**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, Thermoascales 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 (*BT2*), 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 *Orrnati*). Most recently, Houbraken & Samson (2011) also used the sequences of *RPB2* and other structural genes (*RPBI*, 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...
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 Paranaense 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 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, 20 g; gratted carrot, 20 g; agar-agar, 20 g; l- chlorlamphenicol, 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+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 keV.

### 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 12168\(^T\) 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/phylo/treebase study/TB2:S15962).

### Results

#### Phenotypic study

The isolate FMR 11998\(^T\), 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 12168\(^T\) 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 11998\(^T\) (GenBank accession no. HG529487) showed 97% similarity with the sequence of the type strain of

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Leiothecium ellipsoides (FJ358285) whereas isolates FMR 12168\textsuperscript{T} (HG529485) and FMR 12322 (HG529486) showed 99\% similarity with Aspergillus clavatus (JN938924) and the type strain of Aspergillus acanthosphorus (EF669992). The most related member of the order Eurotiales in the ITS BLAST search of isolate FMR 11998\textsuperscript{T} (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\textsuperscript{T} (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. acanthosphorus (EF669992). The BLAST search with the CAL sequences of isolates FMR 12168\textsuperscript{T} (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. acanthosphorus (EU078676).

**Phylogenetic study**

The lengths of the fragments of the three genes used in the combined dataset were 646 bp (Ct8), 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 loci were congruent (P=0.29) and could be combined.

Fig. 1 shows the tree inferred from a ML analysis of the combined dataset. A main clade with a bootstrap support of 100\% grouped the members of the family Aspergillaceae, including the novel isolates. Isolate FMR 11998\textsuperscript{T} grouped in a terminal clade with the type strain of L. ellipsoides (89\% bootstrap support) whereas the isolates FMR 12168\textsuperscript{T} and FMR 12322 grouped with the type strains of A. acanthosphorus and A. clavatus (100\% bootstrap support), despite these two isolates initially being morphologically identified as belonging to the genus Cristaspora.

A phylogenetic analysis of the ITS region (415 bp), CAL (367 bp) and BT2 (381 bp) was carried out in order to assess the genetic relatedness of the isolates FMR 12168\textsuperscript{T} and FMR 12322 with other members of the sect. Clavati of the genus Aspergillus. The ITS and CAL ML trees showed the same topology that was observed in the BT2 ML tree. We only included results of the last locus (Fig. S1) because BT2 was the most phylogenetically informative, and sequences of all species of this section were available in the GenBank database. The tree revealed two main clades (with bootstrap support of 89\% and 93\%, respectively). The first one encompassed three sister clades, all of them with 100\% bootstrap support, corresponding to four isolates of A. clavatus for the first sister clade, two novel isolates (FMR 12168\textsuperscript{T} and 12322) for the second, and four strains of A. acanthosphorus for the third sister clade. In the second main clade of the tree (93\% bootstrap support) other species of this section were located, i.e. Aspergillus rhizophodus, Aspergillus clavatoricus, Aspergillus longivesica and Aspergillus giganteus.

**TAXONOMY**

The previous data demonstrated that isolate FMR 11998\textsuperscript{T} 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\textsuperscript{T} and FMR 12322 are molecularly and morphologically different from A. acanthosphorus and A. clavatus, the nearest phylogenetic species, by the production of non-sclerotioid ascomata 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 (cris.ta’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, ellipsoidal, 6–8.5×4.5–5.5 μm, irregularly reticulated due to the anastomosing low ridges, with two prominent crests of 0.5–1 μm. Chlamydoconidia mostly terminal, sometimes intercalary, hyaline, subserpheral to ellipsoidal, 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; isolate FMR 11998\textsuperscript{T}.

Mycobank accession no. MB803513.

The ex-type culture is FMR 11998\textsuperscript{T} (=CBS 134260\textsuperscript{T}=NBRC 109843\textsuperscript{T}), 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 (pos.adas.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 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 in diameter. Asci eight-spored, globose to subglobose, 9–12.5 × 8.5–10 μm, evanescent at maturity. Ascospores one-celled, hyaline to subhyaline, globose to subglobose, 3.5–4.5 × 3–4 μm, with two equatorial crests, 0.5–1 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. Calduch, 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 Hapsidospora (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 asco-
spores 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 11998\textsuperscript{T}. (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 11998T 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 12168T 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 12168T. (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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