Leiothecium cristatum sp. nov. and Aspergillus posadasensis sp. nov., two species of Eurotiales from rainforest soils in South America

Yasmina Marin-Felix, José Francisco Cano-Lira, Josep Guarro and Alberto Miguel Stchigel

Mycology Unit, Medical School and IISPV, Universitat Rovira i Virgili, C/ Sant Llorenc 21, 43201 Reus, Tarragona, Spain

We describe two novel fungi isolated from soil samples collected in Northern Argentina and belonging to the family Aspergillaceae of the order Eurotiales: Leiothecium cristatum sp. nov. and Aspergillus posadasensis sp. nov. Leiothecium cristatum sp. nov., represented by the ex-type strain FMR 11998T (=CBS 134260T=NBRC 109843T), is distinguishable morphologically from the type species of the genus, Leiothecium ellipsoideum, by the presence of irregular reticulate ascospores with two prominent equatorial crests, and Aspergillus posadasensis sp. nov., represented by the ex-type strain FMR 12168T (=CBS 134259T=NBRC 109845T), is differentiated from Aspergillus acanthosporus, the nearest species phylogenetically, by its non-sclerotoid ascocarps and a lack of an asexual stage on all culture media tested. The taxonomic proposals are supported by the analysis of the sequences of the internal transcribed spacer region, the D1–D2 domains of the 28S rRNA gene, the fragments of the RNA polymerase II largest subunit, and the putative chaperonin complex related to TCP-1, β-tubulin and calmodulin genes.

Introduction

Members of the order Eurotiales G.W. Martin ex Benny & Kimbrough (1980) are mainly characterized by the production of spherical to ovoid, thin-walled evanescent (prototunicate) asci, which arise free on the mycelium or are, more usually, produced within globose, nonostiolate ascomata, and by one-celled, globose or lenticular, smooth-walled or ornamented ascospores (spinulose, reticulate, tuberculate, etc.), frequently with equatorial thickenings or crests. Their asexual stages are mostly phialidic, but can also show a retrogressive conidiogenesis. At the time of writing, the order comprises three monophyletic families, Aspergillaceae, Thermoasacaceae and Trichocomaceae (Houbraken & Samson, 2011).

The genus Aspergillus is the most common and largest of the family Aspergillaceae and of the order Eurotiales. Gams et al. (1985) divided the genus into six subgenera and 18 sections. However, Peterson (2008), using a multigene phylogeny based on sequences of partial fragments of β-tubulin (B2), calmodulin (CAL) and RNA polymerase II (rpb2) genes, and ribosomal [internal transcribed spacer (ITS) and large subunit (LSU)] genes, only accepted five subgenera (Aspergillus, Circumdati, Fumigati, Nidulantes and Ornati). Most recently, Houbraken & Samson (2011) also used the sequences of rpb2 and other structural genes [RPB1, the putative ribosome biogenesis protein (Tsr1) and the putative chaperonin complex component TCP-1 (Cct8)], and concluded that most of the morphospecies traditionally belonging to the genus Aspergillus were included in the Aspergillus s. str. clade, which was divided into four subgenera and 17 sections. The genus Cristaspora has a single species that lacks an anamorph stage (Fort & Guarro, 1984); the genus Phialosimplex has conidiogenous cells consisting of simple phialides, sometimes proliferating to form a second opening (Sigler et al., 2010); and the genus Polypaecilum has

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit; ML, maximum-likelihood; SEM, scanning electron microscope.

The GenBank/EMBL/DDBJ accession numbers for the D1–D2, ITS, Cct8, RPB1 and RPB2 loci sequences of the ex-type strain of Leiothecium cristatum sp. nov. are HG529487, KF732836, HF954979, HF954982, and HF954976, respectively. The GenBank/EMBL/DDBJ accession numbers for the D1–D2, ITS, Cct8, RPB1, RPB2, CAL and BT2 loci sequences of the ex-type strain of Aspergillus posadasensis sp. nov. are HG529485, HG529483, HF954980, HF954983, HF954977, HG529488 and HG529481, respectively, and those of Aspergillus posadasensis sp. nov. FMR 12322, are HG529486, HG529484, HF954981, HF954984, HF954978, HG529489, and HG529482, respectively. The GenBank/EMBL/DDBJ accession number for the D1–D2 locus sequence of the ex-type strain of Leiothecium ellipsoideum is KF732839.

The MycoBank (http://www.mycobank.org) accession numbers of Leiothecium cristatum and Aspergillus posadasensis are MB803513 and MB803514, respectively.

A supplementary table and a supplementary figure are available with the online version of this paper.
conidiogenous cells that are polyphialides (Smith, 1961). All three of these genera are morphologically very dissimilar to the typical Aspergillus and were surprisingly also placed in the mentioned Aspergillus s. str. clade (Houbraken & Samson, 2011).

During a survey on soil-borne ascomycetes from Northern Argentina, two fungi apparently related to some members of the order Eurotiales were isolated in pure culture. These fungi were phenotypically and molecularly characterized and are proposed here as novel species.

### Methods

#### Soil sampling and fungal isolation.

Soil samples were collected in Misiones Province, Argentina, at two locations: the Iguazu National Park (−25° 41′ 28.5″, −54° 26′ 54.9594″) and the Alberto Roth botanical garden (−27° 24′ 28.6092″, −55° 53′ 48.1158″). Both locations are included in the Paranaean phytogeographical province of the Amazonian domain at the neotropical region. They have a hot, wet climate with an average annual temperature of 21 °C, an average maximum temperature of about 32 °C and an average minimum temperature of about 10 °C. The total annual rainfall is about 1900 mm. The Iguazu National Park is situated in the boundaries of the Iguazu River, and has an area of around 550 km². The soil is acidic, red and lateritic. The park has more than 300 species of plants, including trees, ferns, shrubs, lianas, epiphytes and herbs. The Alberto Roth botanical garden is on the south side of the city of Posadas, and has an area of 11 ha. The altitude ranges from 75 to 100 m, and the terrain is mostly basaltic. This location also has a broad diversity of trees, shrubs and herbs, of which 109 are native species.

To carry out the isolation of the soil-borne ascomycetes, we followed a previously described protocol (Schigel et al., 2001). Approximately 1 g of each soil sample was suspended in 5 ml of 5% (v/v) acetic acid, shaken vigorously for 5 min and left for 5 min. The liquid layer was decanted and the residual soil was resuspended in 9 ml sterile water and plated onto three Petri dishes of 9 cm diameter. Melted potato carrot agar (PCA: grated potatoes, 20 g; grated carrot, 20 g; agar-agar, 20 g; l-chloramphenicol, 100 mg; 1% (w/v) dieldrin in dimethyl-ketone, 20 drops; tap water, 1 l) at 50–55 °C was placed on top of the soil suspension and mixed by hand. All cultures were incubated at 15, 25 and 35 °C. The ascomata of the taxonomically interesting fungi were transferred using a sterile needle to two 5 cm-diameter Petri dishes containing oatmeal agar (OA; oatmeal flakes, 30 g; agar-agar, 20 g; tap water, 1 l) and incubated under the same conditions as described above.

### Phenotypic study.

For cultural characterization, the isolates were grown for up to 30 days on OA, PCA, potato dextrose agar (PDA; Pronadisa), Czapek’s yeast extract agar (CYA: sucrose, 30 g; sodium nitrate, 3 g; yeast extract, 5 g; potassium phosphate, 1 g; potassium chloride, 0.5 g; magnesium sulphate, 0.5 g; iron sulphate, 0.01 g; agar, 15 g; tap water, 1 l) and malt extract agar (MEA: bacteriological peptone, 1 g; glucose, 20 g; malt extract, 20 g; agar, 15 g; tap water, 1 l) at 25 °C. Colour notations in parentheses are from Kornerup & Wanscher (1984). To induce the production of asexual reproductive structures, the isolates were grown on MEA plus 40% sucrose (Samson et al., 2007) at 25 and 37 °C. In order to determine the minimum and maximum temperatures of growth of the isolates, a 5 °C increment from 5 to 40 °C, and 2 °C increment from 40 to 50 °C, were used. Fertile fungal structures were mounted and measured in water and in lactic acid. Photomicrographs of the structures were taken with a Zeiss Axio Imager M1 light microscope. The scanning electron microscope (SEM) techniques used were described previously by Figueras & Guarro (1988). SEM micrographs were taken with a JEOL JSM 840 at 15 kV.

### BLAST search and phylogenetic study.

The DNA of the isolates of interest (see Table S1, available in the online Supplementary Material) was extracted and purified directly from fungal colonies according to the Fast DNA kit protocol (MP Biomedicals). D1–D2, ITS, RPB1, RPB2 and Cct8 genes were amplified for all isolates, and BT2 and CAL genes were also amplified for isolates FMR 12168T and FMR 12322, according to Cano et al. (2004) (D1–D2 and ITS), Houbraken & Samson (2011) (RPB1, RPB2 and Cct8), Glass & Donaldson (1995) (BT2) and Hong et al. (2005) (CAL). The sequences of these amplicons were obtained using the protocol of the Taq Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). PCR products were purified and sequenced at Macrogen Europe with a 3730XL DNA analyser (Applied Biosystems). Consensus sequences were obtained using SeqMan (version 7.0.0; DNAStar) and they were aligned using CLUSTAL X (version 1.83) (Thompson et al., 1997) followed by manual adjustments with a text editor. Sequences retrieved from the GenBank database and included in this analysis are also given in Table S1. ITS, D1–D2 and CAL BLAST searches were carried out in order to corroborate the previous taxonomical placement of our isolates. The phylogenetic analyses of the combined dataset (RPB1, RPB2 and Cct8) of our isolates and selected members of the families Aspergillaceae and Trichocomaceae were carried out using MEGA software version 5.05 (Tamura et al., 2011). The combined dataset was tested for incongruence with the partition homogeneity test (PHT) as implemented in PAUP+ (Swofford, 2002). The maximum-likelihood (ML) method using the Tamura–Nei model with gamma distribution, was carried out for the phylogenetic analyses of RPB1, RPB2 and Cct8, and Kimura’s two-parameter model with invariable sites for the ML phylogenetic analysis of BT2 sequences, both with the pairwise deletion of gaps option. The robustness of branches was assessed by bootstrap analysis with 1000 replicates. The sequences generated in this study (see Table S1) were deposited in the GenBank database and the alignments used in the phylogenetic analyses were deposited in TreeBASE: (www.treebase.org, accession URL:http://purl.org/phylo/treebase/phylows/study/TB2:S15962).

### Results

#### Phenotypic study.

The isolate FMR 11998T, from a soil sample of the Iguazu National Park (Table S1), was identified as belonging to the genus Leiothecium based on the presence of typical morphological features, such as spherical, glabrous, dark brown, non-ostiolate ascomata with a peridium of textura angularis; one-celled, hyaline, ellipsoidal, reticulate ascospores; and absence of an asexual stage. Two other isolates, FMR 12168T and FMR 12322, from two soil samples of the Alberto Roth botanical garden were classified as belonging to the genus Cristaspora. They were characterized by the production of orange, spherical, non-ostiolate ascomata covered by a dense mass of aerial hyphae; hyaline to subhyaline ascospores with two equatorial crests and a convex surface verruculose to echinulate and the absence of an asexual stage on all culture media tested.

#### BLAST search.

The BLAST search with the D1–D2 sequence of isolate FMR 11998T (GenBank accession no. HG529487) showed 97% similarity with the sequence of the type strain of...
Leiothecium ellipsoides (FJ358285) whereas isolates FMR 12168<sup>T</sup> (HG529485) and FMR 12322 (HG529486) showed 99 % similarity with Aspergillus clavatus (JN938924) and the type strain of Aspergillus acanthosporus (EF669992). The most related member of the order Eurotiales in the ITS BLAST search of isolate FMR 11998<sup>T</sup> (KF732838) showed a sequence similarity of less than 90 % (Aspergillus fischerianus), but the similarity between the sequence of the former with that of the type strain of L. ellipsoides, sequenced in this study (KF732839), was 92.76 %. The BLAST search of ITS sequences of isolates FMR 12168<sup>T</sup> (HG529483) and FMR 12322 (HG529484) showed 98.19 % and 98.43 % similarity, respectively, with the type strain of A. clavatus, and the same sequence similarity (98.42 %) for the two isolates with the ITS sequence of the type strain of A. acanthosporus (EF669992). The BLAST search with the CAL sequences of isolates FMR 12168<sup>T</sup> (HG529488) and FMR 12322 (HG529489) showed 93 % and 93.2 % similarity, respectively, with the type strain of A. clavatus (EU078665), and 90.87 % for both strains with the type strain of A. acanthosporus (EU078676).

Phylogenetic study

The lengths of the fragments of the three genes used in the combined dataset were 646 bp (Cct8), 695 bp (RPB1) and 887 bp (RPB2), from which 220, 250 and 311 bp were parsimony informative, respectively. The length of the final alignment was 2228 bp. The result of the partition homogeneity test showed that the datasets for the three main clades of the tree (93 % bootstrap support) other species of this section were located, i.e. Aspergillus rhizopodus, Aspergillus clavatonicus, Aspergillus longivesica and Aspergillus giganteus.

TAXONOMY

The previous data demonstrated that isolate FMR 11998<sup>T</sup> belongs to the genus Leiothecium but is distinguishable molecularly from the only species of this genus L. ellipsoides, and also morphologically mainly by the presence of irregular reticulate ascospores with two prominent equatorial crests in our isolate. Our studies also provide evidence that isolates FMR 12168<sup>T</sup> and FMR 12322 are molecularly and morphologically different from A. acanthosporus and A. clavatus, the nearest phylogenetic species, by the production of non-sclerotoid ascocoma and the absence of an anamorphic stage in our isolates. Therefore, we propose the following novel species: Leiothecium cristatum sp. nov. and Aspergillus posadasensis sp. nov.

Description of Leiothecium cristatum Y. Marin, Stchigel & Cano sp. nov. (Fig. 2)

Leiothecium cristatum (crista’tum. L. neut. adj. cristatum referring to the equatorial crests of the ascospores).

Colonies on PDA attaining a diameter of 71–73 mm after 7 days at 25 °C, cottony, white, margins fringed; reverse yellowish-white to pale yellow (M. 3A2 to 3A3). Hyphae thick- and smooth-walled, hyaline to pale brown, septate, 3–9 μm wide. Ascomata initials arising on aerial and submerged hyphae as lateral branches, consisting of single coils. Ascomata superficial and immersed on the medium, spherical, glabrous, dark brown, non-ostiolate, 100–220 μm diameter; peridium brown, three-layered, 15–20 μm thick, textura angularis, composed of polyhedral flattened cells of 10–20 μm diameter. Ascii eight-spored, broadly clavate to spherical, non-catenulate, 12–16 × 10–14 μm, evanescent. Ascospores one-celled, hyaline, elliptoidal, 6.8 × 4.5–5.5 μm, irregularly reticulate due to the anastomosing low ridges, with two prominent crests of 0.5–1 μm. Chlamydoospores mostly terminal, sometimes intercalary, hyaline, subspherical to ellipsoid, smooth and thick-walled, 12–19 × 13–18.5 μm. Anamorph not observed. Colonies on MEA are similar to those on PDA. After 7 days at 25 °C, colonies on OA and PCA of 34–36 and 61–64 mm diameter, respectively. Minimum and maximum growth temperatures are 15 and 35 °C, respectively.

Holotype is CBS-H 21130, a dried culture; isotype FMR 11998<sup>T</sup>.

Mycobank accession no. MB803513.

The ex-type culture is FMR 11998<sup>T</sup> (=CBS 134260<sup>T</sup> = NBRC 109843<sup>T</sup>), isolated from a rainforest soil sample, in Iguazú National Park, Misiones province, Argentina (−25° 41' 28.5" −54° 26' 54.9594", 2 August 1997, M. Calduch, J. Guarro and A. M. Stchigel.)
Description of Aspergillus posadasensis Y. Marin, Stchigel & Cano sp. nov. (Fig. 3)

Aspergillus posadasensis (po.sa.das.en’s.is. N.L. masc. adj. posadasensis belonging to Posadas, capital city of the Misiones province, Argentina).

Colonies on PDA attaining 52–58 mm in diameter after 14 days at 25 °C, velvety, white, irregularly folded and with fringed margins; reverse yellowish-white to pale yellow (M. 3A2 to 3A3). Ascomata superficial, spherical, tomentose, orange to brown at maturity, non-ostiolate, 330–720 µm in diameter; peridium 20–30 µm thick, composed of an outer layer of orange–brown moniliform hyphae, and three to five inner layers of flattened, prismatic, brown cells 6–12 µm wide; convex surface of ascospores ornamented with triangular projections, long ridge lines and microtubercles. Anamorph not observed in any of the culture media tested, including MEA + 40 % sucrose. Colonies on PCA attaining a diameter of 52–58 mm after 14 days at 25 °C, velvety to cottony, with fringed margins, white; reverse white to yellowish-white (M. 2A2). Colonies on MEA attaining 18–20 mm in diameter after 14 days at 25 °C, velvety, white, with orange–grey to brownish-grey (M. 5B2 to 5C2) margins, fimbriate; reverse brownish-orange to yellowish-brown (M. 5C4 to 5E4), white to yellowish-white (M. 4A1 to 4A2) at the margins; ascomata produced. Colonies on CYA attaining 16–20 mm in diameter after 14 days at 25 °C, flattened, mycelium mostly submerged, yellowish-white (M. 2A2); reverse yellowish-white (M. 2A2); ascomata not formed. Minimum and maximum growth temperatures are 15 and 42 °C, respectively.

Holotype is CBS-H 21131, a dried culture; isotype FMR 12168T.

Mycobank accession no. MB803514.

The ex-type culture is FMR 12168T (=CBS 134259T = NBRC 109845T), isolated from a soil sample in Alberto Roth botanical garden, Misiones province, Argentina (−27° 24′ 28.6092″ −55° 53′ 48.1158″) 2 August 1997, M. Caldúch, J. Guarro and A.M. Stchigel.

Other specimen examined: FMR 12322 (from the same origin and source).

Discussion

The genus Leiothecium was erected by Samson & Mouchacca (1975) to include an ascomycete isolated from soil in Greece. Later, this fungus was also reported from soil in South America, Asia and Europe, and from seeds of the capsicum and nest material of a ground-nesting solitary bee in North America, in areas of temperate climate. This fungus shows some similarities with Ascorhiza and Hapsideospora (Samson & Mouchacca, 1975) because of the presence of cleistothecial ascomata and reticulate ascospores. They also mentioned the possible relationship of Leiothecium with Monascus, but they remarked on the
differences among them (ascomata with a very thin, plec-
tenchymatous peridial wall in Monascus vs prosenchymatous
and thickness in Leiothecium; smooth-walled ascospores
in Monascus vs reticulate in Leiothecium; and the presence
of an anamorph with retrogressive ontogeny in Monascus,
which is absent in Leiothecium). Despite Hapsidospora and
Leiothecium producing dark-coloured, closed ascomata,
Leiothecium can be differentiated morphologically from
Hapsidospora because the latter produces dark, globose ascospores of 5–7.5 μm diameter (Guarro et al., 2012), which are
hyaline and ellipsoidal, of 7–8.5 × 4.5–5.5 μm in Leiothecium.

Ascorhiza lacks of original type material, and has a poor
description (Lechtova-Trnka, 1931) lacking of any illustra-
tions, therefore it cannot be compared with Leiothecium, and
its validity as a taxon is doubtful.

A recent phylogenetic study carried out by Houbraken &
Samson (2011), based on the nucleotidic sequences of Cct8,
RPB1, RPB2 and Tsr1 genes demonstrated that the genus
Leiothecium belongs to the family Aspergillaceae, while in
a previous molecular study, based on the analysis of SSU
and LSU rRNA gene sequences (Suh & Blackwell,
1999), Hapsidospora had been placed in the Hypocreales.

**Fig. 2.** Morphology of Leiothecium cristatum sp. nov. FMR 11998T. (a), (b) Ascoma; (c) detail of the peridium; (d) asci and
terminal chlamydospores; (e), (f) ascus; (g) ascospores (SEM). The fungus was grown on PDA at 25°C during two weeks.
Bars, 50 μm (a); 25 μm (b); 20 μm (c); 10 μm (d, e); 5 μm (f, g).
Our molecular analysis, using three of those genes, demonstrates that the isolate FMR 11998⁷ represents a novel species of Leiothecium. This fungus is morphologically distinguishable from L. ellipsoideum by the presence of two prominent equatorial crests (absent in L. ellipsoideum) and an irregular pattern in its ascospore wall ornamentation (which is more regularly reticulate in L. ellipsoideum).

The molecular study of the isolates FMR 12168⁷ and FMR 12322 shows that they are related to A. acanthosporus and A. clavatus. The type strain of A. acanthosporus was isolated from a soil sample in the Solomon Islands, Papua-New Guinea (Udagawa & Takada, 1971), along with another three isolates from the same source of the same country. Houbraken & Samson (2011) placed A. acanthosporus into the section Clavati of Aspergillus subg. Fumigati. Aspergillus posadasensis is easily distinguishable from A. acanthosporus by the non-sclerotioid nature of its ascomata and the absence of an anamorph. Other taxa which are morphologically similar to the novel species and belong to Aspergillus subgenus Fumigati are Aspergillus aureola and Aspergillus spinosus. They also produce ascospores with two equatorial crests and a similar ornamentation to that of A. posadasensis; however, their ascomata are white or very pale yellow, and both produce an anamorph. There are other members of the genus Aspergillus of which no conidiophore structures have been described. Conidiophore structures in Aspergillus monodii, which is accommodated in Aspergillus section Usti, are also not known. However, A. monodii has different ascospores and produces Hülle cells and ascomata in stromata.

Fig. 3. Morphology of Aspergillus posadasensis sp. nov. FMR 12168⁷. (a) Ascoma; (b) detail of the peridium; (c, d) asci; (e, f) ascospores. The fungus was grown on PDA at 25°C during two weeks. Bars, 100 μm (a); 20 μm (b); 10 μm (c, d); 5 μm (e); 2.5 μm (f).
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References


