India, isol ex alkaline soil, 1970
<i>Aspergillus funiculosus</i> 4744 ^T	Nigeria, isol ex soil, 1965
<i>Aspergillus granulosis</i> 1932 ^T	USA, Arkansas, Fayetteville, isol ex soil, isolate ex type
1931	USA, Texas, Greenville, isol ex soil, 1942
<i>Aspergillus insuetus</i> 279 ^T	South Africa, Capetown, isol ex soil, <i>Putterill</i> , isolate ex type
<i>Aspergillus kassunensis</i> 3752 ^T	Syria, isol ex soil, <i>A. Onions</i>
<i>Aspergillus keveii</i> 1974	Panama, isol ex soil, 1942
<i>Aspergillus lucknowensis</i> 3491	India, Lucknow, isol ex soil, 1969, <i>J. N. Rai</i>
<i>Aspergillus pseudodeflectus</i> 6135 ^T	Egypt, isol ex desert soil, 1974.
<i>Aspergillus puniceus</i> 1852	USA, Louisiana, isol ex soil near greenhouse, 1942
5077	Costa Rica, isol ex soil
<i>Aspergillus subsessilis</i> 4905 ^T	USA, California, isol ex Mojave desert soil, 1961, <i>G. F. Orr</i> .
4907	USA, California, isol ex Mojave desert soil, 1961, <i>G. F. Orr</i> .
<i>Aspergillus thesauricus</i> 62485	Spain, Gruta de las Maravillas cave, isol ex cave sediment, April 2012, <i>Vit Hubka</i> .
62484	Spain, Gruta de las Maravillas cave, isol ex cave sediment, April 2012, <i>Vit Hubka</i> .
<i>Aspergillus turkensis</i> 4993 ^T	Turkey, Ankara, isol ex soil, 1950, culture ex type
<i>Aspergillus ustus</i> 275 ^T	USA, Connecticut, isol ex <i>A. sydowii</i> culture, 1914, <i>C. Thom</i> .
4991	Brasil, isol ex soil, 1969, <i>D.I. Fennell</i>

growth, 40 °C no growth/no growth, 43 °C no growth/no growth.

The holotype specimen, BPI-893218, is deposited in The United States National Herbarium, US Department of Agriculture, Beltsville, MD, USA, and was isolated from house air in Pennsylvania, USA. The culture ex type is NRRL 35910^T (=CCF 5174^T =CBS 140842^T). The MycoBank deposit number is 814412.

Morphological comparisons. *Aspergillus asper* morphologically most resembles *A. keveii*, *A. porphyreostipitatus*, *A. pseudodeflectus*, *A. granulatus* and *A. pseudoustus* in terms of rapid growth (30–50 mm diam. 7 days, 25 °C) and colonies appearing greyish or brownish. *A. asper* is distinguished from these species because only *A. porphyreostipitatus*, *A. granulatus* and *A. pseudodeflectus* grow at 37 °C or higher while *A. asper* does not grow at 37 °C. *A. pseudoustus*, *A. keveii* and *A. asper* do not grow at 37 °C, and *A. pseudoustus* and *A. keveii* both have CYA25 colony colours in brownish shades while *A. asper* makes white to grayish-white colonies.

Description of *Aspergillus collinsii* Ž. Jurjević & S. W. Peterson, sp. nov.

Aspergillus collinsii (collins'i.i. N.L. noun in the genitive, honors Dr Ralph P. Collins).

Description of cultures after 7 days (Fig. 3): CYA at 25 °C, conidial heads radiate, grey–green, very good sporulation at the centre of colony approximately 5–7 mm diameter, mycelium yellow to orange or orange–brown with age,

floccose, lightly sulcate, rising approximately 3–4 mm, at margin hyphae subsurface or submerged into medium approximately 2–3 mm, no exudate, soluble pigment when present faint reddish shades, reverse brownish-orange; MEA at 25 °C, conidial heads radiate, buff, with age becoming greyish-olive green at margins, poor to good sporulation, mycelium yellow to buff with orange shades, white at margins, lightly floccose to funiculose, occasionally lightly sulcate, at margin hyphae subsurface or submerged into medium approximately 2–3 mm, no exudate, no soluble pigments, reverse orange–brown; MEA with chloramphenicol at 25 °C, conidial heads orange–yellow to grey–green, sporulation abundant, mycelium yellow–orange, reverse brown; CY20S at 25 °C, conidial heads greenish-gray, poor to good sporulation, mycelium white on surface or submerged in medium, no exudate, no soluble pigments, reverse uncoloured; OM at 25 °C, conidial heads grey–green, buff at margins at early stage, sporulation abundant, mycelium orange, floccose, at margin hyphae subsurface or submerged into medium approximately 3 mm, orange exudate abundant, yellow soluble pigment, reverse brownish-red; PDA at 25 °C, conidial heads buff, very good sporulation, mycelium white to yellowish at margins, orange in the central 7–8 mm, floccose, no exudate or soluble pigment, reverse orange to buff at margins; DG18 at 25 °C, not sporulating, mycelium white, floccose, no exudate or soluble pigments; reverse uncoloured; CYAS at 25 °C, not sporulating, mycelium white, subsurface or submerged into medium, reverse uncoloured; CREA at 25 °C, conidial heads bright green, very good sporulation, mycelium white, subsurface or submerged in medium, no acid production.

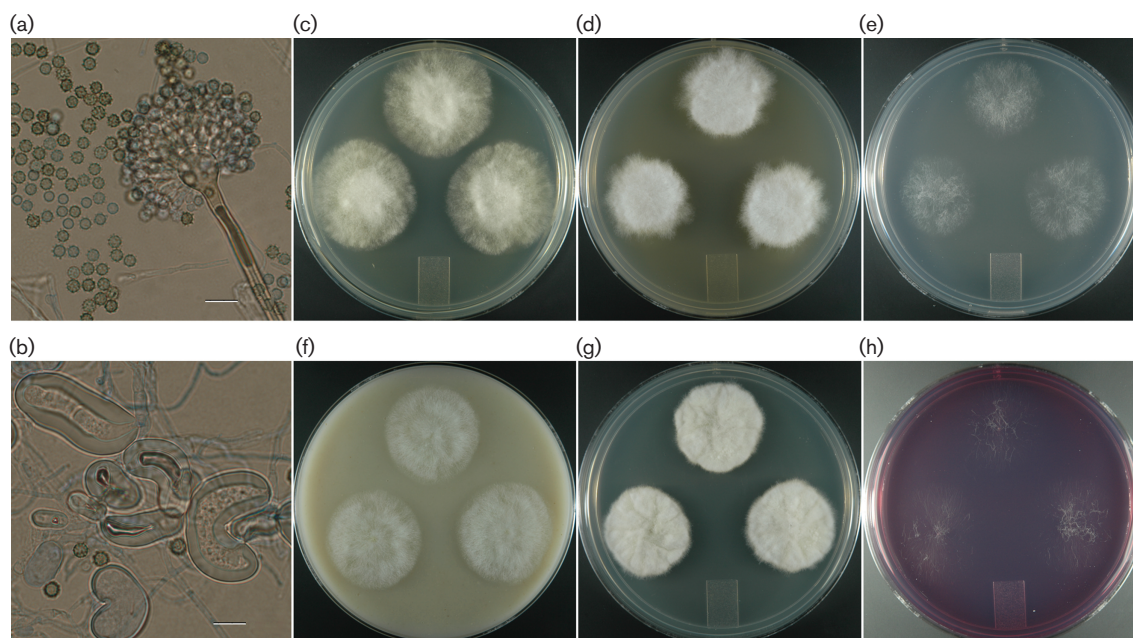


Fig. 2. *Aspergillus asper* NRRL 35910^T. (a) Conidiophore with globose to pyriform vesicle, metulae, phialides and conspicuously roughened conidia, (b) elongated Hülle cells; 7 days of growth at 25 °C on (c) CYA, (d) MEA, (e) CYS20, (f) OA, (g) PDA and (h) CREA. Bar, 10 µm for microphotographs.

Stipes smooth-walled, occasionally finely roughened, rarely septate, hyaline becoming yellowish-orange with age, (45–)75–200(–260) × 3.5–7(–8) μm diameter, occasionally terminating with two vesicles, occasional rope-like structures seen, vesicle pyriform to globose (7–)9–18(–22) μm diam, biseriate, metulae cylindrical (3–)4–6(–7) × 2–4 μm diam, covering ½ to 2/3 of vesicle, phialides ampulliform, (4–)5–7(–8) × 2–2.5(–2.8) μm diameter, conidia globose to subglobose occasionally ellipsoidal, 2–2.5(–3.5) × 2–3 μm diameter, smooth to occasionally finely roughened walls.

Medium-dependent growth characteristics assessed as colony diameter after 7 days of growth at 25 °C on: CYA 14–15 mm; MEA 14–15 mm; CY20S 9–10 mm; CYAS 3–4 mm; DG18 8–9 mm; OA 14–15 mm; PDA 15–16 mm and CREA 13–14 mm. Temperature-dependent growth assessed as colony diameter on CYA and MEA respectively, after 7 days of growth at: 20 °C 8–9 mm/9–10 mm; 30 °C 22–23 mm/23–24 mm; 35 °C 27–28 mm/35–36 mm; 40 °C 14–16 mm/14–15 mm and 43 °C no growth/no growth.

The holotype specimen, BPI 893219, is deposited in The United States National Herbarium, US Department of Agriculture, Beltsville, MD, USA, and was isolated by Ž. Jurjević from an air settle plate exposed in a domestic bathroom, Fair Oaks, California, August 2014. The ex type culture is NRRL 66196^T (=CCF 5175^T =CBS 140843^T). The MycoBank deposit number is MB814413.

Morphological comparisons. Among the species assigned to sect. *Usti* are four species whose colonies on CYA attain 20 mm diameter or less in 7 days at room temperature (22–24 °C) or at 25 °C. Among those four species, pinkish or orange-shaded CYA25 colonies are found in *A. collinsii*,

A. deflectus and *A. turkensis* while *A. lucknowensis* is sulfur-yellow, becoming reddish in age and is thus distinguished from *A. collinsii*. The vesicles of *A. collinsii* are aligned on the same axis with their conidiophores while the *A. deflectus* vesicles are attached to the conidiophores at a very distinctive and noticeable angle to the axis of the conidiophore.

A. collinsii colonies on CREA agar are very thin and composed of yellow hyphae while *A. turkensis* colonies on CREA agar are plane, loose and composed of white hyphae. Neither species makes acid on CREA.

Discussion

In the modern synthesis of species concepts, taxonomists describe species with the fundamental idea that members of species are members of a gene pool distinct from all others (Simpson 1951; de Quiroz, 2005). Other species concepts, e.g. biological species concept, phenetic species concept, or species concepts based on exometabolite profiles, micromorphology, rDNA sequence or genealogical concordance, etc., are not species concepts in the theoretical sense but represent species recognition systems (de Queiroz 2005). Taylor *et al.* (2000) argued for the use of genealogical concordance as the characteristic to determine whether isolates are in the same or different species. We have shown that our novel species are genetically distinct, with statistical support in the bootstrap analysis of *RPB2* DNA sequence data.

Regardless of how one views species concepts, there are several reasons to describe novel species, even when character variation in the species cannot be assessed because the

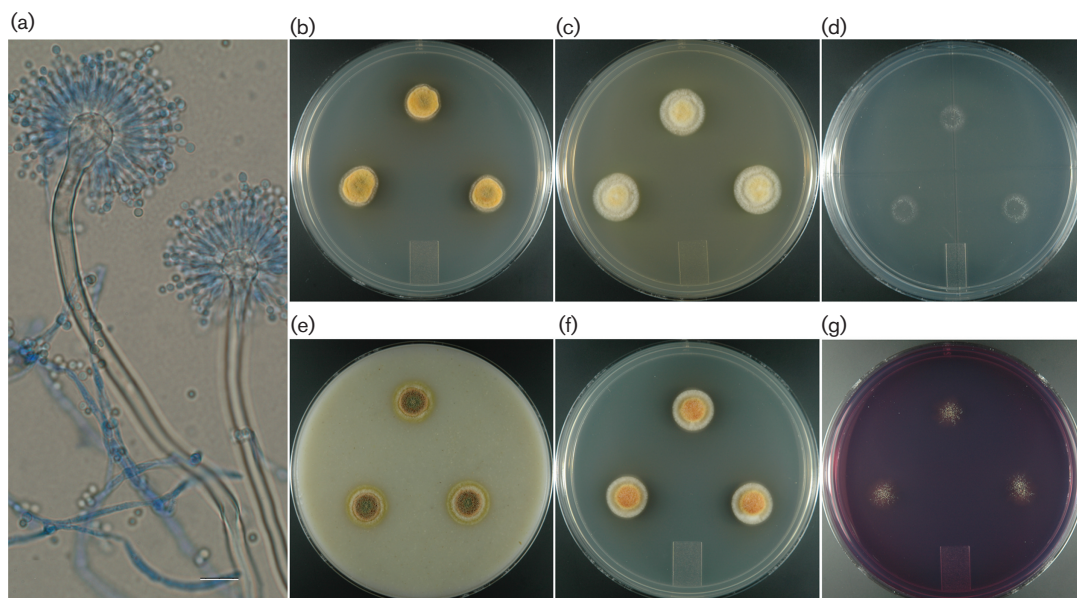


Fig. 3. *Aspergillus collinsii* NRRL 66196^T. (a) Conidiophores with globose to pyriform vesicles, metulae, phialides and smooth to finely roughened spherical conidia; growth for 7 days at 25 °C on (b) CYA, (c) MEA, (d) CYS20, (e) OA, (f) PDA and (g) CREA agar. Bar, 10 μm for microphotograph.

species is currently known only from a single isolate. In determining the phylogeny of a particular group, missing taxa add uncertainty to the analysis, influencing the topology of trees (Rokas & Carroll, 2005). Some taxa are extinct and it may not be possible to include them in analysis, but all extant species should be described and included in analysis to improve our phylogenetic accuracy. Species names are also the reference point for different researchers to accumulate all types of information about the species and organize the specific data sets.

The phylogenetic trees (Figs. 1, S1) show that both *A. asper* and *A. collinsii* are distinct from the other recognized species of sect. *Usti* at the four loci examined with statistical support from the bootstrap. Fig. 1 also reveals representative levels of variation among known sect. *Usti* species having multiple isolates available. Both *A. asper* and *A. collinsii* are on relatively long branches showing that they have no close relatives in the tree. Within the context of Fig. 1, these two species are sufficiently different genetically from other species to conclude that they are distinct. The bootstrap values on the tree provide statistical evidence that the differences did not occur by random chance.

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