***Aspergillus baeticus* sp. nov. and *Aspergillus thesauricus* sp. nov.: two new species in section *Usti* originating from Spanish caves**

Alena Nováková¹, Vit Hubka^{2,3*}, Cesareo Saiz-Jimenez⁴, Miroslav Kolarik^{2,3}

¹ Institute of Soil Biology, Biology Centre AS CR, v.v.i., Na Sádkách 7, 370 05 České Budějovice, Czech Republic, alena@upb.cas.cz

² Laboratory of Fungal Genetics and Metabolism, Institute of Microbiology of the AS CR, v.v.i, Vídeňská 1083, 142 20 Praha 4, Czech Republic,

³ Department of Botany, Faculty of Science, Charles University, Benátská 2, 128 01, Praha 2, Czech Republic

⁴ Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), Consejo Superior de Investigaciones Científicas (CSIC), Av. Reina Mercedes, 10, 41012 Sevilla, Spain

'running title': Two new *Aspergillus* spp. in section *Usti*

contents category: new taxa (eukaryotic micro-organisms)

Abbreviations: CT, Cueva del Tesoro Cave; GM, Gruta de las Maravillas Cave; GSM, Gravity settling method; DPM, Dilution plate method; ISI, in situ isolation; ML, maximum likelihood

***Vit Hubka, Corresponding author**

Department of Botany, Faculty of Science, Charles University in Prague, Czech Republic
Benatska 2, 128 01 Praha 2

Email: vit.hubka@seznam.cz

Fax: (+420) 29644 2347

Phone: (+420) 739663218

The EMBL accession numbers for the ITS, β -tubulin, calmodulin and RNA polymerase II of the type strains of *A. baeticus* sp. nov. and *A. thesauricus* sp. nov. are HE615086, HE615092, HE615117, HE615124 and HE615088, HE615095, HE615120, HE615126, respectively.

The Mycobank (<http://www.mycobank.org>) accession numbers for *A. thesauricus* sp. nov. and *A. baeticus* sp. nov. are MB564187 and MB564188, respectively.

ABSTRACT

Two new species of *Aspergillus* that are clearly distinct from all known species in section *Usti* were revealed during a study of microfungal communities in Spanish caves. The novel species identified in this study and additional species of *Aspergillus* section *Usti* are associated with places and substrates related to human activities in caves. Novel species are described using a polyphasic approach that combines data from four loci (ITS, *benA*, *caM* and *rpb2*), morphology and basic chemical and physiological analyses. *Aspergillus thesaureus* sp. nov. was isolated from various substrates, including the decaying organic matter, cave air and cave sediment of the Cueva del Tesoro Cave (the Treasure cave). The species is represented by twelve isolates and is most closely related to the recently described *A. germanicus*. *Aspergillus baeticus* sp. nov. was isolated from the cave sediment in the Gruta de las Maravillas Cave (the Grotto of the Marvels) and is represented by two isolates. Additional isolate was found in the Cueva del Tesoro Cave and in the Demänovska Peace Cave (Slovakia), suggesting a potentially wide distribution of this microorganism. The species is related to *A. ustus* and *A. pseudoustus*. Both species are unable to grow at 37 °C, and a weakly positive, yellow Ehrlich reaction was observed in *A. thesaureus*. Unique morphological features alone are sufficient to distinguish both species from related taxa.

1 INTRODUCTION

2
3 Caves represent an interesting environment with unique underground communities of
4 organisms. Due to the varied types of speleothems, underground streams and lakes, fossils,
5 paintings and other archaeological elements, caves are very attractive from the perspective of
6 a tourist. Many caves are open to tourists and bring benefits to the local economy, but such
7 commercialisation causes dramatic changes in cave ecosystems, climatic conditions (air
8 temperature and humidity) and cave communities (Pulido-Bosch *et al.*, 1997). The
9 composition of microbial communities can be significantly affected by tourist visits, as
10 specific cave microbiota can be enriched by species transferred from the outside environment,
11 and true cave organisms can be reduced due to microclimate changes. Tourism can result in
12 the enrichment of specific cave biota by species from the outside environment, together with
13 the contamination of the oligotrophic environment by organic materials such as human hairs
14 and textile fibres, which microorganisms can utilise as suitable nutrient sources for their
15 growth. In some cases, microfungus outbreaks have been found in caves visited by tourists
16 (Bastian *et al.*, 2009; Jurado *et al.*, 2010).

17 A study of microfungus communities in selected Andalusian caves focused on microfungus
18 diversity and possible changes of microfungus communities related to tourist visits.
19 Heterogeneous organic material and air in localities affected by human activities
20 (speleologists, tourists) were rich sources of *Aspergillus* isolates which could be classified in
21 *Aspergillus* section *Usti* based on their morphology. Numerous isolates did not fit the
22 description of any known species, suggesting that they have not been previously described in
23 the literature. Other methods used for verifying the status of isolates include PCR-
24 fingerprinting, sequence analysis of the ITS region of rDNA, calmodulin (*cam*), β -tubulin
25 (*benA*) and the second largest subunit of RNA polymerase II (*rpb2*) and basic physiological
26 and chemical analyses. All these strains with unknown species identity represented two
27 species, which are described here as novel using a standard polyphasic approach.

MATERIAL AND METHODS

Description of studied caves. Microscopic fungi were isolated from two Andalusian caves: the Cueva del Tesoro Cave (the Treasure Cave, CT) near Malaga and the Gruta de las Maravillas Cave (the Grotto of the Marvels, GM) in Aracena, Huelva.

The CT, which is a marine cave, has a main gallery of 500 metres and is located 13 km east from Malaga in the hills of El Cantal in El Rincon de la Victoria. The sea formed the caves during the Jurassic Age, and drawings of animals from the Paleolithic Age and ceramics from the Neolithic Age have been discovered inside the cave (Berrocal Pérez & Wallace Moreno, 1988; Giménez Reyna & Laza Palacios, 1964).

The GM cave is located in Aracena. This predominantly horizontal cave with three known levels was developed as a small outcropping of marble from the Lower Cambrian. It was discovered by a shepherd in 1886 and opened to the public in the same year. The total length of the GM is over two kilometres, 1,200 metres of which are open to the public. The cave is particularly well known due to the extraordinary abundance and variety of speleothems, vadose and subaqueous deposits, acicular aragonite and calcite excentrics and underground lakes (Martín-Rosales *et al.*, 1995; Pulido-Bosch *et al.*, 1997).

Isolation techniques. The gravity settling method (GSM) (Buttner & Stetzenbach, 1991) and Dichloran rose bengal chloramphenicol (DRBC) agar (Atlas, 2010) were used for the isolation of airborne microscopic fungi. The dilution plate method (DPM) (Garrett, 1981) and in situ isolation (ISI) on DRBC were used for the isolation of microscopic fungi from cave sediments, various organic materials and from visible microfungial colonies.

Morphology. The strains were grown on Czapek-Dox agar (CZA), Czapek yeast autolysate agar (CYA), malt extract agar (MEA), and yeast extract sucrose agar (YES) and were plated at 25 °C. Agar media formulation was performed according to Frisvad and Samson (2004) and Atlas (2010). Micromorphology was observed and documented on MEA. Colour determination was performed according to the ISCC-NBS Centroid Colour Charts (Foster, J. C., 2004; <http://tx4.us/nbs/nbs-1.htm>).

Physiology and chemical studies. Growth at 37 °C was tested on CYA. The production of cyclopiazonic acid or related alkaloids was tested using the Ehrlich test. The production of

acid compounds into the agar medium was tested on creatine sucrose agar (CREA) following the methods of Samson *et al.* (2007).

Molecular studies. DNA was extracted from seven-day-old colonies using the Microbial DNA Isolation Kit (Mo-Bio Laboratories, Inc.). PCR fingerprinting was performed using the phage M13-core sequence as an oligonucleotide primer (5'- GAGGGTGGCGGTTCT). Amplifications were performed in 18.5 µl volumes, each containing 100 ng of DNA, 25 mM of MgCl₂ (Promega Corp.), 0.2 mM of dNTPs and 1 U of DyNAzyme polymerase (Finnzymes), with the respective buffer. The reaction mixtures were subjected to 32 cycles under the following temperature regime: 94 °C/3 min, 52 °C/1 min, and 65 °C/3 min (1×); 45 °C/40 s, 52 °C/1 min, and 65 °C/3 min (35×) and 94 °C/40 s, 52 °C/1 min, and 65 °C/10 min (1×). The amplified products were subjected to electrophoresis on 1.8 % agarose gels stained with ethidium bromide, and the banding patterns were visualised under ultraviolet light.

For the phylogenetic analysis of the ITS region, partial sequences of *benA* and *caM* were chosen, as they have been used in recent taxonomical monographs (Houbraken *et al.*, 2007; Samson *et al.*, 2011). A partial region of the *rpb2* gene was also amplified, although this fragment was previously amplified only by Peterson (2008) and is not available for some recently described taxa. The Mastercycler Gradient (Eppendorf) was used to amplify the desired regions with the following primer combination: the ITS1-5.8S-ITS2 region of the rDNA using the primers ITS1F (5'- CTTGGTCATTTAGAGGAAGTAA) and NL4 (5'- GGTCCGTGTTTCAAGACGG), the partial *benA* gene using the primers Bt2a (5'- GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (5'- ACCCTCAGTGTAGTGACCCTTGGC), the partial *caM* gene using the primers CMfU (5'- GTYTCYGAGTAYAARGARGCCTT) or CF1L (5'- GCCGACTCTTTGACYGARGAR) and CF4 (5'- TTTYTGATCATRAGYTGGAC) or CMrU (5'- CGGCCRTCRCATCYTGATC) and the partial *rpb2* gene using the primers fRPB2-5F (5'- GAYGAYMGWGATCAYTTYGG) and fRPB2-7cR (5'- CCCATRGCTTGYYTTRCCCAT). The mixture (25 µl) contained 50 ng of genomic DNA, 20 pmol of each primer, 0.2 mM of dNTPs (dNTP master mix, Invitex, Berlin, Germany), and 1 U of DyNAzyme polymerase (Finnzymes) with the respective buffer (Finnzymes, Oy, Finland). The reaction mixtures were subjected to 32 cycles under the following temperature regime: 95 °C/3 min, 55 °C/30 s, and 72 °C/1 min (1×), 95 °C/30 s, 55 °C/30 s, and 72 °C/1 min (30×) and 95 °C/30 s, 55 °C/30 s, and 72 °C/10 min (1×). Sequencing of the purified PCR products was provided by MacroGen Europe, Netherlands. The DNA sequences obtained in this study were deposited in the EMBL

1 database under the accession numbers listed in Table 1. The *CaM* and ITS sequences of type
2 strain *A. calidoustus* CBS 121601, which were previously amplified by Varga *et al.* (2008),
3 were deposited with the permission of authors as HE616559 and HE616558, respectively.

4
5 **Primer design.** The following primers were previously designed for the amplification of the
6 partial *caM* gene in *Aspergillus*: cmd5, cmd6, CF1L, CF1M, and CF4 (Hong *et al.*, 2006;
7 Peterson, 2008). The combination of these primers did not amplify the *caM* gene in *A.*
8 *baeticus* isolates. The CMfU (forward) and CMrU (reverse) primers (for the sequences, see
9 the molecular studies section) were designed in this study based on the sequences of members
10 of section *Usti* deposited by Peterson (2008). This primer pair successfully amplified a *caM*
11 fragment in the *A. baeticus* isolates. The primers were also functional in combination with the
12 previously used primers (see above) and are useful as alternative primers for the amplification
13 of the partial *caM* gene in the *Aspergillus* section *Usti*.

14
15 **Phylogenetic analysis.** The sequences were inspected and assembled using the Bioedit
16 sequence alignment editor v7.0.0 (Hall, 2004). Alignments of the regions were performed
17 using the FFT-NSi strategy, as implemented in MAFFT v6.861b (Katoh *et al.*, 2005). The ITS
18 region (509 sites/134 variable) and the partial *benA* (455 sites/227 variable) and *caM* (485
19 sites/263 variable) sequences were combined. There were a total of 1449 positions in the final
20 dataset. The evolutionary history was inferred with the Maximum Likelihood method (ML)
21 implemented in MEGA5 (Tamura *et al.*, 2011). A discrete gamma distribution was used to
22 model the evolutionary rate differences among the sites. The number of bootstrap replicates
23 was set to 500. *Aspergillus ochraceoroseus* NRRL 28622 was used as an outgroup. The tree
24 with the highest log likelihood (-10158.1031) is shown (Fig. 1). The single locus trees were
25 also constructed and are shown on Supplementary Figures 1-3.

26 The ML tree based on the *rpb2* data with the highest log likelihood (-4894.1928) is shown in
27 Supplementary Fig. 4. A discrete gamma distribution was used to model the evolutionary rate
28 differences among the sites. The rate variation model that allowed for some sites to be
29 evolutionarily invariable was used. There were a total of 902 positions (308 were variable) in
30 the final alignment.

31
32 **Preservation of isolates.** The isolates were deposited into the Collection of Microscopic
33 Fungi of the Institute of Soil Biology at the Academy of Sciences of the Czech Republic in
34 České Budějovice (CMF ISB) and Culture Collection of Fungi in the Department of Botany at

1 Charles University in Prague (CCF) (Table 1). Selected isolates were deposited into the
2 Agricultural Research Service Culture Collection (NRRL) in Peoria, Illinois, USA.
3

RESULTS AND DISCUSSION

Taxonomy and phylogenetic analysis

M-13 fingerprinting of the tested isolates showed four unique patterns (Table 1). Selected isolates that represented different fingerprints were sequenced and compared to type cultures deposited on the GenBank server. The presence of four species was confirmed. The species spectrum comprised *A. insuetus*, *A. cavernicola* and two undescribed species.

The single locus trees of each examined locus were congruent and are shown on Supplementary Figures 1-4. The ITS region was uninformative for many of the species as previously commonly observed across *Aspergillus* spp. (Peterson, 2008), and this region is only recommended for distinguishing the species on the level of section and species complexes.

For all three loci, *A. baeticus* is placed on a branch with *A. ustus* and *A. pseudoustus* as the closest relatives. The creation of the novel species *A. thesaureus* is strongly supported by sequence data from the *benA* and *caM* genes. The ITS sequence of *A. thesaureus* is identical to the ITS sequences of *A. insuetus* and *A. germanicus*.

The genetic differences from the most closely related species for both *A. baeticus* and *A. thesaureus* in the *benA* and *caM* genes range from 2 to 3 %. This value falls within the range of genetic variation between other closely-related *Aspergillus* species in section *Usti*. The data for the *rpb2* gene are incomplete for section *Usti*. The *rpb2* sequence of *A. baeticus* shows 2 % dissimilarity from the most closely related species, *A. ustus*. The *rpb2* sequence difference of *A. thesaureus* is only slightly more than 1 % dissimilar from *A. keveii* NRRL 1954.

The ITS, *benA* and *caM* sequences of *A. insuetus* CCF 4186 were different from those of the type strains NRRL 279 (unknown substrate, South Africa), CCF 48 (industrial material, China, 1955) and CCF 3995 (bronchoalveolar lavage, Czech Republic, 2010) of *A. insuetus* at a number of sites. Also the M-13 fingerprint patterns of the isolates from the caves (CCF 4186, 4224 - type IV, see Table 1) and those of CCF 48 and CCF 3995 (different from type IV) suggest that their genetic lineage could be different from the type strain. Additionally, the morphology of the colonies of both isolates from the caves shows a conspicuous violet-grey colour that has not yet been described for *A. insuetus*. Although the phylogenetic analysis of a combined gene dataset did not show a significantly supported lineage for CCF 4186, suggesting that they likely belong to the variable species *A. insuetus*.

Section *Usti* underwent numerous rearrangements and important taxonomical changes after its establishment (Houbraken *et al.*, 2007; Klich, 1993; Kozakiewicz, 1989; Peterson, 2000; 2008; Peterson *et al.*, 2008; Raper & Fennell, 1965; Samson *et al.*, 2011; Varga *et al.*, 2008). Including the two novel species described here, section *Usti* encompasses 23 species supported by a polyphasic approach of species delineation. The teleomorphic state, a member of the genus *Emericella*, is observed in only two taxa (Samson *et al.*, 2011).

Ecology

As mentioned above, the isolates collected from various substrates in the two studied Andalusian caves comprised four species - *A. insuetus*, *A. cavernicola* and a two distinct taxa proposed as new species. *A. thesauricus* was only found in the CT and was isolated from cave sediment, cave air and was associated with various organic substrates connected to human activities. *A. baeticus* was cultivated from the cave sediment of the GM cave. A morphologically similar isolate was also found in the CT, although later it was not possible to revive it for molecular analysis. An isolate of *A. baeticus* was also revealed among the isolates from the Demänovska Peace Cave (Slovakia), suggesting a wide distribution of this species. This isolate grew on a bat cadaver and is deposited as CCF 4231. Its ITS, *benA* and *caM* sequences showed several changes from the Spanish isolates.

Aspergillus section *Usti* includes widely distributed species commonly isolated from soil, dust, decaying vegetation, food and indoor air (Houbraken *et al.*, 2007; Raper & Fennell, 1965; Samson *et al.*, 2011). Human pathogenicity has been reported for *A. calidoustus*, *A. granulatus* and *A. deflexus* (Fakih *et al.*, 1995; Robinson *et al.*, 2000; Varga *et al.*, 2008). Members of section *Usti* were also repeatedly reported from cave environments (Hermosín *et al.*, 2010; Jurado *et al.*, 2010; Nováková, 2009). Among these species, *A. cavernicola*, *A. ustus*, *A. carlsbadensis* and probably also *A. pseudoustus* (based only on ITS sequence) were identified using sequence data (Samson *et al.*, 2011; Vaughan *et al.*, 2011). Studies related to cave microbiota (Adetutu *et al.*, 2011; Northup & Lavoie, 2001; Nováková, 2009; Rutherford & Huang, 1994; Vaughan *et al.*, 2011) have shown that each cave has a specific microbial community, including microscopic fungi.

Morphology and other characteristics

The micro-morphological features of *A. baeticus* show similarity to *A. ustus*. Differences can be found in the morphology of the conidiophores. The vesicles of *A. baeticus* are typically elongated, while those of *A. ustus* are hemispherical to subglobose. Additionally, the width of

1 the stipes are larger in *A. baeticus* than in *A. ustus*. A macro-morphology of the colonies
2 reveals further significant differences. Hairy brown or olive-grey colony colour of *A. ustus* on
3 MEA is in contrast with the pale yellow colonies of *A. baeticus*. Moreover, the growth of *A.*
4 *ustus* on MEA and YES medium is faster than that of *A. baeticus* (Samson *et al.*, 2011). The
5 conidia of another two related species, *A. pseudoustus* and *A. puniceus*, are considerably
6 smaller than those of *A. baeticus*. Their sizes do not coincide with *A. baeticus* and can be used
7 as a reliable feature for distinguishing these species. The vesicle shapes, growth rates and
8 colony colours on MEA, CYA and YES show further important differences. Hülle cells were
9 not observed in *A. pseudoustus* in contrast with *A. baeticus*, which constantly create yellow
10 masses of Hülle cells.

11 The proposed species, *A. thesauristicus*, shows a number of unique features compared to
12 related taxa. *A. germanicus*, a sibling species, is able to grow at 37 °C (Samson *et al.*, 2011),
13 which is in contrast to *A. thesauristicus*. The conidiophores of *A. thesauristicus* have narrower
14 stipes, and their vesicle diameter is larger. In addition, *A. thesauristicus* shows faster growth on
15 all the tested media. *A. keveii* has much smaller conidia and grows faster on all media,
16 including CREA. The violet Ehrlich reaction in *A. keveii* and *A. insuetus* differs from the
17 weakly positive yellowish reaction in *A. thesauristicus*. Further, *A. insuetus* differs from *A.*
18 *thesauristicus* by greater vesicles diametres, faster growth on MEA and slower growth on YES.

TAXONOMY

Latin diagnosis of *Aspergillus baeticus* A. Nováková & Hubka, sp. nov.

Coloniae flavo-brunneae, margo sulcatus, albus usque gilvus. Cellulae obtegentibae („Hülle“), hyalinae, globosae (12.5–17.0 µm), ovalae usque elongatae, distortae, (26.0–)67.0–81.0(–105.0) × 10.5–20.0 µm. Stipites leves, brunnei, (150–)220–290(–315) µm longi, 5.0–6.0(–8.0) µm lati. Vesiculae late ellipticae usque elongatae, 9.8–15.4 × 16.8–19.6 µm. Metulae praesentes, 4.1–5.2 µm; phialides 7.0–8.4 µm. Conidia globosa conspicue ornamentata, echinulata vel verrucosa, 3.5–4.2 µm. Typus CMF ISB 2153 (= CCF 4226 = NRRL 62501), MB564187.

Description of *Aspergillus baeticus* A. Nováková & Hubka, sp. nov. (Fig. 2)

Aspergillus baeticus (adj. *baeticus* -a -um relating to the Roman province Baetis, Andalusia, the place of isolation).

CYA, 1 wk, 25 °C: 35–38 mm, sporulation heavy in central area, velutinous with a floccose central part, margin wrinkled, yellowish white (ISCC-NBS No. 92), central part brownish pink (No. 33) to light greyish reddish brown (No. 46) (strain No. CMF 5153) to light greyish brown (No. 60) (strain No. CMF 5154), exudate as large droplets coloured light yellow (No. 86) to strong orange yellow (No. 68), later vivid orange yellow (No. 66) to deep orange yellow (No. 69), reverse pale yellow (No. 89), vivid yellow (No. 82) in the centre, strong orange yellow (No. 68) after 10 days, no production of diffusible pigment, Hülle cells, CYA, 1 wk, 37 °C: no growth; MEA, 1 wk, 25 °C: 28–33 mm, sporulation heavy, floccose with umbonate central part (8 mm in diameter), sporulation in pale yellow (No. 89) to greyish greenish yellow (No. 105), no exudate, no diffusible pigmentation, reverse vivid yellow (No. 82); YES, 1 wk, 25 °C: 30–35 mm, floccose in central part, velutinous wrinkled margin, sporulation in light yellow (No. 86) to pale orange yellow (No. 73) to light yellowish brown (No. 76), rare brilliant yellow (No. 83) exudate, no pigmentation, reverse light yellow (No. 86) to strong orange yellow (No. 68) in the centre; CZA, 1 wk, 25 °C: 30–34 mm, no sporulation, no pigmentation, a production of light greenish yellow (No. 101) pigment and reverse after 10 days of cultivation; CREA, 1 wk, 25 °C: good growth (25–27 mm),

sporulation brownish pink (No. 33) to light greyish reddish brown (No. 42), no acid production; Ehrlich reaction: negative.

Colonies yellow brown, Hülle cells on CYA, thick-walled, globose (12.5–17.0 µm), irregularly ovoid to predominantly elongate, twisted, (26.0–)67.0–81.0(–105.0) × 10.5–20.0 µm, usually scattered. Conidiophore stipes smooth, (150–)220–290(–315) µm long, 5.0–6.0(–8.0) µm wide, light brown with darker brown colour in the upper part, conidial heads radiate to short columnar, vesicle broadly elliptical to elongate, 9.8–15.4 × 16.8–19.6 µm in diameter, metulae 4.1–5.2 µm, phialides 7.0–8.4 µm, conidia distinctly ornamented with warts or spines, globose to subglobose, 3.5–4.2 µm.

Type culture CMF ISB 2153 (= CCF 4226 = NRRL 62501), MB564187, isolated from cave sediment of Gruta de las Maravillas cave, Spain, Alena Nováková, 2010.

Additional isolates examined: see Table 1.

Latin diagnosis of *Aspergillus thesauricus* Hubka & A. Nováková, sp. nov.

Coloniae flavoviridae usque griseo flavoviridae, floccosae. Cellulae obtegentibae („Hülle“), hyalinae, globosae (14.0–18.2 µm), pleuries elongatae, directae pleuries distortae, (18.2–21.0–)42.0–57.6(–92.4)µm. Stipites leves, brunnei, 135–180(–220) µm longi, 5.6–7,0(–8.4) µm lati. Vesiculae subglobosae, (9.8–)11.2–16.8 µm diam. Metulae praesentes, 4.2–5.6(–7.0) µm; phialides 4.2–5.6 µm. Conidia conspicue ornamentata, verrucosa vel spinulosa, subglobosa, 4.2–5.6(–6.3) µm. Typus CMF ISB 2155 (= CCF 4166 = NRRL 62485), MB564188.

Description of *Aspergillus thesauricus* Hubka & A. Nováková, sp. nov. (Fig. 3)

Aspergillus thesauricus (adj. *thesauricus* -a -um referring to the origin of the strains, which were isolated in the "Treasure cave", Cueva del Tesoro in Spain).

CYA, 1 wk, 25 °C: 27–30 mm, floccose, good sporulation, medium grey with 2 mm white margin, no exudate, no pigmentation, reverse pale greenish yellow (No. 104) with light greenish yellow (No. 101) in centre, dark greenish yellow (No. 103) after 10 days, CYA, 1 wk, 37 °C: no growth; MEA, 1 wk, 25 °C: 20–25 mm, floccose, good sporulation, light olive grey (No.112) to olive grey (No. 113) in central part, with 2 mm white margin, no exudate

1 and pigmentation, reverse moderate yellow (No. 87) with vivid greenish yellow (No. 90) in
2 centre light olive brown (No. 94); YES, 1 wk, 25 °C: 30–35 mm, floccose in centre, with
3 wrinkled 4 mm white margin, greyish yellowish brown (No. 80) to light greyish brown (No.
4 79), no exudate and pigmentation, reverse dark yellow (No. 88), dark greyish yellow (No. 91)
5 in centre; CZA, 1 wk, 25 °C: 25–30 mm, no sporulation and pigmentation; CREA, 1 wk, 25
6 °C: 18 mm, weak growth and no acid production; Ehrlich reaction: weakly positive, light
7 greenish yellow with some pinkish tint in strain CCF 4154.

8 Colonies grey to greyish green, floccose. Hülle cells rarely observed in some isolates, in
9 others they are abundant, hyaline, thick-walled, globose (14.0–18.2 µm) to predominately
10 elongate, straight to twisted, (18.2–21.0–)42.0–57.6(–92.4) µm. Conidiophore stipes smooth-
11 walled, brown, 135–180(–220) µm long, 5.6–7.0(–8.4) µm wide, vesicle subglobose to
12 globose, (9.8–)11.2–16.8 µm in diameter, metulae 4.2–5.6(–7.0) µm, phialides 4.2–5.6 µm.
13 Conidia verrucose to spinulose, subglobose, 4.2–5.6(–6.3) µm.

14
15 Type culture CMF ISB 2155 (= CCF 4166 = NRRL 62485), MB564188, isolated from indoor
16 air of Cueva del Tesoro, Spain, Alena Nováková, 2010.

17 Additional isolates examined: see Table 1.

ACKNOWLEDGEMENTS

This study was supported by the Research Plan of the ISB BC AS CR, v.v.i., presented results were obtained within bilateral projects between AS CR and CSIC “Fungal diversity in Spanish caves with rock art paintings” and “Early detection of fungal outbreaks in Andalusian caves”. Molecular genetics analyses were supported by projects MSM 6007665801, AV0Z50200510 and GAUK 607812.

1 REFERENCES

- 2
- 3 **Adetutu, E. M., Thorpe, K., Bourne, S., Cao, X., Shahsavari, E., Kirby, G. & Ball, A. S.**
- 4 **(2011).** Phylogenetic diversity of fungal communities in areas accessible and not
- 5 accessible to tourists in Naracoorte Caves. *Mycologia* **103**, 959-968.
- 6 **Atlas, R. M. (2010).** *Handbook of microbiological media*. Boca Raton: CRC Press.
- 7 **Bastian, F., Alabouvette, C. & Saiz-Jimenez, C. (2009).** The impact of arthropods on fungal
- 8 community structure in Lascaux cave. *J Appl Microbiol* **106**, 1456-1462.
- 9 **Berrocal Pérez, J. A. & Wallace Moreno, L. (1988).** *Guía de las cuevas de Málaga*.
- 10 Málaga: Diputación Provincial de Málaga.
- 11 **Buttner, M. P. & Stetzenbach, L. D. (1991).** Evaluation of four aerobiological sampling
- 12 methods for the retrieval of aerosolized *Pseudomonas syringae*. *Appl Environ*
- 13 *Microbiol* **57**, 1268-1270.
- 14 **Fakih, M. G., Barden, G. E., Oakes, C. A. & Berenson, C. S. (1995).** First reported case of
- 15 *Aspergillus granulosis* infection in a cardiac transplant patient. *J Clin Microbiol* **33**,
- 16 471-473.
- 17 **Frisvad, J. C. & Samson, R. A. (2004).** Polyphasic taxonomy of *Penicillium* subgenus
- 18 *Penicillium*: A guide to identification of food and air-borne terverticillate *Penicillia*
- 19 and their mycotoxins. *Stud Mycol* **49**, 1-173.
- 20 **Garrett, S. D. (1981).** *Soil fungi and soil fertility: an introduction to soil mycology*. Oxford:
- 21 Pergamon Press.
- 22 **Giménez Reyna, S. & Laza Palacios, M. (1964).** *Informe de las excavaciones en la Cueva*
- 23 *del Higuerón o del Suizo*. Madrid: Informe de las Excavaciones en la Cueva del
- 24 Higuerón o Suizo.
- 25 **Hall, T. (2004).** *Bioedit 7.0.0*. Raleigh: North Carolina State University.
- 26 **Hermosín, B., Novakova, A., Jurado, V., Laiz Trobajo, L., Porca, E., Rogerio Candelera,**
- 27 **M. A., Sánchez-Moral, S. & Sáiz-Jiménez, C. (2010).** Observatorio microbiológico
- 28 de cuevas: evaluación y control de comunidades fúngicas en cuevas sometidas al
- 29 impacto de actividades turísticas. In *Cuevas: Patrimonio, Naturaleza, Cultura y*
- 30 *Turismo*, pp. 513-520. Edited by J. J. Durán & F. Carrasco Madrid: Asociación de
- 31 Cuevas Turísticas Españolas.
- 32 **Hong, S. B., Cho, H. S., Shin, H. D., Frisvad, J. C. & Samson, R. A. (2006).** Novel
- 33 *Neosartorya* species isolated from soil in Korea. *Int J Syst Evol Microbiol* **56**, 477-
- 34 486.
- 35 **Houbraken, J., Due, M., Varga, J., Meijer, M., Frisvad, J. C. & Samson, R. A. (2007).**
- 36 Polyphasic taxonomy of *Aspergillus* section *Usti*. *Stud Mycol* **59**, 107-128.
- 37 **Jurado, V., Porca, E., Cuezva, S., Fernandez-Cortes, A., Sanchez-Moral, S. & Saiz-**
- 38 **Jimenez, C. (2010).** Fungal outbreak in a show cave. *Sci Total Environ* **408**, 3632-
- 39 3638.
- 40 **Katoh, K., Kuma, K., Toh, H. & Miyata, T. (2005).** MAFFT version 5: improvement in
- 41 accuracy of multiple sequence alignment. *Nucleic Acids Res* **33**, 511-518.
- 42 **Klich, M. A. (1993).** Morphological studies of *Aspergillus* section *Versicolores* and related
- 43 species. *Mycologia*, 100-107.
- 44 **Kozakiewicz, Z. (1989).** *Aspergillus* species on stored products. *Mycol Pap* **161**, 1-188.
- 45 **Martín-Rosales, W., López-Chicano, M., Rodríguez, C. M. & Pulido-Bosch, A. (1995).**
- 46 Morfología, espeleotemas y climatología de la Gruta de las Maravillas, Aracena
- 47 (Huelva). *Espeleotemas* **5**, 1-12.
- 48 **Northup, D. E. & Lavoie, K. H. (2001).** Geomicrobiology of caves: a review. *Geomicrobiol*
- 49 *J* **18**, 199-222.

- 1 **Nováková, A. (2009).** Microscopic fungi isolated from the Domica Cave system (Slovak
- 2 Karst National Park, Slovakia). A review. *Int J Speleol* **38**, 71-82.
- 3 **Peterson, S. W. (2000).** Phylogenetic relationships in *Aspergillus* based on rDNA sequence
- 4 analysis. In *Integration of modern taxonomic methods for Penicillium and Aspergillus*
- 5 *classification*. Edited by R. A. Samson & J. I. Pitt. Amsterdam: Harwood Academic
- 6 Publishers.
- 7 **Peterson, S. W. (2008).** Phylogenetic analysis of *Aspergillus* species using DNA sequences
- 8 from four loci. *Mycologia* **100**, 205-226.
- 9 **Peterson, S. W., Varga, J., Frisvad, J. C. & Samson, R. A. (2008).** Phylogeny and
- 10 subgeneric taxonomy of *Aspergillus*. In *Aspergillus in the genomic era*. Edited by J.
- 11 Varga & R. A. Samson. Wageningen: Wageningen Academic Publishers.
- 12 **Pulido-Bosch, A., Martin-Rosales, W., López-Chicano, M., Rodriguez-Navarro, C. M. &**
- 13 **Vallejos, A. (1997).** Human impact in a tourist karstic cave (Aracena, Spain). *Environ*
- 14 *Geol* **31**, 142-149.
- 15 **Raper, K. B. & Fennell, D. I. (1965).** *The genus Aspergillus*. Baltimore: Williams & Wilkins
- 16 Co.
- 17 **Robinson, W. F., Connole, M. D., King, T. J., Pitt, J. I. & Moss, S. M. (2000).** Systemic
- 18 mycosis due to *Aspergillus deflexus* in a dog. *Aust Vet J* **78**, 600-602.
- 19 **Rutherford, J. M. & Huang, L. H. (1994).** A study of fungi of remote sediments in West
- 20 Virginia caves and a comparison with reported species in the literature. *J Cave Karst*
- 21 *Stud* **56**, 38-45.
- 22 **Samson, R. A., Noonim, P., Meijer, M., Houbroken, J., Frisvad, J. C. & Varga, J. (2007).**
- 23 Diagnostic tools to identify black aspergilli. *Stud Mycol* **59**, 129-145.
- 24 **Samson, R. A., Varga, J., Meijer, M. & Frisvad, J. C. (2011).** New taxa in *Aspergillus*
- 25 section *Usti*. *Stud Mycol* **69**, 81-97.
- 26 **Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011).**
- 27 MEGA5: molecular evolutionary genetics analysis using maximum likelihood,
- 28 evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*
- 29 **doi:10.1093/molbev/msr121**.
- 30 **Varga, J., Houbroken, J., Van Der Lee, H. A. L., Verweij, P. E. & Samson, R. A. (2008).**
- 31 *Aspergillus calidoustus* sp. nov., causative agent of human infections previously
- 32 assigned to *Aspergillus ustus*. *Eukaryot Cell* **7**, 630-638.
- 33 **Vaughan, M. J., Maier, R. M. & Pryor, B. M. (2011).** Fungal communities on speleothem
- 34 surfaces in Kartchner Caverns, Arizona, USA. *Int J Speleol* **40**, 65-77.
- 35

Table 1. Isolates of *Aspergillus* belonging to section *Usti* that were isolated from caves.

species name	collections accession no.	substrate	cave [*]	isolation technique [†]	M13 [‡]	EMBL accession numbers			
						ITS	<i>benA</i>	<i>caM</i>	<i>rpb2</i>
<i>A. baeticus</i>	CMF ISB 2153 ^T = CCF 4226 ^T = NRRL 62501	cave sediment	GM	PDM	I	HE615086	HE615092	HE615117	HE615124
	CMF ISB 2154 = CCF 4150 = NRRL 62487	cave sediment	GM	PDM	I	HE615087	HE615093	HE615118	HE615125
	CDT-SMC5	cave sediment	CT	PDM	-				
	CMF 2181 = CCF 4231	bat cadaver	DPC	ISI	I	HE617298	HE615094	HE615119	
<i>A. thesauroicus</i>	CMF ISB 2155 ^T = CCF 4166 ^T = NRRL 62485	indoor air	CT	GSM	II	HE615088	HE615095	HE615120	HE615126
	CMF ISB 2156 = CCF 4152 = NRRL 62484; CCF 4154	microfungal growth	CT	ISI	II	HE615089	HE615096	HE615121	HE615127
	CCF 4225; CCF 4151; CCF 4185	indoor air	CT	GSM	II				
	CMF ISB 2165 = CCF 4187; CCF 4188	cave sediment	CT	PDM	II				
	CCF 4167; CCF 4155; CCF 4153	organic matter	CT	ISI	II				
	CCF 4189	candle residue	CT	ISI	II				
<i>A. cavernicola</i>	CCF 4165	cave wall	GM	PDM	III	HE615090	HE615098	HE615123	
<i>A. insuetus</i>	CCF 4186 = NRRL 62498	organic matter	CT	PDM	IV	HE615091	HE615097	HE615122	
	CCF 4224 = NRRL 62499	indoor air	CT	GSM	IV				

^{*}GM – Gruta de las Maravillas, CT – Cueva del Tesoro, DPC – Demänovská Peace Cave; [†]PDM – plate dilution method, GSM – gravity settling method, ISI – direct plating; [‡]M13-core fingerprinting pattern

Legend to Figures:

Table 1. Isolates of *Aspergillus* belonging to section *Usti* that were isolated in the caves.

Fig. 1. The maximum likelihood tree with the highest log likelihood score based on a combined data set of ITS, partial *benA* and *caM* gene sequences. Only bootstrap values >70 % are shown; branches supporting a bootstrap value greater than 95 % are thickened. The names of the isolates from the caves where the sequences were obtained for this study are printed in bold.

Fig. 2. *Aspergillus baeticus*: a) 7-days-old colonies on CYA, b) on MEA, c) on YES, d) on CREA; e) reverse of 10-days-old colonies on CYA; f) conidia, g) and h) conidiophores; i) Hülle cells. Bars, 10 µm.

Fig. 3. *Aspergillus thesaureus*: a) 7-days-old colonies on CYA, b) on MEA, c) on YES, d) on CREA; e) reverse of 10-days-old colonies on CYA; f) conidia; g) and h) conidiophores; i) Hülle cells. Bars, 10 µm.

Supplementary Fig. 1. The ML tree with the highest log likelihood score based on the ITS region sequences; 509 sites, 134 variable. Only bootstrap values >70 % are shown. The names of the isolates from the caves where the sequences were obtained for this study are printed in bold.

Supplementary Fig. 2. The ML tree with the highest log likelihood score based on the partial *benA* gene sequences; 455 sites, 227 variable. Only bootstrap values >70 % are shown. The names of the isolates from the caves where the sequences were obtained for this study are printed in bold.

Supplementary Fig. 3. The ML tree with the highest log likelihood score based on the partial *caM* gene sequences; 485 sites, 263 variable. Only bootstrap values >70 % are shown. The names of the isolates from the caves where the sequences were obtained for this study are printed in bold.

Supplementary Fig. 4. The ML tree with the highest log likelihood score based on the partial *rpb2* gene sequences; 902 sites, 308 variable. Only bootstrap values >70 % are shown; branches supported by a bootstrap value greater than 95 % are thickened. The names of the isolates from the caves where the sequences were obtained for this study are printed in bold.

Supplementary Table 1. The GenBank/EMBL accession numbers of the sequences used in this study for phylogenetic tree construction.

0.02

Supplementary Tab. 1 The GenBank/EMBL/DDBJ accession numbers of sequences used in this study for phylogenetic trees construction

Species name	Isolate number	<i>ITS</i>	<i>benA</i>	<i>caM</i>	<i>rpb2</i>
<i>A. baeticus</i>	CMF ISB 2154=CCF 4150=NRRL 62487	HE615087	HE615093	HE615118	HE615125
	CMF ISB 2153 ^T =CCF 4226 ^T =NRRL 62501	HE615086	HE615092	HE615117	HE615124
	CMF 2181	HE617298	HE615094	HE615119	
<i>A. calidoustus</i>	CBS 114380	EF591741	EF591729	EF591716	
	CBS 121601 ^T	HE616558	FJ624456	HE616559	
	NRRL 26162	EF652452	EF652276	EF652364	EF652188
<i>A. californicus</i>	IBT 16748 ^T	FJ531153	FJ531180	FJ531128	
<i>A. carlsbadensis</i>	IBT 14493 ^T	FJ531151	FJ531179	FJ531126	
	IBT 16753	FJ531152	FJ531178	FJ531125	
	IBT 18616	FJ531148	FJ531169	FJ531124	
<i>A. cavernicola</i>	NRRL 5813	EF652503	EF652327	EF652415	EF652239
	NRRL 6327 ^T	EF652508	EF652332	EF652420	EF652244
	CCF 4165	HE615090	HE615098	HE615123	
<i>A. deflectus</i>	NRRL 2206 ^T	EF652437	EF652261	EF652349	EF652173
<i>A. egyptiacus</i>	NRRL 5920 ^T	EF652504	EF652328	EF652416	EF652240
<i>A. elongatus</i>	NRRL 5176 ^T	EF652502	EF652326	EF652414	EF652238
<i>A. germanicus</i>	IBT 29365 ^T	FJ531146	FJ531172	FJ531141	
<i>A. granulosus</i>	NRRL 1932 ^T	EF591737	EF591734	EF591721	EF652166
<i>A. heterothallicus</i>	NRRL 5096 ^T	EF652499	EF652323	EF652411	EF652235
<i>A. insuetus</i>	NRRL 279 ^T	EU076356	EF652281	EU076366	EF652193
	NRRL 4876	EF652481	EF652305	EF652393	EF652217
	CCF 4186	HE615091	HE615097	HE615122	
<i>A. kassunensis</i>	CCF 3995	FR733861	FR851857	FR751455	
	CCF 48	FR733856	FR775315	FR751454	
	NRRL 3752 ^T	EF652461	EF652285	EF652373	EF652197
<i>A. keveii</i>	NRRL 1974	EF652432	EF652256	EF652344	EF652168
	CBS 209.92 ^T	EU076354	EU076376	EU076365	
	CBS 113227	EU076353	EU076373	EU076363	
<i>A. lucknowensis</i>	NRRL 3491 ^T	EF652459	EF652283	EF652371	EF652195
<i>A. monodii</i>	CBS 434.93	FJ531149	FJ531170	FJ531143	
	CBS 435.93 ^T	FJ531150	FJ531171	FJ531142	
<i>A. pseudodeflectus</i>	NRRL 278	EF652456	EF591732	EF652368	EF652192

<i>A. pseudoustus</i>	NRRL 6135 ^T	EF652507	EF652331	EF652419	EF652243
<i>A. puniceus</i>	IBT 28161 ^T	FJ531147	FJ531168	FJ531129	
<i>A. puniceus</i>	NRRL 5077 ^T	EF652498	EF652322	EF652410	EF652234
<i>A. subsessilis</i>	NRRL 4688	EF652469	EF652293	EF652381	EF652205
<i>A. thesauricus</i>	NRRL 4905 ^T	EF652485	EF652309	EF652397	EF652221
	CMF ISB 2155 ^T =CCF 4166 ^T =NRRL 62485	HE615088	HE615095	HE615120	HE615126
	CMF ISB 2156=CCF 4152=NRRL 62484	HE615089	HE615096	HE615121	HE615127
<i>A. turkensis</i>	NRRL 4993 ^T	EF652494	EF652318	EF652406	EF652230
<i>A. ustus</i>	NRRL 275 ^T	EF652455	EF652279	EF652367	EF652191
	CBS 113950	EU076359	EF591728	EF591720	
<i>A. ochraceoroseus</i> (outgroup)	NRRL 28622 ^T	EF661224	EF661113	EF661137	EF661074

0.05