
# SOIL ASCOMYCETES FROM DIFFERENT GEOGRAPHICAL REGIONS.

**Yasmina Marín Félix**

**Dipòsit Legal: T 996-2015**

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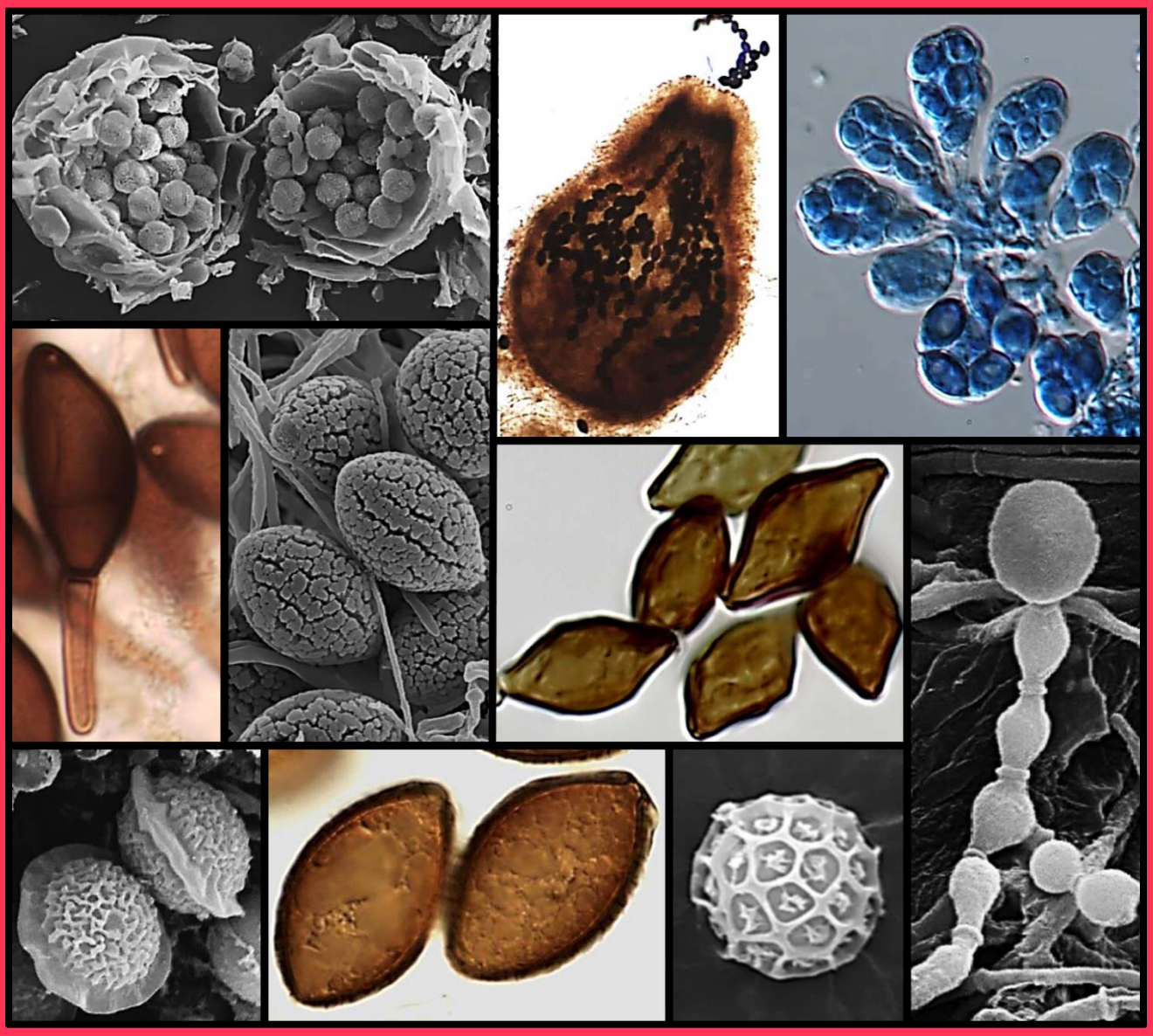

**Yasmina Marín Félix**

**Doctoral Thesis**

**2015**

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**Doctoral Thesis**

**Reus, 2015**

**Directed by Drs. José Francisco Cano Lira and Alberto Miguel Stchigel Glikman**

UNIVERSITAT ROVIRA I VIRGILI

Facultad de Medicina i Ciències de la Salut

Departament de Ciències Mèdiques Bàsiques

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José Francisco Cano Lira and Alberto Miguel Stchigel Glikman, Professors of the department of Basic Health Sciences of the Universitat Rovira i Virgili.

STATE THAT:

The present study, entitled “Soil ascomycetes from different geographical regions”, presented by Yasmina Marín Félix for the award of the degree of Doctor, has been carried out under our supervision at the Department of Basic Health Sciences of this university and and that it fulfills the requirements to obtain the International Doctorate mention.

Reus, March 3, 2015

Dr. José Francisco Cano Lira

Dr. Alberto Miguel Stchigel Glikman

UNIVERSITAT ROVIRA I VIRGILI
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**A mis padres**

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# INDEX

Page

# 1. INTRODUCTION

# 1. INTRODUCTION

## 1.1. ASCOMYCETES

Ascomycota is the largest phylum of fungi with around 65,000 species, which represents approximately 65% of all Fungi described to date (Hawksworth et al., 1995; Kirk et al., 2008). Members of the phylum Ascomycota are characterized by the endogenous formation of the sexual spores (ascospores) inside a sac-like structure called an ascus (Gr. *askos* = sac). The ascus usually contains 2N haploid ascospores, which is the result of karyogamy to form a diploid nucleus, followed by meiosis and one or more mitotic divisions (Tsuneda, 1983; Ingold and Hudson, 1993; Carlile et al., 2001).

### 1.1.1. Vegetative organization

The basic structure of ascomycetes, like the other members of the kingdom Fungi, is a typical eukaryotic cell surrounded by a thick wall composed of glycoproteins and polysaccharides, mainly chitin and beta-glucan. The composition of the cell wall may depend on growth conditions and the stage of development (Bowman and Free, 2006). Some fungal cell walls have melanin cross-linked to polysaccharides – pigments of high molecular weight formed by the oxidative polymerization of phenolic compounds – which provides defense against ultraviolet (UV) light, oxidizing agents and ionizing radiation, occasionally found in the environment. This polymer contributes to the virulence of a large number of pathogenic fungi since it protects the fungal cell against the immune response of hosts such as oxidant substances produced by immune effector cells, and immunomodulatory substances (Jacobson, 2000; Hamilton and Gomez, 2002; Eisenman and Casadevall, 2012).

Ascomycetes can consist of a single cell (unicellular thallus or yeast) or be part of tubular filaments called hyphae (multicellular thallus or filamentous fungi), which are divided by transverse septa. The nuclei, cytoplasm and mitochondria can move from one hyphal compartment to a contiguous one through a central pore in these septa. There is significant structural diversity among ascomycetes, especially in structures with occluded pores. These plugs include non-membranous and membranous materials, such as granular lamellate structures and Woronin bodies, respectively (Alexopoulos et al., 1996; Mouriño-Pérez, 2013).

### 1.1.2. Reproduction

Fungi reproduce by producing mitospores (asexual reproduction) or meiospores (sexual reproduction). Mitospores propagate a progeny that is genetically identical to the parental cell, whereas meiospores are usually the result of outcrossing and generate descendants with genes rearranged into new genotypes (Seifert and Samuels, 2000). In ascomycetes, the mitospores are called conidia, and the meiospores, ascospores. The sexual and asexual stages of a fungus are called teleomorph and anamorph, respectively (Fig. 1). Should a fungus be able to develop two or more anamorphs, these are called synanamorphs. Holomorph is the term used to denote all manifestations of the same genotype: that is, to designate the fungus as a whole, including all its phases and morphologies (the teleomorph and its anamorph or synanamorphs) (Kendrick, 1992; Kirk et al., 2008). The teleomorph and the anamorph may or may not coincide in time or space. For this reason, they are usually collected separately and, in some cases, are treated as different organisms.

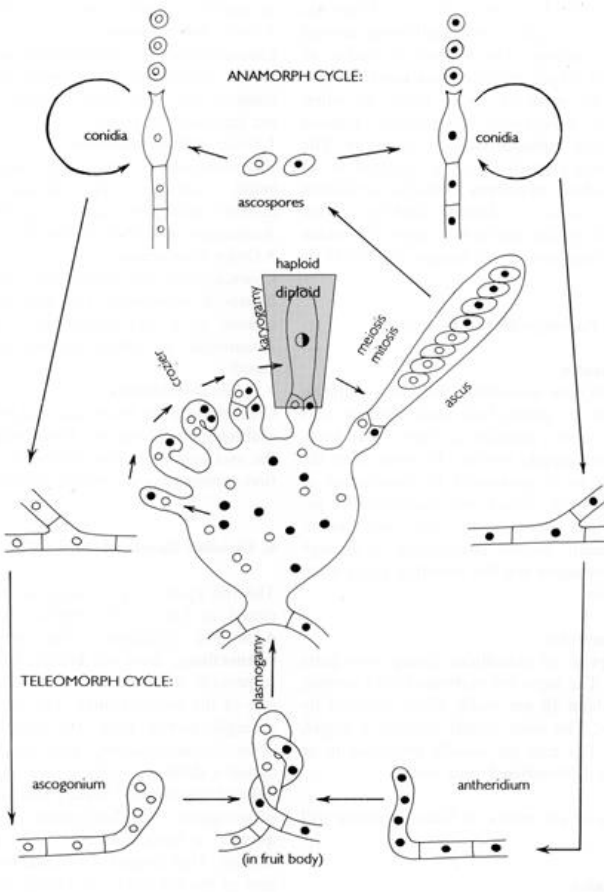



**Fig. 1** Life cycle of the ascomycetes. The lower part shows the sexual stage (teleomorph), while the upper part shows the asexual stage (anamorph). The diploid phase of the life cycle is shaded in gray (de Hoog et al., 2000).

Sexual reproduction plays a key role in guaranteeing genetic diversity in fungal species. It takes place by the fusion of sexually compatible nuclei and the subsequent production of recombinant spores. In ascomycetes and some basidiomycetes, the locus involved in sexual reproduction is known as mating type (*mat*), which presents two alternative forms or idiomorphs. Compatible haploid strains are distinguished only by their mating type, because they are morphologically indistinguishable (Bistis, 1998). The names of the idiomorphs in heterothallic ascomycetes depend on the taxonomic group to which the fungus belongs and can even be specific to some species that were the models on which our understanding of the genetics of these genes was based. The idiomorphs of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were designated α and a, and *h-* (*matM*) and *h+* (*matP*), respectively. The idiomorphs in euascomycetes are referred to as *mat A* and *mat a*, *mat -* and *mat +* or *MAT1-1* and *MAT1-2* (Coppin et al., 1997; Glass and Staben, 1997; Perkins, 1999; Turgeon and Yoder, 2000).

Unlike ascomycetes, the mating system in basidiomycetes involves multiple mating types. Some species can have up to four different types, so the sexual reproduction of these organisms is more complex (Kothe, 2001). In ascomycetes a mating system with multiple mating types has only been reported in *Glomerella cingulata* (Cisar and TeBeest, 1999).

There are three different mechanisms in the sexual reproduction of fungi: heterothallism, homothallism or pseudohomothallism. The strains of the heterothallic species (e.g. *Cochliobolus heterostrophus, Neurospora crassa* and *Gibberella fujikuroi*) have only one of these idiomorphs (*mat-A* or *mat-a*) and sexual spores form only between strains of opposite mating types. Homothallic species do not present a genetically definable mating type and the individuals are self-fertile. Therefore, in these organisms sexual reproduction does not require the interaction of two individuals. Pseudohomothallic species (e.g. *Neurospora tetrasperma* and *Podospora anserina*) develop some spores with two nuclei, one with each idiomorph, so they are capable of completing the sexual cycle themselves. However, other spores only contain a single nucleus (*mat-A* or *mat-a*), requiring another individual with the complementary mating type to complete the sexual cycle (Glass et al., 1990; Alexopoulos et al., 1996.; Pöggeler, 2001).

In mycelial ascomycetes, cytoplasmic fusion (plasmogamy) is followed by a short stage called dikaryophase (in which two genetically different, sexually compatible nuclei coexist in the same segment of hypha). Dikaryotic hyphae are usually protected inside an ascoma, which is formed from the growth and differentiation of haploid vegetative hyphae. Subsequently, nuclear fusion (karyogamy) and meiosis occur and produce

meiospores, which result in new mycelia that can propagate by mitospores and repeat the cycle (de Hoog et al., 2000).

One example of heterothallic reproduction occurs in *Neurospora crassa* (Fig. 2). Haploid ascospores presenting one of the two idiomorphs (*mat A* or *mat a*) germinate to produce a multinucleated mycelium. Both strains, *mat A* and *mat a*, differentiate female sexual organs, the ascogonia, which are surrounded by vegetative hyphae in the form of protoperithecia. Through receptive hyphae (trichogynes), the ascogonia are fertilized by spermatia (spores of the opposite sex) that are also produced by both strains, leading to the formation of the perithecia and initiating the dikaryophase. During this phase the asci are formed inside the perithecia. Nuclear fusion takes place inside the asci between the stem cells of opposing MATs. The meiosis and a postmeiotic division generate 2N haploid (usually 8) ascospores, half of which have *mat A* and the other half *mat a* (Pöggeler, 2001).

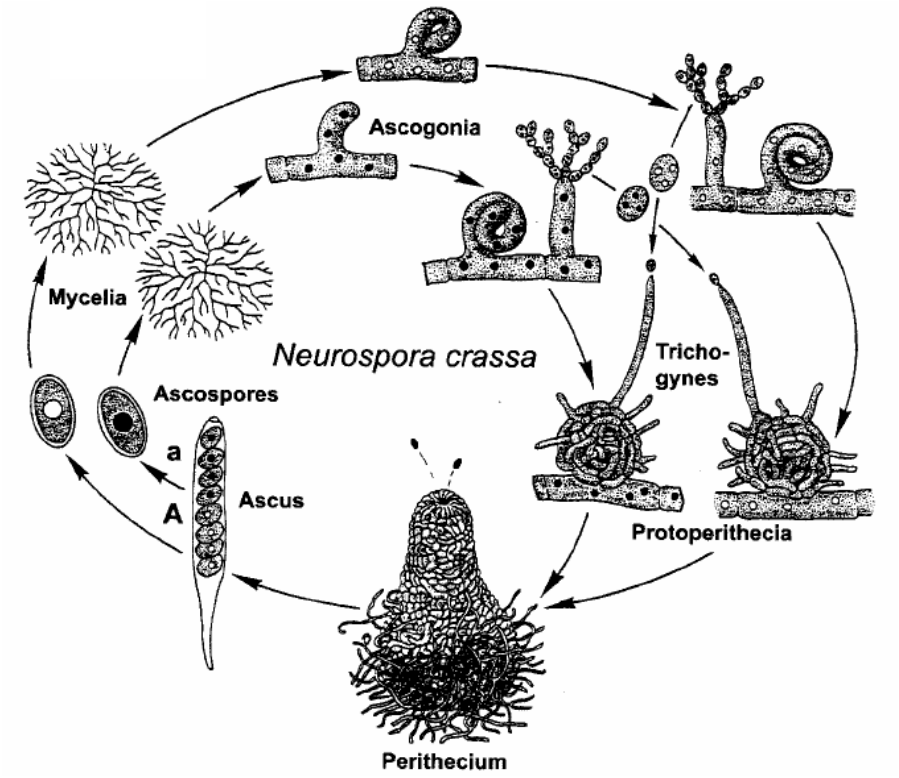



**Fig. 2** Life cycle of heterothallic species *Neurospora crassa* (Pöggeler, 2001).

A study of the mating types of the ascomycete *Cochliobulus* revealed that heterothallism is ancestral to homothallism, and supported the convergent origin for homothallism in this genus (Turgeon, 1998; Yun et al., 1999). In a recent study on *Neurospora*, Nygren et al. (2011) concluded that the ancestor of this genus was probably heterothallic, and that homothallism has evolved independently at least six times in the evolutionary history of the genus. These results agree with those previously obtained for

*Cochliobulus*. They also observed that *N. tetrasperma* and *N. tetraspora*, the two pseudohomothallic taxa of *Neurospora*, represent two independent evolutionary origins. Mating-type sequences can be used as phylogenetic markers to analyse closely related species due to the rapid evolution of mating-type genes (Pöggeler, 2001).

### 1.1.3. Morphology of sexual reproductive structures

As we mentioned above, the main defining feature of ascomycetes is that they produce ascospores inside the asci. The asci are produced from a fertile layer of fungal tissues called a hymenium (Gr. *hymen* = membrane) and, although they are occasionally naked, they usually develop inside "fruiting bodies" or ascocarps (Gr. *askos* = sac + *karpos* = fruit), known as ascomata. Historically, ascomata have been classified according to their morphological and physiological features: they are known as clesitothecia when they are globose and have no openings; gymnothecia when they have clusters of asci surrounded by a loose mesh of hyphae; perithecia when they are pear-shaped (but frequently spherical) fruiting bodies with a (usually pre-formed) apical opening; and apothecia, when they are cup- or disc-shaped and the asci are exposed to the environment (Fig. 3) (Kirk et al., 2008).

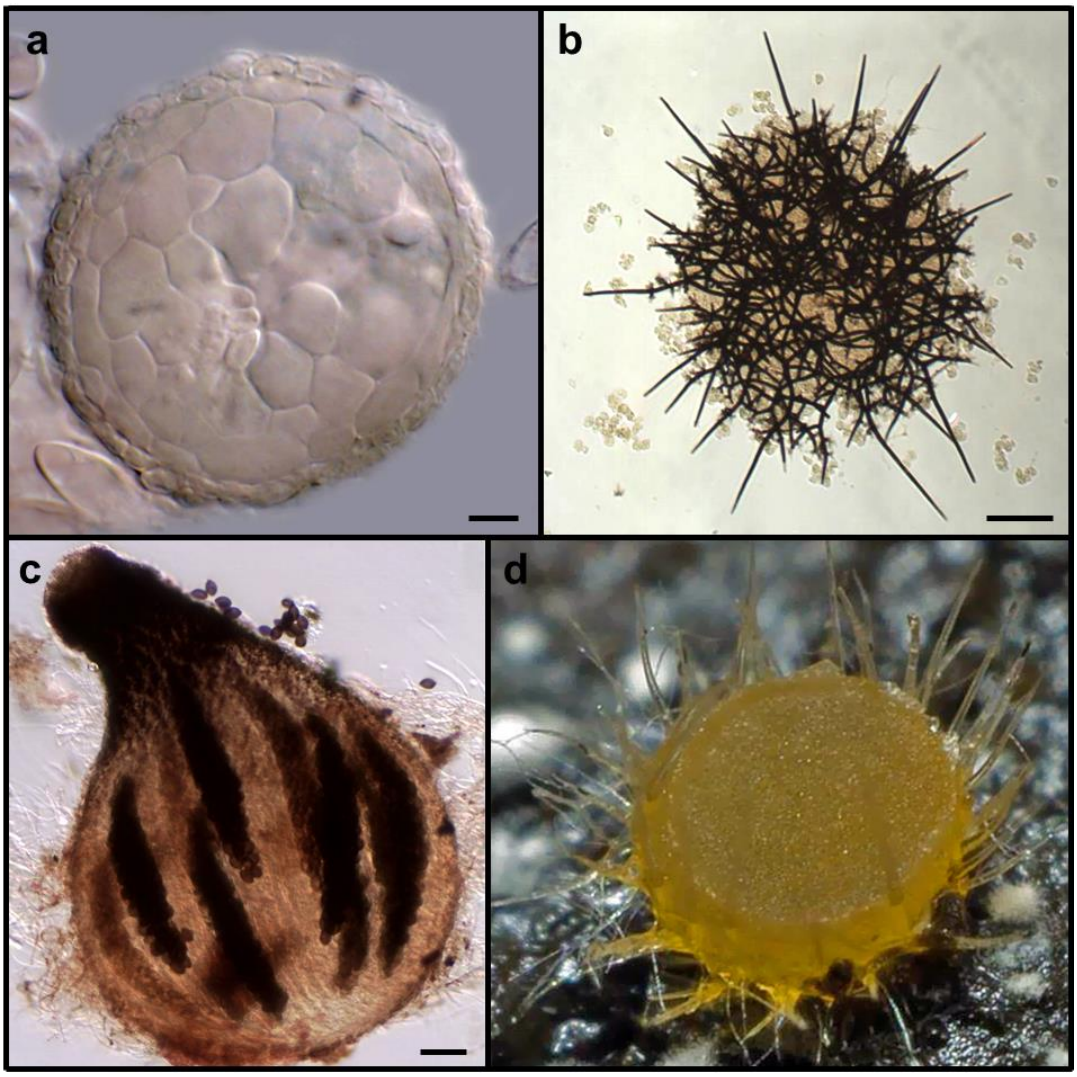



**Fig. 3** Different type of ascoma. a. Cleistothecia (*Corynascus novoguineensis* FMR 6308). b. Gymnothecia (*Myxotrichum stipitatum* FMR 13122). c. Perithecia (*Podospora setosa* FMR 12787). d. Apothecia (*Lasiobulus papillatus*, picture taken from the website http://en.wikipedia.org/wiki/Lasiobolus). Scale bars: a,c, 10 µm; b, 50 µm.

Some types of ascoma (mostly perithecia) can be borne on or embedded into a mass of vegetative hyphae called a stroma (Kirk et al., 2008). The ascoma wall, also known as the peridium, can consist of a variable number of layers of hyphae or cells, with a consistency that can be either membranaceous, coriaceous or stromatic, and have various surface textures (*angularis, epidermoidea, globulosa, intricata*, etc.). Moreover, ascomata can present hyphae-like hairs or true setae, which sometimes help insects and other animals to spread the fungi (Currah, 1985). The perithecial neck can also vary in shape (conical, cylindrical, obvoid, papillate, etc.) (Fig. 4).

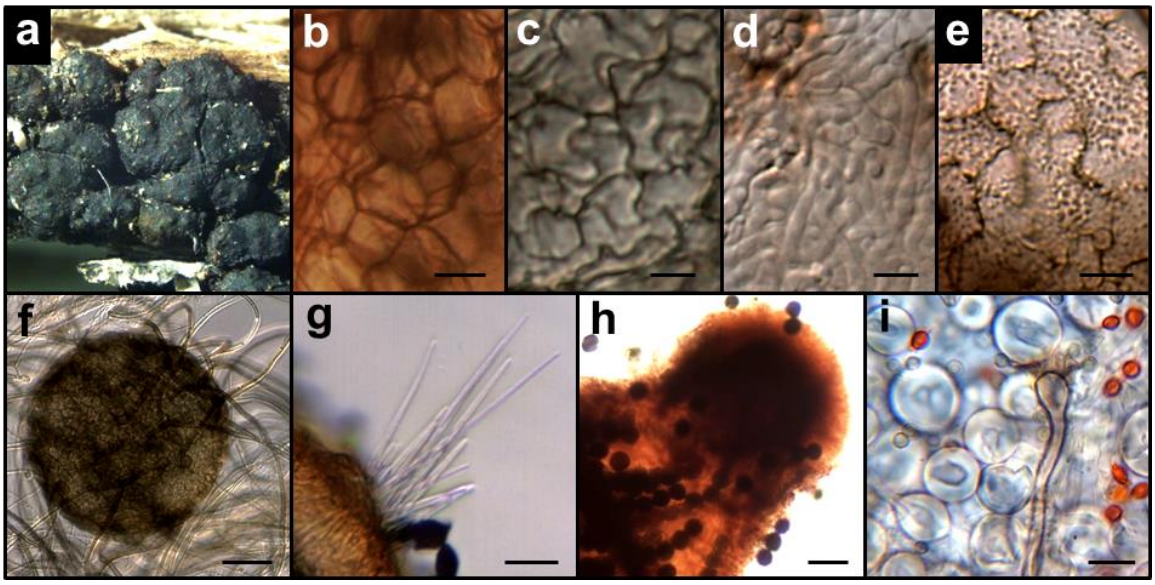



**Fig. 4** Different characteristics of ascomata. a. Stroma (*Nemania illita* ANM 1569). b. *Textura angularis* (*Pseudoneurospora canariensis* FMR 12323). c. *Textura epidermoidea* (*Corynascus sexualis* FMR 5691). d. *Textura intricata* (Xylarial FMR 13615). e. Ornamented peridial cells (*Corynascus fumimontanus* FMR 12372). f. Hyphae-like hairs (*Zopfiella erostrata* FMR 12758). g. Crown of setae around the ostiole (*Microthecium* sp. FMR 7183). h. neck papilliform (*Jugulospora rotula* FMR 12690). i. Hülle cells (*Emericella quadrilineata* FMR 8166). Scale bars: b,g,i, 10 µm; c,d,e, 5 µm; f,h, 50 µm.

Various interascal tissues or sterile hyphae, called hamathecium (Gr. *hama* = together + *theke* = case), can develop in association with the asci in the ascomatal cavity. The fertile and sterile structures in this ascomatal cavity are known as the *centrum*. The most frequent types of sterile interascal tissue in the groups studied are paraphyses (hyphae that develop from the base of the cavity) and periphyses (hyphae which develop inside, or near, the ostiole of the perithecium) (Fig. 5) (Alexopoulos et al., 1996; Kirk et al., 2008).

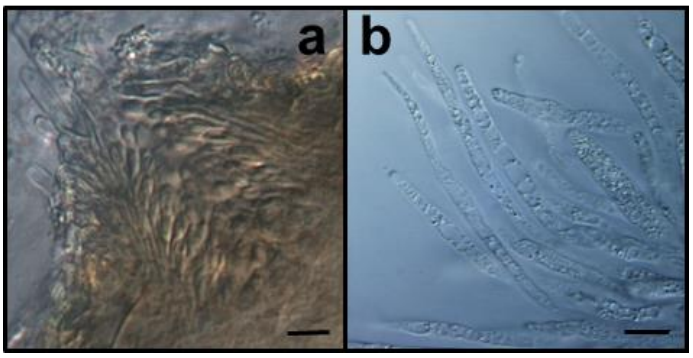



**Fig. 5** Interascal tissues. a. Periphyses (*Microthecium* sp. FMR 7183). b. Paraphyses (*Chaetosphaeria decastyla* SMH 2629). Scale bars: 10 µm.

Asci are classified according to the number of wall layers and how the ascospores are dispersed. There are three main types: protunicate, unitunicate and bitunicate. Protunicate asci have a thin and delicate wall composed of a single layer, and the ascospores are released by deliquescence. Unitunicate and bitunicate asci have two layers: the external wall layer, or exotunica, and the internal wall layer, or endotunica. In unitunicate asci these walls are attached and inseparable, and can be evanescent or persistent. This kind of asci can possess an operculum or an apical pore, through which the ascospores are released. Some groups have a structure known as the apical apparatus in which the pore is surrounded by an apical ring. Apical rings can be amyloid and stain blue in the presence of iodinated dyes (I+) or in amyloid and do not stain any colour in the presence of iodine (I-). In bitunicate asci, the wall is distinctly two-layered. The external wall layer is thin and rigid and the internal wall layer is thick and elastic. In this kind of asci, the endotunica expands and breaks the exotunica, releasing the ascospores through a pore at the apex of the endotunica. (Alexopoulos et al., 1996; Ulloa and Hanlin, 2000; Webster and Weber, 2007). Asci are variable in shape (spherical, cylindrical, ovoid, clavate, etc.), can contain a different number of ascospores (normally eight, but can be anything from one to several thousand), are sessile or stipitate, and are formed at various levels inside the ascoma or in a single level at the base of ascoma (Fig. 6) (Petersen, 2013).

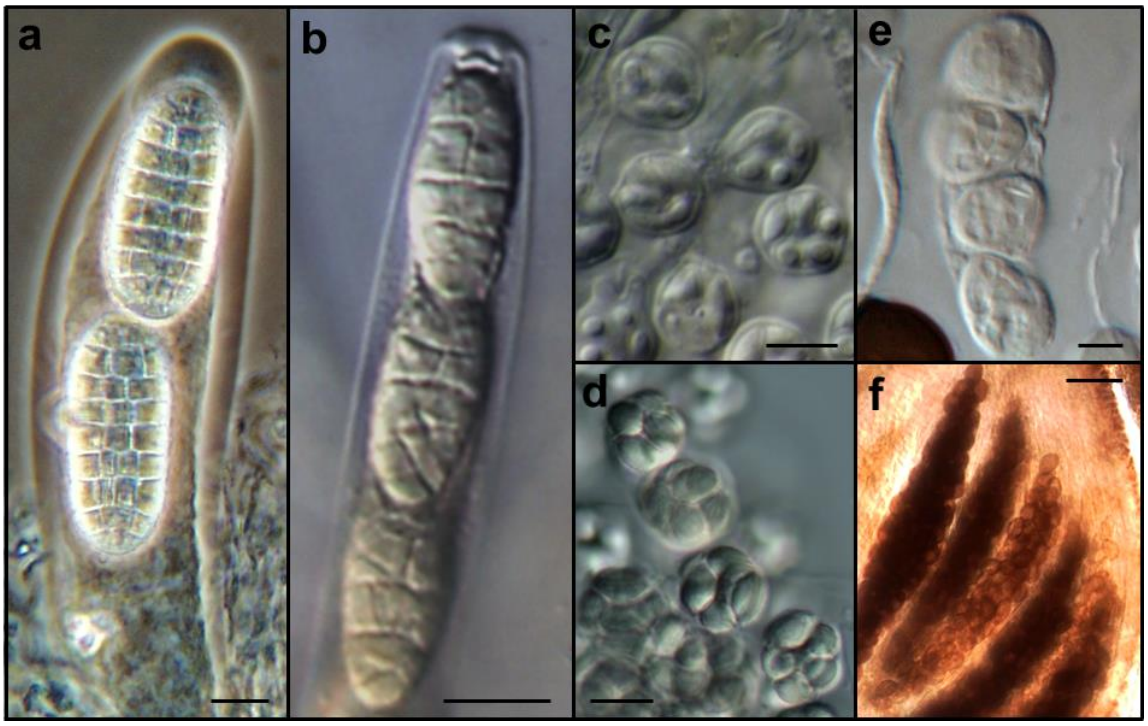



**Fig. 6** Different types of asci. a. Ascus bitunicate (*Boerlagiomyces websteri* AMS-H-2002). b. Ascus unitunicate with apical apparatus (*Rhamphoria* sp. ANM 498-1). c. Asci unitunicate without apical apparatus (*Eremascus fusisporus* FMR 10815). d. Asci prototunicate (*Leiothecium cristatum* FMR 11998). e. Ascus 4-spored (*Coronatomyces cubensis* FMR 7132). f. Asci pluri-spored (*Podospora setosa* FMR 12787). Scale bars: a–d, 10 µm; e, 5 µm; f, 50 µm.

Ascospores have a wide variety of forms. They can be unicellular or multicellular, hyaline or pigmented, smooth-walled or ornamented (spiny, warted, wrinkled, etc.), and may possess germ pores or germ slits, and other structures such as mucilaginous sheaths or appendages (Fig. 7). Ultrastructural studies have demonstrated that ascospores have a cell wall consisting of several layers. The innermost layer, the endospore, covers the cytoplasm, is thin and electron-translucent, and the last to be formed. Outside the endospore there is the epispore, a high electron-dense layer that determines the shape of the ascospore, and which is usually pigmented and may be ornamented. Surrounding the epispore there is the perispore, which can be hyaline or pigmented, smooth-walled or ornamented. The outermost layer is the exospore, the least electron-dense layer, apparently derived from the plasmatic membrane and other components of the ascal cytoplasm. This layer appears in the early stages of ascospore delimitation, together with the epispore (Cailleux, 1971; Webster and Weber, 2007).

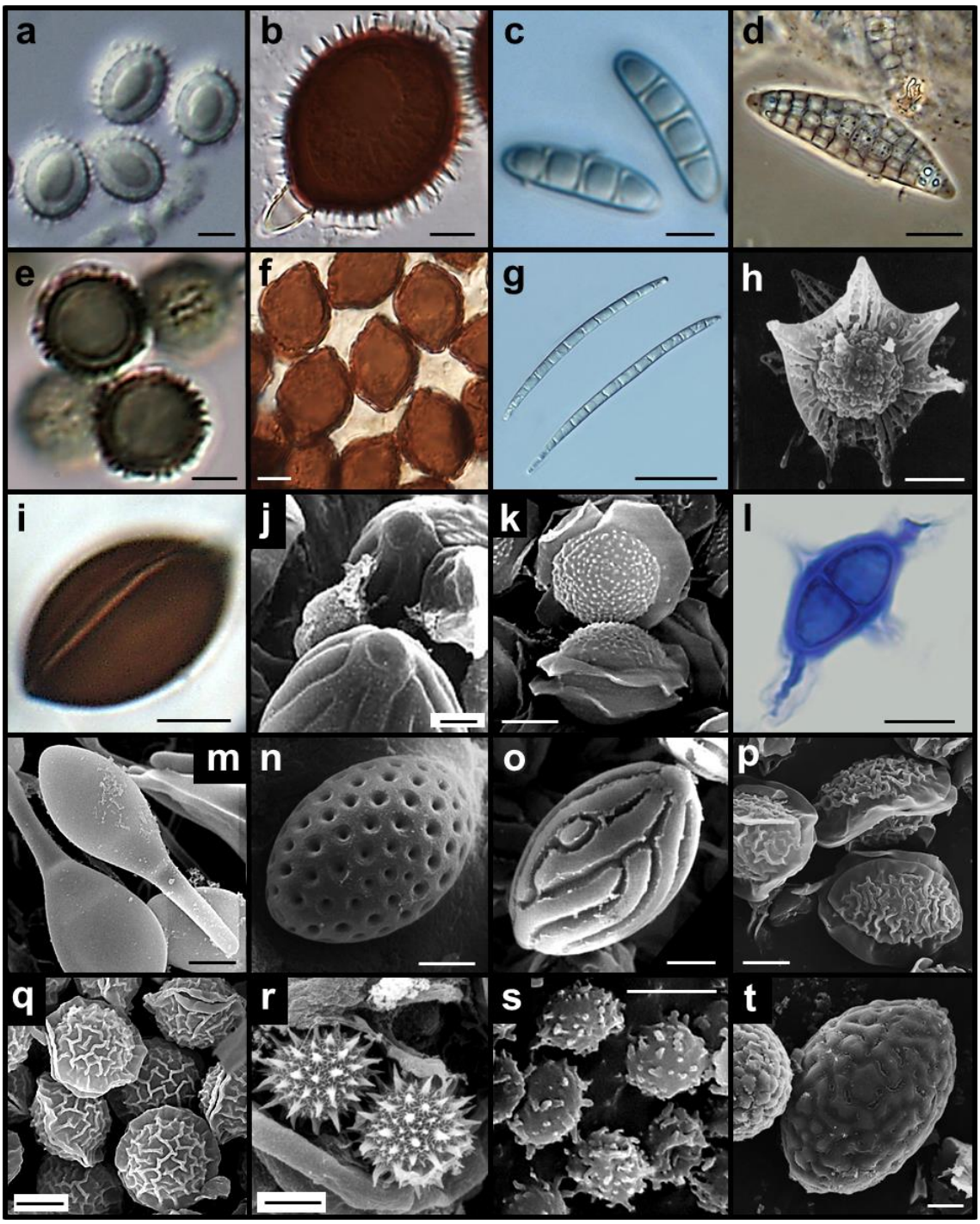



**Fig. 7** Different type of ascospores. a. Unicellular (*Aspergillus spinulosus* NRRL 4376). b. Two-celled (*Apiosordaria nigeriensis* FMR 6363). c. Multicelled (*Endoxylina tehuacanenis* ANM 172). d. Muriform (*Boerlagiomyces costaricensis* INB0003471675). e. Globose (*Hapsidospora irregularis* FMR 13607). f. Citriform (*Melanospora verrucispora* FMR 13064). g. Fusiform (*Chaetosphaeria ellisii* ANM 917). h. Stellate (*Aspergillus pluriseminatus* FMR 5588). i. Germ slit (*Coniolariella limonispora* FMR 8579). j. Germ pores (*Neurospora tetrasperma* FMR 7369). k. Equatorial crests (*Aspergillus* sp. FMR 11861). l. Mucilaginous appendages (*Toriella tubulifera* FMR 9389). m. Smooth-walled (*Naviculispora terrestris* FMR 10060). n. Inwardly pitted (*Neurospora calospora* FMR 7825). o. Ridged (*Neurospora dodgei* FMR 7968). p. Irregularly reticulate (*Leiothecium*

*cristatum* FMR 11998). q. Regularly reticulate (*Aspergillus tatenoi* FMR 11735). r. Spinose (*Eleutherascus lectardii* FMR 5751). s. Verrucose (*Talaromyces flavus* FMR 11810). t. Verrucose to tuberculate (*Pseudoneurospora canariensis* FMR 12156). Scale bars: a–c,h,i,m–o,r,t, 5 µm; d,g, 20 µm; e,j,k,p,q,s, 2.5 µm; f,l, 10 µm.

### 1.1.4. Morphology of asexual reproductive structures

Asexual reproduction in the ascomycetes takes place through the formation of conidia. Conidia are asexual spores and their development is known as conidiogenesis. There are two main ontogenetic processes: blastic and thallic. In blastic conidiogenesis, the conidium elongates and swells from a portion of the conidiogenous cell before it is delimited by a basal septum. There are two types of blastic development: holoblastic and enteroblastic. In holoblastic conidiogenesis, the outer and inner walls of a blastic conidiogenous cell take part in the formation of conidia, while in enteroblastic development the outer layer is perforated when the conidia are formed and is not included in the conidia. Thallic conidiogenesis occurs when a pre-existing segment of a hypha is transformed into a conidium or conidia. There are two different mechanisms in thallic conidiogenesis: holothallic and thallic-arthric. In holothallic development, a hyphal cell is converted into a single conidium, while in thallic-arthric conidiogenesis, the hypha is disarticulated into a series of conidia. Conidiogenous cells are responsible for the production of conidia. They have a wide variety of morphologies and determine the conidial ontogenetic processes. A conidiophore is a hypha bearing one or more conidiogenous cells. Conidia can be produced singly, in chains or in a mucilaginous mass (false heads), and are highly variable in shape (cylindrical, clavate, cuneiform, ellipsoid, spherical, etc.), size (one-celled to multicelled) and ornamentation (spiny, warted, wrinkled, etc.). They can be with or without mucilaginous appendages (Alexopoulos et al., 1996; Webster and Weber, 2007; Kirk et al., 2008) (Fig. 8).

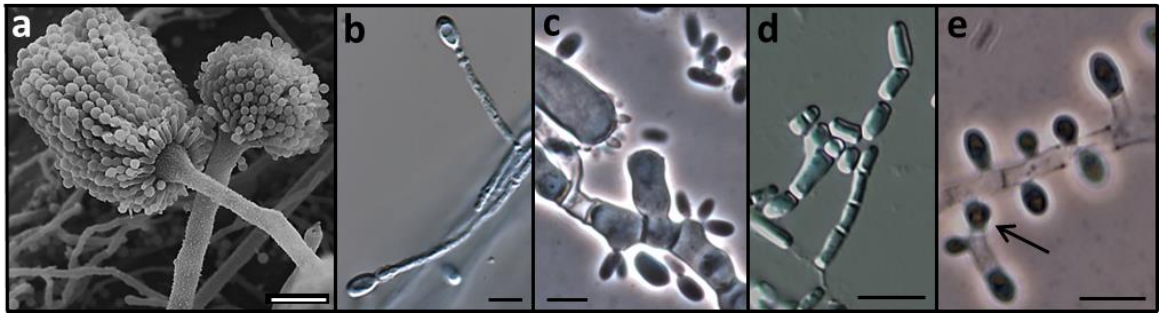



**Fig. 8** Different mechanisms in conidiogenesis. a. Enteroblastic, conidia born in phialides and arranged in chains (*Aspergillus flavus* FMR 9973). b. Enteroblastic, solitary conidia born in annelides (*Scedosporium* sp. FMR 12995). c. Holoblastic, conidia produced synchronously (*Aureobasidium* sp. FMR 13038). d. Thallic-arthric (*Chrysosporium* sp. FMR 10835). e. Holothallic (*Chrysosporium* sp. FMR 12084).Scale bars: a,d, 20 µm; b,e 5 µm; c, 10 µm.

Chlamydospores and sclerotia are resistant, usually dormant structures (memnospores), and ensure fungal survival in extreme conditions. Chlamydospores are thick-walled, swollen, and can have one cell or several. They can form as intercalary, lateral or terminal on the hyphae. A sclerotium is a firm, more or less rounded, structure composed of a melanized pseudoparenchymatous thick-walled outer layer and an inner layer of interwoven hyphae (Alexopoulos et al., 1996; Webster and Weber, 2007; Kirk et al., 2008) (Fig. 9).

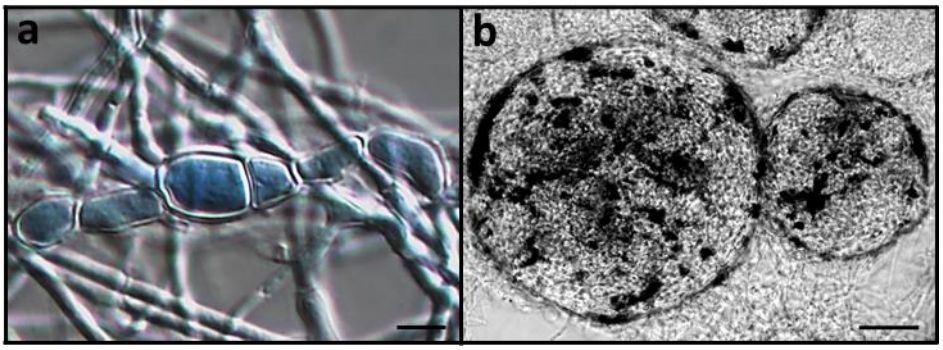



**Fig. 9** Other asexual structures. a. Chlamydospores (*Chrysosporium* sp. FMR 10835). b. Sclerotium (*Penicillium* sp. FMR 5772). Scale bar: a, 10 µm; b, 100 µm.

### 1.1.5. Nutrition

Like other members of the kingdom Fungi, ascomycetes are heterotrophic and obtain essential nutrients from organisms that are dead (saprobes or necrotrophs) or living (biotrophs and parasites) (Carroll and Wicklow, 1992; Griffin, 1994). Saprobes play an important role in the recycling of decomposing plant material. Biotrophic fungi are capable of forming mutualistic symbiosis with algae (leading to the formation of lichens), roots (mycorrhizae) or leaves and/or stems of plants (endophytes or endobionts). Other mutualistic associations are with arthropods, such as ants and ambrosia beetles (Vega and Blackwell, 2005). About 18,000 species of ascomycetes are able to establish some kind of mutual association (Kendrick, 1998). Of all parasitic fungi, ascomycetes are the most important etiological agents of fungal infections in humans and animals. Of the many examples that could be given, *Histoplasma capsulatum* and *Blastomyces dermatitidis* are the etiological agents of histoplasmosis and blastomycosis, respectively (Hoog et al., 2000); *Scedosporium apiospermum* and *Scedosporium prolificans* are the etiological agents of scedosporiosis (Rodriguez-Tudela et al., 2009); and *Aspergillus fumigatus* is the main etiological agent of pulmonary aspergillosis (Hoog et al., 2000). With regard to plant parasites (phytopathogenic fungi), the pathogen *Magnaporthe oryzae,* can have devastating effects on rice crops; *Blumeria graminis* and *Mycosphaerella raminicola* are both important pathogens of cereals; *Fusarium graminearum* is responsible for significant damage to cereals; and *F. oxysporum* can cause considerable losses in such crops as tomato, cotton and banana (Dean et al., 2012).

### 1.1.6. Taxonomy

Taxonomy is the science of classifying, naming and describing organisms. The lowest rank in taxonomy is the species, which is a group of individuals that can breed and produce a fertile progeny. The problem with applying this biological-species concept in fungi is the difficulty of mating and assessing its outcome (Guarro et al., 1999; Katoch and Kappor, 2014). The taxonomical ranks higher than the species are listed in Table 1, with the particular ending of each rank that defines their hierarchical position.

**Table 1.** Main taxonomic ranks used in fungal classification according to Hibbett et al. (2007).

| TAXONOMIC RANK | ENDING |
|---|---|
| Kingdom | Undefined |
| Subkingdom | Undefined |
| Phylum | -mycota |
| Subphylum | -mycotina |
| Class | -mycetes |
| Subclass | -mycetidae |
| Order | -ales |
| Family | -aceae |
| Genus | Undefined |
| Species | Undefined |

The main goal of systematics is to understand the relationships between living organisms and the processes by which these organisms have evolved (Voigt and Kirk, 2011).

### 1.1.6.1. Classical taxonomy

Traditional classification systems are largely based on the morphological features of organisms. They focus on the presence or absence of certain characters, and group different organisms in the same taxonomic category (or taxon) in such a way that all organisms in the same taxon are related to each other by common ancestry (Alexopoulos et al., 1996). However, sometimes this is not possible since morphological characters are not always phylogenetically informative and homologies are not easily distinguishable from analogies. Moreover, different taxonomists regard different features as relevant, which leads to different classifications (Weber, 2009).

The morphological characters that are most commonly used to classify ascomycetes are the structures associated with sexual reproduction: for example, the type of ascoma and its ontogeny, the type of centrum, the morphology and ultrastructure of the asci and their dehiscence or the nature of the pre-formed apical structure for releasing spores, and the main characteristics of the ascospores (presence, type and number of germ structures and mucilaginous layers; appendages; color; ornamentation; and cell number). However, the ontogenetic processes involved in the production of the spores of their anamorphic stage have also been used (Barr, 1990). Although these features have the advantage that they are easily observable, there is little variation in morphology and

some of the characters used have been subjected to convergent evolution for some groups of organisms.

According to Rogers (1994), the taxonomy of ascomycetes has three key problems:

- Few species have been studied in detail.
- The apparent age of ascomycetes and the probability that many of them have not survived has led to evolutionary relationships being interpreted from features which are often lacking in phylogenetic value.
- Taxonomic systems are created on the basis of very few characters.

Recent molecular studies have shown that some of the morphological characters widely used in taxonomy – for example, ascospore ornamentation – are not always phylogenetically informative (García et al., 2004), and other characters not taken into account for certain groups – for example the type of ascomata in Lasiosphaeriaceae – have proved to be useful for elucidating evolutionary relationships (Miller and Huhndorf, 2005). Therefore, characters which are not phylogenetically informative for some taxonomic groups are valuable for others. The problem lies in defining what morphological characters are suitable for designing a classification system. These characters must be homologous to the group of organisms being compared and not homoplastic (i.e. characters that are the result of convergent, parallel, or reversible evolution).

#### 1.1.6.2. Molecular biology

In the past, as discussed in the previous chapter, species of fungi were delimited mostly by their phenotypic traits, so most of the accepted species are known only by their morphological description. The main problem concerning the morpho-species concept is that a correct classification/identification is often time-consuming and laborious, and requires experience, ability and usually the use of different culture media and incubation temperatures, especially for genera with numerous species and/or with poorly differentiated morphological characters. Moreover, fungal structures can vary depending on the incubation time, the substrate and environmental conditions. These variables are minimized when molecular tools are used, which also have the advantage of being much faster, more specific and more accurate. Neither do they need the intervention of specialized taxonomists. Such molecular techniques as Restriction Fragment Length Polymorphism (RFLPs), Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) are still used to type fungal isolates. However, the most widely used tool in fungal taxonomy is DNA sequencing, especially for those genes that encode nuclear ribosomal RNA (nrRNA). These genes

are located in two adjacent transcriptional units that are transcribed separately. One of these units includes the 5S nrRNA (absent from certain fungal groups), and the other is composed of the 18S, 5.8S and 28S nrRNA genes, which are separated by two internal transcribed spacers (ITS1 and ITS2) and flanked by two external transcribed spacers (ETS). The two transcriptional units are separated by the intergenic spacers IGS1 and IGS2 (Fig. 10).

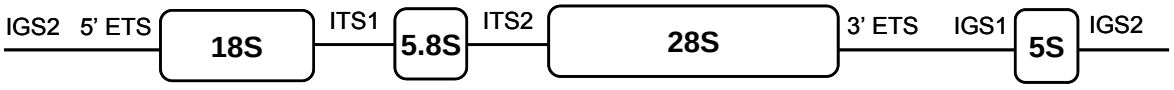



**Fig. 10** Structure of the nuclear ribosomal RNA gene cluster in fungi.

The 18S small subunit (SSU) usually has around 1800 bp and is phylogenetically highly conserved between species of the same genus, and even among different genera. This gene is most commonly used for the taxonomical placement of individuals at order or family level. The 28S large subunit (LSU) has approximately 3400 bp and is usually employed to classify fungi at genus level and above, although it is also useful for species delimitation in some taxa (i.e., Onygenales and Sordariales). The nuclear ribosomal internal transcribed spacer (ITS) region, which includes the 5.8S gene and the internal transcribed spacers (ITS1 and ITS2), was recently designated as the official barcode marker for fungi (Scoch et al., 2012). DNA barcoding uses standardized primers to amplify the widest range of taxonomic groups and produce sequences of 500-800 bp for the identification of species. Several regions were evaluated recently by Scoch et al. (2012), including the mitochondrial cytochrome c oxidase subunit 1 (*CO1*) (which is useful for animal barcoding), the SSU and LSU regions, and several protein encoding gene regions (i.e. the largest subunit of RNA polymerase II (*RPB1*), the second largest subunit of RNA polymerase II (*RPB2*) and the minichromosome maintenance protein (*MCM7*)). The ITS was chosen as the best candidate for barcoding because it is easy to amplify and is a good marker for species-level identification due to the high information content. However, the percentage of identity to be considered as the cut-off for species delimitation remains controversial, because the interspecific variability of this region varies among different fungal taxonomic groups (Stchigel, 2000; Crous et al., 2009). Also, the necessity for another gene to distinguish all fungal species is being discussed because in some fungal groups, ITS can be largely conserved. In these cases, other genes, most of which encode structural proteins, have been used. In 2013, the main purpose of the international meeting "One Fungus = Which Genes" (1F = ?G) was to

explore which additional genes should be sequenced to identify and classify certain groups of fungi.

At present, there are several different sequence databases (i.e. GenBank (http://www.ncbi.nlm.nih.gov/genbank/), the European Molecular Biology Laboratory (EMBL) (http://www.ebi.ac.uk/embl/), the Biological Resource Center (NBRC) (http://www.nbrc.nite.go.jp/)) which contain a variety of gene sequences from a large number of fungi. Thus, comparing the sequences of our fungi to those deposited in the database can provide a great deal of taxonomic information.

#### 1.1.6.3. Molecular phylogeny

The objective of a molecular phylogeny is to reconstruct the evolutionary relationships among organisms by comparing the nucleotide sequences of certain genes. Molecular phylogeny is based on the theory that mutations in a gene sequence constantly accumulate over time and are randomly distributed. Consequently, the differences noticed when comparing homologous nucleotide sequences from several taxa can provide information about the time elapsed since they evolved from a common ancestor, and reveals the shared derived characters that relate these individuals to their theoretical ancestor.

Molecular characters have the advantage over morphological ones that they are exactly defined, quantifiable and highly reproducible. However, they also have some disadvantages, the main one of which is the use of only four “letters” (A, C, T, and G). Molecular homoplasy needs to be reduced because the presence of a common base in two different species may be the result of a random process and not necessarily because they descended from a common ancestor. Another common problem is data saturation. For example, a gene may have varied so much in its evolution that the signs of these changes eventually disappear or overlap. As a result, when one individual is compared with others, there may be a difference of only one nucleotide between them, the result of several past events that cannot be detected with the current sequences analyzed.

Evolutionary distance matrices are constructed from multiple DNA sequence alignments and generated using such computer programs as Clustal, Mafft, Muscle, etc. (Thompson et al., 1997; Edgar, 2004; Katoh et al., 2009). These matrices are subjected to various statistical analyses. The result is a phylogenetic tree which is a graphical representation of the evolutionary relationships among these groups (Fig. 11). The trees are composed of nodes and branches. The external nodes are also called operational taxonomic units (OTUs) and the internal nodes hypothetical taxonomic units (HTUs) (they represent the

hypothetical ancestors of the OTUs). Branches show the relationships among the taxa in terms of descent and ancestry. Usually, trees indicate the length of their branches to show the time of emergence and the degree of sequence divergence. A clade is a cluster of related OTUs that descend from the same HTU.

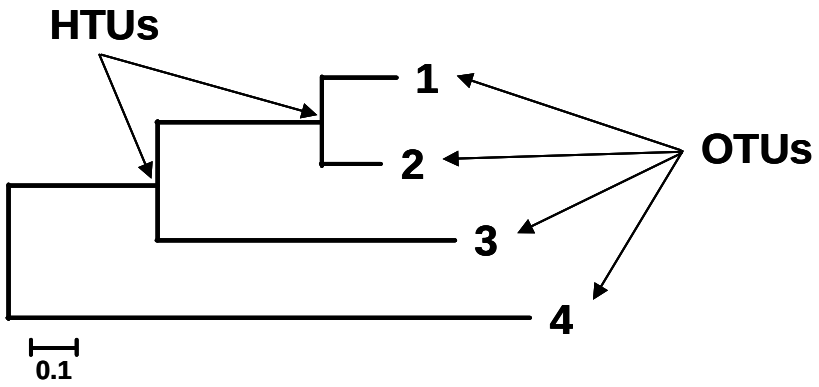



**Fig. 11** Schematic representation of a phylogenetic tree.

The phylogenetic tree can be rooted or unrooted (Fig. 12). An unrooted tree only infers the relationships among the taxonomic units studied and does not give information about the direction of evolution. In a rooted tree, the root represents the common ancestor for all the taxonomic units, which shows the order of sequence inheritance. In order to get a correct rooted tree, an outgroup needs to be added to the analysis. This outgroup can be one or several sequences that are more distantly related to the sequences of the internal group than the OTUs from this internal group are to each other (Podsiadło and Polz-Dacewicz, 2013).

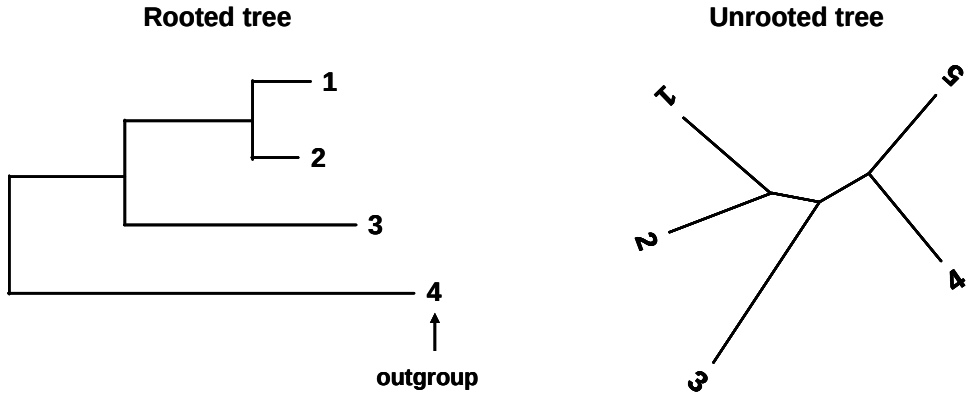



**Fig. 12** Examples of rooted (A) and unrooted trees (B).

In the tree, a group of species that includes a common ancestor and all its descendants is called monophyletic. A group containing some, but not all, descendants of two or more ancestors is called polyphyletic. A group that includes an ancestor, but

not all of its descendants is called paraphyletic (Fig. 13). The natural groups of species represented by genera, families and/or higher taxonomic ranks should be monophyletic. However, many fungal groups are paraphyletic or polyphyletic because most of them have been established on the basis of morphological characters, which do not always reflect the evolution of the species, but are the result of adaptation to their environment. One example of this is the polyphyly exhibited by many genera belonging to the family Lasiosphaeriaceae of the order Sordariales, such as *Cercophora*, *Podospora* and *Zopfiella* (Cai et al., 2006; Chang et al., 2010). It has even been demostrated that this family is paraphyletic, like Chaetomiaceae, another family of the same order (Huhndorf et al., 2004).

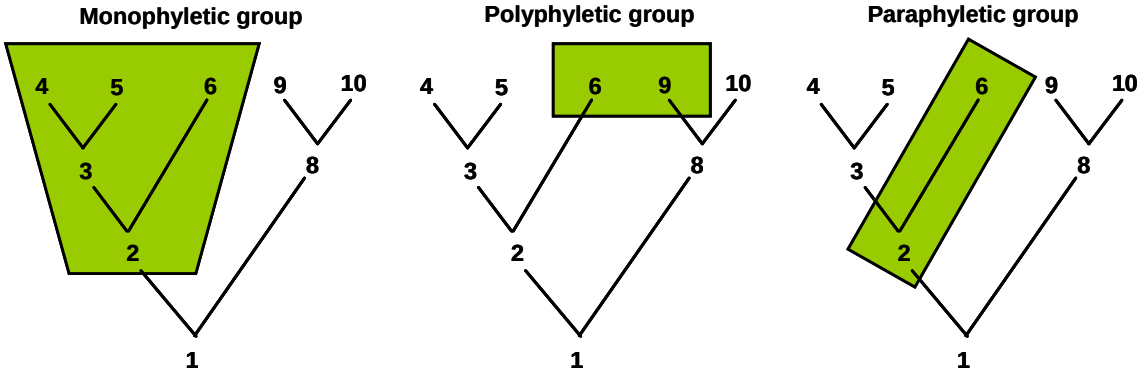



**Fig. 13** Examples of monophyletic, polyphyletic, and paraphyletic groupings.

The methods designed to infer phylogeny aim to be models of a process that reconstructs the evolutionary relationships between a group of individuals. The phylogenetic methods based on the analysis of DNA sequences can be grouped into two categories: distance-based methods and character-based methods (Lemey et al., 2009). Distance-based methods assume that the evolutionary distance between two taxa (defined as the measure of their dissimilarity) is directly related to their phylogenetic relationship. These distances are rebuilt by the comparison of pairs of sequences for which an index of differences is calculated. The distances calculated enable a matrix to be built from which the phylogenetic tree is constructed (Bowman et al., 1992). Neighbor-joining (NJ) is one of the most widely used distance-based methods of phylogeny reconstruction (Saitou and Nei, 1987), which produces trees by forming clusters with the sequences that have the shortest genetic distance between them, following the principle of minimum evolution proposed by Saitou and Imanishi (1989). Thus, the NJ tree obtained is that in which the total sum of the lengths of its branches is shortest. The most significant advantage of NJ over other phylogenetic inference methods is the speed at which it processes data to obtain a relatively credible phylogram, making it one of the

most used methods for inferring phylogenetic relationships (Criscuolo and Gascuel, 2008). Character-based methods consider each nucleotide position as a separate character. From the sequence alignment, this method evaluates the changes for each nucleotide position and establishes the possible phylogenetic relationships (DeSalle et al., 1994). Examples of these methods are: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). The MP method identifies the best tree as that which requires the minimum number of substitutions (Kolaczkowski and Thornton, 2004). Using a suitable computer algorithm, the ML method builds the tree with the biggest value of the reliability algorithm (Guindon and Gascuel, 2003). The BI method does not select the single, most reliable tree but creates a highly reliable set of trees (Podsiadło and Polz-Dacewicz, 2013). Once the phylogenetic tree has been obtained by either method, it is necessary to determine their degree of reliability. There are various methods by which a statistical value that estimates the robustness of the groupings formed can be obtained, the bootstrap analysis being one of the most commonly used (Felsenstein, 1985; Hillis and Bull, 1993). This analysis determines an index of conservation frequency for each branch expressed as a percentage. A branch with a bootstrap value equal to or higher than 70% is considered to be well supported.

Many authors have reported that multilocus analysis is more suitable for species delimitation than an individual marker (Dupuis et al., 2012). The concept of phylogenetic species defines species as the smallest monophyletic group of organisms that can be diagnosed on the basis of one or more derived characters (i.e. unique and exclusive taxon) (Cracraft, 1983). According to this definition individual groups can be diagnosed easily, but the decision on where to place the species limit is subjective (for example, a gene may have two different sequences because there are two alleles in a single population or because there are fixed differences between two different populations). This subjectivity can be avoided by multiple gene studies (concordance between the genealogies of more than one gene). The concordance between the tree branches obtained in the phylogenies based on different genes enables species to be connected. As can be seen in Figure 14, in genetically isolated species there is congruence between their genealogies since their branches overlap, whereas there is incongruence within species, which may be caused, for example, by recombination between individuals of the same species. Therefore, the transition from concordance to conflict marks the species boundaries. For this reason, recent phylogenies are inferred by the sequencing of several gene regions. For example, the sequences of four structural genes have been used in the Eurotiales: *RPB1*, *RPB2*, putative chaperonin complex component TCP-1 (*Cct8*), and putative ribosome biogenesis protein (*Tsr1*) (Houbraken and Samson, 2011).

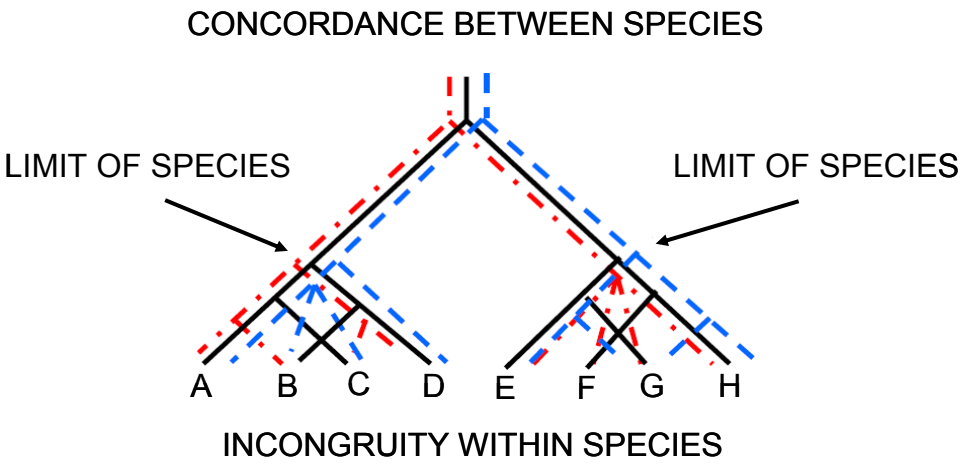



**Fig. 14** Analysis of three genealogies (black, red and blue) (adapted from Taylor et al., 2000).

The interpretation of the results derived from molecular data and the study of morphological characters are complementary methodologies, and using them both enables taxonomic and phylogenetic problems to be solved (Taylor et al., 1994).

### 1.1.6.4. Polyphasic taxonomy

Nowadays, physiological and nutritional abilities, ecological and biogeographic data, and extrolite profiles are also documented for some fungal groups, which are used in combination with molecular and morphological data to build a taxonomical system known as polyphasic taxonomy. Polyphasic studies have recently been developed for the genera *Aspergillus* and *Penicillium* (Frisvad and Samson, 2004; Samson and Varga, 2009; Berni et al., 2011; Samson et al., 2011; Baquião et al., 2013), among others.

### 1.1.6.5. Recent changes in nomenclature

The International Code of Botanical Nomenclature (ICBN) has regulated fungal nomenclature since 1867. Between 1912 and 2011, and on the basis of Article 59 of the ICBN, different scientific names were given to the asexual and sexual morphs of the same species for non-lichenized ascomycetes and basidiomycetes. In the early 1990s, molecular data became available so it was possible to demonstrate that asexual and sexual morphs belong to the same fungus. For this reason, the need to maintain a dual nomenclature for pleomorphic fungi has been questioned (Gams, 2005; Hawksworth, 2005; Rossman and Samuels, 2005). In April 2011 a symposium on fungal taxonomy entitled “One Fungus = One Name (1F = 1N)” was held in the Netherlands, and the ensuing Amsterdam Declaration on Fungal Nomenclature supported the rule that only

one name would be allowed for any fungal species (Hawskworth et al., 2011). However, numerous members of the mycological community disagreed, because they felt that these changes would have chaotic consequences (Gams et al., 2011). The changes proposed in the Amsterdam Declaration were accepted at the 18th International Botanical Congress held in Melbourne in July of the same year. The new code for regulating the fungal nomenclature was approved, Article 59 was replaced by the new rule of only one name for fungal species with pleomorphic life cycles, and the International Code of Botanical Nomenclature was renamed the International Code of Nomenclature for Algae, Fungi and Plants. Once it had been accepted that it would be desirable for each fungus to have only one name, a second symposium entitled "One Fungus = Which Name?" was held in Amsterdam in April 2012, the purpose of which was to address the drastic changes in the naming of pleomorphic fungi adopted in Melbourne (Braun, 2012). This second symposium discussed which name should be chosen for the species that had previously been described. The decision was to maintain the oldest name but to make exceptions in some cases (for example, if the latter name was the most widely used). It was also proposed to create lists of the names that had been accepted and rejected so as to minimize the impact that these changes would have on, for example, clinicians and clinical microbiologists. No restriction was placed on who might be involved in producing these lists except for the fact that they would have to be approved by several committees. The lists of names will be submitted for formal adoption in the next International Botanical Congress in China in 2017 (Hawskworth et al., 2013).

### 1.1.7. Taxonomy of ascomycetes. Phylogenetic relationships with other taxa of the kingdom Fungi

The phylum Ascomycota is phylogenetically related to Basidiomycota and both are classified in the subkingdom Dikarya (Fig. 15), which reflects their ability to form a dikaryotic phase during a part of their life cycles (Hibbet et al., 2007).

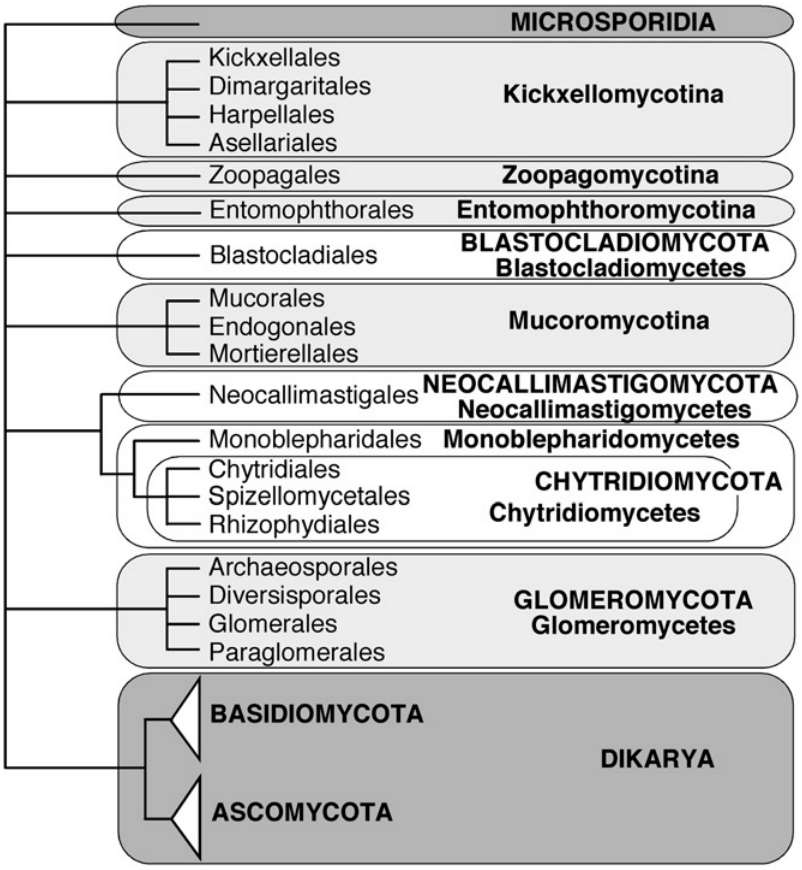



**Fig. 15** Phylogeny of kingdom Fungi. Branch lengths are not proportional to genetic distances. Picture taken from Hibbett et al., 2007.

Ascomycetes are divided into three subphyla (i.e. Taphrinomycotina, Saccharomycotina and Pezizomycotina). Taphrinomycotina includes members belonging to the earliest divergence, Saccharomycotina comprises most of the yeasts, and Pezizomycotina is the largest subphylum and includes approximately 90% of all reported species of ascomycetes and the vast majority of filamentous, ascoma-producing species (Spatafora et al., 2006; Hibbett et al., 2007). Figure 16 shows the phylogeny and classification of the phylum Ascomycota, including all the subphylums, classes and orders.

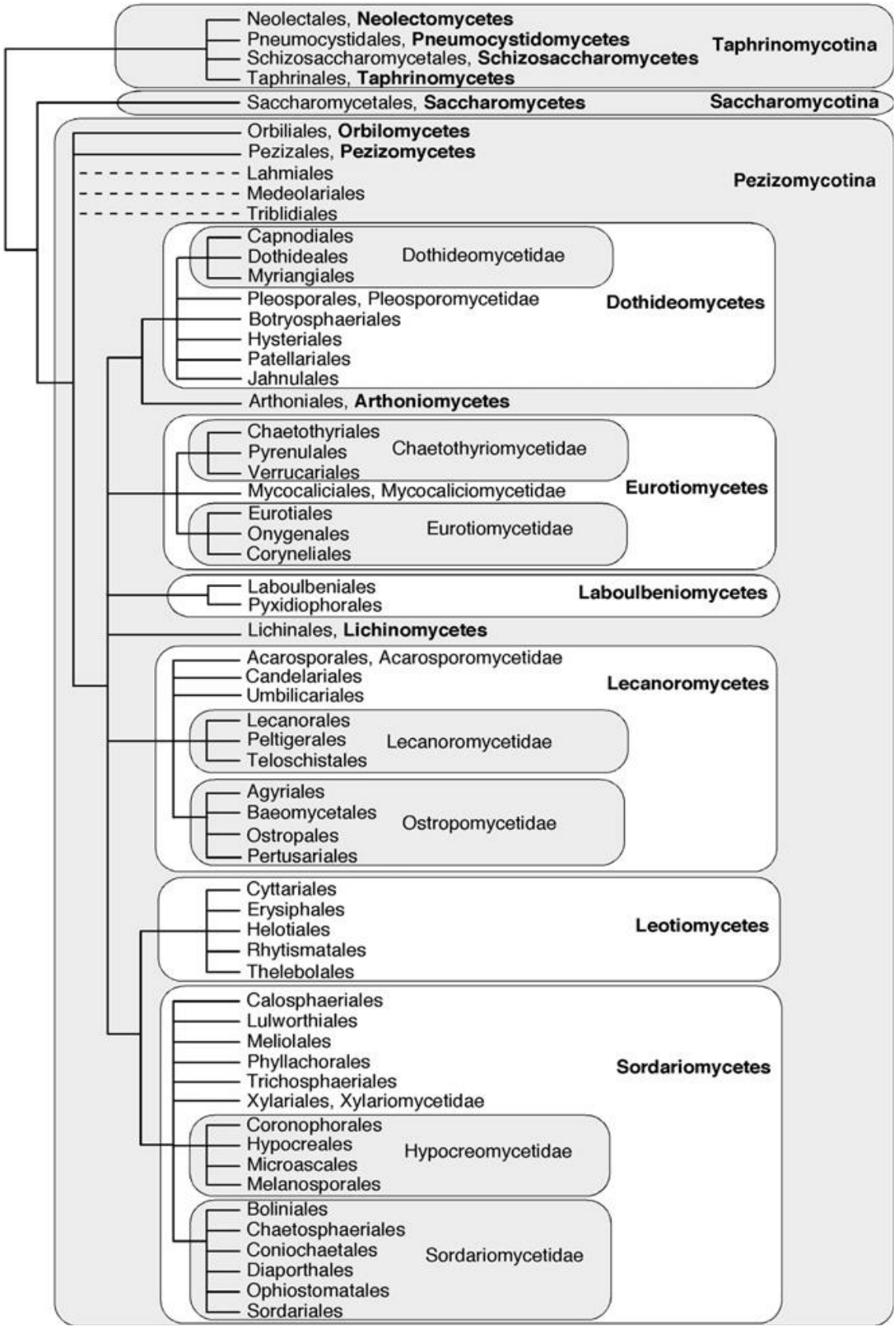



**Fig. 16** Phylogeny of the phylum Ascomycota. Picture taken from Hibbet et al. 2007.

## 1.2. SOIL ASCOMYCETES

Soil is an environment for living organisms on the earth's surface. It is made up of a mixture of mineral and organic constituents that are in solid, gaseous and aqueous states (Voroney, 2006). The proportions of the main soil components are shown in Figure 17 and their distribution in Figure 18. The organic soil matter is the factor that plays the most important role in determining the structure of the microbial community because it contains large amounts of carbon, and directly supports microbial, plant and animal life by retaining essential nutrients and water in the soil (Simpson and Simpson, 2012; García-Orenes et al., 2013).

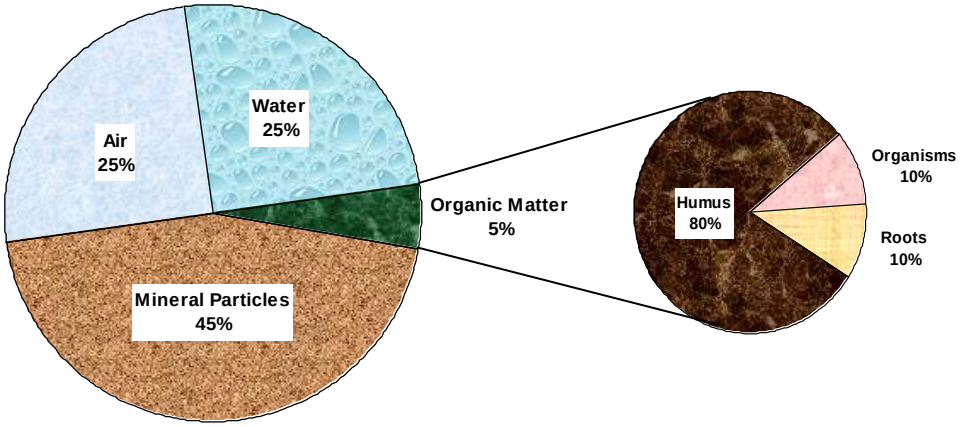
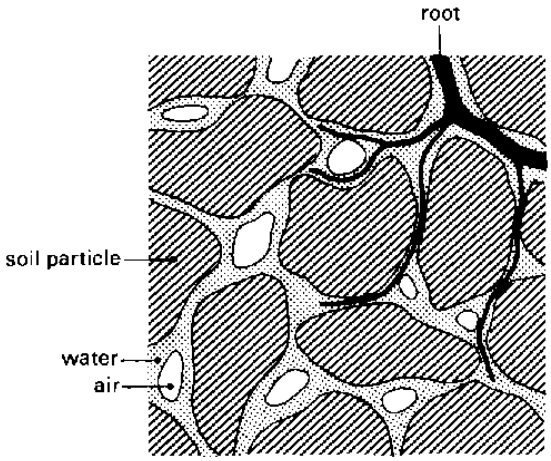



**Fig. 17** Composition and percentage of each soil component.




**Fig, 18** Soil composition and structure. Picture taken from the website http://www.fao.org/docrep/r4082e/r4082e03.htm.

The soil habitat is defined as the totality of living organisms inhabiting the soil, including plants, animals and microorganisms, and their abiotic environment. It is characterized by heterogeneities because its chemical, physical and biological characteristics vary in space and over time (Voroney, 2006).

Soil is the habitat of widely heterogeneous and extremely diverse microbial communities, which are complex and dynamic, and vary in composition depending on whether they are in one compartment or level or another (Alexander, 1977; Trabelsi and Mhamdi, 2013) (explained in Figure 19). The microorganisms in soil are involved in the biogeochemical cycling of carbon, nitrogen, sulfur, phosphorus, and metals, and the biodegradation or stabilization of environmental contaminants (Zhang et al., 2014).

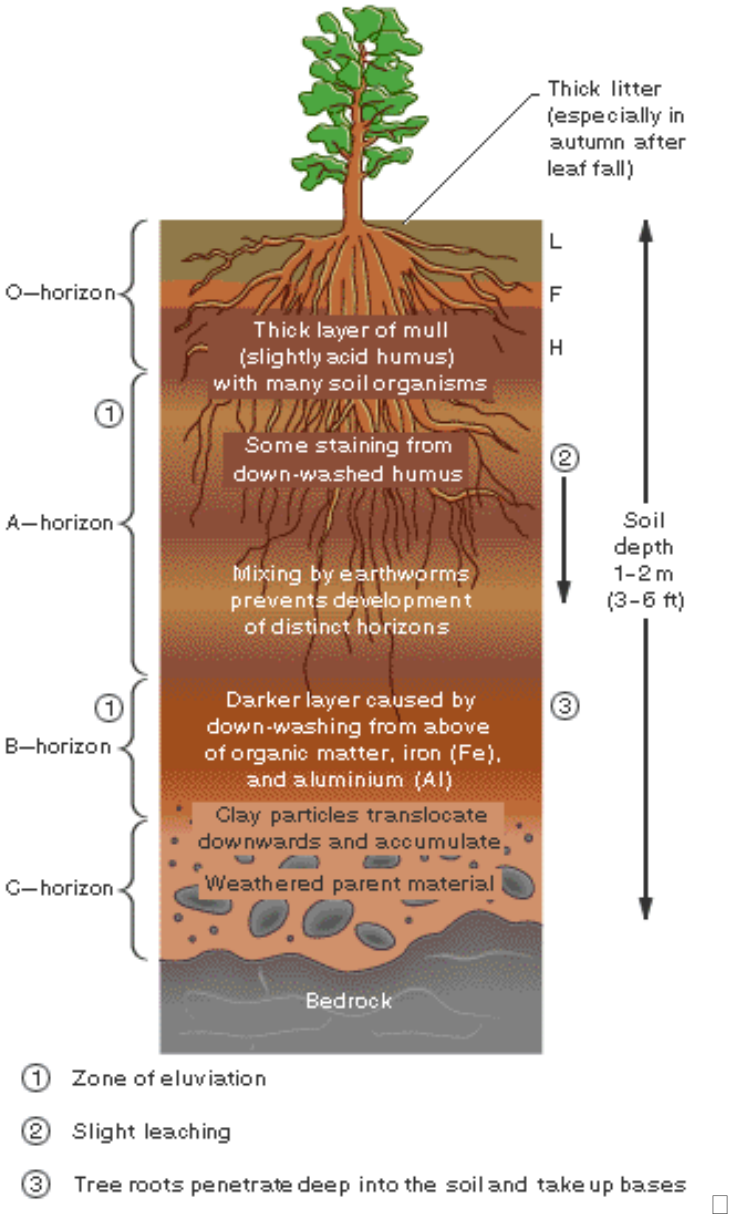



**Fig. 19** Vertical section of soil profile showing its different horizons**.** Scheme taken from the website http://www.geogonline.org.uk/soil_profile.htm.

Soil is a dynamic ecosystem in which sources of organic and inorganic materials are transformed and renewed continuously by the physiological/metabolic activity of microorganisms (Ingold and Hudson, 1993; Kendrick, 1992). There are several million

prokaryotic and eukaryotic microorganisms in a single gram of soil (Curtis and Sloan, 2005), and fungi are the dominant eukaryotic lineage in terms of biomass (Mueller and Schmit, 2007). Moreover, soil is a highly biodiverse habitat and the wide variety of microhabitats means that fungal communities are very diverse (Waksman, 1922; Domsch et al., 2007). Fungi grow in the spaces between the particles that make up the soil, such as the organic material and/or the roots, which provide an appropriate substrate for development. The fungal species present in this ecosystem are called soil fungi (Watanabe, 2002). They play a key role in the decomposition and mineralization of complex, recalcitrant compounds from plants and animals, such as cellulose, hemicellulose, lignin and chitin (Boddy et al., 2007). The metabolic activity of fungi promotes the redistribution of nutrients and contributes to the formation of humus, which immobilizes large amounts of nitrogen and thus maintains fertility and soil structure (Hawksworth, 1993; Kirk et al., 2008; Watanabe, 2002). Furthermore, fungi contribute to the structural development of soil by hyphal growth and the production of coagulating substances like glomalin, which can help the adhesion of soil microaggregates (Aspiras et al., 1971; Wright and Upadhyaya, 1998). In the soil, fungi establish complex relations with each other and with such other inhabitants as bacteria, algae, protozoa, helminthes and arthropods. The mutualistic associations of fungi and plants, which are called mycorrhizal symbioses, are widely known and have a determinant effect on plant nutrition, plant health, soil structure and even water uptake (Finlay, 2008). The spatial distribution of soil fungal communities depends on their response to environmental factors, such as soil nutrients, water availability, soil texture and vegetation (Wubet et al., 2012).

Many soil ascomycetes produce compounds that inhibit other organisms, such as antibiotics, carbon dioxide, ethylene or ammonium. Antibiotics are secondary metabolism substances (also called extrolytes), and occur when carbon sources are abundant but growth is limited by the absence of other essential nutrients, mostly nitrogen (Carlile et al., 2001). Many of the chemical compounds produced by fungi (for example, aflatoxins and ochratoxins) are highly toxic to humans, but others have been of great therapeutic value (for example, penicillins and cephalosporins).

Some soil ascomycetes tolerate thermal shocks, desiccation and ultraviolet radiation better than other groups of fungi because of the high content of melanic substances in the cell walls of their conidia, ascospores and/or ascomata, which protect them from ultraviolet radiation. Some of them also tolerate short exposures to high temperatures probably due to the chemical composition and structure of the fungal walls, or are even thermotolerant or thermophilic. The occasional warming of their ecosystem is a natural

phenomenon for many soils (for example, when they are exposed to fire or solar irradiation) (Dix and Webster, 1995; Sussman, 1981).

### 1.2.1. Mycostasis and dormancy phenomena

The rapid disappearance of nutrients is probably one of the main causes of the soil's inhibiting germination of fungal spores (Carlile et al., 2001; Dobbs and Hinson, 1953; Kendrick, 1992; Lockwood, 1964, 1977; Steiner and Lockwood, 1969). This phenomenon is known as mycostasis or fungistasis. The presence of inhibitory substances produced by different microorganisms (i.e. actinomycetes, fungi) is another determining factor in soil mycostasis (Bristow and Lockwood, 1975; Gupta and Tandon, 1977), which can be interpreted as an advantageous adaptation because it delays ascospore germination until the activity of the microorganisms in the ecosystem is reduced, or until the nutritional status is restored (Carlile et al., 2001).

In addition to being the primary habitat of geophylic fungi that are metabolically active, soil is also a reservoir of latent spores. Dormancy is the stage that usually precedes germination, and during this period spores undergo no morphological changes and they maintain basal metabolism. For example, in *Neurospora,* latent ascospores have rates of carbon dioxide production and consumption of oxygen that represent between 1 and 4% of somatic cells, respectively. The latent period depends on many factors and can last from a few hours to several years (Carlile et al., 2001).

There are two types of dormancy: exogenous and endogenous (or constitutive) (Sussman, 1965). The first is a condition of the environment in which the propagules are a part. For example, the temperature may be inappropriate for germination or lack the essential nutrients. Because nutrients are quickly removed, soil is usually a nutritionally poor medium. It is probably for this reason that many soil fungi are latent for most of their life cycle, and develop only when nutrients are occasionally available. Endogenous dormancy depends on the structure and/or metabolism of the propagule, and requires precise and unusual conditions for disruption. The mechanisms involved in this kind of dormancy are diverse in nature, such as the low permeability of the cell wall or the production of germination self-inhibitors (Cooke and Whipps, 1993). The distinction between exogenous and endogenous dormancy is unclear, and both mechanisms are probably present in many spores.

Spore germination involves the initiation of biochemical activity (absent or much reduced during dormancy), a gradual increase in metabolic rate, and morphological changes such as the enlargement and emission of the germ tube. The circumstances that determine the completion of the dormancy of the fungal spores vary among species

and among spore types from the same species (ascospores, macro- and microconidia, or chlamydospores) (Carlile et al., 2001). However, some general factors influence spore germination: for example, the presence of water or high humidity, the presence of oxygen or carbon dioxide, and variations in temperature. Some propagules of thermophilic fungi, such as conidia and ascospores, need a heat shock to break the dormancy (Dix and Webster, 1995). For some saprophytic fungi the appearance of water-soluble nutrients of low molecular weight (sugars and amino acids) is essential to germination. Sugars, alcohols, ketones, aldehydes and terpenes produced by plants activate the germination of a large number of fungal species. Moreover, the spores of coprophilous fungal species are activated by passing through the digestive tract of herbivorous animals, where they are exposed to a variety of chemical compounds (enzymes and acids) at elevated temperatures.

### 1.2.2. Methodology for studying soil fungi

Many studies have been undertaken on microorganisms in natural habitats, including techniques for the direct examination of samples by using the microscope, which reveals the distribution of microbial populations in soil particles (Gams et al., 1987; Garrett, 1981). However, if a mycelial fungus is to be identified, the morphology of the structures that produce the fungal propagules must be observed. In the natural medium, these may be absent, inconspicuous or hardly visible. Thus, they can only be identified if the fungus can be induced to grow and multiply in a culture medium, or if some genetic regions can be amplified and sequenced. Isolation methods, which enable fungi to grow in pure culture, can be direct or indirect (Carlile et al., 2001). Direct methods transfer spores or mycelium from the natural substrate to a sterile culture medium, while indirect methods inoculate a fraction of the soil sample directly onto the culture medium, and the fungal colonies that develop are then transferred to a new culture medium (which may or may not be the same as the primary isolation medium). The techniques based on direct extractions, which consist of directly isolating from soil without culture media or substrates, are more complex and difficult to perform. The differences in size and density of fungal propagules enable them to be separated from soil particles or other microorganisms using centrifugation, decantation or flotation in density gradients (Davet and Rouxel, 2000).

### 1.2.3. Importance of the study and conservation of soil fungi

Several fungi are pathogens for humans, animals and plants. In humans, *Candida* spp. and *Aspergillus* spp. cause the most common opportunistic invasive mycoses that are associated with high rates of mortality in immunocompromised patients (Kück et al., 2014). Pathogenic fungi are usually prevalent in soils but are generally suppressed by high fungal diversity (Brussaard et al., 2007). Moreover, fungi pose the greatest threat of infection to animals and plants, being the cause of 72% and 64% of all extinctions, respectively, and the risk appears to be increasing (Fisher et al., 2012). In agriculture, filamentous fungi destroy over 125 million tons of rice, wheat, maize, potatoes and soybeans every year (Fisher et al., 2012). However, most fungi have a wide range of advantages for man. For example, interest in fungi as a food and feed is increasing, because they have considerable nutritional value, are regarded as a delicacy, and are used in a variety of processing industries to produce bakery foodstuffs, cheese and alcoholic beverages (Ghorai et al., 2009). Fungi can also produce a spectrum of highly beneficial drugs and antibiotics because they have a widespread metabolism and produce a wealth of bioactive compounds, mostly secondary metabolites such as antifungals, antibacterials and insecticidal agents. One example is the cephalosporin group of antibiotics produced by *Acremonium chrysogenum*, and the lovastatin which is a statin cholesterol-lowering drug produced by *Aspergillus terreus* (Kück et al., 2014). Fungi are also used in modern agriculture as agents for the bio-control of plant diseases (for example, *Trichoderma* spp. and *Gliocladium* spp.) (Singh and Sachan, 2013), the cosmetic industry (Hyde et al., 2010), bioremediation (Harms et al., 2011), and plant biomass degradation (van den Brink et al., 2013).

Bearing in mind that there are probably more than 5 million species of fungi, of which only about 99,000 have been described (Blackwell, 2011), surely there are numerous possibilities for discovering new fungal metabolites and new applications for these industrial needs. The first step in the investigation of biologically active compounds, or valuable organisms, is to isolate, identify and preserve them. The advantage of soil fungi is that the effort and cost involved in isolation, cultivation and preservation is minimum. Preserving fungi is also important because the earth's habitats and biotas are being lost or biologically impoverished, largely as a result of human action (Novacek and Cleland, 2001). This is happening to such an extent extinction rates are up 1000 times (Brooks et al., 2006).

### 1.2.4. Previous studies on soil fungi and current knowledge of soil ascomycetes

Adametz (1886), one of the pioneers of the study of soil fungi, isolated and named four species of yeast and eleven species of filamentous fungi. Subsequently, researchers from several countries gradually increased knowledge about soil mycobiota. Of these, Butler (1907), Hagem (1908), Jensen (1912), Oudemans and Koning (1912), Waksman (1916, 1917), Takahashi (1919), Chesters (1949) and Warcup (1959) are worthy of particular mention. However, the first important step in the study of soil fungi was in 1945, when Joseph C. Gilman published *A manual of soil fungi*, which reported about 600 species of fungi, about 200 of which belonged to the class Phycomycetes (not currently a valid class, and characterized by filamentous fungi with a coenocytic thallus that produced zygospores) and 32 to the class Ascomycetes (now Ascomycota). The remaining 385 were mitosporic (anamorphic) fungi. After this publication, which is the first compendium of soil fungi, an extensive series of monographs on the taxonomy and ecology of soil fungi were published. For example, Litvinov (1967) published a manual of soil fungi belonging to the order Moniliales (now invalid, mostly anamorphic Ascomycota) from the Soviet Union (USSR); Barron (1968) studied hyphomycetes (anamorphic fungi); and Domsch and Gams (1972), who described in detail 204 species belonging to the classes Ascomycetes, Basidiomycetes (now Basidiomycota), Deuteromycetes (anamorphic fungi), Oomycetes (not regarded as true fungi now) and Zygomycetes (a non valid class), including their geographic distribution, type of soil and/or the substrate on which they can develop, the optimal growth temperature, the use of certain nutrients, etc. The *Compendium of soil fungi* (Domsch et al., 1980), which picked up where a *Manual of soil fungi* left off, described 389 species, and is still frequently cited today. In the 1980s, Joseph von Arx published the monograph *Fungi sporulating in pure culture* (Arx, 1981), a reference work for identifying soil ascomycetes among other fungi, and Randolph S. Currah published *Taxonomy of the Onygenales: Arthrodermataceae, Gymnascaceae, Myxotrichaceae and Onygenaceae*, an exhaustive review of keratinolytic soil fungi (Currah, 1985). In the 1990s, the monographs by Abdel-aal H. Moubasher (1993) and Tsuneo Watanabe (1994) were published, although they are only of relative importance because of the limited geographical area taken into account. In this period Richard T. Hanlin also published three volumes of his work *Illustrated genera of Ascomycetes*, which describes and illustrates 200 genera of ascomycetes, and includes a dichotomous key for their identification (Hanlin, 1990, 1998a, 1998b). Particular emphasis should be given to the contributions made by Roy F. Cain, Paul F. Canon, Kouhei Furuya, Josep Guarro, David L. Hawksworth, Yoshikazu Horie, John C.

Krug, David W. Malloch, Takashi Matsushima, Robert A. Samson, Amelia C. Stolk and Shun-ishi Udagawa, who described a large number of new taxa for science. In Spain, several PhD theses have contributed to the knowledge of ascomycetes present in the soil of regions around the world by undertaking taxonomic studies, reassessing the previous taxonomy and describing new species (Punsola, 1985; Cano, 1989; Gené, 1994; Stchigel, 2000; Rodríguez, 2003; Solé, 2004; García, 2005; Madrid, 2011). The most recent monograph on soil-borne ascomycetes is the *Atlas of Soil Ascomycetes*, authored by Guarro et al. in 2012, and which discusses the sexual morph of ascomycetes reported in soil throughout the world since 2011, with their respective phenotypic and geographic distribution.

In recent years, the diversity, richness and distribution of fungal communities in different types of soil and geographical areas have been studied using new molecular tools based on high-throughput sequencing (for example, pyrosequencing, a non-electrophoretic method of DNA-sequencing). These studies have made it possible to make a real estimation of the number of species that exist and also increase our knowledge of soil fungi and ascomycetes. Two examples of this kind of study are those undertaken by Jumpponen et al. (2010) and Porras-Alfaro et al. (2011), who studied fungal communities from different types of soil. Figure 20 shows the distribution of ascomycetes found in tallgrass prairie soil. There are more than 14,000 fungal sequences distributed across Basidiomycota, Ascomycota, basal fungal lineages and Glomeromycota. The first of these two studies also investigated the vertical distribution of fungi. It found that the richness and diversity of fungal communities declines with soil depth, and that different communities are found in different strata. Despite this, some members are found in increasing numbers at greater depths. Some studies have also been made of fungi in different locations (for example, Orgiazzi et al. (2012) characterized soil fungal communities from different Mediterranean land uses). On the basis of the results of recent studies, we can conclude that fungal communities vary in diversity and abundance between geographical regions, soil types and even between strata in the same soil (different vertical distribution).

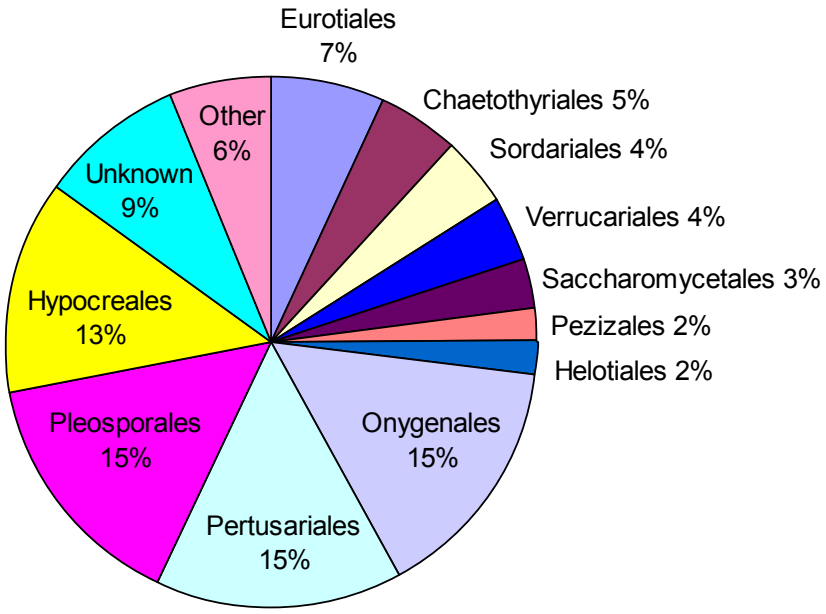



**Fig. 20** Distribution of ascomycetes by orders from a tallgrass prairie soil (adapted from Jumpponen et al., 2010).

Recently, several studies have been undertaken to determine how latitude, temperature and precipitation influence the composition of fungal communities and the relationships between the evolutionary history of soil fungi and their biogeographical patterns (Sun et al., 2012; Coince et al., 2014; Treseder et al., 2014).

# 2. INTEREST AND OBJECTIVES

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Interest in fungi is increasing because of their potential as producers of bioactive molecules that can be used in medicine, veterinary and plant pathologies, and chemical and pharmaceutical industrial processes. Also, because they are eukaryotic organisms, they have structures and a genome organization similar to that of plants and animals, so they are easily manipulated and the filamentous ascomycetes are models for studying the basic aspects of cell regulation. One example is the work done by George Beadle and Edward Tatum on *Neurospora crassa* that was awarded the Nobel Prize in Physiology and Medicine in 1958, and opened up a new research field, genetic biochemistry.

To date, about 99,000 fungal species have been described (Kirk et al., 2008). However, it was recently estimated that the approximate total is over five million species (Blackwell, 2011). Therefore, less than 2% of the extant fungi are known and thousands of species are waiting to be discovered.

A total of 65% of the species described are ascomycetes (Kirk et al., 2008). However, sexual morphs have hardly been investigated. They are difficult to isolate in culture because the dormancy of their ascospores needs to be interrupted if they are to germinate and produce colonies with fertile structures.

The soil is a huge reservoir for biodiversity, and fungi are the dominant eukaryotic lineage in terms of biomass (Mueller and Schmit, 2007). For both these reasons, it has been the subject of a great deal of research. Of the large number of studies on soil fungi, particular mention should be made of the recently published atlas that compiles all the information on taxonomy and geographical distribution for the sexual morphs of the phylum Ascomycota isolated from soil prior to 2011 (Guarro et al., 2012).

The development and improvement of molecular biology tools allowed us to reassess the classical taxonomy, previously based on phenotypic (mostly morphological) characters, and to build a less subjective taxonomic system.

Therefore, the main objectives of this thesis are:

- To isolate members of the phylum Ascomycota from a variety of soil samples using different selective techniques.
- To characterize phenotypically the noteworthy ascomycetes isolated from the soil samples processed.
- To establish the phylogenetic relationships among members of the Ascomycota, particularly between the isolates derived from this thesis and related taxa, by analysing nucleotide sequences of different loci.

- To apply a polyphasic approach to reassess the taxonomy of certain groups of the phylum Ascomycota.

# 3. MATERIALS AND METHODS

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## 3.1. FIELD TECHNIQUES

### 3.1.1. Origin of soil samples

Soil samples were collected in different surveys done mostly by the scientific staff of the Mycology Unit of the Faculty of Medicine (*Universitat Rovira i Virgili*) of Reus (FMR), Spain. The soil samples were collected in tropical and subtropical (Argentina, Colombia, Costa Rica) and in temperate (Chile, Spain and USA) regions of the world. The places selected for collection were chosen based on the poor knowledge or the absence of data about the soil-borne fungi in the literature. The collecting period spanned from 1997 to 2012, and a total of 140 samples were analyzed. Climatologic and biogeographic features of the sampled areas where the new taxa were recovered are described in detail in the “Materials and Methods” section of each article.

### 3.1.2. Sampling and preservation of soil samples

The collection of samples was accomplished by taking approximately 50−100 g of the most superficial layer of soil without the organic material (horizon A-$A_0$ ~1 cm deep). The samples were placed in sterile polyethylene bags, which were sealed with a rubber band and labelled. On returning to the laboratory, these were unsealed and stored at room temperature until processed.

## 3.2. LABORATORY TECHNIQUES

### 3.2.1. Methods for activation of latent spores

After collection, soil samples were processed in the laboratory to break down the dormancy of the resting spores and to induce their germination. The methods for the metabolic activation of dormant spores were based on subjecting a small amount of each soil sample to aqueous solutions of selected chemical agents, or heating a suspension of a small quantity of the soil sample in water for a short time (30 minutes).

#### 3.2.1.1. Spore activation using chemical agents

##### 3.2.1.1.1 Activation by acetic acid

Approximately 1 g of each soil sample was suspended in 5 mL of 5 % v/v acetic acid (Panreac, Barcelona, Spain), shaking vigorously for 5 min and left for 5 min. The liquid layer was removed by decantation and the solid residue was resuspended in 10 mL of sterile water and plated onto three Petri dishes of 9 cm diameter in equal volumes (approx. 3 mL each). 10–15 mL of melted potato carrot agar (PCA; grated potatoes, 20 g; grated carrot, 20 g; agar-agar, 20 g; distilled water, 1 L) with L-chloramphenicol (200 mg) and 1% w/v dieldrin™ in dimethyl-ketone (20 drops), which is used to inhibit the growth of bacterial colonies and the presence of mites, was placed on top of the soil suspension at 50□55 ºC and mixed by hand. All cultures were incubated at 15, 25 and 35 ºC (Stchigel, 2000).

##### 3.2.1.1.2. Activation by ethanol

Approximately 1 g of each soil sample was suspended in 5 mL of 65 % v/v ethanol (Panreac, Barcelona, Spain), shaking vigorously for 5 min and left for 5 min. The following steps were the same as those for the acetic acid activation technique (Stchigel, 2000).

##### 3.2.1.1.3. Activation by phenol

Approximately 1 g of each soil sample was suspended in 5 mL of 2 % w/v phenol (Panreac, Barcelona, Spain), shaking vigorously for 5 min and left for 5 min. The following steps were the same as those described for the activation by acetic acid (Stchigel, 2000).

#### 3.2.1.2. Activation by heat

Approximately 1 g of each soil sample was suspended in 10 mL of sterile water and homogenized by shaking vigorously. It was incubated in the water bath for 30 min at 60 °C. Once the incubation was completed, the content of the tube was shaken again and plated onto three 9 cm diameter Petri dishes. The following steps were the same as those in the activation techniques using chemical agents (Stchigel, 2000).

### 3.2.2. Examination and isolation of taxonomically interesting fungi

The fungal colonies that developed were examined under a stereo microscope (Cambridge Instruments Z45L). Several ascoma were taken using sterile hypodermic needles and deposited, usually on a drop of lactic acid (Panreac, Barcelona, Spain) or lactophenol (lactic acid, 20 g; phenol, 20 g; glycerol, 40 g; distilled water, 0.02 L), to a slide. When the presence of a capsule or gelatinous appendages, apparati at the apex of the asci, or dextrinoid / amyloid reactions were suspected, the mountings were also performed in distilled water, lactophenol cotton blue (Panreac, Barcelona, Spain) and Melzer reagent (chloral hydrate, 100 g; KI, 5 g; $I_2$, 1.5 g; distilled water, 0.1 L), respectively. The sexual reproductive structures (ascomata, asci and ascospores) were observed and, when the specimens resulted of taxonomic interest, small portions of the colony from the primary culture were transferred to a couple of 5 cm diameter Petri dishes containing oatmeal agar (OA; oatmeal flakes, 30 g; agar-agar, 20 g; distilled water, 1L) using a sterile needle. The incubation conditions were the same as in the original plate culture where the specimens were recovered. If the specimen of interest was contaminated with another fungi, before the inoculation onto OA, some ascomata were removed with a needle and decontaminated by immersion into a drop of 1% sodium hypochlorite (NaClO) solution for different time periods (1, 2 or 5 min), and then washed with sterile distilled water to remove residual hypochlorite.

### 3.2.3. Phenotypic identification of the ascomycetes isolated in pure culture

The specimens isolated in pure culture were identified according to the criteria established for each genus, through the study of the fungal colonies growing in different culture media and at different temperatures, and by the morphological characterization of the somatic and reproductive structures. For some taxa, a biochemical characterization was also carried out.

#### 3.2.3.1. Cultural characterization

For cultural characterization of the isolates of interest, the colonies were grown and incubated on different culture media (see below) at different temperatures (5, 15, 25, 35 and 40 ºC) and times (from two weeks to one month), depending on the nature of the taxa: Czapek's agar (Cz; Difco, Becton Dickinson, France), Czapek-Yeast Extract agar (CYA; $K_2HPO_4$, 1 g; Czapek's concentrated solution, 0.01 L; extract or autolyzed yeast, 5 g; sucrose, 30 g; agar-agar, 15 g; distilled water, 1 L), malt extract agar (MEA; meal extract, 20 g; agar-agar, 20 g; distilled water, 1 L), and malt extract agar with 40%

sucrose (MEA 40%; meal extract, 20 g; sucrose, 400 g; agar-agar, 20 g; distilled water, 1 L), for members of the order Eurotiales; phytone yeast extract agar (PYE; papaic digest of soybean meal, 10 g; yeast extract, 5 g; dextrose, 40 g; streptomycin, 0.03 g; chloramphenicol, 0.05 g; agar-agar, 17 g; distilled water, 1 L), for members of the order Onygenales; and OA, PCA and potato dextrose agar (PDA, Pronadisa, Madrid, Spain), for ascomycetes in general. Colour notations were taken from the "Methuen handbook of colour" (Kornerup and Wanscher, 1984). The cultural characteristics were described every week up to one month, using the assistance of a stereomicroscope. An example of this is shown in Figure 21. The features of interest were:

- Size of the colony (diameter in millimeters)
- Shape/Form (lobulate, punctiform, circular, irregular, filamentous, rhizoid)
- Colour of the surface and the reverse
- Surface (smooth, veined, rough, cerebriform, radially folded, dull, wrinkled / shriveled, glistening)
- Texture (mucoid, brittle, butyrous, dry, coriaceus, felted/felty, velvety, fasciculate, floccose, cottony, woolly, powdery, granulose)
- Topography (flat, raised, convex, pulvinate, umbonate, crateriform)
- Margins (regular, fringed, arachnoid, fimbriate, undulate, lobate, filamentous, curled, filiform)
- Growing zones (bands, sectors, concentric circles)
- Presence of exudates
- Presence of fertile ascomata

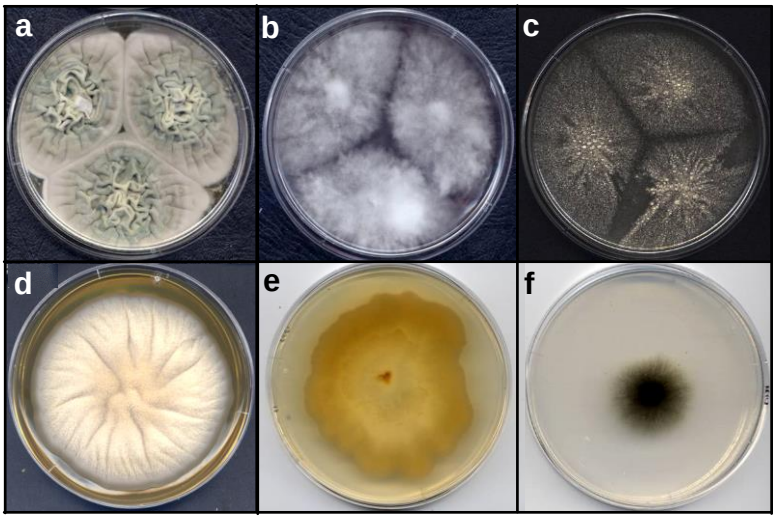


**Fig. 21** Different types of colonies. a. velvety, cerebriform. b. cottony, raised. c. granulose, flat. d. woolly, radially folded, crateriform. e. lobulate, margins regular. f. filamentous, margins fringed.

### 3.2.3.2. Biochemical tests

The following biochemical tests were carried out for some taxa belonging to the Onygenales studied during the development of this thesis:

- Resistance to cycloheximide was evaluated by growing on Mycosel agar (MYC; papaic digest of soybean meal, 10 g; dextrose, 10 g; cycloheximide, 0.4 g; chloramphenicol, 0.05 g; agar-agar, 15.5 g; distilled water, 1 L).
- Growth rate, and alkalinization or acidification was tested on bromcresol purple milk solids glucose agar (BCP-MS-G; skim milk powder, 40 g; glucose, 20 g; agar-agar, 15 g; bromocresol purple solution [bromocresol purple, 0.16 g; ethanol 95 % solution, 10 mL] 1 mL; distilled water, 1L).The acidification of the medium is observed by a colour change, from bluish to yellow, and the alkalinization, from bluish to deep purple.
- Urease production on Christensen's urea agar (Merck, Darmstadt, Germany). The splitting of the urea by urease produces a colour change of the medium, from yellow or yellowish-orange to fuchsia.
- Lipase activity on Tween 80 opacity test medium (TOTM; bacteriological peptone, 10 g; NaCl, 5 g; $CaCl_2$, 0.1 g; Tween 80, 5 ml; agar-agar, 15 g; distilled water, 1 L). The lipase activity is observed by opacity around the colony.
- Keratinolytic capability was evaluated by culturing the fungus on blonde, sterile, prepubesence child's hair on tap water agar (TWA; agar-agar, 20 g; tap water, 1 L) in a 9 cm diameter Petri dish, incubated for four weeks at 25 ºC in darkness, and examining the hairs under a bright-field microscope by mounting them in a drop of 20% KOH.

### 3.2.3.3. Morphological characterization

The main features of the vegetative and reproductive structures are follows:

- Hyphae: colour, shape, diameter, presence of septa and anastomosis.
- Ascomata: kind, colour, shape, size, initial forms; presence, kind, colour, shape, ornamentation and size of peridial hairs; thickness, number of layers and texture of the peridium; colour, shape, ornamentation and size of peridial cells; presence of paraphyses and periphyses and their colour, shape, branching pattern and disposition of the septa.
- Asci: number of ascospores per ascus, shape and size, longevity (evanescent or persistent), presence of apical structures and staining reactions (amyloids, non-amyloids or dextrinoid), presence and size of stipe.

- Ascospores: colour, shape and size, cell wall ornamentation, disposition in the asci, presence and number of septa and their disposition; presence, number and position of germ pores or furrows; presence of a mucilaginous layer or a sheath; presence, disposition, shape and size of mucilaginous appendages.
- Anamorph: kind, colour, shape, size and ornamentation of the conidiophores and conidiogenous cells; kind of conidiogenesis; colour, shape, size, ornamentation, disposition and number of cells of the conidia.
- Chlamydospores: presence, colour, shape, size, ornamentation and disposition.

The descriptions of the new taxa, found during the development of this doctoral thesis, were also deposited in MycoBank (http://www.mycobank.org/).

#### 3.2.3.3.1. Microscopic mountings

For microscopic examination and characterization of the fungal structures, the techniques listed below have been used:

- Direct mounting: reproductive structures from a colony of the fungus of interest were removed using a hypodermic needle, and deposited onto a drop of mounting medium previously deposited in a slide. Then, it was covered by a coverslip and sealed with nail polish to prevent the drying of the preparation.
- Microculture: into a Petri dish containing a culture medium suitable for fungal growth, blocks of 1 $cm^2$ were cut and deposited aseptically on the surface of tap water agar (TWA). Subsequently, the four corners of the blocks were inoculated and each block covered with a sterile coverslip. When the fertile structures of the fungus were observed, the coverslip was carefully removed from the agar block and placed onto a drop of mounting medium previously deposited in a slide (Fig. 22).

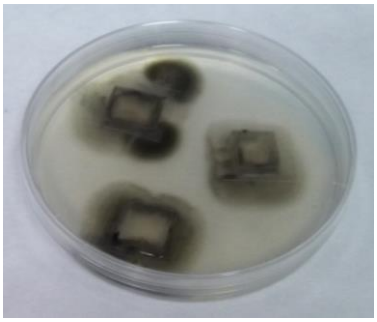

**Fig. 22** Example of microculture

## 3.2.4. Microscopic examination of the specimens

Light field (with Nomarski and phase contrast condensers) and scanning electron microscopes were used to study in detail and to document the characteristics (including the measures) of the vegetative and reproductive structures of the specimens for their identification.

### 3.2.4.1. Light field microscopy

To study the morphological features of the taxonomically interesting taxa, the following microscopes were used:

- Olympus BH2
- Olympus CH2
- Zeiss Axio Imager M1

Images captation of the fungal structures were taken by Zeiss Axio Imager M1 light microscope.

### 3.2.4.2. Scanning electron microscopy (SEM)

In order to study more in detail the ornamentation of several fungal structures particularly of conidia and ascospores, the specimens were studied under scanning electron microscope (SEM) following the protocol described in Figueras and Guarro (1988 a, b) with some modifications (Stchigel 2000). A scanning electron microscope Jeol JSM-6400 of the Scientific-Technique Service of the University Rovira i Virgili (Tarragona, Spain) was employed. The working distance to the sample varied from 8 to 39 mm, and the applied voltage ranged between 10 and 20 keV.

## 3.2.5. Molecular study

The molecular study was carried out to corroborate the morphological identification of the specimens, to assess the taxonomical placement of *insertae sedis* taxa, and to clarify the phylogenetic relationships of the fungi of taxonomic interest with their relatives.

### 3.2.5.1. DNA extraction

DNA extraction was performed directly from fungal colonies on PDA incubated at 25 °C during a period of time to obtain enough fungal material (three days to two weeks). Young colonies were used to minimize the presence of pigments which might inhibit the

following PCR reactions. We used the Fast DNA kit protocol (Bio 101, Inc., Joshua Way, Vista, California, USA) according to the manufacturer.

### 3.2.5.2. Amplification and sequencing

Amplification and sequencing of the 5.8S nrRNA gene and the internal transcribed spacers 1 and 2 (ITS region) were performed with the primer pair ITS5/ITS4 as described by White et al. (1990). The D1 and D2 domains of the 28S large subunit of the nrRNA gene (D1−D2) were amplified using primers NL1/NL4 following the protocol of O`Donnell (1993). A larger fragment of the 28S which includes the D1, D2 and D3 domains (D1−D3) was amplified with the LR0R/LR5 pairs of primers following the protocol published by Vilgalys and Hester (1990). Amplification and sequencing of the 18S small subunit of the nrDNA gene was performed with the NS1/NS4 primer pair, according to Wu et al. (2002). A fragment of the actin (*ACT*) was amplified and sequenced with the Act-1/Act-4R pairs of primers following the protocols published by Voigt and Wöstemeyer (2000), and a fragment of the calmodulin (*CAL*) using the Cmd5/Cmd6 primer pair according to Hong et al. (2005). The fragments of the RNA polymerase II largest subunits (*RPB1* and *RPB2*) and of the putative chaperonin complex related to TCP-1 (*Cct8*) were amplified with primer pairs RPB1-F1843/RPB1-R3096, RPB2-5F/RPB2-7R and Cct8-F660/Cct8-R1595, respectively (Houbraken and Samson, 2011). Amplification of two different fragments of the β-tubulin (*BT2*) and of elongation factor (*EF1*) genes was performed with the Bt2a/Bt2b and BT2916/BT1819R primer pairs according to Glass and Donaldson (1995) and EF983F/EF2218R and EF-1H/EF-2T according to Sung et al. (2007) and O'Donnell (2000), respectively. The nucleotidic sequences of the primers indicated in this paragraph and the size of the amplification product are shown in the table 2. The PCR reaction was carried out in a final volume of 40 µL that contained 20 a 60 ng of genomic DNA, 10X PCR Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), forward and reverse primers in a concentration of 10 µM, $MgCl_2$ 50 mM, dNTP´s 0.2 mM (of each nucleotide), Taq DNA polimerase 5 U/µL (Invitrogen, Holanda), all diluted in sterile miliq water. The amplification program was: pre denaturation at 94 ºC, 5 min; 35 cycles of: 95 ºC, 30 s (denaturation), variable temperature depending on the gene (Table 2), 1 min (annealing) and 72 ºC, 1.5 min (extension); final extension at 72 ºC, 7 min.

**Table 2.** Details of the primers used to amplify and sequence the genes used in the molecular studies of this thesis.

| Locus | Primer forward (5' → 3') | Primer reverse (5' → 3') | Annealing T[a] (ºC) | Product size (pb) |
|---|---|---|---|---|
| ITS | ITS 5<br>GGAAGTAAAAGTCGTAACAAGG | ITS 4<br>TCCTCCGCTTATTGATATGC | 53 | 650–750 |
| D1-D2 | NL1<br>GCATATCAATAAGCGGAGGAAAAG | NL4<br>GGTCCGTGTTTCAAGACGG | 53 | 600–650 |
| D1-D3 | LROR<br>ACCCGCTGAACTTAAGC | LR5<br>TCCTGAGGGAAACTTCG | 53 | 800–900 |
| SSU | NS1<br>GTAGTCATATGCTTGTCTC | NS4<br>CTTCCGTCAATTCCTTTAAG | 53 | 1,050–1,100 |
| *ACT* | Act-1<br>TGGGACGATATGGAIAAIATCTGGCA | Act-4R<br>TCITCGTATTCTTGCTTIGAIATCCACAT | 56 | 850–950 |
| *BT2* | Bt2a<br>GGYAACCARATHGGTGCYGCYTTC | Bt2b<br>ACCCTCRGTGTAGTGACCCTTGGC | 58–60 | 450–500 |
| | BT1819R<br>TTCCGTCCCGACAACTTCGT | BT2916<br>CTCAGCCTCAGTGAACTCCAT | 50–55 | 900–1,050 |
| *CAL* | Cmd5<br>CCGAGTACAAGGAGGCCTTC | Cmd6<br>CCGATAGAGGTCATAACGTGG | 55–58 | 550–650 |
| *Cct8* | Cct8-F660<br>GIGTKGTBAAGATCATGGGWGG | Cct8-R1595<br>RTCMACRCCNGTIGTCCAGTA | 50 | 700–850 |
| *EF1* | EF983F<br>GCYCCYGGHCAYCGTGAYTTYAT | EF2218R<br>ATGACACCRACRGCRACRGTYTG | 58–60 | 900–1,050 |
| | EF-1H<br>ATGGGTAAGGARGACAAGAC | EF-2T<br>GGARGTACCAGTSATCATGTT | 53 | 500–650 |

| | | | | |
|---|---|---|---|---|
| *RPB1* | RPB1-F1843<br>ATTTYGAYGGTGAYGARATGAAC | RPB1-R3096<br>GRACRGTDCCRTCATAYTTRACC | 49 | 850–950 |
| *RPB2* | RPB2-5F<br>GGGGWGAYCAGAAGAAGGC | RPB2-7R<br>CCCATRGCTTGYTTRCCCAT | 56–62 | 1,000–1,100 |

Once obtained a correct amplification following the protocols mentioned for each locus and verified using agarose gel electrophoresis at 2% (Pronadisa, Madrid, Spain), followed by RedSafe DNA Stain (iNtRON Biotechnology, Seoul, Korea) staining, the amplified products were sent to Macrogen (Netherlands and Korea) for sequencing these using a 3730 XL DNA analyzer (Applied Biosystems).

The software SeqMan version 7.0.0 (Lasergene, Madison, Wisconsin) were used to obtain consensus sequences from the complementary sequences of each isolate.

#### 3.2.5.3. Molecular identification

BLAST searches (Altschul et al., 1990) were performed to compare molecular data of the isolates studied with those of other fungi deposited in the GenBank database (http://blast.ncbi.nlm.nih.gov). The genes usually used were 28S and ITS.

#### 3.2.5.4. Sequence alignment

Nucleotide sequence alignments were performed with ClustalX version 1.81 (Thompson et al. 1997), followed by manual adjustments with MEGA5 and MEGA6 (Tamura et al. 2011, 2013) or with a text editor.

#### 3.2.5.5. Phylogenetic analyses

For the phylogenetic inference, the following methods were used:

- Neighbor-joining (NJ) and Maximum likelihood (ML): trees were inferred using MEGA5 and MEGA 6 (Tamura et al. 2011, 2013), with partial deletion of gaps, the substitution model proposed by the program, and 1000 bootstrap replicates. Bootstrap support values ≥ 70% were considered significant.
- Maximum parsimony (MP): this analyses were performed using the PAUP* version 4.9b10 software (Swofford, 2002), obtaining the trees after 100 heuristic searches with random sequence addition and tree bisection-reconnection branch-swapping algorithms, collapsing zero-length branches and saving all minimal length trees. The robustness of branches was assessed by bootstrap analysis of 1000 replicates, considering significant bootstrap support values ≥ 70%.
- Bayesian inference (BI): the bayesian analyses were performed in the MrBayes (version 3.1) computer program (Huelsenbeck and Ronquist, 2001), running 1.000.000 generations in four chains, saving the trees every 100 generations. The 25% of all trees obtained were used to construct a 50% majority-rule consensus tree. Bayesian posterior probability scores ≥0.95 were considered significant.

#### 3.2.5.6. Archive of nucleotide sequences and alignments

The sequences generated during our research were deposited in the European Molecular Biology Larboratory (EMBL) (http://www.ebi.ac.uk/embl/Submission/) and in the The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The alignments used in the cladistic analyses were deposited in TreeBASE (www.treebase.org).

### 3.2.6. Conservation of the isolates of interest

The fungi isolated in pure culture were preserved using different methods of conservation and in different culture collections.

#### 3.2.6.1. Conservation in slants

Melted PCA, PDA and OA were introduced aseptically into polyethylene sterile tubes and inclined to form a slant after sealed by a plastic cap. Once the medium gelled and the control of sterility passed, the slants were inoculated with the fungus of interest. The fungi were grown at optimum temperature in an incubator (Selecta, Barcelona, Spain) during the time required for the ascomata formation. Finally, to guarantee the conservation, the cultures were totally covered with sterile mineral oil (petrolate or liquid paraffin), and preserved at room temperature.

#### 3.2.6.2. Conservation in sterile water

Colony blocks about 5 $mm^2$ of sporulated cultures on agar medium were transferred to glass vials containing 5 mL of sterilized distilled water. Then they were stored at room temperature.

#### 3.2.6.3. Conservation by lyophilisation

The strains were cultured on agar medium to obtain abundant sporulation. The colonies were scraped and mixed with 3 mL of a 10% skim milk solution (Difco, USA), a cryoprotectant, which was previously sterilized at 115 °C for 15 min. The plates were slightly agitated and, then 1 mL of this solution was dispensed in 3 mL sterile glass vials and placed in the lyophilizer (Advantage 2.0 Series; Virtis Company Gardiner, USA). Sublimation was achieved when the temperature of the condenser reached 45° C, and after the vacuum of 200 mTorr was made, the following lyophilization cycle was

performed: -30° C (240 min), -10ºC (240 min), +10 °C (300 min), and +30°C (300 min). After the process was complete, the vials were sealed under vacuum and stored at room temperature.

#### 3.2.6.4. Dried cultures

The holotypes, which consisted of the primary isolate of the new taxon, were dried in a heater at 45–55 °C for several days. These were sent for deposit to the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. The isotypes (cultures derived from the type culture, which were also dried in the heater) where deposited in the Faculty of Medicine, Reus (FMR), Tarragona, Spain.

#### 3.2.6.5. Preservation of living cultures

The living cultures of the new or interesting taxa were deposited in the following collections:

- Biological Resource Center (NBRC), Japan
- Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands
- Faculty of Medicine, Reus (FMR)

# 4. RESULTS AND DISCUSSION

# 4. RESULTS AND DISCUSSION

The fungal taxonomy was traditionally based on the description and comparison of the reproductive (asexual and sexual) and vegetative structures and, secondary, on cultural characterization of the specimens. However, with the advent of molecular techniques (the PCR-based amplification of genomic loci and their sequencing) in the 1990s, it has changed considerably. Molecular studies have revealed that the use of morphological features to infer phylogenetic relationships between the taxa included in certain families of the Ascomycota can lead to erroneous conclusions because, as was mentioned previously, these taxonomic criteria have some disadvantages. Because of the building of a stable taxonomic system implies that it must be as objective as possible, both molecular and morphological data must be taken into account. Greater use is also being made of polyphasic studies, which combine morphological and molecular data with physiological and nutritional abilities, ecological and biogeographic information, and the analysis of extrolites profiles.

During the developing of this thesis, a total of 171 ascomycetes were isolated in pure culture and morphologically characterized and presumptively identified, amplifying and sequencing the domains D1–D3 of the 28S nrRNA gene and the ITS region of the nrRNA of 123 of them (Table 3). Both loci were used to verify the morphological identification by BLAST search, and also to display their phylogenetic relationships with other Ascomycota. Occasionally other loci were amplified and sequenced when the phylogeny obtained using the ribosomal genomic regions did not have sufficiently resolution, or to reinforce that. Other type and reference strains were obtained from different collections to incorporate in the phylogenetic analysis (Table 4).

A phylogenetic analysis of the D1–D3 sequences of all the isolates molecularly studied and of the reference strains for other collections was carried out to verify their taxonomic placement and study the phylogenetic relationships among them. The length of the amplicon used was 821 bp, of which 344 bp were parsimony informative. Figure 23 shows the Maximum Likelihood tree inferred.

In the phylogenetic study, our isolates were located in five well-supported main clades, belonging to the classes Dothideomycetes (100% bs / 1 pp), Eurotiomycetes (97% bs / 1 pp), Leotiomycetes (93% bs / - pp), Sordariomycetes (100% bs / 1 pp) and Pezizomycetes (100% bs / 1 pp). We observed that a large number of isolates were located in Eurotiomycetes and Sordariomycetes (43% and 47% of the isolates, respectively), which suggested that the techniques for spore activation employing chemical agents and heat were especially useful for recovering such taxa.

Our molecular and phenotypic results (developed below) provided new and noteworthy taxonomic/systematic information about certain known taxa and led us to propose 5 new genus, 13 new species and 47 new combinations.

**Table 3.** Isolates sequenced during the development of the thesis.

| Taxa | FMR | Origin | GenBank accession # | |
|---|---|---|---|---|
| | | | ITS | LSU |
| *Achaetomium* sp. | 13001 | Argentina, Iguazu National Park | | |
| *Anthracobia muelleri* | 13609 | Spain, Gran Canaria, Barranco Laurel | | |
| *Aphanoascella galapagosensis* | 12019 | Lesions on the carapace of a Galapagos tortoise, South Texas zoological collection | JQ864081 | JQ864082 |
| *Apiosordaria backusii* | 12439 | Tennessee, Great Smoky Mountains National Park | | KP981423 |
| *Apiosordaria backusii* | 13591 | Spain, Tarragona, “Els Gorgs de la Febró” | | KP981424 |
| *Arnium* sp. | 13412 | India, Gualior | | KP981428 |
| *Aspergillus auratus* | 11604 | Colombia, Bogotá, Natural Park | | |
| *Aspergillus auratus* | 11802 | Colombia, Isla de los Micos | | |
| *Aspergillus fischeri* | 11606 | Colombia, Bogotá, Natural Park | | |
| *Aspergillus fischeri* | 11804 | Colombia, Isla de los Micos | | |
| *Aspergillus fischeri* | 11803 | Colombia, Bogotá, Natural Park | | |
| *Aspergillus fischeri* | 11860 | Colombia, Bogotá, Natural Park | | |
| *Aspergillus fischeri* | 11862 | Colombia, Leticia, “Parque de los Loros” | | |
| *Aspergillus fischeri* | 11912 | Colombia, Leticia, “Parque de los Loros” | | |
| *Aspergillus fischeri* | 11921 | Colombia, Leticia, “Parque de los Loros” | | |
| *Aspergillus fischeri* | 12006 | Argentina, Misiones, Iguazu National Park | | |
| *Aspergillus fischeri* | 13506 | Argentina, Posadas, Alberto Roth botanical garden | | |
| *Aspergillus fischeri* | 13512 | Argentina, Misiones, Iguazu National Park | | |
| *Aspergillus fischeri* | 11922 | Colombia, Leticia, “Parque de los Loros” | | |
| *Aspergillus fischeri* | 11923 | Colombia, Leticia, “Parque de los Loros” | | |
| *Aspergillus fischeri* | 11924 | Colombia, Bogotá, Natural Park | | |
| *Aspergiilus fischeri* | 13512 | Argentina, Misiones, Iguazu National Park | | |
| *Aspergillus laciniosus* | 11807 | Colombia, Bogotá, Natural Park | | |
| *Aspergillus laciniosus* | 13504 | Argentina, San Ignacio | | |
| *Aspergillus laciniosus* | 13515 | Argentina, Posadas, Alberto Roth botanical garden | | |
| *Aspergillus quadricinctus* | 13511 | Spain, Mallorca, Trenc | | |
| *Aspergillus tsunodae* | 13605 | Colombia, Bogotá, Natural Park | | |
| *Aspergillus nidulans* | 13507 | Spain, Malaga, “Sierra de las Nieves” | | |
| *Aspergillus posadasensis* | 12168 | Argentina, Posadas, Alberto Roth botanical garden | HG529483 | HG529485 |
| *Aspergillus posadasensis* | 12322 | Argentina, Posadas, Alberto Roth botanical garden | HG529484 | HG529486 |
| *Aspergillus tatenoi* | 11735 | Colombia, Leticia, “Parque de los Loros” | | |
| *Aspergillus tatenoi* | 12010 | Argentina, Misiones, Iguazu National Park | | |
| *Aspergillus tsunodae* | 13605 | Argentina, Misiones, Iguazu National Park | | |

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|---|---|---|---|---|
| *Auxarthron longisporum* | 12768[I] | Portugal, Beja, Castro Verde | HG326873 | HG326874 |
| *Auxarthron ostraviense* | 14372 | Colombia. Amacayacu | | |
| *Auxarthron umbrinum* | 13614 | Spain, Madrid | | |
| *Chaetomidium arxii* | 12364 | Spain, Gran Canaria | KP204014 | |
| *Chaetomium bostrychodes* | 12161 | Colombia, Leticia, “Parque de los Loros” | | |
| *Chaetomium brasiliense* | 11849 | Colombia, Leticia, “Parque de los Loros” | | |
| *Chaetomium flavigenum* | 13488 | Tennessee, Great Smoky Mountains National Park | | |
| *Chaetomium fusiforme* | 13491 | Colombia, Leticia, “Parque de los Loros” | | |
| *Chaetomium gracile* | 13489 | Tennessee, Great Smoky Mountains National Park | | |
| *Chaetomium indicum* | 13610 | Tennessee, Great Smoky Mountains National Park | | |
| *Chaetomium megasporum* | 11909 | Colombia, Leticia, “Parque de los Loros” | | |
| *Chaetomium robustum* | 12163 | Spain, Gran Canario, Pico de Osorio | | |
| *Chaetomium robustum* | 13487 | Tennessee, Great Smoky Mountains National Park | | |
| *Chaetomium spiralotrichum* | 12004 | Spain, Gran Canaria, Pico de Osorio | | |
| *Corynascella inaequalis* | 12440 | Spain, Gran Canaria | | |
| *Corynascus fumimontanus* | 12372[I] | Tennessee, Great Smoky Mountains National Park | LK932694 | LK932706 |
| *Corynascus verrucosus* | 12369 | Tennessee, Great Smoky Mountains National Park | LK932699 | LK932704 |
| *Corynascus verrucosus* | 12783 | Tennessee, Great Smoky Mountains National Park | LK932695 | LK932705 |
| *Diplogelasinospora moalensis* | 13034[I] | Principado de Asturia, Cangas del Narcea, Veiga de Rengos, Moal | HG514152 | KP981430 |
| *Diplogelasinospora princeps* | 12784 | Tennessee, Great Smoky Mountains National Park | | |
| *Diplogelasinospora princeps* | 13414 | Tennessee, Great Smoky Mountains National Park | | KP981431 |
| *Diplogelasinospora princeps* | 13415 | Tennessee, Great Smoky Mountains National Park | | KP981432 |
| *Diplogelasinospora princeps* | 13665 | Spain, Galicia, Betanzos, Mandeo river | | |
| *Emmonsiellopsis coralliformis* | 4024[T] | Spain, Girona, Empuriabrava | KP101585 | KP101585 |
| *Emmonsiellopsis terrestris* | 4023 | Spain, Girona, Estartit | KP101582 | KP101583 |
| *Hamigera avellanea* | 11805 | Colombia, Leticia, “Parque de los Loros” | | |
| *Hamigera avellanea* | 13505 | Argentina, Misiones, Iguazu National Park | | |
| *Hamigera avellanea* | 13513 | Tennessee, Great Smoky Mountains National Park | | |
| *Hamigera paravellanea* | 11605 | Colombia, Bogotá, Natural Park | | |
| *Hamigera paravellanea* | 13503 | Tennessee, Great Smoky Mountains National Park | | |
| *Hapsidospora irregularis* | 13607 | Spain, Mallorca, Sa Calobra | | |
| *Jugulospora rotula* | 12428 | Tennessee, Great Smoky Mountains National Park | | KP981436 |
| *Jugulospora rotula* | 12690 | North Carolina, Great Smoky Mountains National Park | | KP981437 |
| *Jugulospora rotula* | 12781 | Tennessee, Great Smoky Mountains National Park | | KP981438 |
| *Lasiobolidium orbiculoides* | 13606 | Chile | | |
| *Leiothecium cristatum* | 11998 | Argentina, Misiones, Iguazu National Park | KF732838 | HG529487 |

| | | | | |
|---|---|---|---|---|
| *Leiothecium ellipsoideum* | 11997 | Argentina, Misiones, Iguazu National Park | | |
| *Leucothecium emdenii* | 12366 | Tennessee, Great Smoky Mountains National Park | | |
| *Medusitheca citrispora* | 12767[I] | North Carolina, Great Smoky Mountain National Park, Cartoogechaye Creek Campground | KP981477 | KP981453 |
| *Microthecium fayodii* | 12363 | Tennessee, Great Smoky Mountains National Park | KP981482 | KP981460 |
| *Microthecium fimicola* | 5483 | Australia, Moara | KP981485 | KP981463 |
| *Microthecium fimicola* | 12370 | Tennessee, Great Smoky Mountains National Park | KP981486 | KP981464 |
| *Microthecium fimicola* | 13148 | Spain, Aragon, Los Valles Occidentales | KP981487 | KP981465 |
| *Microthecium japonicum* | 12371 | Spain, Gran Canaria, Pico de Osorio | KP981488 | KP981467 |
| *Microthecium levitum* | 6218 | Nepal, Bhadgaon | KP981489 | KP981468 |
| *Microthecium levitum* | 10098 | Nigeria, Enugu, Nsukka | KP981490 | KP981469 |
| *Microthecium levitum* | 13884 | Spain, Catalonia, Vall Fosca | KP981491 | KP981470 |
| *Microthecium* sp. | 6725 | Egypt, Sinai | KP981494 | KP981474 |
| *Microthecium* sp. | 7183 | New South Wales, Sydney, Blue Mountains | KP981495 | KP981475 |
| *Microthecium* sp. | 12373 | Forest soil, USA, North Carolina, Great Smoky Mountain | | |
| *Myxotrichum* sp. | 13210 | Spain, Navarra, Baraibar | | |
| *Myxotrichum stipitatum* | 13122 | Spain, Galicia, Betanzos, Mandeo river | | |
| *Myxotrichum stipitatum* | 13664 | Spain, Galicia, "Parque natural de Olveira" | | |
| *Naviculispora terrestris* | 10060[I] | Argentina, Tucumán province, Tafí del Valle | | KP981439 |
| *Neurospora dictyophora* | 12886 | Tennessee, Great Smoky Mountains National Park | | |
| *Neurospora novoguineensis* | 12697 | Argentina, Posadas, Alberto Roth botanical garden | | |
| *Neurospora indica* | 12429 | Tennessee, Great Smoky Mountains National Park | | |
| *Paecilomyces niveus* | 11806 | Colombia, Leticia, "Parque de los Loros" | | |
| *Podospora setosa* | 12787 | Spain, Gran Canaria | | KP981441 |
| *Preussia flaganni* | 13603 | Spain, Gran Canaria, Los Picos de Gáldar | | |
| *Preussia funicola* | 13611 | Argentina, Posadas, Alberto Roth botanical garden | | |
| *Preussia funicola* | 14373 | Argentina, Posadas, Alberto Roth botanical garden | | |
| *Pseudallescheria fusoidea* | 12001 | Colombia, Leticia, "Parque de los Loros" | | |
| *Pseudallescheria fusoidea* | 13416 | Spain, Tarragona, Prades | | |
| *Pseudeurotium ovale* | 13600 | Tennessee, Great Smoky Mountains National Park | KP686192 | KP686193 |
| *Pseudoneurospora canariensis* | 12156[T] | Spain, Gran Canaria, Pico de Osorio | | HG326871 |
| *Pseudoneurospora canariensis* | 12323 | Spain, Gran Canaria, Pico de Osorio | | HG326872 |
| *Oidiodendron flavum* | 13883 | Spain, Galicia, Betanzos, Mandeo river | | |
| *Rinaldiella pentagonospora* | 12018[T] | Contaminated human lesion, USA, Georgia, Dahlonega | KC702789 | KP981442 |
| *Sordaria prolifica* | 13599 | Spain, Mallorca, Cap de les salines | | |
| *Talaromyces bacillisporus* | 11600 | Colombia, Bogotá, National Park | | |

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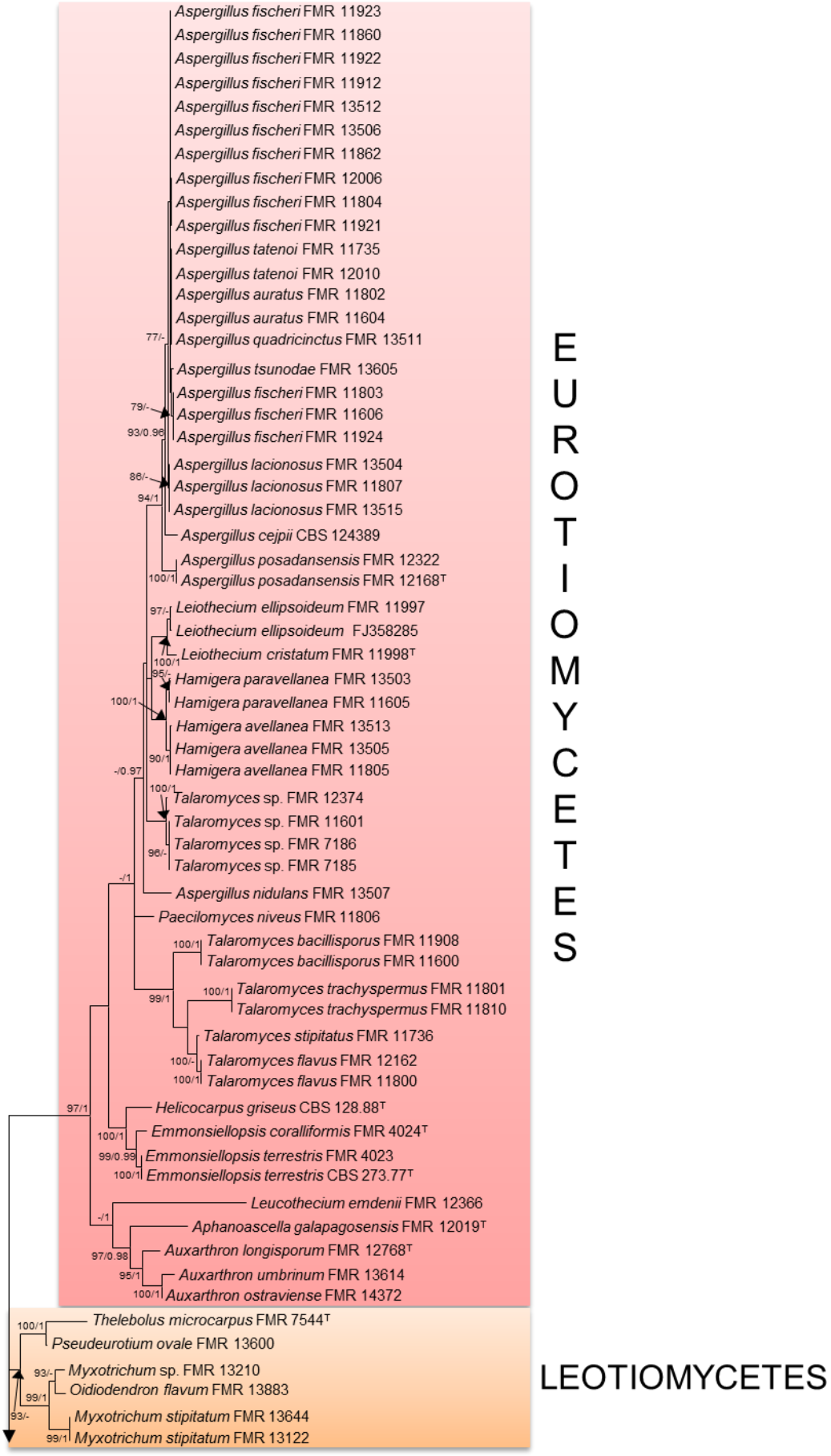
|---|---|---|---|---|
| *Talaromyces bacillisporus* | 11908 | Colombia, Leticia, “Parque de los Loros” | | |
| *Talaromyces flavus* | 11800 | Colombia, Bogotá, National Park | | |
| *Talaromyces flavus* | 12162 | Colombia, Leticia, “Parque de los Loros” | | |
| *Talaromyces* sp. | 7185 | Australia, Sidney, Blue Montains | | |
| *Talaromyces* sp. | 7186 | Spain, Zamora, Sanabria lake | | |
| *Talaromyces* sp. | 11601 | Colombia, Bogotá, National Park | | |
| *Talaromyces* sp. | 12374 | Spain, Gran Canaria, Pico de Osorio | | |
| *Talaromyces stipitatus* | 11736 | Colombia, Isla de los Micos | | |
| *Talaromyces trachyspermus* | 11801 | Colombia, Leticia, “Parque de los Loros” | | |
| *Talaromyces trachyspermus* | 11810 | Colombia, Leticia, “Parque de los Loros” | | |
| *Thelebolus microcarpus* | 7544 | Argentina, Tucumán, Tafí el Valle, Abra del Infiernillo | LN609269 | LN609269 |
| *Thermothelomyces heterothallica* | 5174 | Spain | | LK932692 |
| *Thermothelomyces heterothallica* | 5175 | Spain | | LK932693 |
| *Thermothelomyces heterothallica* | 13215 | Tennessee, Great Smoky Mountains National Park | LK932697 | LK932703 |
| *Thielavia terricola* | 12785 | Tennessee, Great Smoky Mountains National Park | | |
| *Thielavia terricola* | 12786 | Spain, Gran Canaria | LK932696 | |
| *Warcupia terrestris* | 12689 | Tennessee, Great Smoky Mountains National Park | | |
| *Zopfiella longicaudata* | 12365 | Tennessee, Great Smoky Mountains National Park | | KP981448 |
| *Zopfiella longicaudata* | 12782 | Spain, Gran Canaria | | KP981449 |

**Table 4.** Type and reference strain obtained from different collections sequenced by us.

| Taxa | Strain | GenBank accession # | |
|---|---|---|---|
| | | ITS | LSU |
| *Annulispora ellipsospora (=Sphaerodes ellipsospora)* | NBRC 31376$^{T}$ | | KP981451 |
| *Annulispora singaporensis (=Sphaerodes singaporensis)* | NBRC 30865$^{I}$ | | KP981452 |
| *Apiosordaria sacchari* | CBS 713.70 | | KP981425 |
| *Apiosordaria striatispora* | CBS 154.77$^{I}$ | | KP981426 |
| *Apiosordaria vermicularis* | CBS 303.81$^{T}$ | | KP981427 |
| *Aspergillus cejpii* | CBS 124389 | | |
| *Cercophora mirabilis* | CBS 120402 | | KP981429 |
| *Corynascella humicola* | CBS 337.72$^{T}$ | | |
| *Corynascella inaequalis* | CBS 331.75$^{T}$ | LK932700 | |
| *Corynascella inquinata* | CBS 155.80$^{T}$ | | |
| *Corynascus novoguineensis* | NBRC 9556 | LK932698 | |
| *Corynascus sepedonium (=Corynascus similis)* | IMI 378521 | | |
| *Corynascus verrucosus* | IMI 378522$^{T}$ | | |
| *Corynascus sexualis* | IMI 378520$^{T}$ | | LK932708 |
| *Emmonsiellopsis terrestris* | CBS 273.33$^{T}$ | | |
| *Helicocarpus griseus (=Ajellomyces griseus)* | CBS 128.88$^{T}$ | KP686191 | |
| *Jugulospora carbonaria* (**=***Apiosordaria antarctica*) | IMI 381338 | | KP981433 |
| *Jugulospora rotula* (**=***Apiosordaria hispanica*) | CBS 110112 | | KP981434 |
| *Jugulospora rotula* (**=***Apiosordaria globosa*) | CBS 110113 | | KP981435 |
| *Melanospora damnosa* | CBS 113681 | KP981478 | KP981454 |
| *Melanospora kurssanoviana* | NBRC 8098 | KP981479 | KP981455 |
| *Melanospora verrucispora* | NBRC 31375$^{I}$ | KP981480 | KP981456 |
| *Melanospora zamiae* | NBRC 7902 | | KP981457 |
| *Microthecium ciliatum (=Pteridiosperma ciliatum)* | NBRC 9829 | KP981481 | KP981458 |
| *Microthecium compressum (=Sphaerodes compressa)* | NBRC 8627 | | KP981459 |
| *Microthecium fimbriatum (=Melanospora fimbriata)* | NBRC 8523 | KP981483 | KP981461 |
| *Microthecium fimicola (=Sphaerodes fimicola)* | NBRC 8354 | KP981484 | KP981462 |
| *Microthecium fusisporum (=Melanospora fusispora)* | NBRC 8806 | | KP981466 |
| *Microthecium quadrangularum (=Sphaerodes quadrangularis)* | CBS 112763$^{T}$ | KP981492 | KP981471 |
| *Microthecium retisporum (=Sphaerodes retispora)* | NBRC 8366 | | KP981472 |
| *Microthecium sepedonioides (=Papulaspora sepedonioides)* | FMR 11933 | KP981493 | KP981473 |
| *Microthecium zobelii (=Melanospora zobelii)* | NBRC 9442 | | KP981476 |
| *Myceliophthora lutea* | MUCL 10070 | LK932701 | LK932707 |
| *Myceliophthora lutea* | MUCL 10071 | LK932702 | |
| *Podospora fimiseda* | CBS 482.64 | | KP981440 |
| *Triangularia batistae* | CBS 381.68$^{T}$ | | KP981443 |
| *Triangularia mangenotii* | CBS 419.67$^{I}$ | | KP981444 |
| *Zopfiella attenuata* | CBS 266.77$^{T}$ | | KP981445 |
| *Zopfiella karachiensis* | CBS 657.74 | | KP981447 |
| *Zopfiella pleuropora* | CBS 518.70$^{I}$ | | KP981450 |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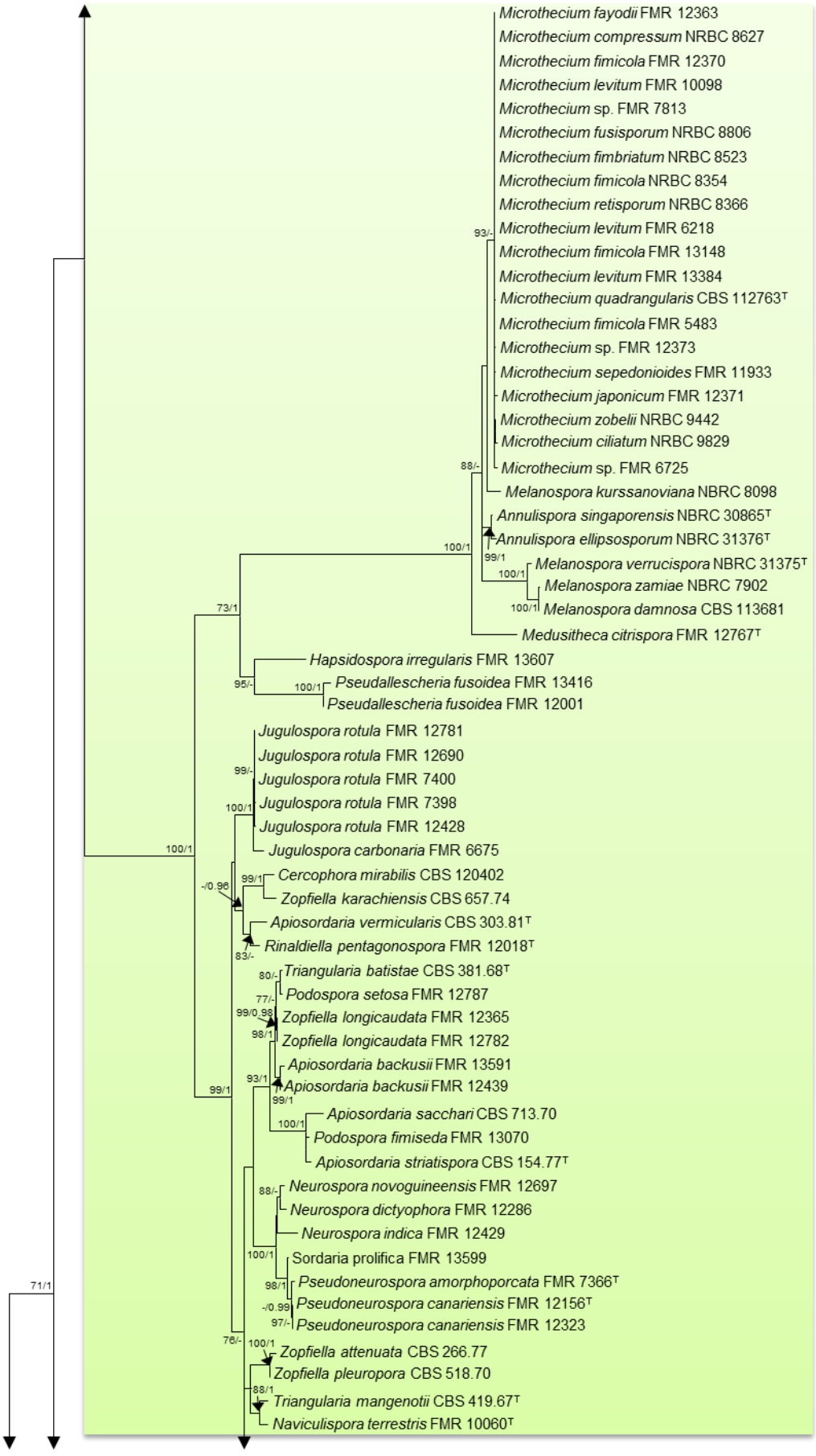
Aspergillus fischeri FMR 11922
Aspergillus fischeri FMR 11912
Aspergillus fischeri FMR 13512
Aspergillus fischeri FMR 13506
Aspergillus fischeri FMR 11862
Aspergillus fischeri FMR 12006
Aspergillus fischeri FMR 11804
Aspergillus fischeri FMR 11921
Aspergillus tatenoi FMR 11735
Aspergillus tatenoi FMR 12010
Aspergillus auratus FMR 11802
Aspergillus auratus FMR 11604
Aspergillus quadricinctus FMR 13511
Aspergillus tsunodae FMR 13605
Aspergillus fischeri FMR 11803
Aspergillus fischeri FMR 11606
Aspergillus fischeri FMR 11924
Aspergillus lacionosus FMR 13504
Aspergillus lacionosus FMR 11807
Aspergillus lacionosus FMR 13515
Aspergillus cejpii CBS 124389
Aspergillus posadansensis FMR 12322
Aspergillus posadansensis FMR 12168T
Leiothecium ellipsoideum FMR 11997
Leiothecium ellipsoideum FJ358285
Leiothecium cristatum FMR 11998T
Hamigera paravellanea FMR 13503
Hamigera paravellanea FMR 11605
Hamigera avellanea FMR 13513
Hamigera avellanea FMR 13505
Hamigera avellanea FMR 11805
Talaromyces sp. FMR 12374
Talaromyces sp. FMR 11601
Talaromyces sp. FMR 7186
Talaromyces sp. FMR 7185
Aspergillus nidulans FMR 13507
Paecilomyces niveus FMR 11806
Talaromyces bacillisporus FMR 11908
Talaromyces bacillisporus FMR 11600
Talaromyces trachyspermus FMR 11801
Talaromyces trachyspermus FMR 11810
Talaromyces stipitatus FMR 11736
Talaromyces flavus FMR 12162
Talaromyces flavus FMR 11800
Helicocarpus griseus CBS 128.88T
Emmonsiellopsis coralliformis FMR 4024T
Emmonsiellopsis terrestris FMR 4023
Emmonsiellopsis terrestris CBS 273.77T
Leucothecium emdenii FMR 12366
Aphanoascella galapagosensis FMR 12019T
Auxarthron longisporum FMR 12768T
Auxarthron umbrinum FMR 13614
Auxarthron ostraviense FMR 14372
Thelebolus microcarpus FMR 7544T
Pseudeurotium ovale FMR 13600
Myxotrichum sp. FMR 13210
Oidiodendron flavum FMR 13883
Myxotrichum stipitatum FMR 13644
Myxotrichum stipitatum FMR 13122
EUROTIOMYCETES
LEOTIOMYCETES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Microthecium compressum NRBC 8627
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Microthecium levitum FMR 10098
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Microthecium fimbriatum NRBC 8523
Microthecium fimicola NRBC 8354
Microthecium retisporum NRBC 8366
Microthecium levitum FMR 6218
Microthecium fimicola FMR 13148
Microthecium levitum FMR 13384
Microthecium quadrangularis CBS 112763T
Microthecium fimicola FMR 5483
Microthecium sp. FMR 12373
Microthecium sepedonioides FMR 11933
Microthecium japonicum FMR 12371
Microthecium zobelii NRBC 9442
Microthecium ciliatum NRBC 9829
Microthecium sp. FMR 6725
Melanospora kurssanoviana NBRC 8098
Annulispora singaporensis NBRC 30865T
Annulispora ellipsosporum NBRC 31376T
Melanospora verrucispora NBRC 31375T
Melanospora zamiae NBRC 7902
Melanospora damnosa CBS 113681
Medusitheca citrispora FMR 12767T
Hapsidospora irregularis FMR 13607
Pseudallescheria fusoidea FMR 13416
Pseudallescheria fusoidea FMR 12001
Jugulospora rotula FMR 12781
Jugulospora rotula FMR 12690
Jugulospora rotula FMR 7400
Jugulospora rotula FMR 7398
Jugulospora rotula FMR 12428
Jugulospora carbonaria FMR 6675
Cercophora mirabilis CBS 120402
Zopfiella karachiensis CBS 657.74
Apiosordaria vermicularis CBS 303.81T
Rinaldiella pentagonospora FMR 12018T
Triangularia batistae CBS 381.68T
Podospora setosa FMR 12787
Zopfiella longicaudata FMR 12365
Zopfiella longicaudata FMR 12782
Apiosordaria backusii FMR 13591
Apiosordaria backusii FMR 12439
Apiosordaria sacchari CBS 713.70
Podospora fimiseda FMR 13070
Apiosordaria striatispora CBS 154.77T
Neurospora novoguineensis FMR 12697
Neurospora dictyophora FMR 12286
Neurospora indica FMR 12429
Sordaria prolifica FMR 13599
Pseudoneurospora amorphoporcata FMR 7366T
Pseudoneurospora canariensis FMR 12156T
Pseudoneurospora canariensis FMR 12323
Zopfiella attenuata CBS 266.77
Zopfiella pleuropora CBS 518.70
Triangularia mangenotii CBS 419.67T
Naviculispora terrestris FMR 10060T
SORDARIOMYCETES

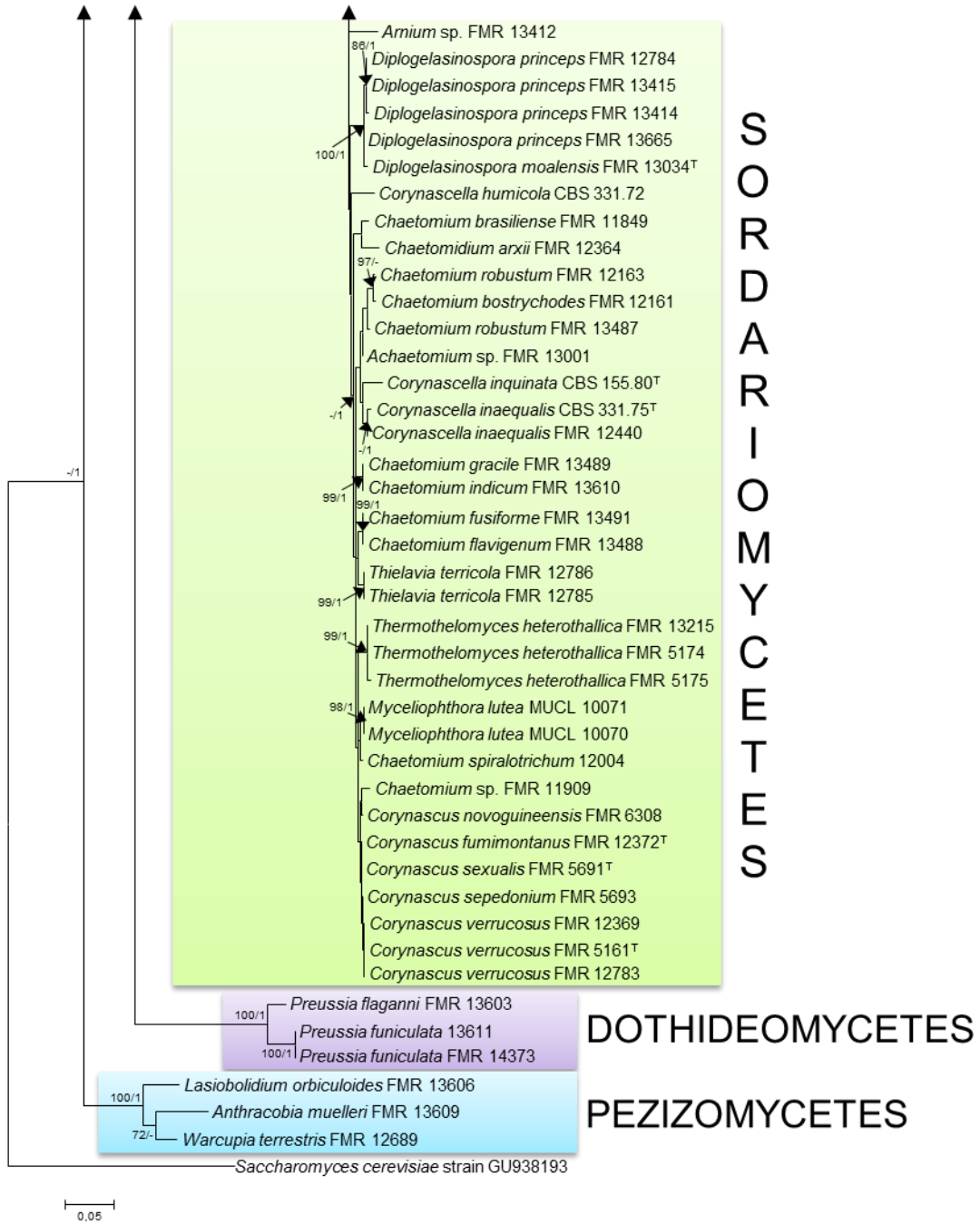
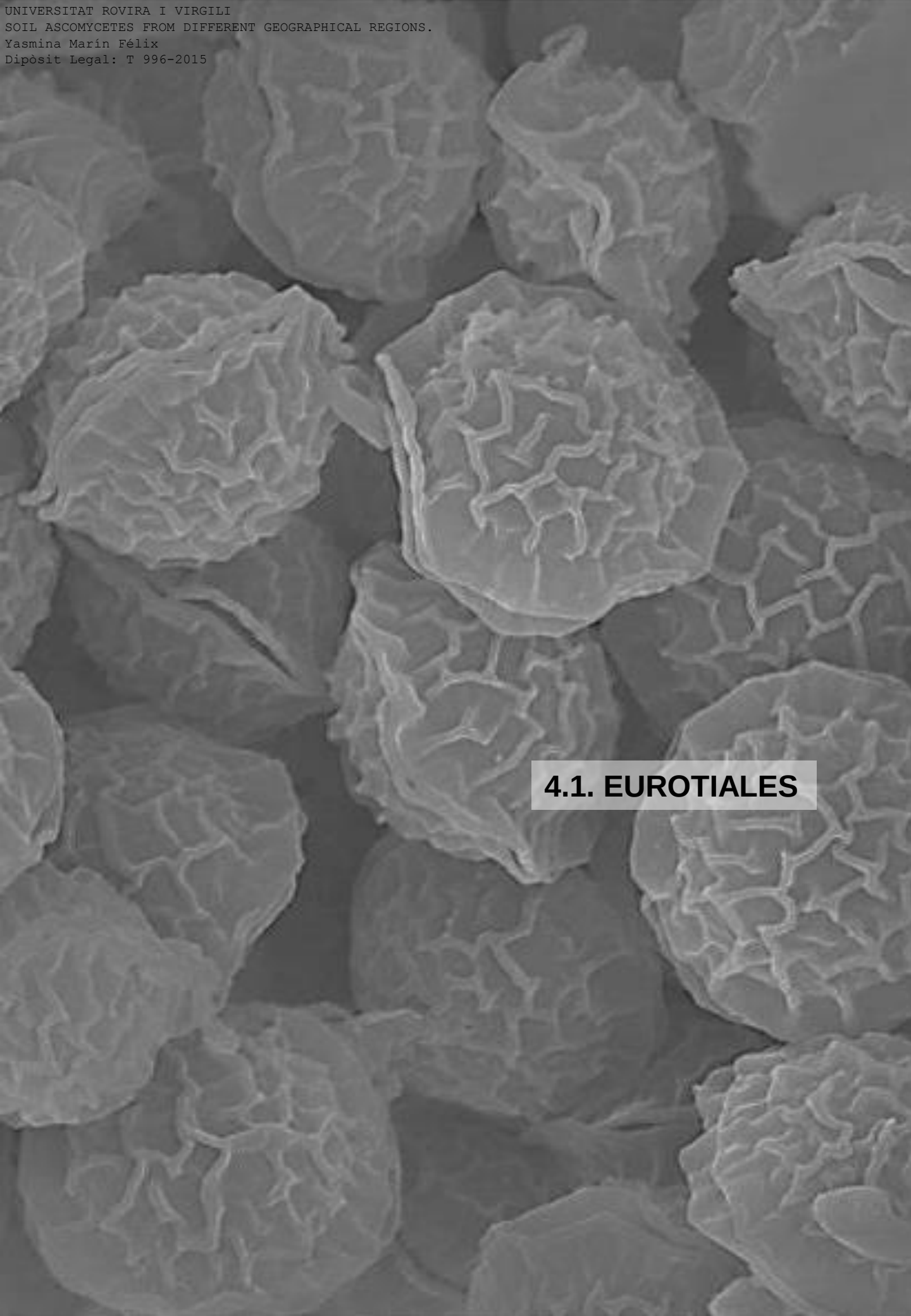



**Fig. 23** Maximum-likelihood (ML) tree obtained from D1–D3 sequences of our isolates and type and reference strains of ascomycetes. *Saccharomyces cerivisiae* was used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Branch lengths are proportional to distance. Type strains of the different species are indicated with $^{T}$.

UNIVERSITAT ROVIRA I VIRGILI
SOIL ASCOMYCETES FROM DIFFERENT GEOGRAPHICAL REGIONS.
Yasmina Marín Félix
Dipòsit Legal: T 996-2015



# 4.1. EUROTIALES

## 4.1. EUROTIALES

The order Eurotiales (Benny and Kimbrough, 1980) includes the families Aspergillaceae, Elaphomycetaceae, Thermoascaceae and Trichocomaceae (Houbraken and Samson, 2011; Castellano et al., 2012). It has been widely studied because it encompasses the economically important genera *Aspergillus* and *Penicillium*. Both genera have a worldwide distribution and a high social impact because they include pathogenic species causing diseases in both humans and animals, and spoilage and production of toxic secondary metabolites (mycotoxins) on food, but other species are involved in such industrial processes as the production of enzymes (e.g. amylases), antibiotics (e.g. penicillin), food preservatives (e.g. citric and gluconic acids), fermented foods (e.g. cheeses and sausages) and drugs (e.g. lovastatin) (Frisvad et al. 2004, Papagianni, 2007; Perrone et al., 2006; Wösten et al., 2007; Baker and Bennett, 2008; Giraud et al., 2010; Ludemann et al., 2010; Goswami et al., 2012; Visagie et al., 2014a,b). Recent changes in fungal nomenclature, which have led to use a single name for each fungus (McNeill et al., 2012), resulted in the renaming of a high number of sexual morphs as *Aspergillus* and *Penicillium* species. Nowadays, *Aspergillus* is composed of 339 species and *Penicillium* of 354 (Samson et al., 2014; Visagie et al., 2014b).

Figure 24 shows the result of the phylogenetic study based on D1−D3 sequences of our isolates belonging to the order Eurotiales plus other type and reference strains belonging to the same order. The length of the amplicons used in the combined data set was 785 bp, 192 bp of which were parsimony informative.

In Figure 24, our isolates were distributed among the families Aspergillaceae, Thermoascaceae and Trichocomaceae; however, most of them belonged to the Aspergillaceae, specifically to the genus *Aspergillus*.

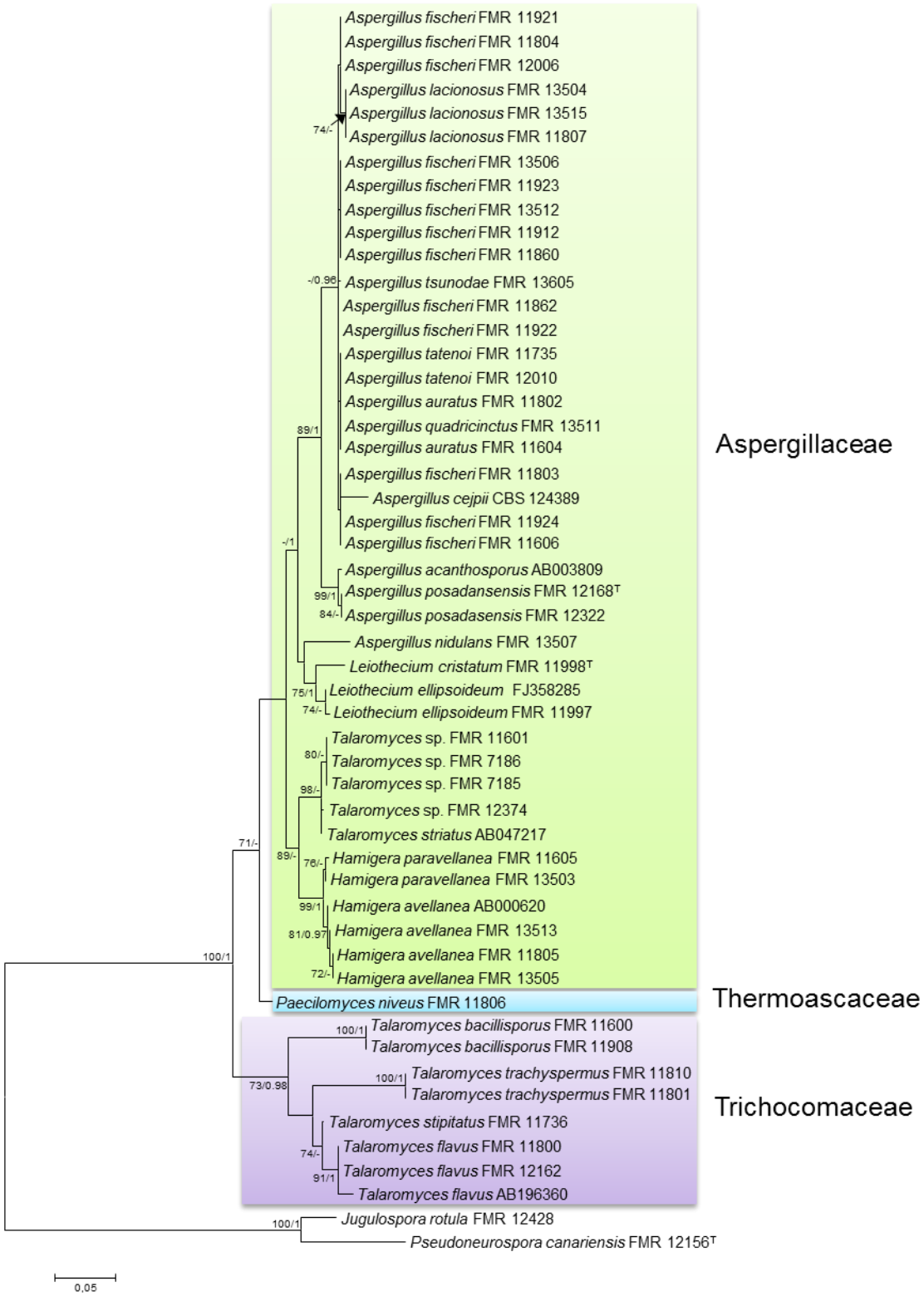



**Fig. 24** Maximum-likelihood (ML) tree obtained from D1–D3 sequences of our isolates, and type and reference strains of the Eurotiales. *Jugulospora rotula* and *Pseudoneurospora canariensis* were used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Branch lengths are proportional to distance. Type strains of the different species are indicated with $^{T}$.

The isolates FMR 12168 and FMR 12322 were displayed as a new species of that genus (*Aspergillus posadasensis*), whereas the isolate FMR 11998 represented a new species of the genus *Leiothecium* (*Leiothecium cristatum*), which also belongs to the Aspergillaceae and was monospecific until this new species was described. Both new taxa are fully described and illustrated in section 4.1.1.

The type strain of *Talaromyces striatus* grouped in the same well-supported clade (98% bs / - pp) that four of our isolates, e.g. FMR 7185, FMR 7186, FMR 11601 and FMR 12374). This clade was related to the genus *Hamigera* and phylogenetically distant from the clade of *Talaromyces* spp. including their type species. A phylogenetic study performed by Houbraken and Samson (2011) also placed *T. striatus* in a clade independent from *Talaromyces*, and it was recently excluded from this genus (Yilmaz et al., 2014). Although these recent studies regarded *T. striatus* as a *Hamigera* species (*Hamigera striata*), this taxon was not located in the *Hamigera* spp. clade. This was observed by Peterson et al. (2010), who excluded *T. striatus* from the genus *Hamigera*. Consequently, the correct taxonomical placement of this taxon has still to be clarified.

On the basis of our molecular results, *T. striatus* represents a new genus of the family Aspergillaceae. This taxon can be differentiated from the closest genus *Hamigera* by their ascospore ornamentation, consisting on longitudinal ridges while it is warted in *Hamigera* spp (Fig. 25). The isolates FMR 7185, FMR 7186 and FMR 11601 represent a new species of this new genus, which can be distinguished from *T. striatus* by the size and the colour of the ascospores, as well as the pattern of their ridges. This proposal is actually in preparation.

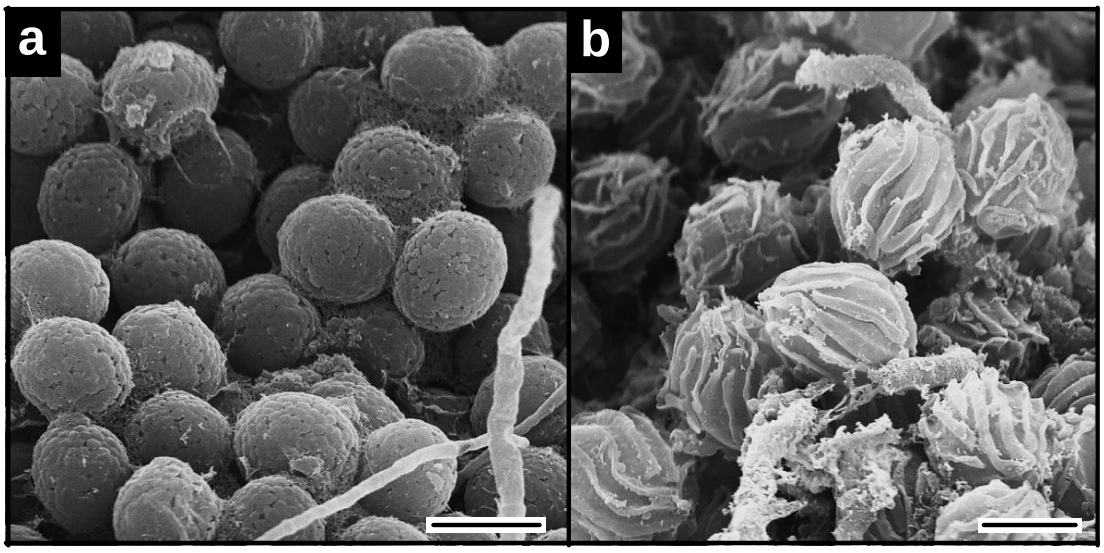



**Fig. 25** Comparison of ascospore morphology of *Hamigera* spp. and *Talaromyces striatus*. a. *Hamigera paravellanea* FMR 11605. b. *Talaromyces striatus* FMR 8816. Scale bars: 5 µm.

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

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

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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 11998$^T$ (=CBS 134260$^T$=NBRC 109843$^T$), 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 12168$^T$ (=CBS 134259$^T$=NBRC 109845$^T$), is differentiated from *Aspergillus acanthosporus*, the nearest species phylogenetically, by its non-sclerotioid ascomata 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, $\beta$-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, Thermoascaceae 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 $\beta$-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 *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, KF732838, 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 Iguazú 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 Iguazú National Park is situated in the boundaries of the Iguazú River, and has an area of around 550 km$^2$. 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 (Stchigel *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 + 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/phylows/study/TB2:S15962).

## Results

### Phenotypic study

The isolate FMR 11998$^T$, from a soil sample of the Iguazú 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

*Leiothecium ellipsoideum* (FJ358285) whereas isolates FMR 12168$^T$ (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$^T$ (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. ellipsoideum*, sequenced in this study (KF732839), was 92.76 %. The BLAST search of ITS sequences of isolates FMR 12168$^T$ (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. acanthosporus* (EF669992). The BLAST search with the *CAL* sequences of isolates FMR 12168$^T$ (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. 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 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$^T$ grouped in a terminal clade with the type strain of *L. ellipsoideum* (89 % bootstrap support) whereas the isolates FMR 12168$^T$ and FMR 12322 grouped with the type strains of *A. acanthosporus* 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$^T$ 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$^T$ and 12322) for the second, and four strains of *A. acanthosporus* 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 rhizopodus*, *Aspergillus clavatonicus*, *Aspergillus longivesica* and *Aspergillus giganteus*.

## TAXONOMY

The previous data demonstrated that isolate FMR 11998$^T$ belongs to the genus *Leiothecium* but is distinguishable molecularly from the only species of this genus *L. ellipsoideum*, 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$^T$ and FMR 12322 are molecularly and morphologically different from *A. acanthosporus* 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. Marín, 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 polyhedric flattened cells of 10–20 µm diameter. Asci 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. Chlamydospores mostly terminal, sometimes intercalary, hyaline, subspherical 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; isotype FMR 11998$^T$.

Mycobank accession no. MB803513.

The ex-type culture is FMR 11998$^T$ (=CBS 134260$^T$= NBRC 109843$^T$), 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.

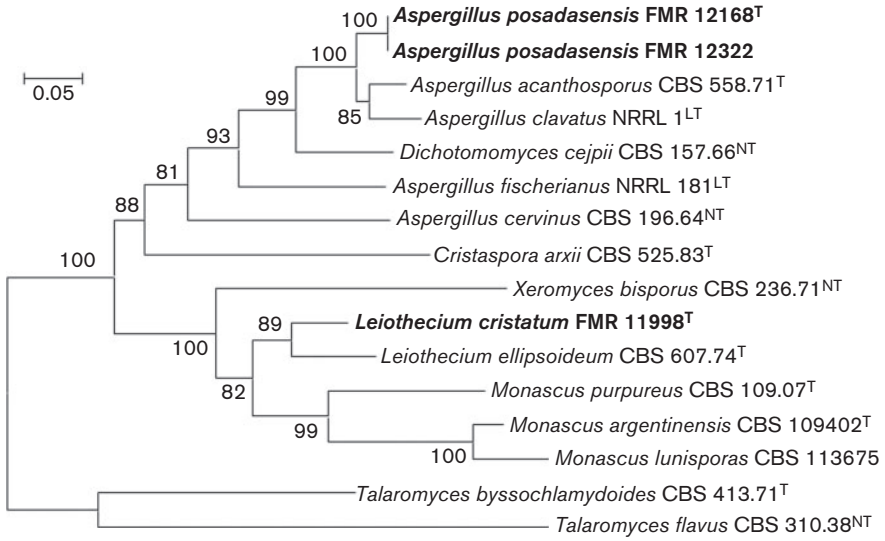


**Fig. 1.** Maximum-likelihood (ML) rooted tree obtained from the combined DNA sequence data from three loci (*Cct8*, *RPB1* and *RPB2*) of the novel isolates and 11 selected species belonging to the family Aspergillaceae, chosen because of their molecular or morphological similarity to the novel isolates. *Talaromyces byssochlamydoides* CBS 413.71$^T$ and *Talaromyces flavus* CBS 310.38$^T$ (family Trichocomaceae) were used as outgroups. Bootstrap support values $\geqslant$70 % are indicated at the nodes. Branch lengths are proportional to distance. Type and Neotype strains of the different species are indicated with $^T$ and $^{NT}$ respectively. Bar, 0.05 substitutions per nucleotide position.

### Description of *Aspergillus posadasensis* Y. Marín, Stchigel & Cano sp. nov. (Fig. 3)

*Aspergillus posadasensis* (po.sa.das.en′sis. 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 12168$^T$.

Mycobank accession no. MB803514.

The ex-type culture is FMR 12168$^T$ (=CBS 134259$^T$= NBRC 109845$^T$), 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

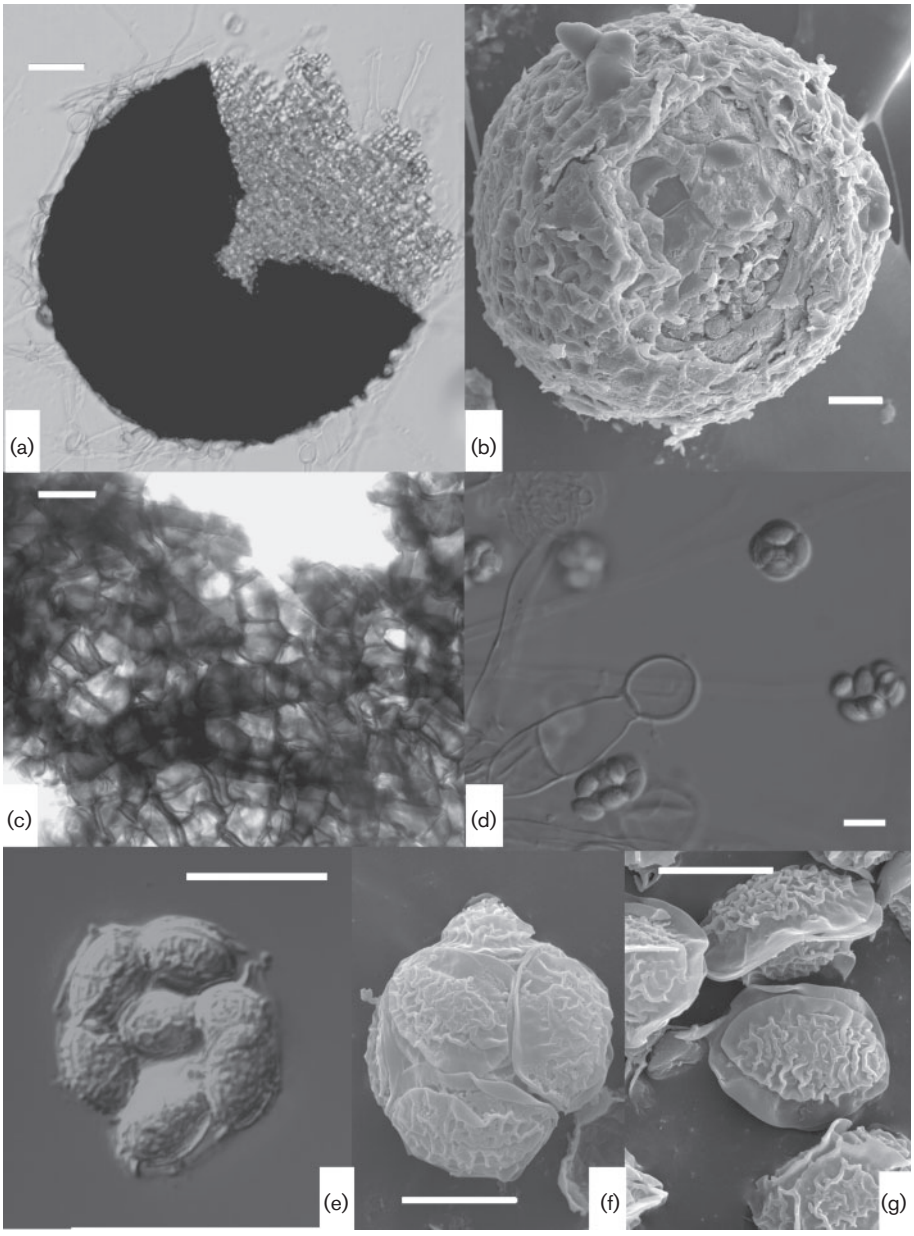


**Fig. 2.** Morphology of *Leiothecium cristatum* sp. nov. FMR 11998$^T$. (a), (b) Ascoma; (c) detail of the peridium; (d) asci and terminal chlamydospores; (e), (f) ascus; (g) ascospores (SEM). The fungus was grown on PDA at 25°C during two weeks. Bars, 50 μm (a); 25 μm (b); 20 μm (c); 10 μm(d, e); 5 μm (f, g).

differences among them (ascomata with a very thin, plectenchymatous 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 illustrations, 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.

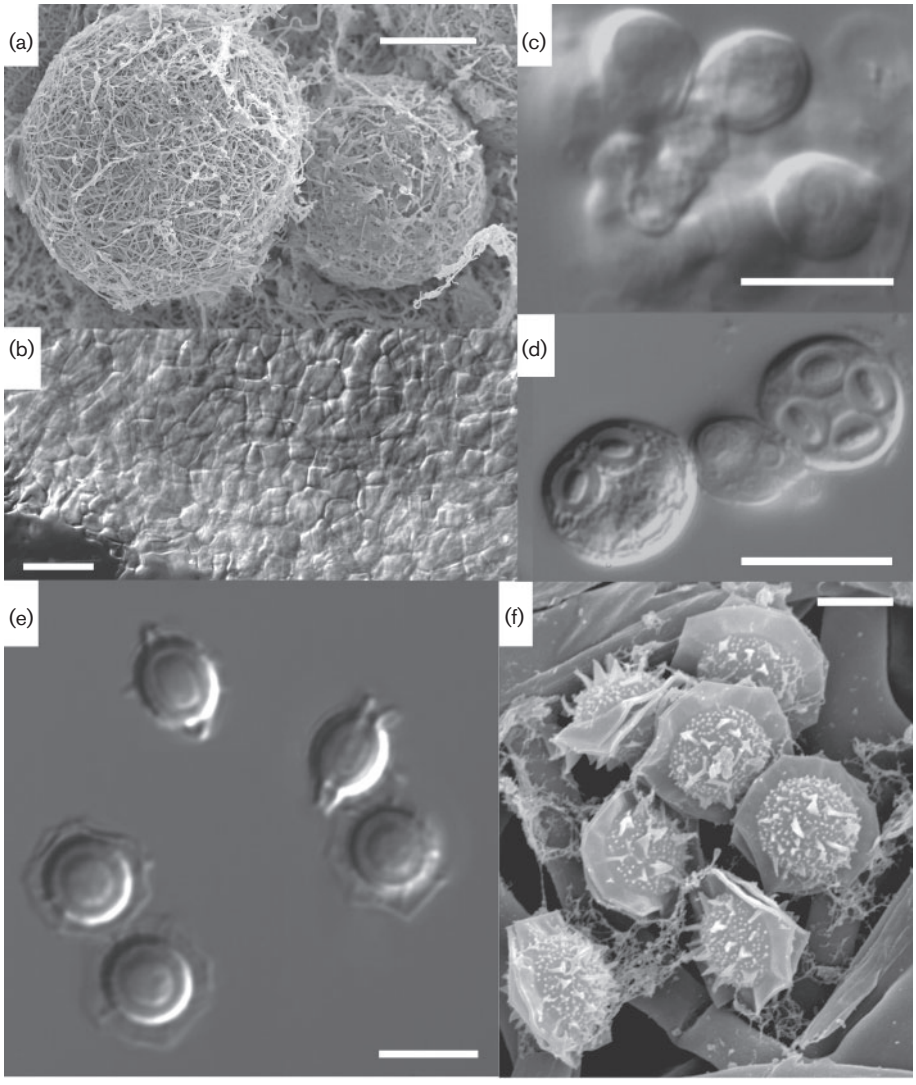


**Fig. 3.** Morphology of *Aspergillus posadasensis* sp. nov. FMR 12168$^T$. (a) Ascoma; (b) detail of the peridium; (c, d) asci; (e, f) ascospores. The fungus was grown on PDA at 25°C during two weeks. Bars, 100 μm (a); 20 μm (b); 10 μm (c, d); 5 μm (e); 2.5 μm (f).

Our molecular analysis, using three of those genes, demonstrates that the isolate FMR 11998$^T$ 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 12168$^T$ 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.

## Acknowledgements

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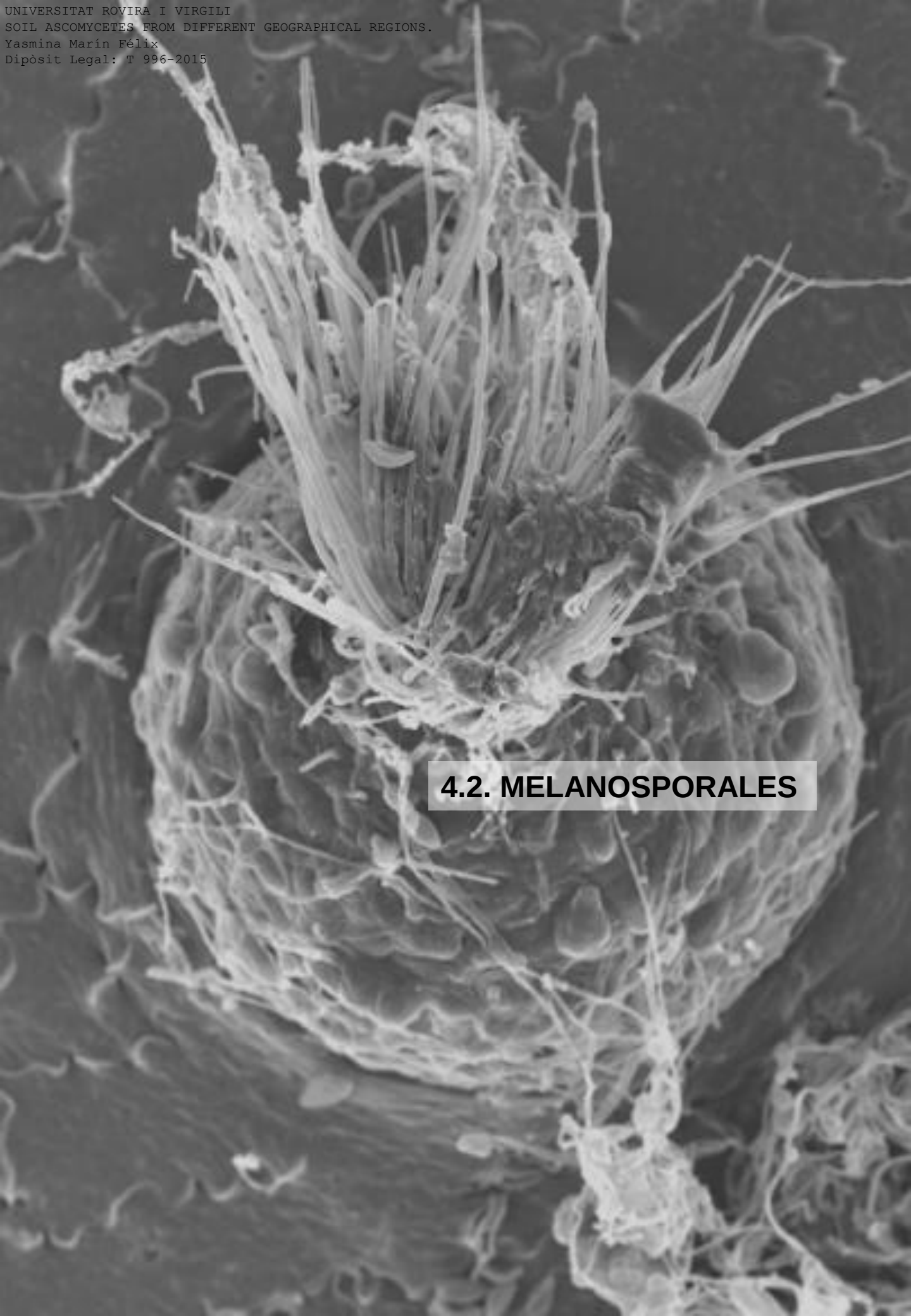
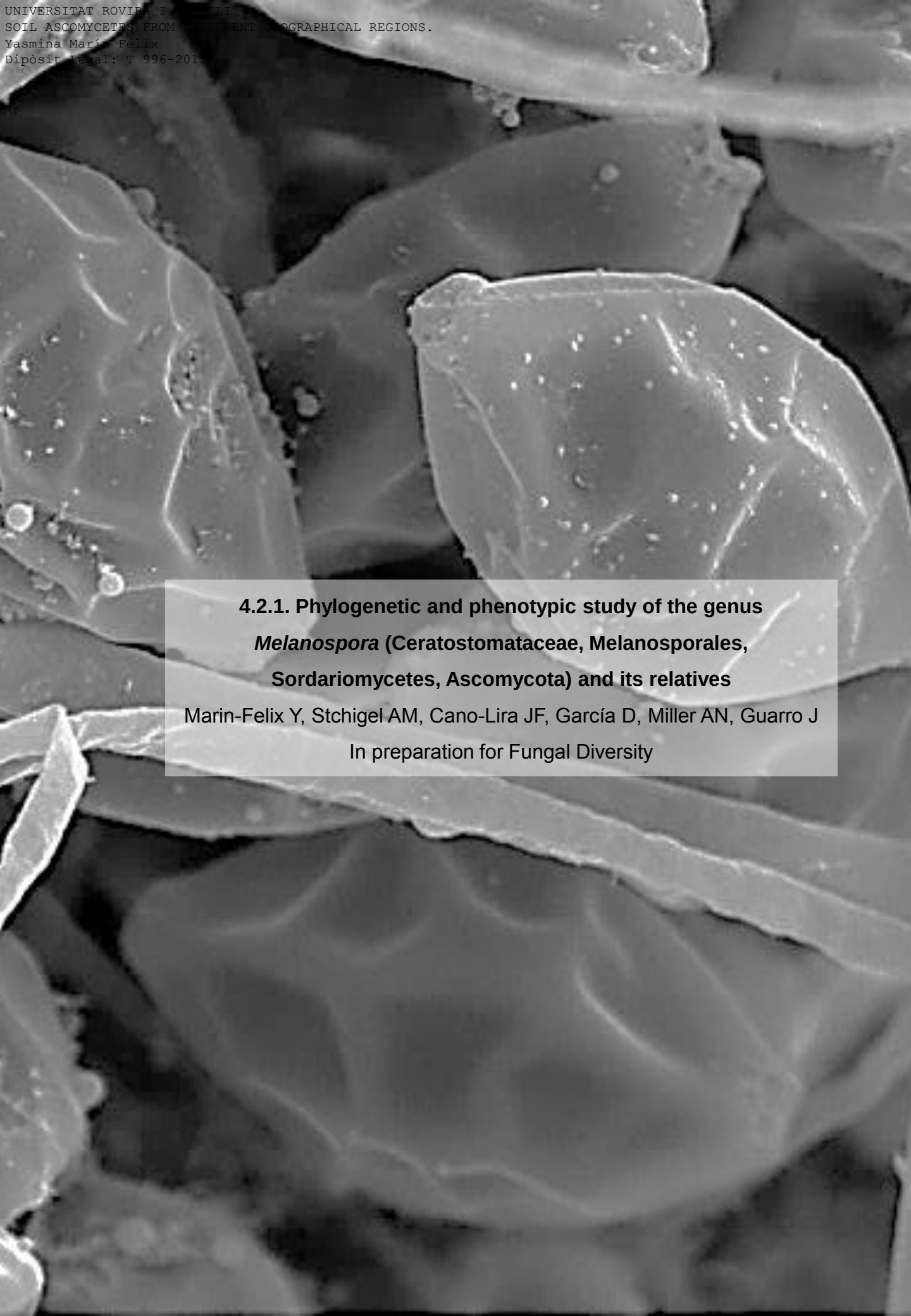


# 4.2. MELANOSPORALES

## 4.2. MELANOSPORALES

The order Melanosporales was recently introduced by Zhang and Blackwell in a phylogenetic study based on the genes SSU, LSU, *RPB2* and *EF1* (Hibbett et al., 2007). This order consists of only one family, Ceratostomataceae, which groups the genera *Annulispora, Arxiomyces*, *Medusitheca, Melanospora*, *Microthecium, Pseudomicrothecium, Pustulipora*, *Rhytidospora, Scopinella*, *Syspastospora* and *Vittatispora*. While this thesis was being drafted, the genus *Melanospora* was redefined, *Microthecium* re-established and *Annulispora, Pseudomicrothecium* and *Medusitheca* introduced (see the article in section 4.2.1. for more details). The taxa belonging to this order are frequently isolated from soils, but they have always been difficult to study molecularly because most of them are mycoparasites, and are consequently very difficult to isolate and grow in pure culture. This is why many sequences of the Melanosporales deposited in databases really correspond to the host. This also explains why it is very difficult to obtain the reproductive structures *in vitro*: in many cases isolates cannot grow without their host or lose their ability to develop reproductive structures, thus hindering the morphological study.

As part of this thesis, we used sequences of four loci (SSU, D1−D3, ITS, *ACT* and *EF*1) to make the first phylogenetic study of a large number of taxa belonging to the Melanosporales (section 4.2.1.). This work revealed that ascomatal morphology is more phylogenetically informative than the ornamentation of the ascospores, even though the latter has traditionally been used to delimit genera within the Melanosporales, as was previously reported for the Sordariales by Miller and Huhndorf (2005). The greatest difficulty during the phylogenetic study was the low interspecific molecular variability of *Microthecium*. All the loci sequenced did not show enough variability to differentiate species. We also sequenced fragments of *BT*2 and *RPB*2, but they showed a high molecular interspecific identity (100% or close to it) and were of no use for our study.



**4.2.1. Phylogenetic and phenotypic study of the genus *Melanospora* (Ceratostomataceae, Melanosporales, Sordariomycetes, Ascomycota) and its relatives**

Marin-Felix Y, Stchigel AM, Cano-Lira JF, García D, Miller AN, Guarro J

In preparation for Fungal Diversity

Phylogenetic and phenotypic study of the genus *Melanospora* (Ceratostomataceae, Melanosporales, Sordariomycetes, Ascomycota) and its relatives.

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***Abstract***: The order Melanosporales comprisses a large group of ascomycetes, most of them mycoparasites, characterized by ascomata usually ostiolate and translucent, unitunicate asci and unicellular, pigmented ascospores with germ pores or germ slits. The largest and most studied genera are *Melanospora* and *Sphaerodes*, but their circumscriptions and boundaries with other related genera are not yet resolved. In this study the taxonomy of *Melanospora* and related taxa have been re-evaluated based on the phylogenetic analyses of nuclear rRNA, actin and elongation factor genes sequences of fresh isolates and numerous type and reference strains. As a result of this analysis the genus *Melanospora* has been restricted to species with ostiolate ascomata with a neck composed of intermixed hyphae and a phialidic asexual state. The genus *Microthecium* has been re-established and circumscribed to the species of *Melanospora* and *Sphaerodes* with absence of neck in the ascoma, or, if present, short and composed of angular cells similar to those of the peridium, and usually producing bulbils. Three new genera have

been proposed: *Dactylidispora,* showing ascospores with a raised rim surrounding both terminal germ pores; *Medusitheca*, with densely setose, dark ascomata; and *Pseudomicrothecium*, characterized by ascospores with indistinct germ pores. Dichotomous keys to identify the species of *Melanospora* and *Microthecium*, as well as a brief description of the accepted species of both genera are provided.

***Keywords*:** *Dactylidispora*, Ceratostomataceae, *Medusitheca*, *Melanospora*, Melanosporales, *Microthecium*, *Pseudomicrothecium*, soil, *Sphaerodes*.

## INTRODUCTION

The family Ceratostomataceae (Winter 1887) includes near a hundred species, often mycoparasitic and characterized by ostiolate and rostrate, or less frequently non-ostiolate, translucent ascomata, unitunicate and evanescent asci, brown, exceptionally hyaline, unicellular ascospores with a germ pore at each end, less frequently with only one germ pore or germ slits, and phialidic asexual morphs. Currently, the family Ceratostomataceae is included in Melanosporales (Chaudhary et al. 2006; Zhang et al. 2006; Hibbett et al. 2007), although historically it had been placed in other orders such as Aspergillales (Gaüman 1964), Hypocreales (Alexopoulos 1962; Spatafora and Blackwell 1994a; Rehner and Samuels 1995; Jones and Blackwell 1998; Zhang and Blackwell 2002) and Sphaeriales (Bessey 1950; Dennis 1968). This family comprises eleven genera that produce sexual morph, i.e. *Arxiomyces*, *Melanospora, Persiciospora*, *Pteridiosperma*, *Pustulipora, Rhytidospora*, *Scopinella*, *Setiferotheca*, *Sphaerodes*, *Syspatospora* and *Vittatispora*. *Melanospora,* the largest genus of this family, with more than fifty species was erected by Corda (1837) to accommodate *Ceratostoma chionea* and two new species, *Melanospora zamiae* and *Melanospora leucotricha*, *M. zamiae* chosen later as type species

of the genus (Kowalski 1965). *Melanospora* is characterized by usually perithecial ascomata with a long neck and a translucent, pale yellow to reddish-brown peridium, and mostly smooth-walled, brown, ellipsoidal to citriform, rarely discoid or fusiform ascospores, with a depressed germ pore at each end, occasionally surrounded by a raised rim (Guarro et al. 2012). Related genera are *Microthecium* and *Sphaerodes.* The former was erected in 1842 by Corda to place *M. zobelii* and distinguishable from *Melanospora* by the presence of non-ostiolate, usually immersed ascomata. *Sphaerodes*, introduced in 1909 by Clements to accomodate *Melanospora episphaeria*, is similar to *Melanospora* but encompassing species with reticulate ascospores. However, the boundaries between *Melanospora* and its relatives remained confuse. Doguet (1955) carried out a revision of *Melanospora*, synonymizing several species and transferring additional species from other genera, mostly from *Sphaeroderma*, which had been proposed by Fuckel (1877) and distinguished from *Melanospora* by the absence of ascomatal neck. Doguet (1955) considered the production of the neck as a non stable taxonomic character influenced by culture media. Doguet divided *Melanospora* into several sections on the basis of the morphology of the ascospores (shape and ornamentation) and ascomata (presence or absence of neck, and its size when present). The most comprehensive revision of *Melanospora* and related genera was carried out by Cannon and Hawksworth (1982), mainly based on the structure of the ascospore wall under SEM, the species of *Microthecium* being transferred to *Melanospora* and *Sphaerodes.* However, more recent molecular studies demonstrated that *Melanospora* and *Sphaerodes* are both polyphyletic (Zhang and Blackwell 2002; Fan et al. 2012). Other genera included in the family are *Arxiomyces*, which produces ovoid to ellipsoidal ascospores with a rounded apex and a truncate base, and a large sunken germ pore (Cannon and Hawksworth 1982, 1983); *Persiciospora*, characterized by ascospores with a pitted wall and a faint reticulation

(Cannon and Hawksworth 1982); *Pteridiosperma*, with ascospores ornamented with longitudinal wing-like appendages (Krug and Jeng 1979); *Pustulipora*, distinguished by its ascospores with a germ pore at each end surrounded by a cushion-like structure showing an irregular pustulate appearance (Cannon 1982); *Rhytidospora*, characterized by non-ostiolate ascomata with a cephalothecoid peridium (Krug and Jeng 1979); *Scopinella*, producing brown, cuboid-ellipsoidal ascospores with two prominent longitudinal germ slits (Cannon and Hawksworth 1982); *Setiferotheca*, which produces ascospores similar to those of *Arxiomyces* and ascomata with a crown of dark brown setae surrounding the ostiole; *Syspastospora*, with ascomata with a long neck composed of parallelly arranged hyphae and cylindrical ascospores with a large terminal slightly sunken germ pore at each end (Cannon and Hawksworth 1982); and *Vittatispora*, which produces ascomata similar to those of *Syspatospora* and citriform ascospores with a longitudinal, thick, hyaline ridge (Chaudhary et al. 2006). Practically all the taxonomic studies performed about these fungi have been exclusively based on the morphological characterization of the reproductive structures based on fungarium specimens. Mainly due to their mycoparasitism, these fungi do not grow in pure culture and not fructify in absence of their hosts. The obtention of reliable sequences from Melanosporales is also difficult because the massive interfering presence of DNA of their hosts. Based on the study of several fresh soil-borne isolates and reference and type strains obtained from different culture collections, and sequences retriewed from the GenBank and NBRC databases, we have revised the phylogenetic relationships of the most relevant genera of the Ceratostomataceae. Consequently, the genus *Melanospora* has been redefined, *Microthecium* re-established, and three new genera proposed.

## MATERIALS AND METHODS

### *Fungal isolates*

The strains included in this study are listed in Table 1. The fresh isolates included in the study have been isolated from soil. For their isolation, we followed previously described procedures for activation of dormant ascospores in soil, using acetic acid and phenol solutions (Stchigel et al. 2001; García et al. 2003). Ascomata were transferred to 5 cm diam Petri dishes containing oatmeal agar (OA: oatmeal flakes, 30 g; agar-agar, 20 g; distilled water, 1 L) using a sterile needle, which were incubated at 15, 25 and 35ºC.

### *Morphological study*

For cultural characterization, the isolates were grown for up to 30 d on OA, potato carrot agar (PCA: grated potatoes, 20 g; grated carrot, 20 g; agar-agar, 20 g; L-chloramphenicol, 100 mg; distilled water, 1 L), and potato dextrose agar (PDA; Pronadisa, Madrid, Spain) at 5, 10, 15, 20, 25, 30, 35 and 40ºC. Color notations in parentheses are from Kornerup and Wanscher (1984). Vegetative and reproductive structures were examined under an Olympus BH-1 light field microscope, by direct mounting of the ascomata or microcultures performed on OA and PDA, on lactic acid and water. Pictures were obtained by a Zeiss Axio Imager M1 lightfield microscope. The samples for scanning electron microscope (SEM) were processed according to Figueras and Guarro (1988), and SEM micrographs taken by using a Jeol JSM 840 at 15 keV microscope.

### *Molecular study*

The DNA of the fungal isolates (Table I) was extracted and purified directly from the colonies according to the Fast DNA Kit protocol (MP Biomedicals, Solon, Ohio). The amplification of the 18S small subunit (18S) and D1−D3 domains of the 28S large

subunit (28S) of the nuclear rRNA genes, internal transcribed spacer region (ITS) of the nuclear rDNA, and partial segments of actin (*ACT*) and elongation factor (*EF*1) loci were performed according to White et al. (1990) (18S), Vilgalys and Hester (1990) (28S), Cano et al. (2004) (ITS), Voigt and Wöstermeyer (2000) (*ACT*) and Houbraken et al. (2007) (*EF*1). The sequences of the amplicons were obtained using the protocol of the Taq Dye-Deoxy Terminator Cycle Sequencing Kit. PCR products were purified and sequenced at Macrogen Europe (Amsterdam, The Netherlands) with a 3730XL DNA analyzer (Applied Biosystems), and the consensus sequences were obtained using SeqMan (version 7.0.0; DNASTAR, Madison, WI, USA). The sequences were aligned using the Clustal W implemented in MEGA v. 6.06 (Tamura et al. 2013), followed by a manual adjustment under the same software platform. Sequences retrieved from GenBank and NBRC included in this analysis are in Table I. Phylogenetic analyses were carried out using MEGA v. 6.06. We performed a first analysis based on 18S sequences of our isolates and type and reference strains of members of Melanosporales and of some members of the orders Chaetosphaeriales, Coniochaetales, Coronophorales, Hypocreales, Microascales, Sordariales and Xylariales, using *Thelebolus ellipsoideus* (Thelebolales) as outgroups, in order to determine the taxonomic placement of our isolates. A second study, carried out to infer the phylogenetic relationships among the members of *Melanosporales*, was based in the analysis of a combined data set including the ITS, 28S, *ACT* and *EF1* sequences of our isolates and type and reference strains of a large number of Melanosporales, and including *Nectria cinnabarina* and *Pseudallescheria fusoidea* as outgroups. Because the living strains of certain members of the Melanosporales included in previous works were not available for the present study, we built a phylogenetic tree using their 28S sequences and those of representatives of all terminal clades displayed in the previous multilocus trees. Maximum Likelihood (ML) method using Kimura 2-parameter model and Tamura-

Nei, both with gamma distribution and the pair-wise deletion of gaps option, were used for the phylogenetic analysis of 18S and, of the combined dataset and 28S, respectively. The robustness of branches was assessed by bootstrap analysis with 1,000 replicates. Bayesian inference (BI) was carried out using MrBayes v. 3.1 following the parameters detailed in Alvarez et al. (2010). The sequences generated in this study were deposited in GenBank database (Table I) and the alignments used in the phylogenetic analyses are deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S17079).

## RESULTS

The length of the 18S alignment used in this study was of 970 bp, 186 bp of which were parsimony informative. All the members of Melanosporales included in the 18S ML phylogenetic tree, including our isolates, were placed in a main clade, with a bootstrap support (bs) of 99% and bayesian posterior probability (pp) of 1 (Fig. 1). Within this main clade, our isolate CBS 137837, whose morphological features did not match with any previously described taxon, was clearly separated from the other strains included in the study constituting a basal branch representing a new genus. The other fungi included in Melanosporales were grouped together with a highly support (79% bs / 1 pp) and divided in three subclades. The first one (94% bs / - pp), grouped most of the isolates morphologically identified as *Melanospora* spp., *Persiciospora* spp. and *Sphaerodes* spp., including reference strains of *Melanospora brevirostris, M. fimbriata, M. fusispora, M. zobelii, Papulaspora sepedonioides, Pteridiosperma ciliatum, Sphaerodes compressa, S. fimicola, S. tenuissima* and *S. quadrangularis*, without significant genetic distances among them. The second subclade (81% bs / 0.95 pp) comprised the type strains of *Sphaerodes ellipsospora* and *Sphaerodes singaporensis* and a reference strain of *Melanospora kurssanowiana*, the latter one clearly separated from *S. ellipsospora* and *S.*

*singaporensis*, which were grouped with high support (98% bs / 1 pp). In the last subclade (100% bs / 1 pp) were nested the type species of the genus *Melanospora* (*M. zamiae*), the type strains of *Melanospora verrucosa* and *Sphaerodes mycoparasitica*, and reference strains of *Melanospora damnosa* and *Melanospora tiffanii*.

The lengths of the individual alignments used in the combined data set were 795 bp (28S), 535 bp (ITS), 727 bp (*ACT*) and 846 bp (*EF*1), and the final total alignment was 2903 bp, 555 bp of which were parsimony informative. In the tree derived from the combined data set (Fig. 2), the Melanosporales were divided into five monophyletic clades. The first one (93% bs/ 1 pp; Clade *Microthecium*) grouped all our isolates except the isolate CBS 137837, and type or reference strains of *Melanospora fimbriata, M. fusispora, M. zobelii, Papulaspora sepedonioides, Pteridiosperma ciliatum, Sphaerodes compressa, S. fimicola*, *S. tenuissima* and *S. quadrangularis*. All the fungi belonging to this clade have cleistothecial ascomata without neck or when present it is short and composed of angular cells as the rest of the ascomatal peridium. Bulbils (microsclerotial-like asexual propagules) are present in most of these species (Fig. 3). In spite of the high morphological variability showed by the members of this clade, the loci used in the phylogenetic analysis were not able to separate the species from each others. The second clade (99% bs/ 1 pp; Clade *Melanospora*) comprissed the type species *M. zamiae* and reference strains of *M. verrucosa* and of *M. damnosa.*The members of this clade showed ostiolate ascomata with a long neck composed of hyphae irregularly arranged, and with a crown of setae at the apex. In addition, an asexual morph characterized by solitary, sessile, flask-shaped phialides was commonly present (Fig.4). The reference strain of *Melanospora kurssanoviana*, which did not sporulate, and our isolate CBS 137837 formed two independent branches. The isolate CBS 137837 produced globose, non-ostiolate dark ascomata densely setose, and smooth-walled ascospores with a depressed

germ pore at each end (Fig. 5). The last clade (99% bs/ 1pp; Clade *Dactylidispora*) was composed by the type strains of *Sphaerodes ellipsospora* and *S. singaporensis*, both characterized by translucent ascomata and ascospores with a raised rim surrounding the germ pores.

The length of the 28S alignment was of 808 pb, 111 pb of which were parsimony informative. The five monophyletic well-supported clades (*Dactylidispora*, *Medusitheca*, *Melanospora*, *Melanospora kurssanoviana* and *Microthecium* clades) obtained in the other two phylogenetic trees were also represented in the 28S ML tree (Fig. 6). Apart from these clades, two independent terminal branches corresponding to the type strains of *Melanospora subterranea* and of *Vittatispora coorgii*, respectively were also shown. Both taxa showed distinctive morphological features unique in Melanosporales, e.g. ascospores with indisctinct germ pores in *M. subterranea* and with a longitudinal thick hyaline ridge in *V. coorgii*.

***Taxonomy***

Key to the accepted genera of the Melanosporales producing sexual morph (adapted from Cannon and Hawksworth 1982)

1. Ascospores with longitudinal germ slits.....*Scopinella*

1. Ascospores with germ pores.....2

2. Ascospores with a broad germ pore and a small basal appendage.....3

2. Ascospores with a germ pore at each end.....4

3. Ascomata with a crown of dark brown setae surrounding the ostiole.....*Setiferotheca*

3. Ascoma without setae.....*Arxiomyces*

4. Ascospores oblong or cylindric-fusiform, and germ pores crateriform…..*Syspastospora*

4. Ascospores ellipsoidal to citriform.....5

5.Ascoma ostiolate; neck long, composed of hyphae irregularly arranged.....*Melanospora*

5. Ascoma non-ostiolate or ostiolate; neck absent or short, conical, composed of angular cells as in the peridium.....6

6. Ascospores with indistinct germ pores.....*Pseudomicrothecium*

6. Ascospores with conspicuous germ pores.....7

7. Ascospores with a longitudinal hyaline ridge.....*Vittatispora*

7. Ascospores without ridges.....8

8. Germ pores surrounded by hyaline structures.....9

8. Germ pores without such structures.....10

9. Germ pores with a raised rim.....*Dactylidispora*

9. Germ pores with a blistered, rarely cushion-like, structure.....*Pustulipora*

10. Peridium cephalothecoid.....*Rhytidiospora*

10. Peridium not cephalothecoid.....11

11. Ascomata densely setose; peridium dark.....*Medusitheca*

11. Ascomata glabrous or surrounded by hyphae-like hairs; peridium translucent.....*Microthecium*

***Dactylidispora*** Y. Marín, Stchigel, Guarro, Cano, **gen. nov.**

MycoBank MB812079.

*Type species*. *Dactylidispora ellipsospora* (Takada) Y. Marín, Stchigel, Guarro & Cano.

*Etymology*. From Latin *annulus*–, ring, and –*spora*, spore, due to the raised rim that surround the germ pores of the ascospores.

*Diagnosis*: This genus is characterized by the production of smooth-walled ascospores with a germ pore, surrounded by a raised rim, at each end.

*Ascomata* superficial, globose to pyriform, ostiolate or not, yellowish-brown, appearing dark brown when the ascospores are mature, glabrous or setose; neck cellular, short, conical, with a crown of setae surrounding the ostiole; *peridium* membranaceous, of *textura angularis*. *Asci* 8-spored, broadly clavate, short-stipitate, evanescent. *Ascospores* one-celled, at first hyaline, becoming brown to dark brown, fusiform or citriform, umbonate and truncate at the ends, smooth-walled, with one germ pore at each end; germ pores depressed, surrounded by a raised rim. *Asexual morph* absent, or phialidic; *phialides* solitary, flask-shaped, arising on fertile hyphae; *conidia* hyaline, subglobose to ovoid, smooth-walled.

***Dactylidispora collipora*** (Stchigel & Guarro) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812080.

*Basionym*. *Melanospora collipora* Stchigel & Guarro, in Stchigel, Guarro & Figueras, Mycol. Res. 101: 446 (1997)

Notes — This species produces ostiolate ascomata with a crown of setae around the ostiole, ellipsoidal ascospores, and bulbils.

***Dactylidispora ellipsospora*** (Takada) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812081.

*Basionym*. *Microthecium ellipsosporum* Takada, in Kobayasi et al., Bull. natn. Sci. Mus., Tokyo 16: 527 (1973)

≡ *Sphaerodes ellipsospora* (Takada) Dania García, Stchigel & Guarro, Stud. Mycol. 50: 67 (2004)

Notes — *Dactylidispora ellipsospora* is characterized by non-ostiolate ascomata, fusiform ascospores and absence of an asexual morph.

***Dactylidispora singaporensis*** (Morinaga, Minoura & Udagawa) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812082.

*Basionym*. *Melanospora singaporensis* Morinaga, Minoura & Udagawa, Trans. Mycol. Soc. Japan 19: 142 (1978)

≡ *Sphaerodes singaporensis* (Morinaga, Minoura & Udagawa) Dania García, Stchigel & Guarro, Stud. Mycol. 50: 67 (2004)

Notes — *Dactylidispora singaporensis* is distinguished by its ostiolate ascomata, citriform ascospores, and a phialidic asexual morph.

***Medusitheca*** Y. Marín, Stchigel, D. García, Guarro & Cano, **gen. nov.** Fig. 4

MycoBank MB812084.

*Type species*. *Medusitheca citrispora* Y. Marín, Stchigel, D. García, Guarro, A.N. Mill. & Cano.

*Etymology*. From Greek μέδουσα–, jellyfish, and –τείχος, wall, because of the resemblance of the ascomata to a jellyfish due to the abundant presence of setae.

*Diagnosis*: This genus is characterized by dark and strongly setose non-ostiolate ascomata.

*Ascomata* superficial or immersed, solitary o gregarious, globose, non-ostiolate, strongly setose, semi-translucent, pale brown to brown, appearing black when ascospores are mature; setae right, becoming sinouse toward the apex, pale brown to brown, non-septate, rarely 1-septate, thick-walled, verrucous to tuberculate, sometimes branched; *peridium* membranaceous, of *textura angularis* to *textura globulosa*. *Asci* 8-spored, globose to subglobose. *Ascospores* 1-celled, at first hyaline, becoming brown to dark brown, ellipsoidal, one-celled, smooth-walled, with a depressed germ pore at each end.

***Medusitheca citrispora*** Y. Marín, Stchigel, D. García, Guarro, A.N. Mill. & Cano, **sp. nov.** Fig. 4

MycoBank MB812085.

*Etymology*. From Latin *citrum*-, lemon, and -*spora*, spore, referring to the lemon-shaped ascospores.

*Colonies* on PDA attaining a diam of 70–75 mm after 14 d at 35ºC, cottony and granulose (due to the presence of a high number of ascomata), white with grey to black dots, depressed at the centre, margins fringed; reverse yellowish-white to pale yellow (M. 4A2 to 4A3) and with olive brown (M. 4F2) dots. *Mycelium* composed of hyaline to pale yellow, septate, branched, smooth-walled hyphae, 1–3 µm diam. *Ascomata* immersed in the mycelium, solitary or gregarious, globose, cleistothecial, 130–280 µm, setose, semi-translucent, pale brown to brown, appearing black when ascospores are mature; setae right, becoming sinuous toward the apex, 20–200 µm long, 5–20 µm wide at the base, tapering gradually to a rounded tip of 2–5 µm diam, pale brown to brown, non-septate, rarely 1-septate, thick-walled, verrucose to tuberculate, sometimes branched; *peridium* membranaceous, composed of 5-6 layers of flattened cells, 30–40 µm thick, of *textura angularis* to *textura globulosa*, peridial cells of 5–30 µm diam. *Asci* 8-spored, globose to subglobose, 20–25 x 15–20 µm, soon evanescent, without apical structures, disposed at the centrum. *Ascospores* irregularly arranged into the asci, 1-celled, at first hyaline, becoming brown to dark brown with the age, smooth- and thick-walled, ellipsoidal, 20–27 x 10–15 µm, with one germ pore at each end; germ pores 0.75–2 µm diam, depressed. *Asexual morph* absent.

Culture characteristics — Colonies on OA attaining a diam of 50–60 mm in 14 d at 35°C, white to orange white (M. 5A2) with brownish grey dots (M. 5F2), cottony and granulose due to the presence of numerous ascomata, margins arachnoid; reverse

yellowish-white to golden grey (M. 4A2 to 4C2). Minimum and maximum temperature of growth 20 and 40ºC, respectively. Optimum temperature of growth 35ºC.

*Specimens examined*. USA, North Carolina, Great Smoky Mountains National Park, Cartoogechaye Creek Campground (35.137548; -83.491525), forest soil, 15-VII-2008, col. A. N. Miller, M. Calduch and A. M. Stchigel (holotype CBS H-21596, cultures ex-type CBS 137837 = FMR 12767).

***Melanospora*** Corda, Icon. fung. (Prague) 1: 24. 1837, emend. Fig. 3

*Type species*. *Melanospora zamiae* Corda, Icon. fung. (Prague) 1: 24 (1837)

*Ascomata* superficial to immersed, globose to subglobose, ostiolate, yellowish-orange or reddish, tomentose or glabrous, with, usually, a long neck composed of intermixed hypha, with a ring of rigid, hyaline, septate, smooth- and thick-walled setae; *peridium* membranaceous, translucent, of *textura angularis*. *Periphysis* present. *Paraphyses* absent. *Asci* 8-spored, clavate, rounded at the apex, without apical structures, thin-walled, evanescent. *Ascospores* 1-celled, at first hyaline but later becoming brown to dark brown, fusiform, ellipsoidal or citriform, smooth-walled or reticulate or verrucose, with a terminal apiculate germ pore at each end. *Asexual morph* phialidic, hyaline. *Bulbils* uncommon.

Notes — This genus is distinguished by ascomata translucent with a neck composed of intermixed hyphae and with an apical crown of setae, smooth or ornamented ascospores with an apiculate germ pore at each end, and a phialidic asexual morph.

Key to the species of *Melanospora*

1. Ascospores ornamented.....2

1. Ascospores smooth-walled.....4

2. Ascospores irregularly verrucose.....*M. verrucispora*

2. Ascospores reticulate.....3

3. Ascospores coarsely reticulate, 18–24 × 9–12 µm.....*M. mycoparasitica*

3. Ascospores slightly reticulate, 19–24 × 8–10 µm.....*M. tiffanii*

4. Ascospores discoid-ellipsoidal.....5

4. Ascospores otherwise.....7

5. Ascus 4-spored; ascospores 14–19 x 12–14 x 8–9 µm.....*M. longisetosa*

5. Ascus 8-spored; ascospores smaller.....6

6. Neck 250–400 µm; ascospores 7.5–16 x 6–12 x 4–7 µm.....*M. chionea*

6. Neck 150–200(–260) µm; ascospores 10.5–12(–13.5) x 9–10.5(–12) x 7–9 µm.....*M. washingtonensis*

7. Ascomata usually narrower than 100 µm; ascospores citriform to rhomboidal, 18–25 x 10–14 µm.....*M. damnosa*

7. Ascomata usually broader than 100 µm; ascospores ellipsoidal to citriform….....8

8. Ascomata strongly tomentose; neck 1500–2000 µm long.....….....*M. caprina*

8. Ascomata weakly or not tomentose, neck shorter than 1500 µm.....9

9. Neck shorter than 250 µm long; setae 40–80(–200) µm long.....*M. zamiae*

9. Neck longer than 800 µm.....10

10. Setae longer than 100 µm.....*M. arenaria*

10. Setae up to 50 µm long.....*M. lagenaria*

***Melanospora arenaria*** L. Fisch. & Mont., in Montagne, Annls. Sci. Nat., Bot., sér. 4 5: 337 (1856)

Notes — *Melanospora arenaria* is characterized by ascomata with a long neck and ellipsoidal to citriform, smooth-walled ascospores. It is similar to *Melanospora caprina*, but this has more tomentose ascomata.

***Melanospora caprina*** (Fr.) Sacc., Syll. fung. (Abellini) 2: 462 (1883)

*Basionym*. *Sphaeria caprina* Fr., Fl. Danic. 11: tab. 1859, fig. 2 (1825)

≡ *Ceratostoma caprinum* (Fr.) Fr., Summa veg. Scand., Section Post. (Stockholm): 396 (1849)

≡ *Cerastoma caprinum* (Fr.) Quél., Mém. Soc. Émul. Montbéliard, Sér. 2 5: 522 (1875)

= *Sphaeria vervecina* Desm., Annls Sci. Nat., Bot., sér. 2 17: 13 (1842)

≡ *Melanospora vervecina* (Desm.) Fuckel, Jb. nassau. Ver. Naturk. 23-24: 126 (1870)

= *Melanospora vervecina f. arundinis* Sacc., *Syll. fung.* (Abellini) 2: 461 (1883)

Notes — *Melanospora caprina* is distinguished from the other species of the genus by its larger, densely white-tomentose, ascomata with a very long neck, and the ellipsoidal to citriform, smooth-walled ascospores with slightly apiculate germ pores.

***Melanospora chionea*** (Fr.) Corda, Icon. fung. (Prague) 1: 24 (1837)

*Basionym*. *Ceratostoma chioneum* Fr., Observ. mycol. (Havniae) 2: 340 (1818)

≡ *Sphaeria chionea* (Fr.) Fr., Syst. mycol. (Lundae) 2: 446 (1823)

≡ *Melanospora chionea* var. *chionea* (Fr.) Corda, Icon. fung. (Prague) 1: 24, tab. 7, fig. 297 (1837)

= *Sphaeria biformis* var. *brachystoma* Pers., Syn. meth. fung. (Göttingen) 1: 60 (1801)

≡ *Melanospora chionea* var. *brachystoma* (Pers.) Sacc., Syll. fung. (Abellini) 2: 461 (1883)

= *Sphaeria leucophaea* Fr., Elench. fung. (Greifswald) 2: 92 (1828)

≡ *Ceratostoma leucophaeum* (Fr.) Fr., Summa veg. Scand., Section Post. (Stockholm): 396 (1849)

≡ *Melanospora chionea* var. *leucophea* (Fr.) Sacc., Syll. fung. (Abellini) 2: 461 (1883)

= *Melanospora antarctica* Speg., Boln Acad. nac. Cienc. Córdoba 11: 233 (1888)

Notes — This species is characterized by white-tomentose ascomata and discoid, smooth-walled ascospores with depressed germ pores.

***Melanospora damnosa*** (Sacc.) Lindau, in Engler & Prantl, Nat. Pflanzenfam., Teil. I (Leipzig) 1: 353 (1897). Fig. 3a,d

*Basionym*. *Sphaeroderma damnosum* Sacc., in Berlese, Riv. Patol. veg., Pavia 3: tabs VII-VIII, nos 1-6 (1896)

Notes — *Melanospora damnosa* is distinguised by ascomata with a short neck and citriform to rhomboidal, smooth-walled ascospores with a slightly apiculate germ pore at each end.

***Melanospora lagenaria*** (Pers.) Fuckel, Jb. nassau. Ver. Naturk. 23-24: 126 (1870)

*Basionym*. *Sphaeria lagenaria* Pers., Syn. meth. fung. (Göttingen) 1: 58 (1801)

≡ *Ceratostoma lagenaria* (Pers.) Fr. [as 'lagenarium'], Syst. veg., Edn 16: 392 (1827)

≡ *Auerswaldia lagenaria* (Pers.) Rabenh., Hedwigia 1: 116 (1857)

≡ *Cerastoma lagenaria* (Pers.) Quél., Mém. Soc. Émul. Montbéliard, Sér. 2 5: 522 (1875)

≡ *Phaeostoma lagenaria* (Pers.) Munk [as 'lagenarium'], Dansk bot. Ark. 17: 82 (1957)

= *Sphaeria vervecina* Desm., Annls Sci. Nat., Bot., sér. 2 17: 13 (1842)

= *Melanospora lagenaria* var. *tetraspora* Rehm, Hedwigia 30: 259 (1891)

Notes — *Melanospora lagenaria* is similar to *M. caprina*, but the former has less tomentose ascomata, shorter necks with a poorly developed crown of setae.

***Melanospora longisetosa*** P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 130 (1982).

Notes — This species is characterized by 4-spored asci and discoid, smooth-walled ascospores.

***Melanospora mycoparasitica*** (Vujan.) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812086

*Basionym*. *Sphaerodes mycoparasitica* Vujan., Mycol. Res. 113: 1173 (2009)

Notes — *Melanospora mycoparasitica* is distinguished by fusiform and coarsely reticulate ascospores.

***Melanospora tiffanii*** Kowalski, Mycologia 57: 279 (1965)

Notes — This species produces fusiform and slightly reticulate ascospores.

***Melanospora verrucispora*** Takada, in Kobayasi et al., Bull. natn. Sci. Mus., Tokyo 16: 525 (1973). Fig. 3e,f

Notes — This species is distinguished by its irregularly verrucose ascospores.

***Melanospora washingtonensis*** Nitzan, J.D. Rogers & D.A. Johnson, Sydowia 56: 282 (2004)

Notes — This species is similar to *M. chionea*, but they differ in the length of the neck (150–200(–266) μm in *M. washingtonensis* and 250–400 μm in *M. chionea*) and in the size the ascospores (10.5–12(–13.5) x 9–10.5(–12) x 7–9 μm in *M. washingtonensis* and 7.5–16 x 6–12 x 4–7 μm in *M. chionea*), as well as in the presence of phialidic asexual morph in *M. washingtonensis*.

***Melanospora zamiae*** Corda., Icon. fung. (Prague) 1: 24 (1837). Fig. 3b,c

= *Melanospora leucotricha* Corda, Icon. fung. (Prague) 1: 25 (1837)

= *Melanospora coemansii* Westend., Bull. Acad. R. Sci. Belg., Cl. Sci.: 579 (1857)

= *Melanospora cirrhata* Berk. in Cooke, Grevillea 16: 102 (1888)

= *Melanospora globosa* Berl., Malpighia 5: 409 (1891)

= *Melanospora pampeana* Speg., Anal. Mus. nac. Hist. nat. B. Aires 6: 287 (1898)

= *Melanospora townei* Griffiths, Bull. Torrey bot. Club 26: 434 (1899)

= *Melanospora rhizophila* Peglion & Sacc., Annls mycol. 11: 16 (1913)

= *Melanospora mattiroloana* Mirande [as 'mattiroliana'], Bull. Soc. mycol. Fr. 32: 72 (1916)

= *Melanospora schmidtii* Sacc., Syll. fung. (Abellini) 24: 650 (1926)

= *Melanospora asclepiadis* Zerova, J. Inst. Bot. Acad. Sci. Ukraine 12: 155 (1937)

Notes — *Melanospora zamiae* is characterized by the production of ellipsoidal to citriform and smooth-walled ascospores with a depressed germ pore at each end. Doguet (1955) described the presence of bulbils; however, later studies on this fungus did not mention the presence of those propagules (Calviello 1973, Cannon and Hawksworth 1982). In any case, the bulbils rarely occur in the genus.

We could not include the species *Melanospora arenaria, M. caprina, M. chionea, M. lagenaria, M. tiffanii* and *M. washingtonensis* in the present study since their type species are not available; however, these species are well described in the protologues and their inclusion in *Melanospora* seems undoubtable.

DOUBTFUL SPECIES

***Melanospora aculeata*** (E.C. Hansen), Vidensk. Meddel. Dansk Naturhist. Foren. Kjøbenhavn 59: 15 (1877)

Notes — Cultures of this species were not available, but this species was described producing small asci (18–21 x 7–8 μm) and ascospores (4–6 x 3–4 μm). This species produced ostiolate ascomata without a neck, typical of *Microthecium*; however, so small ascospores have never seen in that genus.

***Melanospora cervicula*** Hotson, Proc. Amer. Acad. Arts & Sci. 48: 254 (1912)

Notes — Cultures were not available, but jutging from the protologue, it could be a species of *Melanospora* since it shows a long neck (85–140 μm), which is a typical distinction for the genus; however, in the original description the structure of the neck is not mentioned.

***Melanospora endobiotica*** Woron., Notul. syst. Inst. cryptog. Horti bot. petropol. 3: 31 (1924)

Notes — Cultures were not available, and pictures or drawings were not included in the protologue. It was reported as morphologically related to *Melanospora rhizophila* (now considered a synonym of *Melanospora zamiae* (Doguet 1955)) when was described (Woronichin 1924).

EXCLUDED SPECIES

***Melanospora arachnophila*** Fuckel, Jb. nassau. Ver. Naturk. 23-24: 127 (1870)

Notes — This species shows cylindrical asci and hyaline ascospores, features never seen in *Melanospora.* It was already excluded fron *Melanospora* by Doguet (1955).

***Melanospora argadis*** Czerepan., Nov. sist. Niz. Rast. 3: 177 (1966)

Notes — This species shows morphological features never observed in *Melanospora*, e.g. the small size of their asci (10–14 x 5–6.5 μm) and of their olivaceous ascospores (5–5.5 x 3–3.5 μm). The original description is not enough detailed to ascertain its possible taxonomical placement.

***Melanospora exsola*** Bat. & H.P. Upadhyay, Atas Inst. Micol. Univ. Recife 2: 331 (1965)

Notes — This species is excluded from *Melanospora* because its dark brown, setose ascomata and small ascospores (4.5–12 x 4–7 μm) which seems to indicate a closer relationship with *Chaetomium*.

***Melanospora gigantea*** (Massee & Crossl.) Massee & Crossl., Fungus Flora of Yorkshire (Leeds): 215 (1905)

Notes — Descriptions of this species and of its basyonym, *Sphaeroderma gigantea*, were not found.

***Melanospora lucifuga*** (Jungh.) Sacc., Syll. fung. (Abellini) 2: 464 (1883)

Notes — Cultures were not available and the original description lacks of asci and ascospores description. Therefore, we agree with Doguet (1955) in the exclusion of this fungus from *Melanospora*.

***Melanospora kurssanoviana*** (Beliakova) Czerepan., Notul. syst. Sect. cryptog. Inst. bot. Acad. Sci. U.S.S.R. 15: 84 (1962)

Notes — In this phylogenetic study, *M. kurssanoviana* was placed in a lineage far from the Melanosporales. Unfortunately, the only living culture available apparently has lost its

ability to develop reproductive structures, and we did not find in the description and the drawing provided when was originally described as a new *species* of *Chaetomium* any distinctive morphological feature to differentiate and delimitate this species from the other members of Melanosporales.

***Melanospora macrospora*** P. Karst., Hedwigia 30: 299 (1891)

Notes — Doguet (1955) excluded this species due to the production of very large (480–500 x 33–36 μm) cylindrical asci and ascospores (42–52 x 28–35 μm), morphological features not observed in members of *Melanosporales*.

***Melanospora octahedrica*** Pat., Cat. Rais. Pl. Cellul. Tunisie (Paris): 109 (1897)

Notes — This species is transferred to *Scopinella* by the shape of their ascospores.

***Scopinella octahedrica*** (Pat.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB812087.

***Melanospora pascuensis*** Stchigel & Guarro, Mycol. Res. 103: 1305 (1999)

Notes — This species is excluded from *Melanospora* since its neck is cellular or absent. It is characterized by dark ring-like structure around the germ pores of the ascospores. This species could be a new genus since this kind of structure around the germ pore has never seen in other members of *Melanosporales*, considering that this structure could be phylogenetically informative as in the case of *Dactylidispora*, which is distinguished by its ascospores with a raised rim around the germ pores. However, the living culture of the type strain of this fungus was contaminated with other fungus and it could not be included in the molecular study.

***Melanospora setchellii*** (Harkn.) Sacc. & P. Syd., Syll. fung. (Abellini) 16: 564 (1902)

Notes — This species is excluded from *Melanospora* since it produces cylindrical asci within the ascospores are linearly disposed, feature never observed in the species of that genus.

***Melanospora similis*** Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 126: 343 (1917)

Notes — There are not cultures of this species, and was described in German, without Latin diagnosis. This species is morphologically similar to *Pustulipora corticola*, differing only by the shape of the ascospores (symmetric in *Melanospora similis* and assymetric in *Pustulipora corticola*). Neither pictures nor drawings were included in the protologue to determine if cushion-like structures surrounding the germ pore, typical of *Pustulipora*, were produced.

***Melanospora vitrea*** (Corda) Sacc., Syll. fung. (Abellini) 2: 463 (1883)

*Basionym*. *Sphaeronaema vitreum* Corda, Icon. fung. (Prague) 1: 25 (1837)

Notes —Doguet (1955) excluded this species because it produces oblate and pale yellow ascospores.

***Microthecium*** Corda, Icon. fung. (Prague) 5: 30, 74 (1842), emend. Fig. 2

*Type species*. *Microthecium zobelii* Corda, Icon. fung. (Prague) 5: 74. 1842.

= *Sphaerodes* Clem., Gen. fung. (Minneapolis): 44, 173. 1909.

= *Pteridiosperma* J.C. Krug & Jeng, Mycotaxon 10: 44. 1979.

= *Persiciospora* P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 133. 1982.

*Ascomata* superficial or immersed, globose to subglobose or pyriform, ostiolate or not, yellowish-orange or reddish, tomentose or glabrous; neck short or absent, conical, composed of angular cells similar to those of the peridial cells, usually with a crown of hyaline, septate, smooth- and thick-walled setae around the ostiole; *peridium* membranaceous, translucent, of *textura angularis*. *Periphysis* present. *Paraphyses* absent. *Asci* 8-spored, clavate, rounded at the apex, without apical structures, thin-walled, evanescent. *Ascospores* 1-celled, at first hyaline becoming brown to dark brown with the age, ellipsoidal, fusiform, navicular or citriform, smooth, reticulate, pitted or wrinkled,

with a terminal apiculate or depressed germ pore at each end. *Asexual morph* phialidic, hyaline. *Bulbils* usually produced, pale orange to reddish-orange.

Notes — *Microthecium* has translucent ascomata of *textura angularis*, cellular necks short or absent, ascospores smooth-walled or ornamented with a depressed or apiculate germ pore at each end, often producing bulbils and a phialidic asexual morph.

Key to the species of *Microthecium*

1. Sexual morph absent, only producing of bulbils.....*M. sepedonioides*

1. Sexual morph present.....2

2. Ascomata non-ostiolate.....3

2. Ascomata ostiolate.....13

3. Ascospores with the surface ornamented.....4

3. Ascospores smooth or nearly so.....9

4. Ascospores pitted and with wing-like ridges, 17–21 x 8–10 µm.....*M. foveolatum*

4. Ascospores coarsely reticulate.....5

5. Asci 4-spored.....6

5. Asci 8-spored.....7

6. Ascospores (25–)28–34(–40) x 14–18(–20) µm.....*M. beatonii*

6. Ascospores 22–28 x 12–15 x 9–11 µm, 1/3 of which are coarsely reticulate and remaining smooth-walled the rest.....*M. perplexum*

7. Ascospores 25–34 x 12–18 µm.....*M. episphaerium*

7. Ascospores 17–20 x 10–12 x 7–9 µm.....*M. retisporum*

8. Ascomata smaller than 120 µm; ascospores finely reticulated under SEM, 19–23 x (12–)14–15(–17) x 10–13 µm.....*M. tenuissimum*

8. Ascomata larger than 120 µm.....9

9. Ascospores shorter than 20 µm.....10

9. Ascospores longer than 20 µm.....11

10. Ascospores 15–19 x 11–13 x 8–9 µm, with the narrow faces coarsely reticulate and the rest smooth.....*M. compressum*

10. Ascospores 10–17 x 8–12 x 9–10 µm, completely smooth-walled.....*M. levitum*

11. Ascospores fusiform, 20–21 x 8 x 7 µm.....*M. hypomyces*

11. Ascospores citriform.....12

12. Ascospores 28–30 x 12–13(–15) µm.....*M. geoporae*

12. Ascospores 18–25 x 8.5–12 x 6–9 µm.....*M. zobelii*

13. Ascospores ornamentated with wing-like appendages.....14

13. Ascospores otherwise.....15

14. Ascospores with wrinkles, (12–)13–18 x (7–)8–10 µm.....*M. ciliatum*

14. Ascospores pitted-walled, (17–)20–22(–24) x 12–14 x 10–12.....*M. lenticulare*

15. Ascospores with the surface ornamentated.....16

15. Ascospores smooth-walled.....23

16. Ascospores punctate or punctate-reticulate.....17

16. Ascospores reticulate or striate-reticulate.....19

17. Ascospores punctate, ellipsoidal, 17–21 x 13–15 µm.....*M. africanum*

17. Ascospores punctate-reticulate, ellipsoidal-fusiform.....18

18. Ascospores delicately punctate, asexual morph and bulbils present.....*M. japonicum*

18. Ascospores coarsely punctate, asexual morph and bulbils absents.....*M. moreaui*

19. Ascospores striate-reticulate.....20

19. Ascospores reticulate.....21

20. Ascospores with incospicuous ridges forming a very coarse reticulum, 18–22(–28) x 9.5–11(–13) x 8–9 µm.....*M. micropertusum*

20. Ascospores without ridges or reticulum, 26–36 x 13–17µm…….....*M. masonii*

21. Ascospores with 4–6 prominent longitudinal ribs, 23–28(–30) x 10–12 x 8–10 µm.....*M. quadrangulatum*

21. Ascospores without longitudinal ribs, coarsely reticulate…….....22

22. Ascospores spindle-shaped, 19.5–22 x 8.5–11 µm.....*M. internum*

22. Ascospores citriform to fusiform, 14–20 x 10–17 µm.....*M. fimicola*

23. Crown of setae absent.....*M. nectrioides*

23. Crown of setae present around the ostiole.....24

24. Ascospores citriform, 20–24 x 11–14 µm.....*M. marchicum*

24. Ascospores otherwise.....25

25. Ascospores ellipsoid to citriform, often somewhat plataniform.....26

25. Ascospores otherwise.....28

26. Bulbils present; ascospores 20–32 x 10–16 µm.....*M. fallax*

26. Bulbils absents.....27

27. Ascospores 21–34 x 11–17 µm.....*M. brevirostris*

27. Ascospores 18–22 x 9–11 µm.....*M. fimbriatum*

28. Ascospores ellipsoid to fusiform, 20–25 x 7–12 µm.....*M. fusisporum*

28. Ascospores ellipsoid to navicular.....29

29. Ascospores (9.5–)11–12(–13) x 4–4.5 µm.....*M. pegleri*

29. Ascospores longer tan 15 µm.....30

30. Ascospores 16–24 x 8–12 µm.....*M. fayodii*

30. Ascospores 25–30 x 11–15 µm.....*M. brevirostratum*

***Microthecium africanum*** (J.C. Krug) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812088.

*Basionym*. *Persiciospora africana* J.C. Krug, Mycologia 80: 416 (1988)

Notes — *Microthecium africanum* is characterized by ostiolate ascomata and punctate ellipsoidal ascospores. Although it was reported the presence of asexual morph with two different sort of conidia (i), 1–4(–5)-celled, globose and smooth-walled at first but becoming cylindrical and coarsely verrucose later; (ii), 1–2-celled, large, usually cylindrical and smooth-walled), probably the strain was not a pure culture because the 18S and 28S sequences match with *Fusarium* spp. and the pictures of those conidia resemble the chlamydospores produced by several species of this genus.

***Microthecium beatonii*** D. Hawksw., Trans. Mycol. Soc. Japan 18: 145 (1977)

Notes — This species is characterized by non-ostiolate ascomata, 4-spored asci and very coarsely reticulate, citriform ascospores. These morphological features are also observed in *Microthecium perplexum*, but this species produces only a third of the ascospores coarsely reticulated remaining smooth-walled the rest. *Microthecium episphaerium* and *Microthecium retisporum* differs from *M. beatonii* because both produce 8-spored asci. Both species can be distinguished by the size of their ascospores (25–34 x 12–18 µm in *M. episphaerium* and 17–20 x 10–12 x 7–9 µm in *M. retisporum*). Moreover, *M. retisporum* produces a phialidic asexual morph and bulbils, absent in *M. beatonii, M. episphaerium* and *M. perplexum.*

***Microthecium brevirostratum*** (Moreau) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

Mycobank MB812089.

*Basionym*. *Melanospora brevirostrata* Moreau, Bull. trimest. Soc. mycol. Fr. 61: 59 (1945)

Notes — *Microthecium brevirostratum* together with *Microthecium fayodii* and *Microthecium pegleri* produces ostiolate ascomata, smooth-walled, ellipsoidal to

navicular or citriform ascospores and bulbils. *Microthecium brevirostratum* is easily distinguished by its ascospores with apiculate germ pores and the presence of a phialidic asexual morph (ascospores showing depressed germ pores and lacking of an asexual morph in the other species). *Microthecium fayodii* and *M. pegleri* differ in the size of the ascospores, being *M. pegleri* the species with the smaller ascospores in *Microthecium* ((9.5–)11–12(–13) x 4–4.5 µm).

***Microthecium brevirostre*** (Fuckel) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812090.

*Basionym*. *Teichospora brevirostris* Fuckel, Jb. nassau. Ver. Naturk. 23-24: 161 (1870)

≡ *Strickeria brevirostris* (Fuckel) G. Winter, Rabenh. Krypt.-Fl., Edn 2 (Leipzig) 1.2: 283 (1885)

≡ *Melanospora brevirostris* (Fuckel) Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 123: 94 (1914)

= *Sphaeria brevirostris* Fr., Syst. mycol. (Lundae) 2: 474 (1823)

≡ *Cerastoma brevirostre* (Fr.) Quél., Mém. Soc. Émul. Montbéliard, Sér. 2 5: 521 (1875)

≡ *Ceratostoma brevirostre* (Fr.) Sacc., Syll. fung. (Abellini) 1: 216 (1882)

= *Ceratostoma helvellae* Cooke, Grevillea 1: 175 (1873)

≡ *Melanospora helvellae* (Cooke) Sacc., Syll. fung. (Abellini) 2: 462 (1883)

= *Melanospora sphaerodermoides* Grove, J. Bot., Lond. 23: 4 (1885)

= *Melanospora sphaerodermoides var. sphaerodermoides* Grove, J. Bot., Lond. 23: 4 (1885)

= *Thielavia soppittii* Crossl., Naturalist, London: 7 (1901)

= *Rosellinia aurea* McAlpine, Fungus Diseases of stone-fruit trees in Australia: 102 (1902)

≡ *Melanospora aurea* (McAlpine) Doguet, Botaniste 39: 124 (1955)

= *Melanospora sphaerodermoides var. rubella* Pidopl., Fungus flora of coarse fodders: 69 (1948)

= *Melanospora camelina* Faurel & Schotter, Revue Mycol., Paris 30: 144 (1965)

= *Melanospora tulasnei* Udagawa & Cain, Can. J. Bot. 47: 1932 (1970)

Notes — *Microthecium brevirostris*, *Microthecium fallax* and *Microthecium fimbriatum* produce ostiolate ascomata and ellipsoidal to citriform, often plataniform, smooth-walled ascospores with an apiculate germ pore at each end. *M. fimbriatum* is easily distinguished

by its smaller (100–110 µm) and reddish ascomata and *M. fallax* differs in the production of bulbils.

***Microthecium ciliatum*** Udagawa & Takada, Trans. Mycol. Soc. Japan 15: 23 (1974)

≡ *Pteridiosperma ciliatum* (Udagawa & Y. Takeda) J.C. Krug & Jeng, Mycotaxon 10: 45 (1979)

Notes — This species is characterized by non-ostiolate ascomata and ellipsoidal to fusiform ascospores ornamented with wing-like appendages and wrinkles, and the production of a phialidic asexual morph and bulbils. *Microthecium lenticulare* and *Microthecium foveolatum* also present ascospores with wing-like appendages, but these are pitted. Moreover, both species do not produce bulbils. *Microthecium foveolatum*, such as *M. ciliatum*, is characterized by non-ostiolate ascomata and the production of a phialidic asexual morph, whereas *Microthecium lenticulare* has ostiolate ascomata and lacks asexual morphs.

***Microthecium compressum*** Udagawa & Cain, Can. J. Bot. 47: 1921 (1970)

≡ *Sphaerodes compressa* (Udagawa & Cain) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 145 (1982)

Notes — This species is distinguished by non-ostiolate ascomata and citrifom, bilaterally flattened ascospores, with the narrow faces coarsely reticulate and the widest ones smooth or nearly so, plus the production of a phialidic asexual morph.

***Microthecium episphaerium*** (W. Phillips & Plowr.) Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 123: 98 (1914)

*Basionym*. *Melanospora episphaeria* W. Phillips & Plowr., Grevillea 10: 71 (1881)

≡ *Sphaeroderma episphaerium* (W. Phillips & Plowr.) Sacc., *Syll. fung.* (Abellini) 2: 460 (1883)

≡ *Sphaerodes episphaerium* (W. Phillips & Plowr.) Clem. [as 'episphaericum'], Gen. fung. (Minneapolis): 1–227 (1909)

≡ *Vittadinula episphaeria* (W. Phillips & Plowr.) Clem. & Shear, *Gen. fung.*, Edn 2 (Minneapolis): 281 (1931)

= *Sphaeroderma epimyces* Höhn., Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften Math.-naturw. Klasse Abt. I 116: 103 (1907)

≡ *Melanospora epimyces* (Höhn.) Doguet, Botaniste 39: 125 (1955)

***Microthecium fallaciosum*** (Zukal) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812772.

*Basionym*. *Melanospora fallax* Zukal, Ascomyceten: 28 (1889)

= *Melanospora anomala* Hotson, Proc. Amer. Acad. Arts & Sci.: 257 (1912)

= *Melanospora papillata* Hotson, Proc. Amer. Acad. Arts & Sci.: 251 (1912)

= *Melanospora phaseoli* Roll-Hansen, Blyttia 6: 73 (1948)

***Microthecium fayodii*** (Vuill.) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.** Fig. 2b, f, m

MycoBank MB812091.

*Basionym*. *Melanospora fayodii* Vuill. [as 'fayodi'], Bull. Séanc. Soc. Sci. Nancy, Sér. 2 8: 33 (1887)

***Microthecium fimbriatum*** (Rostr.) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812092.

*Basionym*. *Sphaeroderma fimbriatum* Rostr., Oest. Grönl. Svampe: 25 (1894)

≡ *Melanospora fimbriata* (Rostr.) Petch, Trans. Br. mycol. Soc. 21: 253 (1938)

***Microthecium fimicola*** (E.C. Hansen) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

Fig. 2c, k, n

MycoBank MB812093.

*Basionym*. *Melanospora fimicola* E.C. Hansen, Vidensk. Meddel. Dansk Naturhist. Foren. Kjøbenhavn: 15 (1876)

≡ *Sphaeroderma fimicola* (E.C. Hansen) Sacc., Syll. fung. (Abellini) 2: 460 (1883)

≡ *Sphaerodes fimicola* (E.C. Hansen) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 146 (1982)

= *Melanospora ornata* Zukal, Verh. zool.-bot. Ges. Wien 35: 340 (1886)

≡ *Sphaerodes ornata* (Zukal) Arx, Gen. Fungi Sporul. Cult., Edn 3 (Vaduz): 156 (1981)

= *Sphaeroderma hulseboschii* Oudem., Contrib. Flora Mycol. d. Pays-Bas 11: 23 (1886)

≡ *Melanospora hulseboschii* (Oudem.) Doguet, Botaniste 39: 121 (1955)

= *Melanospora affine* Sacc. & Flageolet, Bull. Soc. mycol. Fr. 12: 67 (1896)

= *Melanospora manginii* Vincens [as 'mangini'], Bull. Soc. mycol. Fr. 33: 69 (1917)

≡ *Sphaerodes manginii* (Zukal) Arx, Gen. Fungi Sporul. Cult., Edn 3 (Vaduz): 156 (1981)

Notes — *Microthecium fimicola* is characterized by ostiolate ascomata and coarsely reticulate ascospores with a strongly apiculate germ pore at both ends. The other species with ostiolate ascomata and reticulate ascospores are *Microthecium internum* and *Microthecium quadrangularis.* The main differences among them are the shape and the size of the ascospores, being citrifrom in *M. fimicola*, spindle-shaped in *M. internum* and fusiform in *M. quadrangularis*. The production of bulbils has been only observed in *M. fimicola*, although this production was not reported before.

***Microthecium foveolatum*** Udagawa & Y. Horie, in Hawksworth & Udagawa, Trans. Mycol. Soc. Japan 18: 149 (1977)

≡ *Pteridiosperma foveolatum* (Udagawa & Y. Horie) J.C. Krug & Jeng, Mycotaxon 10: 45 (1979)

***Microthecium fusisporum*** (Petch) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812094.

*Basionym*. *Sphaeroderma fusisporum* Petch, Naturalist, London: 58 (1936)

≡ *Melanospora fusispora* (Petch) Doguet, Botaniste 39: 215 (1955)

= *Melanospora fusispora var. fusispora* (Petch) Doguet, Botaniste 39: 215 (1955)

= *Melanospora fusispora var. parvispora* Matsush., Matsush. Mycol. Mem. 8: 24 (1995)

Notes — *Microthecium fusisporum* is related to *Microthecium nectrioides*, both showing ostiolate ascomata and smooth-walled, fusiform ascospores. However, *M. nectrioides* can be distinguished by the absence of the crown of setae around the ostiole and its more asymmetric ascospores.

***Microthecium geoporae*** (W. Oberm.) Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 123: 98 (1914)

*Basionym*. *Guttularia geoporae* W. Oberm., Mykol. Zentbl. 3: 9 (1913)

Notes — This species produces non-ostiolate ascomata and smooth-walled, citrifrom ascospores. Other species of *Melanospora* characterized by the production of non-

ostiolate ascomata and smooth-walled ascospores are *Microthecium hypomyces*, *Microthecium levitum* and *Microthecium zobelii*. *Microthecium hypomyces* is distinguished by its fusiform ascospores (citriform in the other species) and *M. levitum* by the presence of bulbils and a phialidic asexual morph. *Microthecium geoporae* and *M. zobelii* are distinguished by the size of their ascospores (28–30 x 12–13(–15) µm in *M. geoporae* and 18–25 x 8.5–12 x 6–9 µm in *M. zobelii*). *Microthecium tenuissimum* shows similar morphological features to these species but its ascospores are finely reticulate under SEM and its ascomata are smaller than the other species (less than 120 µm).

***Microthecium hypomyces*** (Höhn.) Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 123: 50 (1914)

*Basionym*. *Sphaeroderma hypomyces* Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 116: 102 (1907)

≡ *Melanospora hypomyces* (Höhn.) Doguet, Botaniste 39: 215 (1955)

***Microthecium internum*** (Tehon & G.L. Stout) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812095.

*Basionym*. *Melanospora interna* Tehon & G.L. Stout, Mycologia 21: 181 (1929)

***Microthecium japonicum*** (Y. Horie, Udagawa & P.F. Cannon) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.** Fig. 2h

MycoBank MB812096.

*Basionym*. *Persiciospora japonica* Y. Horie, Udagawa & P.F. Cannon, Mycotaxon 25: 233 (1986)

Notes — *Microthecium japonicum* is characterized by ostiolate ascomata and punctate-reticulate, ellipsoidal to fusiform ascospores, similar to *Microthecium moureai*. However, *M. japonicum* produces a phialidic asexual morph and bulbils (which are absent in *M. moureai*) and delicately reticulate ascospores (coarsely reticulate in *M. moureai*).

***Microthecium lenticulare*** (Udagawa & T. Muroi) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812097.

*Basionym*. *Pteridiosperma lenticulare* Udagawa & T. Muroi [as 'lenticularis'], Trans. Mycol. Soc. Japan 22: 20 (1981)

***Microthecium levitum*** Udagawa & Cain, Can. J. Bot. 47: 1917. 1970. Fig. 2a, e, i

≡ *Sphaerodes levita* (Udagawa & Cain) Dania García, Stchigel & Guarro, Stud. Mycol. 50: 67 (2004)

***Microthecium marchicum*** (Lindau) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812099.

*Basionym*. *Chaetomium marchicum* Lindau, Hedwigia 35: 56 (1896)

≡ *Sphaeroderma marchicum* (Lindau) Sacc. & P. Syd., Syll. fung. (Abellini) 14: 627 (1899)

Notes — *Microthecium marchicum* is characterized by ostiolate ascomata and smooth-walled citrifrom ascospores. Its ascospores are similar to those of *M. geoporae*, *Microthecium hypomyces*, *Microthecium levitum* and *Microthecium zobelii*, but all of them produce non-ostiolate ascomata.

***Microthecium masonii*** (Kirschst.) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812100.

*Basionym*. *Ceratostoma masonii* Kirschst., Trans. Br. mycol. Soc. 18: 306 (1934)

≡ *Persiciospora masonii* (Kirschst.) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 135 (1982)

Notes — *Microthecium masonii* is characterized by ostiolate ascomata and faintly striate-reticulate, ellipsoidal to fusiform ascospores. The same sort of ascospore ornamentation is also observed in *Microthecium micropertusum*, but it is easily distinguished by the presence of inconspicuous ridges forming a very coarse reticulum, and a phialidic asexual morph.

***Microthecium micropertusum*** (Petch) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB812101.

*Basionym*. *Sphaerodes micropertusa* Y. Horie, Udagawa & P.F. Cannon, Mycotaxon 25: 236 (1986)

***Microthecium moreaui*** (P.F. Cannon & D. Hawksw.) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812102.

*Basionym*. *Persiciospora moreaui* P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 134 (1982)

***Microthecium nectrioides*** (Marchal) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812103.

*Basionym*. *Sphaeroderma nectrioides* Marchal, Bull. Soc. R. Bot. Belg. 23: 25 (1884)

≡ *Melanospora nectrioides* (Marchal) Doguet, Botaniste 39: 121 (1955)

= *Melanospora asparagi* G. Arnaud, Ann. Serv. Epiph. 2: 273 (1915)

***Microthecium pegleri*** (D. Hawksw. & A. Henrici) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812104.

*Basionym*. *Melanospora pegleri* D. Hawksw. & A. Henrici, Kew Bull. 54: 795 (1999)

***Microthecium perplexum*** D. Hawksw., Trans. Mycol. Soc. Japan 18: 151 (1977)

≡ *Sphaerodes perplexa* (D. Hawksw.) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 148 (1982)

***Microthecium quadrangulatum*** (E.C. Hansen) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.** Fig. 2d, j

MycoBank MB812105.

*Basionym*. *Sphaerodes quadrangularis* Dania García, Stchigel & Guarro, Stud. Mycol. 50: 64 (2004)

***Microthecium retisporum*** Udagawa & Cain, Can. J. Bot. 47: 1926 (1970). Fig. 2g, l

≡ *Sphaerodes retispora* (Udagawa & Cain) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 149 (1982)

= *Microthecium retisporum var. inferius* Udagawa & Cain [as 'inferior'], Can. J. Bot. 47: 1928 (1970)

≡ *Sphaerodes retispora var. inferior* (Udagawa & Cain) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 149 (1982)

= *Microthecium retisporum var. retisporum* Udagawa & Cain, Can. J. Bot. 47: 1926 (1970)

≡ *Sphaerodes retispora var. retispora* (Udagawa & Cain) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 149 (1982)

***Microthecium sepedonioides*** (Preuss) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

Fig. 2o

MycoBank MB812106.

*Basionym*. *Papulaspora sepedonioides* Preuss, Linnaea 24: 112 (1851)

Notes — *Microthecium sepedonioides* only produces bulbils. The sexual morph has never been observed.

***Microthecium tenuissimum*** (Dania García, Stchigel & Guarro) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812107.

*Basionym*. *Sphaerodes tenuissima* Dania García, Stchigel & Guarro, Stud. Mycol. 50: 65. 2004.

***Microthecium zobelii*** Corda, Icon. fung. (Prague) 5: 74 (1842).

≡ *Sphaeria zobelii* (Corda) Tul. & C. Tul., Fungi hypog.: 186 (1851)

≡ *Ceratostoma zobelii* (Corda) Berk., Journal of the Royal Horticultural Society 4: 402 (1860)

≡ *Melanospora zobelii* (Corda) Fuckel, Jb. nassau. Ver. Naturk. 23-24: 127 (1870)

= *Melanospora zobelii var. zobelii* (Corda) Fuckel, Jb. nassau. Ver. Naturk. 23-24: 127 (1870)

= *Melanospora coprophila* Zukal, Verh. zool.-bot. Vereins Wien 37: 25 (1887)

= *Melanospora marchicum* Lindau, Hedwigia 35: 56 (1896)

= *Melanospora zobelii var. minor* Pidopl. Fungus flora of coarse fodders: 68 (1953)

The species *M. africanum, M. beatonii, M. brevirostratum, M. episphaerium, M. foveolatum, M. geoporae, M. hypomyces, M. internum, M. lenticulare, M. marchicum, M. masonii, M. micropertusum, M. moureai, M. nectrioides, M. pegleri and M. perplexum* were not included in the present study because we could not locate the specimens since the holotype or live cultures of most of them are not available. However, these species were transferred to *Microthecium*, based on their complete and well illustrated descrptions.

EXCLUDED SPECIES

***Microthecium ryvardenianum*** Aramb. & Gamundí, Agarica 6: 124 (1985)

Notes — This species is excluded from *Microthecium* because presents morphological features atypical of *Melanosporales*, e.g. allantoid ascospores when immature and striate when mature.

***Pseudomicrothecium*** Y. Marín, Stchigel, Guarro, Cano, **gen. nov.**

MycoBank MB812108.

*Type species*. *Pseudomicrothecium subterraneum* (L. Fan, C.L. Hou, P.F. Cannon & Yong Li) Y. Marín, Stchigel, Guarro & Cano.

*Etymology*. The name refers to the morphological resemblance to *Microthecium*.

*Diagnosis*: This genus is characterized by non-ostiolate ascomata, 2-spored asci and smooth-walled ascospores with an indistinct germ pore at each end.

*Ascomata* immersed, growing in internal tissues of *Tuber* spp. ascomata, globose, non-ostiolate, translucent, light brown to mid brown, appearing dark brown when the ascospores are mature, glabrous or setose; *peridium* membranaceous, *textura angularis*. *Asci* 2-spored, clavate, short-stipitate, evanescent. *Ascospores* one-celled, at first hyaline, becoming dark brown to blackish, ellipsoidal to citriform, umbonate and truncate at both ends, germ pores indistinct, terminal. *Asexual morph* absent.

***Pseudomicrothecium subterraneum*** (L. Fan, C.L. Hou, P.F. Cannon & Yong Li) Y. Marín, Stchigel, Guarro & Cano, **comb. nov.**

MycoBank MB812109.

*Basionym. Melanospora subterranea* L. Fan, C.L. Hou, P.F. Cannon & Yong Li, Mycologia 104: 1434 (2012)

## DISCUSSION

We have revised the taxonomy of relevant members of the family *Ceratostomataceae* based on the analysis of 18S, 28S, ITS, *ACT* and *EF* sequences. The study strongly supported the order *Melanosporales* proposed by Zhang and Blackwell in 2007 (Hibbett et al. 2007). The phylogenetic inference showed five lineages corresponding to the genera *Dactylidispora, Medusitheca, Melanospora* and *Microthecium*, and to *Melanospora kurssanoviana*. Our results agree with previous works (Zhang and Blackwell 2002; Fan et al. 2012) which demonstrated that the ornamentation of the ascospores under SEM, which had been traditionally used to delimitate most of the genera of *Melanosporales*, is not useful to establish phylogenetic relationships among these fungi. Similarly, the morphology of the ascospores resulted also to be a poor predictor for the generic delimitation in the family Sordariaceae, and consequently the genera *Gelasinospora* and *Neurospora* being synonymyzed (Dettman et al. 2001; García et al. 2004; Nygren et al. 2011). In our case, two of the most relevant genera of Melanosporales, *Melanospora* and *Microthecium* grouped species with both smooth and ornamented cell walls. By contast, a phylogenetic study of the Lasiosphaeriaceae (Miller and Huhndorf 2005) revealed that the nature of the ascomal wall (peridium) is much more phylogenetically informative than the ornamentation of the ascospores, being proposed several new genera, i.e. *Immersiella, Lasiosphaeria* and *Lasiosphaeris,* or emending others, such as *Schizothecium,* based on this fact (Miller and Huhndorf 2004; Cai et al. 2005). In our study, the new genus *Medusitheca* is a clear example of the relevance of the ascomal morphology for phylogenetic relationships. *Arxiomyces, Medusitheca* and *Scopinella* are the only genera into the Melanosporales characterized by the production of dark ascomata. *Medusitheca* constitutes the lineage phylogenetically most distant from the rest of the genera included in this study, although its ascospores are similar to those of *Melanospora* and

*Microthecium*. *Scopinella* can be easily distinguished from *Medusitheca* by its cuboid-ellipsoidal ascospores with two prominent longitudinal germ slits, and *Arxiomyces* by its ellipsoidal ascospores, rounded at the apex and truncated at the base, with a broad germ pore that bears a small appendage.

*Melanospora* resulted restricted to the species with the ascoma bearing a neck composed of interwoven hyphae and, mostly, with a crown of setae at the top of the neck. This kind of neck differentiates this genus from *Microthecium* that has a neck composed by angular cells similar to those of the peridium, and showing a crown of setae surrounding the ostiole rather than disposed at the apex of the neck. The only exception was *Melanospora mycoparasitica* that does not show that kind of neck. Nevertheless, it could be due to this species was described and illustrated at an early stage of the ascoma development. In a study on the development and the cytology of *Melanospora tiffanii,* other species accepted in *Melanospora* based on the morphology, Kowalski (1965) displayed some figures of that species in early stages of development, and the neck seems similar to the neck of *M. mycoparasitica*. *Melanospora arenaria, Melanospora caprina*, *Melanospora chionea, Melanospora langenaria*, *Melanospora longisetosa* and *Melanospora washingtonensis* are the other species that produce long hyphal necks; therefore these have been kept in the emended genus *Melanospora* although it could not be included in the phylogenetic study.

The neck of *Melanospora* spp. is morphologically similar to those of the genera *Syspastospora* and *Vittatispora*, which are also composed of hyphae. *Syspastospora* was introduced in 1982 by Cannon and Hawksworth to re-accommodate *Melanospora parasitica*, with three additional species described later (*S. boninensis, S. cladoniae* and *S. tropicalis*). This genus differs from *Melanospora* in the structure of the ascomatal neck composed of hyphae in a parallel arrangemend (interwoven hyphae in *Melanospora*) and

the cylindrical to barrel-shaped ascospores with a large, slightly sunken germ pore at both ends (which are ellipsoidal, citriform or fusiform, having much smaller, apiculate or depressed germ pores, in *Melanospora*). *Vittatispora* was proposed by Chaudhary et al. (2006) and characterized by ascospores with a thick hyaline longitudinal ridge. In the ML phylogenetic tree based on LSU sequences, this taxon constituted a lineage independent from the other members of *Melanosporales*. *Vittatispora* is morphologically related to *Dactylidispora*, both characterized by a raised rim surrounding the germ pores of the ascospores. The presence of a raised rim was also described in *Melanospora collipora* (Stchigel et al. 1997), being consequently transfered to *Dactylidispora*. *Pustulipora* is also morphologically similar to *Dactylidispora* being characterized by blistered, rarely cushion-like, structures surrounding the germ pore (Cannon 1982), although unfortunately it could not be included into the phylogenetic study since living cultures are not available.

The new genus *Pseudomicrothecium* is proposed here to accommodate *Melanospora subterranea*, since in the LSU phylogenetic tree it demonstrated to be a separate lineage. The ascomata of *Pseudomicrothecium* are similar to those of *Microthecium* (globose and non-ostiolate), but the former produces 2-spored asci and ascospores with indistinct germ pores. Asci containing two ascospores have been only observed in some species of *Scopinella*, i.e. *Scopinella gallicola* and *S. sphaerophila*.

*Melanospora pascuensis*, which now is excluded from that genus, is characterized by dark ring-like structures around the germ pores of the ascospores (Stchigel et al. 1999). Because the only available strain is contaminated by other fungus (probably the host), it was not included in the phylogenetic study.

The phylogenetic inference demonstrated that *Melanopora kurssanoviana* represented a new lineage located far from the other species of *Melanosporales* which suggests that this

species represent a new genus. However, it is not proposed because the colonies of this fungus in spite of all the attempts to induce sporulation remained sterile and a detailed morphological study was not possible. The infertility of the cultures is probably due to the fact that these species are mycoparasites, needing the presence of the host to develop the reproductive structures. The mycoparasitism of *Melanospora*, *Syspastospora* and the species previously belonged to *Persiciospora* and *Sphaerodes* have already been demonstrated by numerous authors (Doguet 1955; Calviello 1973; Jordan and Barnett 1978; Harveson and Kimbrough 2000; Harveson and Kimbrough 2001), and even this ability is exploited in biocontrol of phytopathogenic fungi (Vujanovic and Goh 2009; Goh and Vujanovic 2010).

*Sphaeronaemella* and *Viennotidia*, both characterized by pale and translucent ascomata, have been related to *Melanospora* (Cannon and Hawksworth 1982). However, they differ from the genera of Melanosporales because both produce hyaline ascospores, while in the Melanosporales they are pigmented. Our results agree with other authors that have demonstrated a closer phylogenetic relationship of the two mentioned genera with the order *Microascales* (Spatafora and Blackwell 1994b; Hausner and Reid 2004). Our SSU tree inference seems to demonstrate that *Sphaeronaemella* and *Viennotidia* together with the genus *Gabarnaudia* (which only displays an asexual morph) could represent a new order of the *Sordariomycetes*. However, further studies including more genes are needed to confirm more accurately their taxonomic status.

The placement of our isolate of *Persiciospora japonicum* in the *Microthecium* clade demonstrated that the ornamentation of the ascospores, which is pitted in *Persiciospora* spp., is of poor taxonomic value, and all the species of *Persiciospora* should be transferred to *Microthecium* because the presence of a typical cellular neck of the ascomata in both genera. Surprisingly, in previous phylogenetic studies members of

*Persiciospora* were placed in the *Hypocreales*, closely related to *Nectria* (Zhang and Blackwell 2002; Maharachchikumbura et al. 2015). However, probably this was due to the probable contamination of the cultures of *Persiciospora* with sequences of the hypocrealean host (Fan et al. 2012). The same occurs, probably, with the sequences of *Scopinella* and *Syspastospora*, which led to a possible erroneous classification of both taxa in the *Hypocreales* (Zhang and Blackwell 2002; Chaudhary et al. 2006; Fan et al. 2012; Maharachchikumbura et al. 2015).

*Pteridiosperma ciliatum*, a member of the Melanosporales with ascospores with longitudinal wing-like ridges anastomosing to form a reticulum (a relevant feature of *Pteridiosperma*), was also located in the *Microthecium* clade, proving once again that the ascospore ornamentation is not phylogenetically informative. Consequently, we have synonymyzed the genus *Pteridiosperma* with *Microthecium* since *Pteridiosperma* spp. show non-ostiolate ascomata, or if ostiolate they show a short neck composed of angular cells.

The genus *Sphaerodes* is also synonymized here with *Microthecium* because its type species, *S. episphaerium*, shows morphological features (non-ostiolate ascomata) that fit with such re-established genus. Most of the species of *Sphaerodes*, with the exception of *S. ellipsospora* and *S. singaporensis*, which are now located in the new genus *Dactylidispora*, are also transferred to *Microthecium* since these produces non-ostiolate or ostiolate ascomata without neck, or less frequently with a short neck composed of angular cells like the ascomata ones. Other relevant feature of the genus *Microthecium* is the production of bulbils. These propagules are typical of *Papulaspora*, an anamorphic genus that encompasses more than fourty species. Although it was initially established as a genus without a sexual stage (Hotson 1912), its link with species of *Melanospora* and of *Chaetomium* has been reported (Roll-Hansen 1948; Zhang et al. 2004). In our

phylogenetic study *Papulaspora sepedonioides*, the type species of the genus was nested in the *Microthecium* clade, and therefore transferred to this genus. The relationship of this species with Melanosporales had already previously demonstrated by Davey et al. (2008). However, *Papulaspora* is a polyphyletic genus, and other species of the genus have been reported as belonging to the classes Leotiomycetes and Sordariomycetes (Ascomycota). The relationship of some species of *Papulaspora* with the *Melanosporales* is also suggested by the production of similar phialidic asexual morphs (Van Beyma 1931; Hotson 1942).

There are important morphological differences among the strains of *Microthecium* that suggest the presence of several species into the genus; however, our phylogenetic study, in spite of having used five loci, was not able to resolve the boundaries among them. Further studies are needed looking for appropriate molecular loci for this purpose.

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**Table 1** Isolates and reference strains of members of Melanosporales included in this study.

| Taxa | Strain | Source | GenBank accession # | | | |
|---|---|---|---|---|---|---|
| | | | LSU | ITS | ACT | EF1 |
| *Annulispora ellipsospora* | NBRC 31376$^{T}$ | Forest soil, Papua New Guinea, Buin, Bougainville Island | KP981451 | 03137601* | KP981545 | KP981579 |
| *Annulispora singaporensis* | NBRC 30865$^{T}$ | Soil, Singapore | KP981452 | 03086502* | KP981546 | KP981580 |
| *Nectria cinnabarina* | CBS 127383 | Austria, Niederösterreich, Litschau | HM534894 | HM534894 | - | HM534873 |
| *Medusitheca citrispora* | CBS 137837$^{T}$ (=FMR 12767$^{T}$) | Forest soil, USA, North Carolina, Great Smoky Mountain National Park, Cartoogechaye Creek Campground | KP981453 | KP981477 | KP981547 | KP981581 |
| *Melanospora damnosa* | CBS 113681$^{NT}$ | Soil, France, Pont d'Espagne | KP981454 | KP981478 | KP981543 | KP981582 |
| *Melanospora kurssanoviana* | NBRC 8098 | Unknown | KP981455 | KP981479 | KP981548 | KP981583 |
| *Melanospora verrucispora* | NBRC 31375$^{T}$ | Forest soil, Papua New Guinea, Kebil, Chimb Dist. | KP981456 | KP981480 | KP981549 | KP981584 |
| *Melanospora zamiae* | NBRC 7902 | Unknown | KP981457 | 00790201* | KP981544 | KP981585 |
| *Microthecium ciliatum* | NBRC 9829 | Soil, unknown | KP981458 | KP981481 | KP981524 | KP981586 |
| *Microthecium compressum* | NBRC 8627 | Unkown | KP981459 | 00862701* | KP981525 | KP981587 |
| *Microthecium fayodii* | FMR 12363 | Soil, Tennessee, Great Smoky Mountains National Park, Cosby Creek trail | KP981460 | KP981482 | KP981526 | KP981588 |
| *Microthecium fimbriatum* | NBRC 8523 | Unknown | KP981461 | KP981483 | KP981527 | KP981589 |
| *Microthecium fimicola* | NBRC 8354 | Unknown | KP981462 | KP981484 | KP981528 | KP981590 |
| *Microthecium fimicola* | FMR 5483 | Soil, Moara, Australia | KP981463 | KP981485 | KP981529 | KP981591 |
| *Microthecium fimicola* | FMR 12370 | Soil, Spain, Gran Canaria, x | KP981464 | KP981486 | KP981530 | KP981592 |
| *Microthecium fimicola* | FMR 13418 | Soil, Spain, Aragon, Los Valles Occidentales | KP981465 | KP981487 | KP981531 | KP981593 |
| *Microthecium fusisporum* | NBRC 8806 | Unknown | KP981466 | 00880601* | KP981532 | KP981594 |
| *Microthecium japonicum* | FMR 12371 | Soil, Spain, Gran Canaria, Pico de Osorio | KP981467 | KP981488 | KP981533 | KP981595 |
| *Microthecium levitum* | FMR 6218 (=CBS 966.97) | Soil, Nepal, Bhadgaon | KP981468 | KP981489 | KP981534 | KP981596 |
| *Microthecium levitum* | FMR 10098 | Soil, Nigeria, Enugu. Nsukka | KP981469 | KP981490 | KP981535 | KP981597 |
| *Microthecium levitum* | FMR 13884 | Soil, Spain, Catalonia, Vall Fosca | KP981470 | KP981491 | KP981536 | KP981598 |
| *Microthecium quadrangulatum* | CBS 112763$^{T}$ | Soil, Spain, Asturias, Muniellos Biological Absolute Reserve | KP981471 | KP981492 | KP981537 | KP981599 |
| *Microthecium retisporum* | NBRC 8366 | Soil, Japan | KP981472 | 00836601* | KP981538 | KP981600 |
| *Microthecium sepedonioides* | FMR 11933 | Forest soil, Spain, Aragón, valle de Ordesa | KP981473 | KP981493 | KP981539 | KP981601 |
| *Microthecium* sp. | FMR 6725 (=CBS 102190) | Desert soil, Egypt, Sinai | KP981474 | KP981494 | KP981540 | KP981602 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Microthecium* sp. | FMR 7183 (=CBS 108937) | Forest soil, New South Wales, Sydney, Blue Mountains | KP981475 | KP981495 | KP981541 | KP981603 |
| *Microthecium* sp. | FMR 12373 | Forest soil, USA, North Carolina, Great Smoky Mountain | Pendent | Pendent | Pendent | Pendent |
| *Microthecium zobelii* | NBRC 9442 | Decaying carpophore, *Coriolus flabelliformis* | KP981476 | 00944201* | KP981542 | KP981604 |
| *Pseudallescheria fusoidea* | CBS 106.53$^{T}$ | Soil, Panama, Guipo | EF151316 | AY878941 | - | - |

$^{T}$ and $^{NT}$ Type and neotype strains, respectively; * sequences retrieved from NBRC database; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; FMR, Facultat de Medicina, Reus, Spain; NBRC, Biological Resource Center, Chiba, Japan.

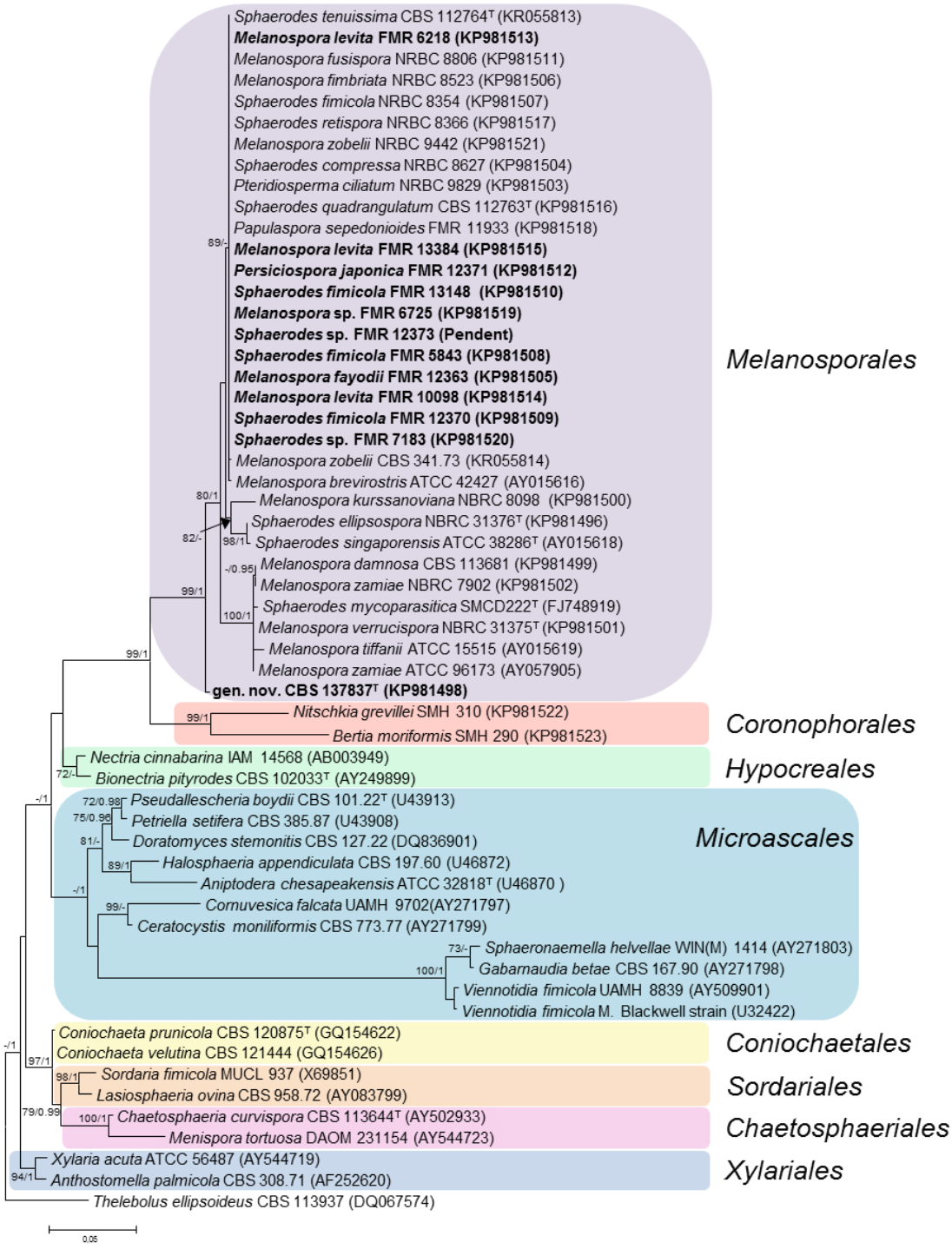


Fig. 1 Maximum-likelihood (ML) tree obtained from 18S sequences of our isolates and reference strains included in *Melanosporales*, and strains belonging to the orders *Chaetosphaeriales, Coniochaetales, Coronophorales, Halosphaeriales, Hypocreales, Microascales, Sordariales* and *Xylariales. Thelebolus ellipsoideus* was used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Type strains of the different species are indicated with $^{T}$.

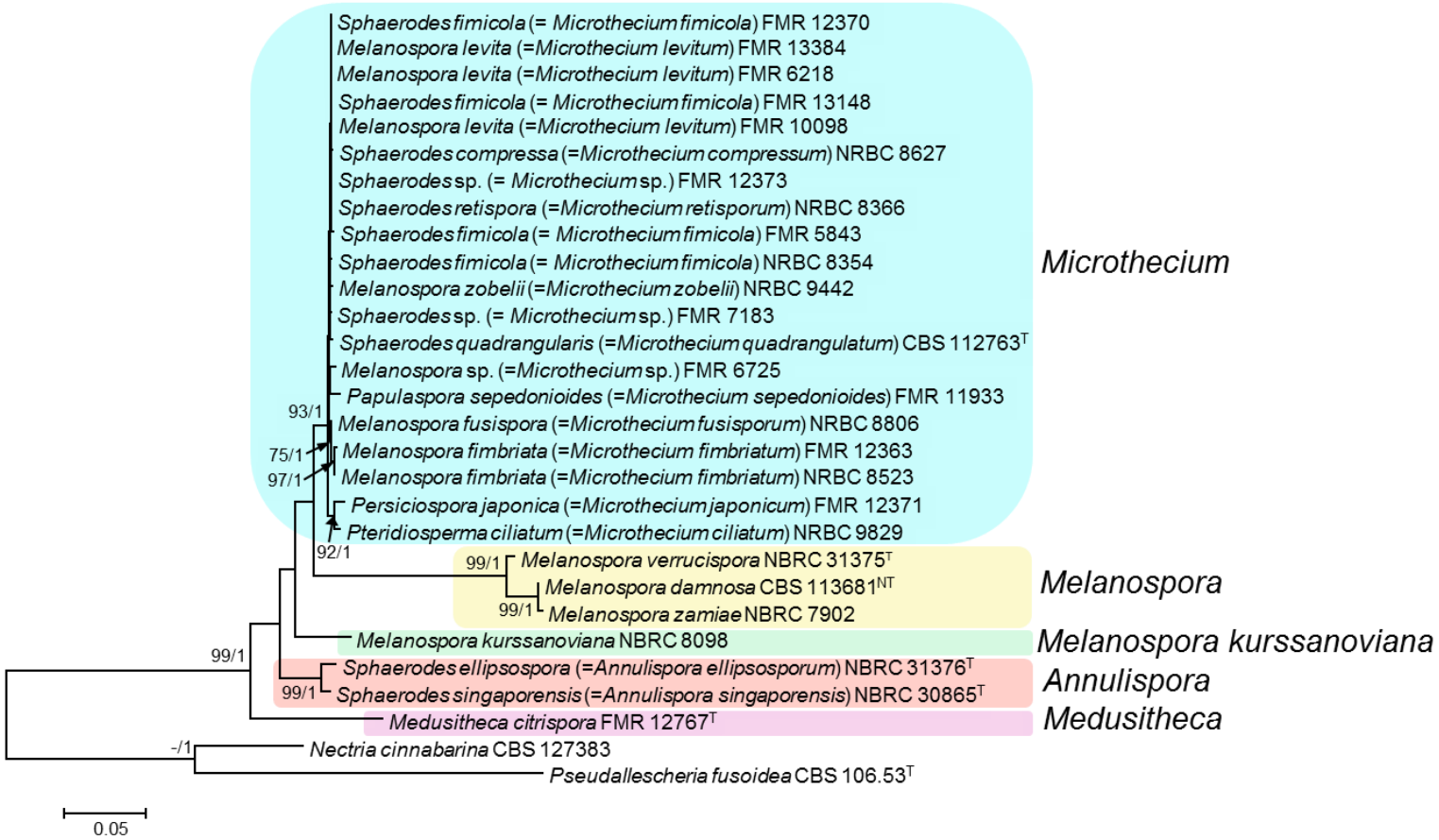


Fig. 2 Maximum-likelihood (ML) tree obtained from the combined DNA sequence data from three loci (ITS, *act* and *EF1*) of our isolates and selected strains of the order *Melanosporales*. *Nectria cinnabarina* and *Pseudallescheria fusoidea* were used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Branch lengths are proportional to distance. Type strains of the different species are indicated with $^{T}$.

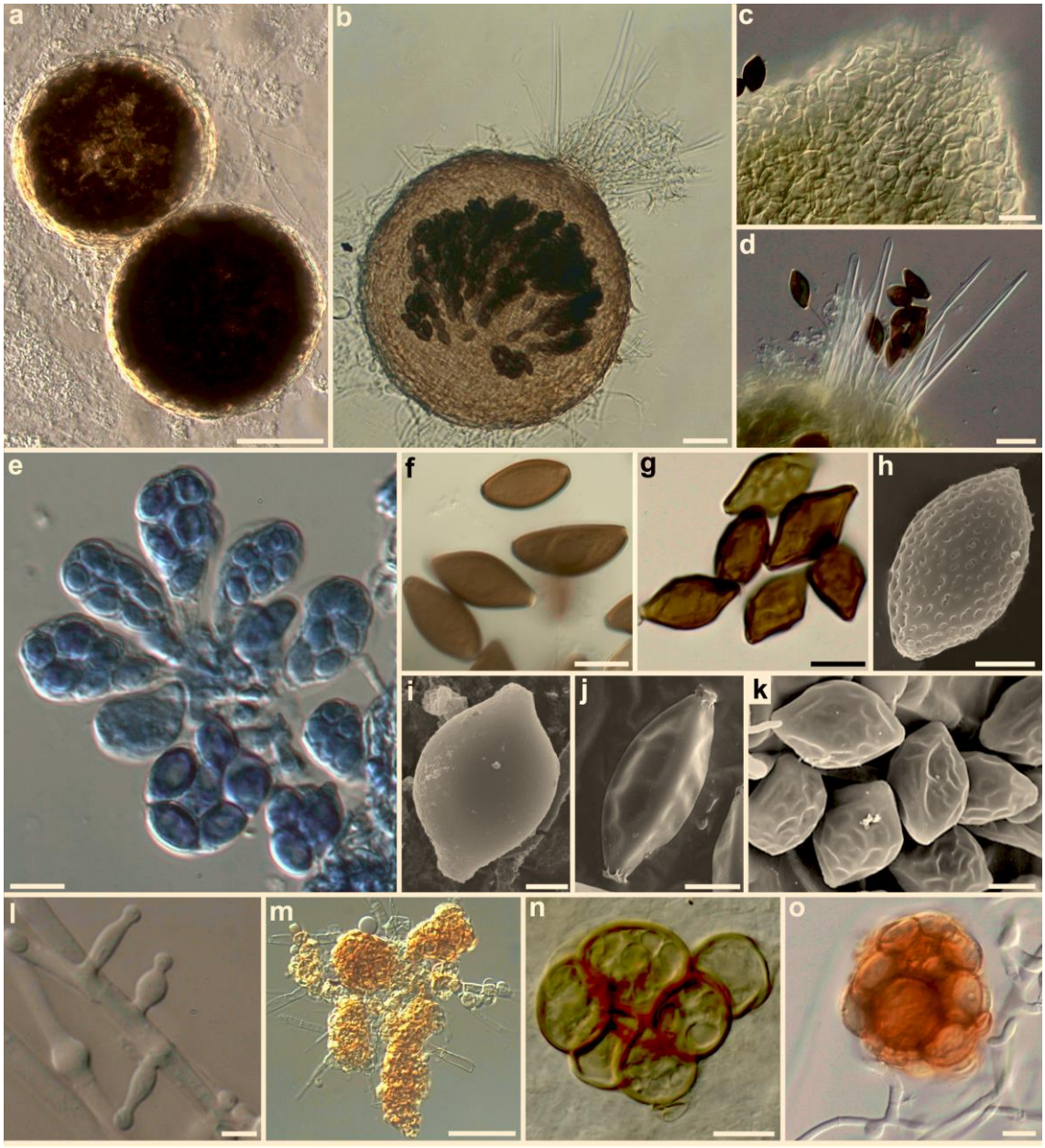


Fig. 3 Morphological features of the genus *Microthecium*. *Microthecium levitum* FMR 10098. a. Non-ostiolate ascoma; e. asci; i. ascospore (SEM). *Microthecium fayodii* FMR 12363. b. Ostiolate ascomata; f. ascospores; m. variable shaped bulbils. *Microthecium fimicola* FMR 5483. c. Detail of cellular neck. k. ascospores; n. bulbil. *Microthecium quadrangulatum* CBS 112763$^{T}$. d. Crown of setae around the ostiole. j. ascospore SEM. *Microthecium retisporum* NBRC 8366. g. Ascospores. l. asexual morph. *Microthecium japonicum* FMR 12371. h. Ascospore SEM. *Microthecium sepedonioides* FMR 11933. o. Bulbil. Scale bars: a, b, m = 50 µm; c, d = 20 µm; e–g, n, o = 10 µm; h, j–l = 5 µm; i = 2.5 µm.

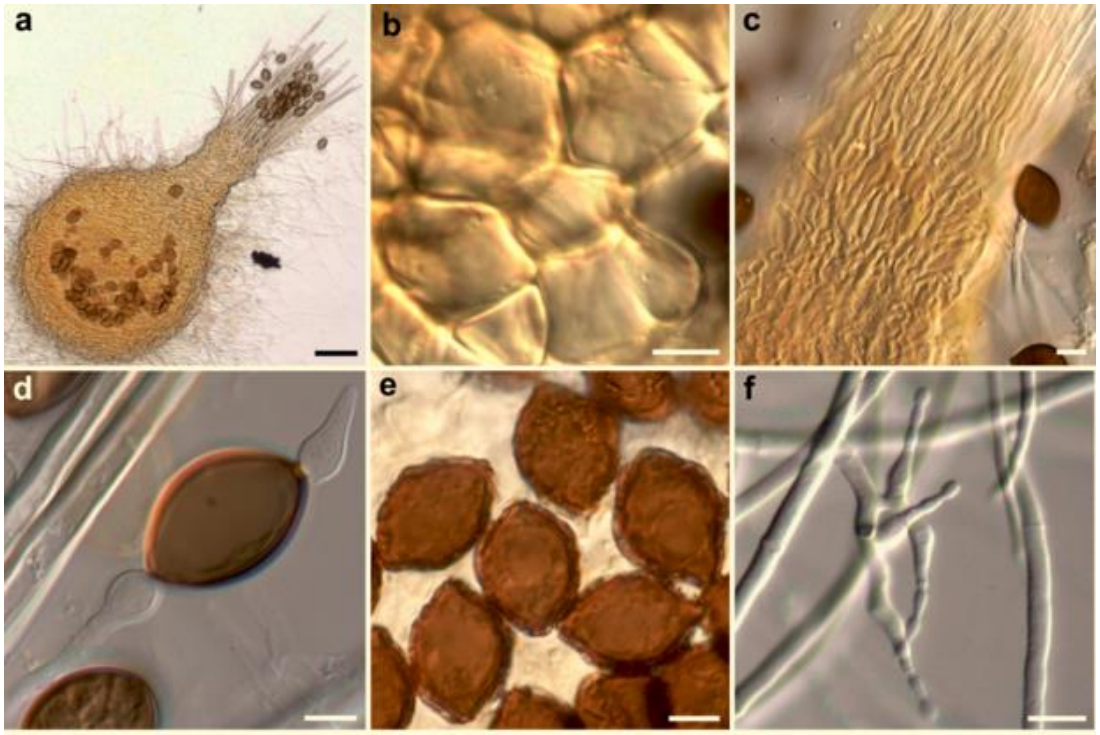


Fig. 4 Morphological features of the genus *Melanospora*. *Melanospora damnosa* CBS 113681. a. Ascoma; d. ascospore germinating. *Melanospora zamiae* NBRC 7902. b. Detail of peridium; c. detail of hyphal neck. *Melanospora verrucispora* NBRC 31375$^{T}$. e. Ascospores; f. phialidic asexual morph. Scale bars: a = 50 µm; b ,c, f = 10 µm; d, e = 5 µm.

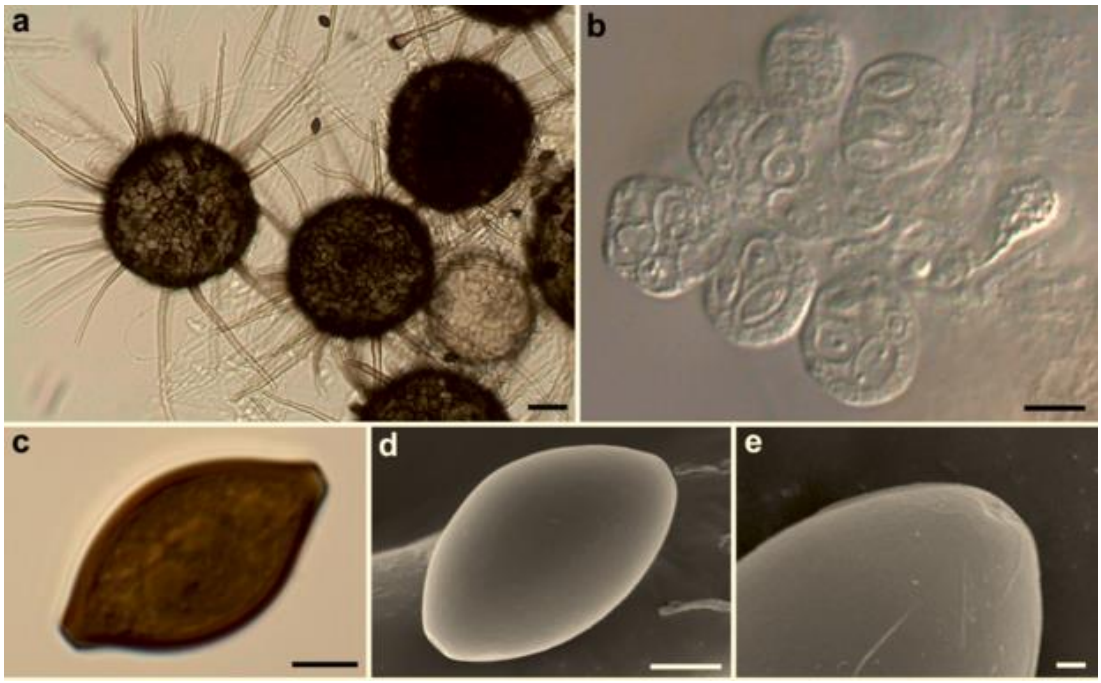


Fig. 5 Medusitheca citrispora CBS 137837$^{T}$. a. Ascomata; b. asci; c. ascospore; d. ascospore (SEM); e.depressed germ pore. Scale bars: a = 50 µm; b = 10 µm; c, d = 5 µm; e = 1 µm.

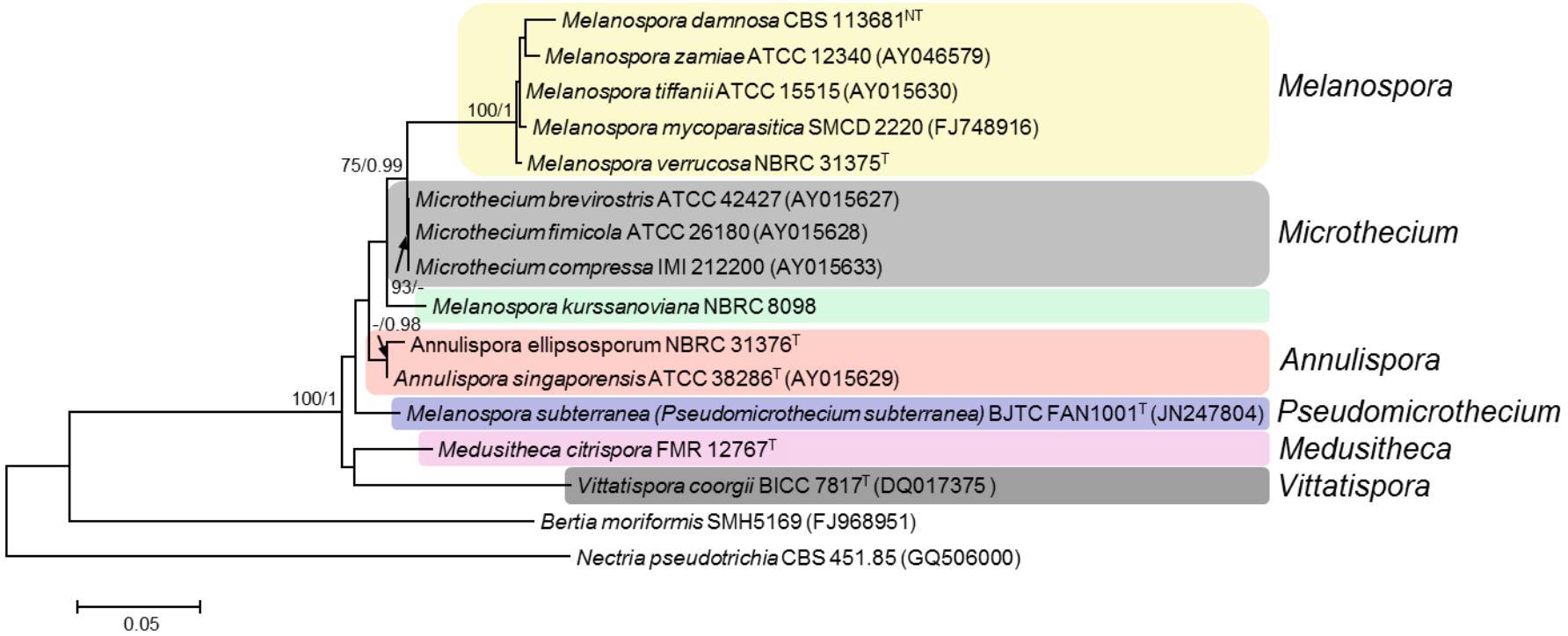


Fig. 6 Maximum-likelihood (ML) tree obtained from 28S sequence of representants of the lineage obtained in the other two phylogenetic studies and the type strains of *Melanospora subterranea* and *Vittatispora coorgii*. *Bertia moriformis* and *Nectria pseudotrichia* were used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Branch lengths are proportional to distance. Type strains of the different species are indicated with $^{T}$.

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Yasmina Marín Félix
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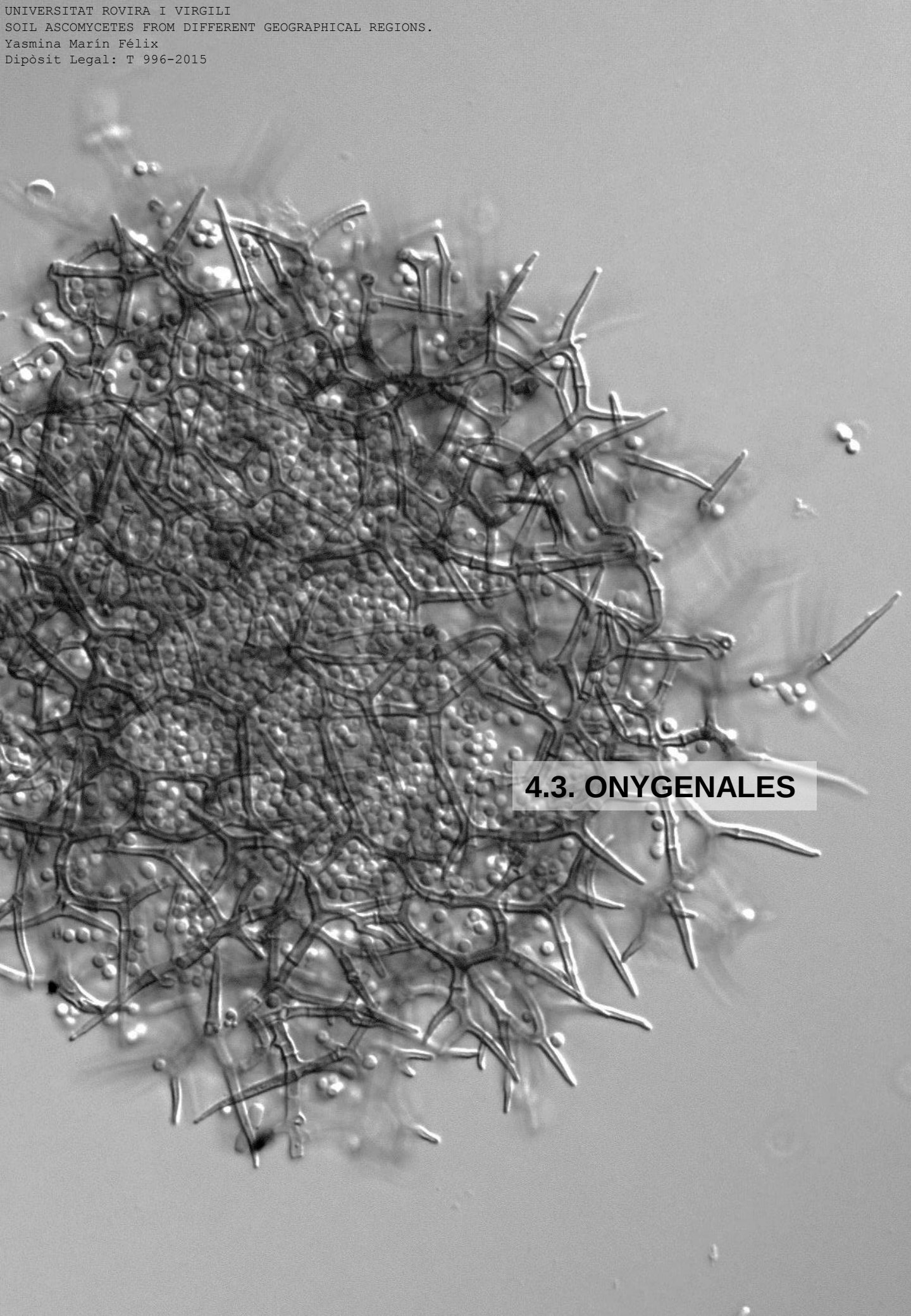

# 4.3. ONYGENALES

## 4.3. ONYGENALES

The order Onygenales encompasses members of the Ascomycota which produce gymnothecial or, less frequently, cleistothecial ascomata, prototunicate evanescent asci, unicellular ascospores and blastic and/or arthric conidia. Recently, extensive molecular studies involving taxa of the class Eurotiomycetes have been performed to clarify the phylogenetic relation between taxa included in the Onygenales. These studies have led to some changes being proposed: the exclusion of the genera *Arachnomyces* and *Spiromastigoides* (formally named *Spiromastix*), and the resulting establishment of the new orders Arachnomycetales and Spiromastixales (Gibas et al., 2002; Rizzo et al., 2014); and the proposal of the family Nannizziopsiaceae within the Onygenales to contain the species of the genus *Nanniziopsis,* which have the particularity of causing skin infections in reptiles (Stchigel et al., 2013). All these phylogenetic studies were based on the analysis of only one genomic locus (SSU or D1–D2).

Figure 26 shows the tree based on ITS and D1–D2 sequences of our taxa belonging to the Onygenales (indicated in bold). The lengths of the individual alignments used in the combined data set were 465 bp (ITS) and 456 bp (D1–D2). The total alignment was 921 bp, 339 bp of which were parsimony informative. Our isolates were located in the families Onygenaceae and Ajellomycetaceae. We observed that the family Onygenaceae was polyphyletic being divided in two main well-supported clades (99% bs / 0.98 pp and 99% bs / 1 pp). Two of the isolates included in the family Onygenaceae – FMR 12019 and FMR 12768 – corresponded to undescribed taxa. Therefore, we introduced the new genus *Aphanoascella* to place the new species *Aphanoascella galapagosensis* (section 4.3.1), and we proposed the new species *Aurxarthron longisporum* (section 4.3.2). Moreover, during the phenotypic and molecular study of *Aphanoascella*, the genus *Aphanoascus* was redefined, being restricted to those species with reticulate ascospores and without an equatorial rim, and the genus *Keratinophyton* re-established to incorporate species whose ascospores are pitted and display a conspicuous equatorial rim.

Our study also demonstrated that the family Ajellomycetaceae was in a terminal clade independent from that of the Onygenales, suggesting that it could represent a new order. Most of the taxa belonging to this family are thermally dimorphic and pathogenic for animals, including humans. Under environmental conditions they grow as molds with a filamentous thallus, but in physiological conditions, when the spores are inhaled into the lungs of the mammalian hosts, they turn into yeast-like structures (Klein and Tebbets, 2007). These cause systemic mycoses called adiaspiromycosis, blastomycosis, histoplasmosis and paracoccidioidomycosis, depending on the nature of the etiologic

agent. Within the Ajellomycetaceae clade, the isolates FMR 4023 and FMR 4024, together with the isolates CBS 273.77 and UAMH 141, which were previously identified as *Emmonsia parva*, were located in a well-supported subclade representing a new genus, *Emmonsiellopsis*, and two new species, *E. coralliformis* and *E. terrestris*. This new genus, unlike the other taxa in the first main clade, did not produce the yeast-like morph or adiaspores in the *in vitro* or *in vivo* studies carried out by us. Moreover, the type and a reference strain of *Ajellomyces griseus* were also grouped in a well-supported subclade independently from the other genera. Therefore, we introduced the genus *Helicocarpus* to accommodate this taxon on the basis of the molecular results and morphological differences since unlike the other species of the Ajellomycetaceae, it does not have an asexual morph or yeast-like structures. The sexual morph also differs from those of the other species, producing lenticular to oblate and sparingly pitted ascospores that are not minute, whereas the other species present minute, globose to subglobose, finely spinulose or roughened ascospores. Finally, the type species of *Blastomyces* and *Emmonsia* clustered in the same well-supported clade suggesting that both genera should probably be synonymized, even though there are a few morphological differences. *Blastomyces dermatitidis,* for instance, is not inhibited by cycloheximide and its conidia are usually solitary and sessile, or produced on conidiophores that are only slightly swollen or not swollen at all. On the other hand, *Emmonsia* spp. are more or less sensitive to the cycloheximide, and the conidia are produced on swollen conidiophores and frequently in chains. However, these differences are not enough to regard both genera as different. Moreover, the sexual morph produced by both taxa is almost identical. For more details about members of the family Ajellomycetaceae see section 4.3.3.

The low number of taxa recovered from these families during the developing of this thesis is probably related to the isolation techniques used (i.e. acetic acid, ethanol and phenol “activations”). Their ascospores probably have a thinner cell wall than other taxa of the Ascomycota (i.e., Coniochaetales, Eurotiales, Sordariales and Xylariales), and their ascospores do not resist the treatment with these chemicals. The same occurs with the members of the family Myxotrichaceae, which has hardly been isolated either. These fungi can only be recovered from soil using methods such as those used by Punsola (1985) and Cano (1989). One example is the “*ToKaVa hair-baiting method*” described by Vanbreuseghem (1952).

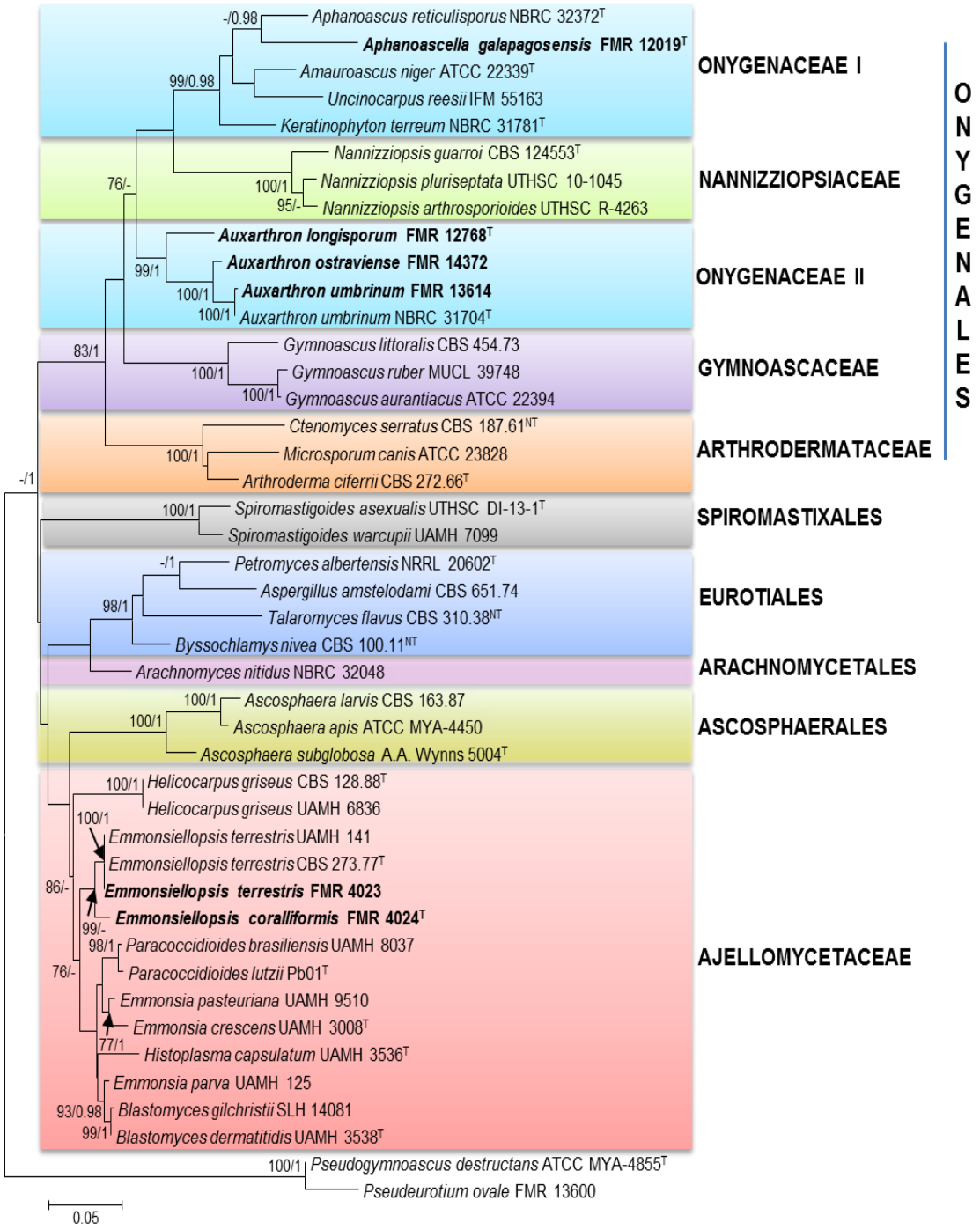


**Fig. 26** Maximum-likelihood (ML) tree obtained from the ITS and D1–D2 sequence data of our isolates and selected strains of the subclass Eurotiomycetidae. *Pseudogymnoascus destructans* and *Pseudeurotium ovale* were used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Branch lengths are proportional to distance. Type and neotype strains of the different species are indicated with $^{T}$ and $^{NT}$, respectively.

**4.3.1. Isolation and characterization of a new fungal genus and species, *Aphanoascella galapagosensis*, from carapace keratitis of a Galapagos tortoise (*Chelonoidis nigra microphyes*)**

Sutton DA, Marín Y, Thompson EH, Wickes BL, Fu J, García D, Swinford A, de Maar T, Guarro

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# Isolation and characterization of a new fungal genus and species, *Aphanoascella galapagosensis*, from carapace keratitis of a Galapagos tortoise (*Chelonoidis nigra microphyes*)

D. A. SUTTON*, Y. MARÍN#, E. H. THOMPSON*, B. L. WICKES†, J. FU†, D. GARCÍA#, A. SWINFORD‡, T. DE MAAR§ & J. GUARRO#

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A new fungal genus and species, *Aphanoascella galapagosensis,* recovered from carapace keratitis in a Galapagos tortoise residing in a south Texas zoological collection, is characterized and described. The presence of a pale peridium composed of textura epidermoidea surrounded by scarce Hülle cell-like chlamydospores, and the characteristic reticulate ascospores with an equatorial rim separates it from other genera within the Onygenales. The phylogenetic tree inferred from the analysis of D1/D2 sequences demonstrates that this fungus represents a new lineage within that order. As D1/D2 and ITS sequence data also shows a further separation of *Aphanoascus* spp. into two monophyletic groups, we propose to retain the generic name *Keratinophyton* for species whose ascospores are pitted and display a conspicuous equatorial rim, and thereby propose new combinations in this genus for four *Aphanoascus* species.

**Keywords** *Onygenales, Aphanoascella, Aphanoascella galapagosensis*, Galapagos tortoise, *Keratinophyton*

## Introduction

In recent years there have been anecdotal reports of a form of keratitis affecting carapaces (shells) of dry land tortoises kept in captivity and residing in the wild on the Galapagos Islands. The syndrome is commonly seen in tortoises living in zoological gardens in Florida and the Gulf Coast states and may be associated with prolonged exposure to moisture. In these cases, the keratin in the scute sutures of the carapaces turns white and powdery, and is easily scraped away to reveal normal black keratin or underlying bone. It has been hypothesized that the etiology is potentially mycotic.

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A wild caught, Volcan Darwin Tortoise, also called a Galapagos Tortoise (*Chelonoidis nigra microphyes*), estimated to be approximately 50 years old and residing in a zoological collection in south Texas, was observed to develop white discoloration in lower areas of the carapace. The lesions were first noted during a summer with significant rainfall. Only one animal in eight was affected. These lesions slowly expanded over the next 3 years across the lower quadrants of the costal scutes, upper quadrants of the marginal scutes and the plastral bridge (Fig. 1). Lesions were most severe along scute sutures, areas of the newest keratin growth. All lesions were situated below the high water mark of the animal's mud wallow. The affected keratin developed an eroded shale-like pattern. Scraping of the crumbling surface revealed healthy keratin or bone underneath. Samples of the scraped material were harvested and submitted to a veterinary diagnostic laboratory for fungal culture.


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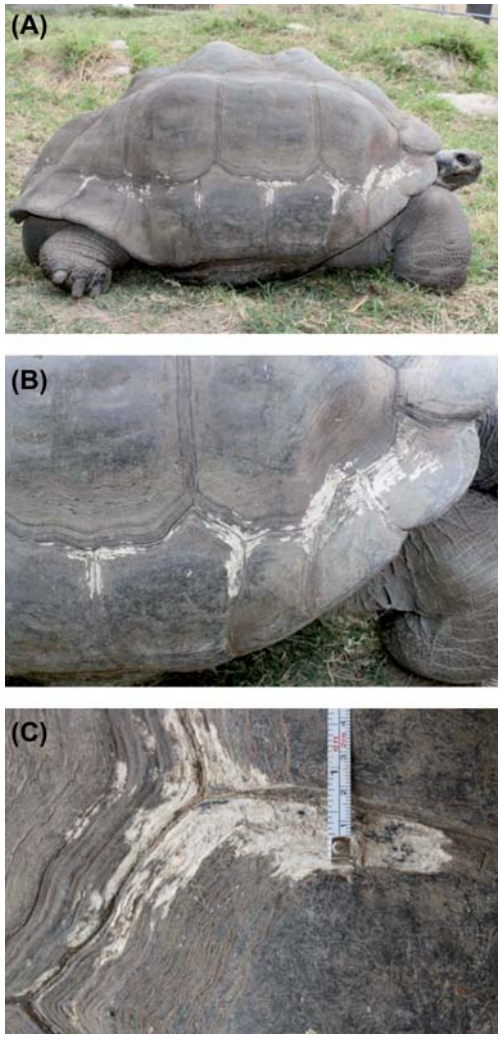


**Fig. 1** Lesions on the carapace of a 50-year-old Galapagos Tortoise (*Chelonoidis nigra microphyes*) due to *Aphanoascella galapagosensis*. Extent of lesions (A), close up of right side (B), and depth of erosion (C).

## Materials and methods

### *Fungal isolation and initial identification*

The carapace scrapings were inoculated onto Sabouraud dextrose, potato dextrose, Mycobiotic, and dermatophyte test medium agars (Remel, Lenexa, KS) and incubated at 25°C for 3 weeks. Heavy growth of a white to buff-colored fungus was observed on all media. The colony morphology of the isolate was downy to cottony and resembled a dermatophyte, but could not be identified by conventional laboratory methods, and was forwarded to the Fungus Testing Laboratory, Department of Pathology, The University of Texas Health Science Center at San Antonio, San Antonio, TX, for further attempts at identification. There the isolate, Fig. 2, was accessioned into their collection as UTHSC 11-1518 and tentatively identified as an *Aphanoascus* species based on ascomata and ascospore formation resembling those seen in this genus. However, the isolate was subsequently referred to Spain for more in-depth study when a BLAST search of the ITS and D1/D2 sequence data in GenBank failed to return an identification with any significant percent identity.

**Fig. 2** Colony of *Aphanoascella galapagosensis* on potato flakes agar after 3 weeks incubation at 25°C, measuring approximately 20 µm in diameter.

*Morphologic identification.* The fungal isolate was grown on oat meal agar (OA), potato carrot agar (PCA), potato dextrose agar (PDA) and Czapek agar (Cz) plates at 15°C, 25°C and 35°C. Color notations in parentheses are from Kornerup and Wanscher [1]. The fungal structures were measured after 2 months of growth on Cz using lactophenol-stained mounts (Fig. 3). Photomicrographs were obtained with a Leitz Dialux 20 EB microscope. Scanning electron microscopy techniques were described previously by Figueras and Guarro [2].

*DNA extraction and sequencing.* Template DNA was prepared from a 24 h culture of UTHSC 11-1518 (= R-4747) grown on potato dextrose agar at 30°C as previously described [3]. PCR reactions were then performed in a 50 µl volume using 3 µl of template DNA, 5 µl 10 × PCR buffer, 5 µl of a 10 µM stock solution of each primer (ITS-1 forward primer [4] and NL-4 reverse primer [5,6]), 1.5 µl of 10 mM dNTP (Invitrogen, Carlsbad, CA), and 2.5 U of *Taq* Extender (Fisher Scientific, Pittsburgh, PA). PCR reactions were performed in an Eppendorf Master Thermocycler (Eppendorf) and were run with a temperature profile of 2 min at 94°C followed by 30 cycles of 20 s at 94°C, 20s at 60°C, and 1 min at 72°C. The 30 cycles were followed by 5 min at 72°C.

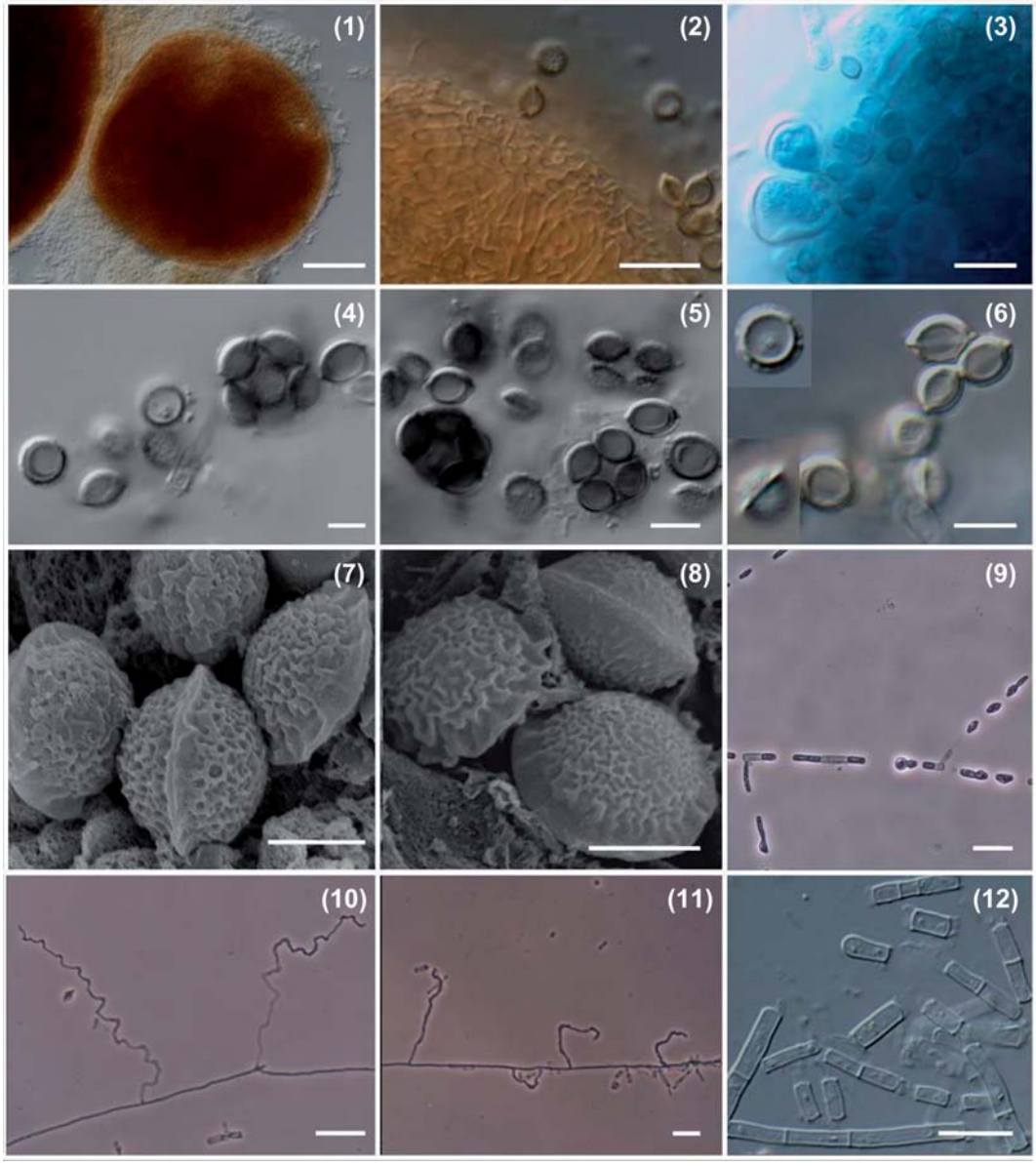


**Fig. 3** Microscopic features of *Aphanoascella galapagosensis* (Czapek, 2 months, 25°C). (1) Non-ostiolate, spherical ascoma, bar = 50 µm; (2) *textura epidermoidea*, bar = 10 µm; (3) Hülle cells (chlamydospore-like) covering the ascoma, bar = 10 µm; (4, 5 & 6) asci and ascospores, bar = 5 µm; (7 & 8) scanning electron microscopy of oblate ascospores demonstrating irregular reticulate wall, anastomosing ridges and an equatorial ridge, bar = 2.5 µm; (9) intercalary arthroconidia, bar = 20 µm; (10 and 11) arthroconidia borne on straight primary hyphae or on short loosely curved or sinuous lateral branches, bar = 20 µm; (12) alternating or adjacent cylindrical to slightly barrel-shaped arthroconidia, bar = 20 µm.

PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced on both strands using the two flanking primers (ITS-1 and NL-4), as well as two internal primer runs (ITS-4 and NL-1) [5,6]. Sequencing was performed at the UTHSCSA Advanced Nucleic Acids Core Facility and data were edited using Sequencing Analysis Software v5.3.1 (Applied Biosystems, Foster City, CA).

*Sequence analysis.* The sequence data were assembled and analyzed using MacVector software (MacVector, Inc, Cary, NC) and then searched using the ITS-1 and ITS-4 primer sequences to delineate the ITS region, as well as the NL-1 and NL-4 sequences to delineate the D1/D2 region. The ITS and D1/D2 regions were then used in separate BLASTn searches of GenBank at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequence-based identities with a cutoff of 97% or greater and query length of 90% or greater were considered significant.

*Alignment and phylogenetic reconstruction.* Phylogenetic analyses of the two regions selected for study were performed using the neighbor-joining (NJ) method with the MEGA 2.1 computer program. The NJ tree was constructed using maximum composite likelihood method [7] with the pairwise deletion of gaps option. The robustness of branches was assessed by bootstrap analysis with 1000 replicates.

*Nucleotide sequence accession numbers.* GenBank nucleotide sequence accession numbers for the case isolate and morphologically similar species are listed in Table 1.

## Results

A BLAST search using the ITS region (18S partial, ITS1, 5.8S, ITS2, 28S partial) provided only insignificant hits from GenBank, the top three of which were *Aphanoascus foetidus* (accession# AJ439448.1, 88% identity), *Chrysosporium lucknowense* (accession# AJ131682.1, 88% identity), and *Chrysosporium mephiticum* (accession# AJ131683.1, 87% identity). None of which were considered significant for a conspecific isolate (>97% identity). The D1/D2 region (28S partial sequence) also did not return a significant BLAST hit, with the top three closest identities being *Chrysosporium keratinophilum* (accession# AB359446.1, 94% identity), *Chrysosporium keratinophilum* (accession# AB359445.1, 94% identity), and *Aphanoascus verrucosus* (accession# AB075348.1,

**Table 1** LSU and ITS DNA sequences included in the phylogenetic analyses.

| | | GenBank no. | |
|---|---|---|---|
| Family | Species | LSU | ITS |
| Arthrodermataceae | *Arthroderma ciferrii* | EF413625 | |
| | *Ctenomyces serratus* | AY176733 | |
| Gymnoascaceae | *Gymonascus aurantiacus* | AY176747 | |
| | *Gymnoascus littoralis* | FJ35827 | |
| | *Gymnoascus ruber* | AY176746 | |
| Onygenaceae | *Amauroascus niger* | AY176706 | |
| | *Aphanoascus canadensis* | | AJ439435 |
| | *Aphanoascus clathratus* | | AJ439436 |
| | *Aphanoascus cubensis* | | AJ439432 |
| | *Aphanoascus durus* | AB075345 | AJ439434 |
| | *Aphanoascus foetidus* | | AJ439448 |
| | *Aphanoascus fulvescens* | JN941548 | AF038357 |
| | *Aphanoascus hispanicus* | | AJ439438 |
| | *Aphanoascus keratinophilus* | | AJ133436 |
| | *Aphanoascus mephitalis* | AY176725 | AJ439439 |
| | *Aphanoascus orissi* | | AJ315843 |
| | *Aphanoascus pinarensis* | | AJ439433 |
| | *Aphanoascus punsolae* | | AJ439440 |
| | *Aphanoascus reticulisporus* | JN941550 | JN943435 |
| | *Aphanoascus saturnoideus* | AB075347 | AJ439442 |
| | *Aphanoascus terreus* | JN941552 | JN943438 |
| | *Aphanoascus verrucosus* | JN941554 | JN943439 |
| | *Aphanoascella galapagosensis* | JQ864082 | JQ864081 |
| | *Chrysosporium* sp. | | AJ439445 |
| | *Chrysosporium evolceanui* | | AJ005368 |
| | *Chrysosporium fluviale* | | AJ005367 |
| | *Chrysosporium indicum* | | AJ439446 |
| | *Chrysosporium keratinophilum* | | AJ131681 |
| | *Chrysosporium lucknowense* | | AJ131682 |
| | *Chrysosporium minutisporosum* | | AJ131689 |
| | *Chrysosporium siglerae* | | AJ131684 |
| | *Chrysosporium submersum* | | AJ131686 |
| | *Chrysosporium tropicum* | | AJ131685 |
| | *Chrysosporium zonatum* | | AJ390393 |
| | *Uncinocarpus queenslandicus* | AB075358 | AB361646 |
| | *Uncinocarpus reesii* | AY176724 | |
| Trichocomaceae | *Byssochlamys nivea* | AY176750 | |
| | *Eurotium herbariorum* | AY176751 | |
| Sordariaceae | *Neurospora nigeriensis* | FR774265 | |

94% identity). The phylogenetic tree inferred from the analysis of the D1/D2 sequences showed a clear genetic separation between *Aphanoascus* spp. and other genera of Onygenales included in the study (Fig. 4). It also demonstrated that the species of *Aphanoascus* included in the study were grouped in two highly supported clades (98% and 94%, respectively). One clade included those species displaying reticulate-walled ascospores without an equatorial rim, and the other clade consisted of isolates with pitted ascospores with a prominent equatorial rim. The new fungus described here represents a new lineage phylogenetically distant from the two mentioned clades. The ITS phylogenetic tree (Fig. 5) showed a similar topology, confirming that there is insufficient evidence to place the currently accepted *Aphanoascus* spp. into the same genus and that the new fungus is not related with any of the two clades of *Aphanoascus*.

## Taxonomy

***Aphanoascella*** D.A. Sutton, Y. Marín, E.H. Thompson et Guarro, gen. nov.

Anamorph: *Malbranchea* sp.
Etym: Similar to *Aphanoascus*
MycoBank: MB 564389

Ascomata superficialia, sphaerica, non-ostiolata, cum chlamydosporae 'hülle' cellulis similis, aurantiaca vel brunnea; peridium cum textura epidermoidea. Asci octospori, subglobosi vel ellipsoidei, muris evanescentibus. Ascosporae unicellulares, oblatae, reticulatae, cum crista equatoriali, subhyalinae vel aurantiaca. Anamorphosis: Arthroconidia hyalina, tenuitunicata, laevia.
Species typica: *Aphanoascella galapagosensis* D.A. Sutton, Y. Marín, E.H. Thompson et Guarro

Ascomata superficial, spherical, non-ostiolate, orange to brown at maturity, surrounded by Hülle cell-like chlamydospores; peridium pale, with textura epidermoidea. Asci 8-spored, subglobose to oblate, evanescent. Ascospores one-celled, oblate, reticulate, with an equatorial rim, subhyaline to orange in mass. Anamorph: Arthroconidia hyaline, thin-walled, smooth.
Type species: *Aphanoascella galapagosensis* D.A. Sutton, Y. Marín, E.H. Thompson & Guarro

***Aphanoascella galapagosensis*** D.A. Sutton, Y. Marín, E.H. Thompson et Guarro sp. nov.

Anamorph: *Malbranchea* sp.
Etym.: *galapagosensis* latinized from the name Galapagos Islands referring to the type locality.
MycoBank: MB 564390

Hyphae vegetativae hyalinae, ramosae, 2–4 crassae. Ascomata superficialia, sphaerica, non-ostiolata, cum chlamydosporae 'hülle' cellulis similis, 170–270 µm diam, aurantiaca vel brunnea; peridium 4–7 µm crassi,

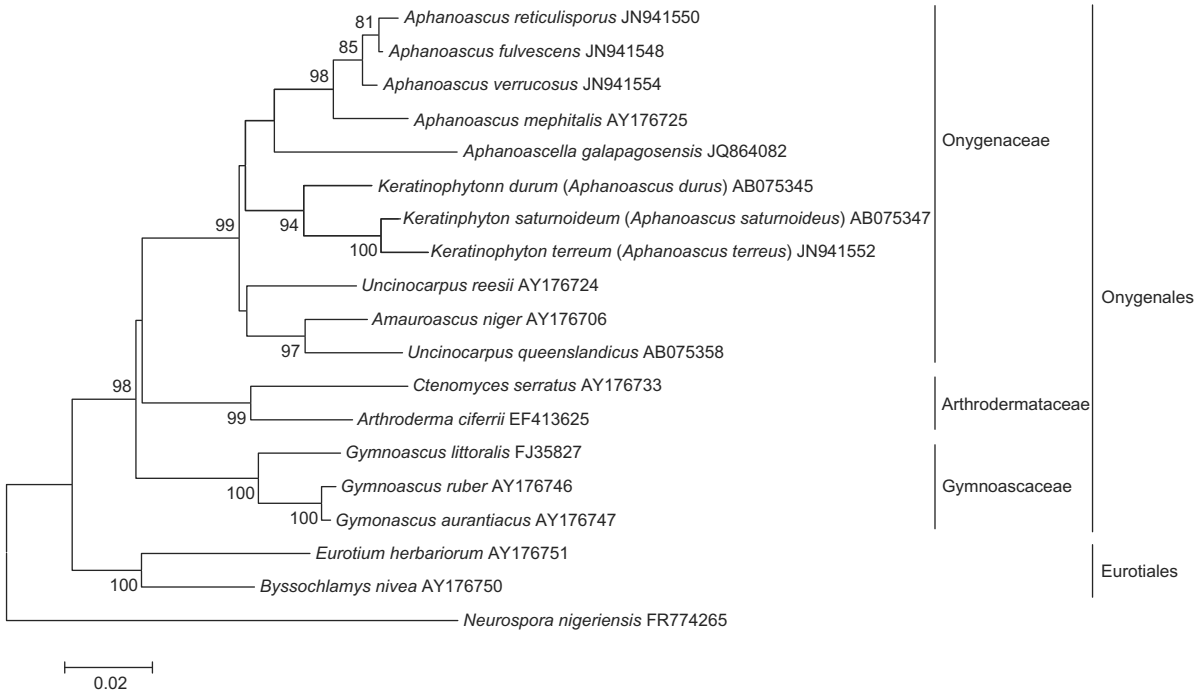


**Fig. 4** NJ tree based on LSU rDNA sequences, including 19 taxa belonging to Onygenales, two taxa belonging to Eurotiales and *Neurospora nigeriensis* as outgroup. Bootstrap values of 70% or greater are indicated above the internodes.

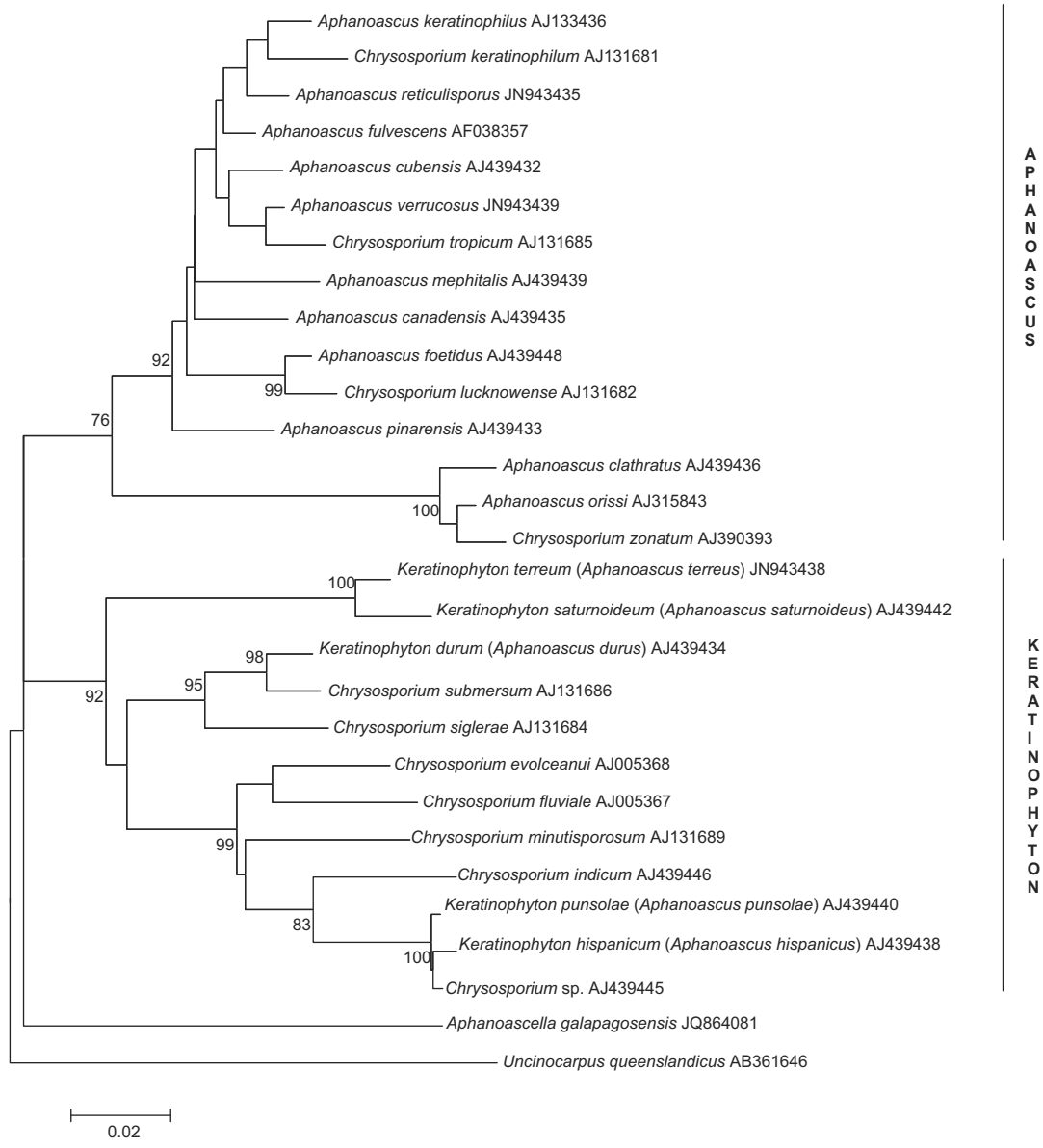


**Fig. 5** NJ tree based on ITS sequences from *Aphanoascus* and *Chrysosporium* species and our isolated *Uncinocarpus queenslandicus* as outgroup. Bootstrap values of 70% or greater are indicated above the internodes.

et 3–4 stratiorum compositum, ex textura epidermoidea. Asci octospori, subglobosi vel ellipsoidei, 10–14.5 × 7–11 µm, evanescentes. Ascosporae oblatae, reticulatae, cum crista equatoriali, hyalinae vel aurantiaca in massa, 5–6 × 3–4.5 µm. Arthroconidia cylindrica vel subdoliformia, laevia, hyalina, 4–11(–13) × 2–4 µm, in senectute inflata.

Colonies on potato carrot agar (PCA) reaching 12–13 mm in diameter after 14 days at 25°C, white, velvety to cottony, margins fringed; reverse uncolored. Growth at 15 and 35°C was very restricted on all media tested. Vegetative hyphae hyaline, branched, smooth, septate, 2–4 µm broad, thin-walled. Ascomata superficial, spherical, non-ostiolate, surrounded by Hülle cell-like chlamydospores, 170–270 µm diam, orange to brown at maturity, ascoma wall pale, 4–7 µm thick, composed of 3–4 layers of flattened cells, *textura epidermoidea.* Asci numerous, 8-spored, subglobose to ellipsoidal, 10–14.5 × 7–11 µm, evanescent. Ascospores oblate, with an irregularly reticulate wall formed by inconspicuous and anastomosed ridges, with an equatorial rim 0.5–1 µm broad, sub-hyaline to orange in

mass, 5–6 × 3–4.5 μm (including rim). Arthroconidia borne on the straight primary hyphae or on short loosely curved or sinuous lateral branches, separated by one or more alternate empty cells, or rarely, formed immediately adjacent to each other. Arthroconidia cylindrical or barrel-shaped, slightly broader than the width of the interconnecting hyphae, hyaline, smooth, 4–11(–13) × 2–4 μm.

Holotype: CBS H-20943 (ex-type strains CBS 132345, FMR12019, UTHSC 11-1518)

## Discussion

The ascomycete genus *Aphanoascus* (Onygenaceae, Onygenales) encompasses a large number of species characterized by spherical, pale to dark brown ascomata, lenticular ascospores, either discoid or oblate, with or without an equatorial rim, pale to dark brown with a reticulate, pitted or verrucose wall, and with anamorphs belonging to the genera *Chrysosporium* or *Malbranchea*. Members of the genus are found in soil or dung. While some species are keratinophilic [8,9,10], rarely do they cause human infections. Although poorly supported genetically, two morphologically well-differentiated groups were defined within the genus, i.e., one comprising species with reticulate ascospores and without a rim, and a second with pitted ascospores and with an equatorial rim [9]. This second group included two species that previously belonged to the genus *Keratinophyton*, i.e. *A. terreus* and *A. durum*. The new fungus shows unique morphological characteristics which are intermediate between the two mentioned groups and is also genetically unrelated. The ascospores of *Aphanoascus pinarensis* and *Aphanoascus cubensis*, when observed under light microscopy, appear to have equatorial crests, but when examined by SEM, such structures are in fact prolongations of the reticules of the surface. The genus *Aphanoascella* is characterized by the presence of a pale peridium composed of textura epidermoidea surrounded by scarce Hülle cell-like chlamydospores, and by its characteristic reticulate ascospores with an equatorial rim. These features separate it from other genera within the Onygenales.

In Onygenales ribosomal genes have been commonly used to infer molecular phylogenies [11–15]. In general, the genetic distances among the genera of Onygenales are considerably large and probably numerous species could be proposed as new genera, although most of them would be monotypic. The percent similarity between the type strains of *Aphanosacus fulvescens* and *Aphanoascus terreus* (*Keratinophyton terreum*), which are the type species of *Aphanoascus* and *Keratinophyton*, is 81.3%. Between these species and the new fungus, the percent similarity is 78.7% and 80.35%, respectively.

Reconsidering the phenotypic and molecular data concerning these fungi we believe it is more appropriate to maintain the generic name *Keratinophyton* for those species with ascospores with a pitted wall and a conspicuous equatorial rim, which were clearly separated from the clade where the type species of *Aphanoascus*, *A. fulvescens*, was nested. Therefore, the new combinations are proposed.

***Keratinophyton multiporum*** (Cano & Guarro) Guarro & Y. Marín, comb. nov.

Basyonym *Aphanoascus multiporus* Cano & Guarro, Mycol. Res. 366. 1990.

MycoBank: MB 800127

***Keratinophyton hispanicum*** (Cano & Guarro) Guarro & Y. Marín, comb. nov.

Basyonym *Aphanoascus hispanicus* Cano & Guarro, Mycol. Res. 94: 364. 1990.

MycoBank: MB 800128

***Keratinophyton punsolae*** (Cano & Guarro) Guarro & Y. Marín, comb. nov.

Basyonym *Aphanoascus punsolae* Cano & Guarro, Mycotaxon 38: 162. 1990.

MycoBank: MB 800129

***Keratinophyton saturnoideum*** (Cano & Guarro) Guarro & Y. Marín, comb. nov.

Basyonym *Aphanoascus saturnoideus* Cano & Guarro, Mycol. Res. 94: 370. 1990.

MycoBank: MB 800130

Various disease syndromes involving the shells of tortoises and turtles (*Chelonia*) have been described since the 1980s [16,17]. The chelonian shell is composed of bony plates with intercalating areas of epidermis (scutes). Just as the integumentary systems of other animals can become diseased due to a wide range of causes, from nutritional deficiencies to infectious agents, the shells of tortoises can be similarly afflicted, though the etiologies are less well-documented. Two studies of populations of wild desert tortoises in California suffering from high morbidity rates and shell disease [18,19] failed to demonstrate a single definitive cause for necrotic lesions, despite extensive pathological and microbiologic evaluations. The authors speculated that in these tortoises, the shell lesions might be attributable to chronic toxicoses or nutrient deficiencies. Conversely, in a study of Texas tortoises (*Gopherus berlandieri*) demonstrating scute necrosis, the fungus *Fusarium semitectum* was isolated and believed to have been the etiologic agent [20].

It is unknown at this time if the newly-characterized fungal isolate in this report, *Aphanoascella galapagosensis*, is a primary pathogen of the scute disease observed in this

tortoise, or represents an opportunistic, secondary pathogen. Lesions similar to the ones seen in this tortoise have been observed in multiple tortoises of another species at the same zoological park, and a similar fungus was isolated. These animals demonstrate a different pattern of lesion but share the possibility of prolonged exposure to moisture. Attempts are currently underway to identify these strains and determine potential commonality with our case isolate in the Galapagos tortoise.

While there is no present indication that this fungus affects mortality in tortoises, any disease process causes some morbidity. Morbidity may decrease resistance to other diseases, longevity or reproductive success. In the husbandry and breeding of endangered species any increase in morbidity may affect the progress of the species' recuperation. Further research into the identity, prevalence, epidemiology and possible treatment of this fungus is proposed to reduce any negative effects on these rare animals.

***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the writing and content of the paper.

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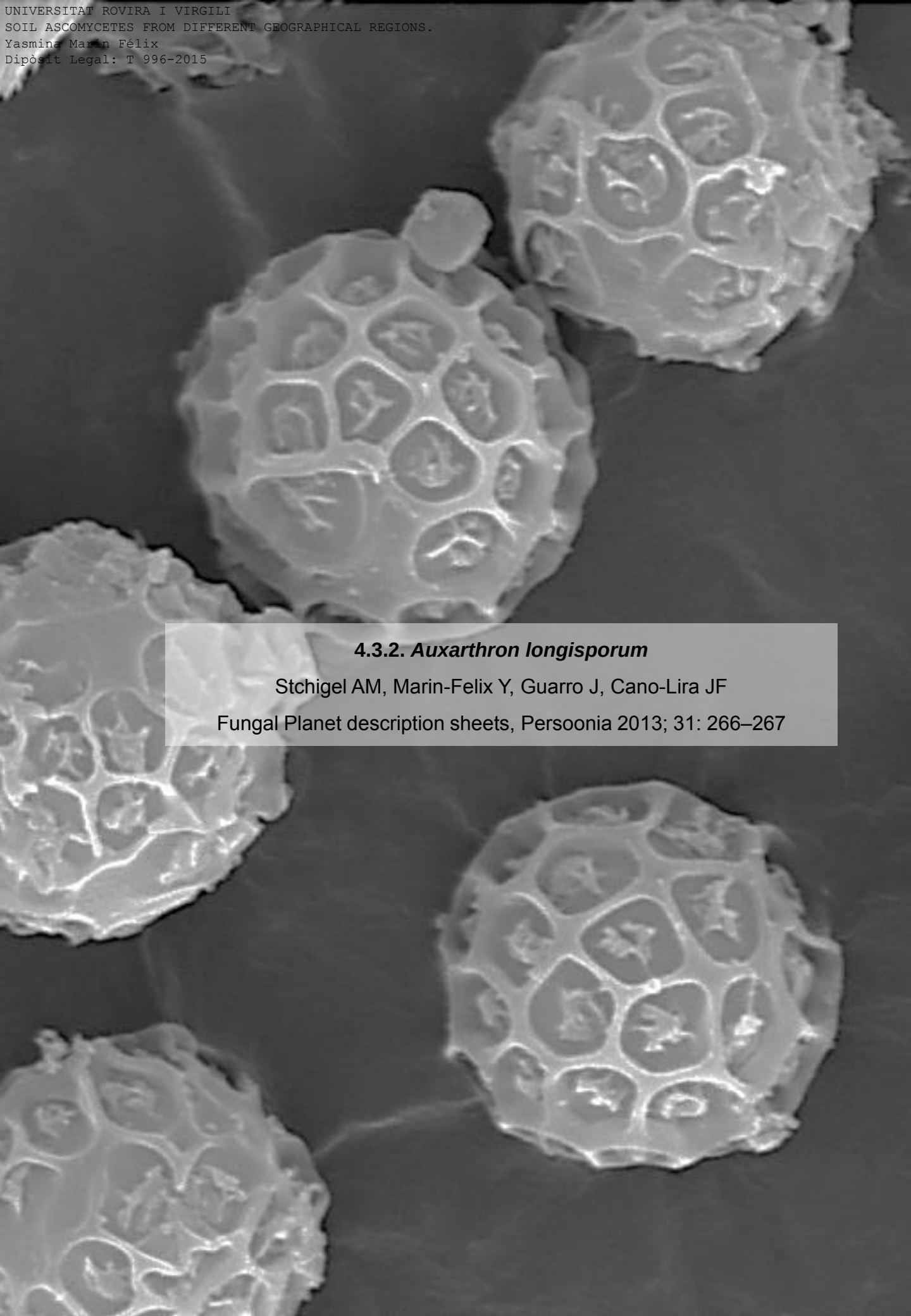
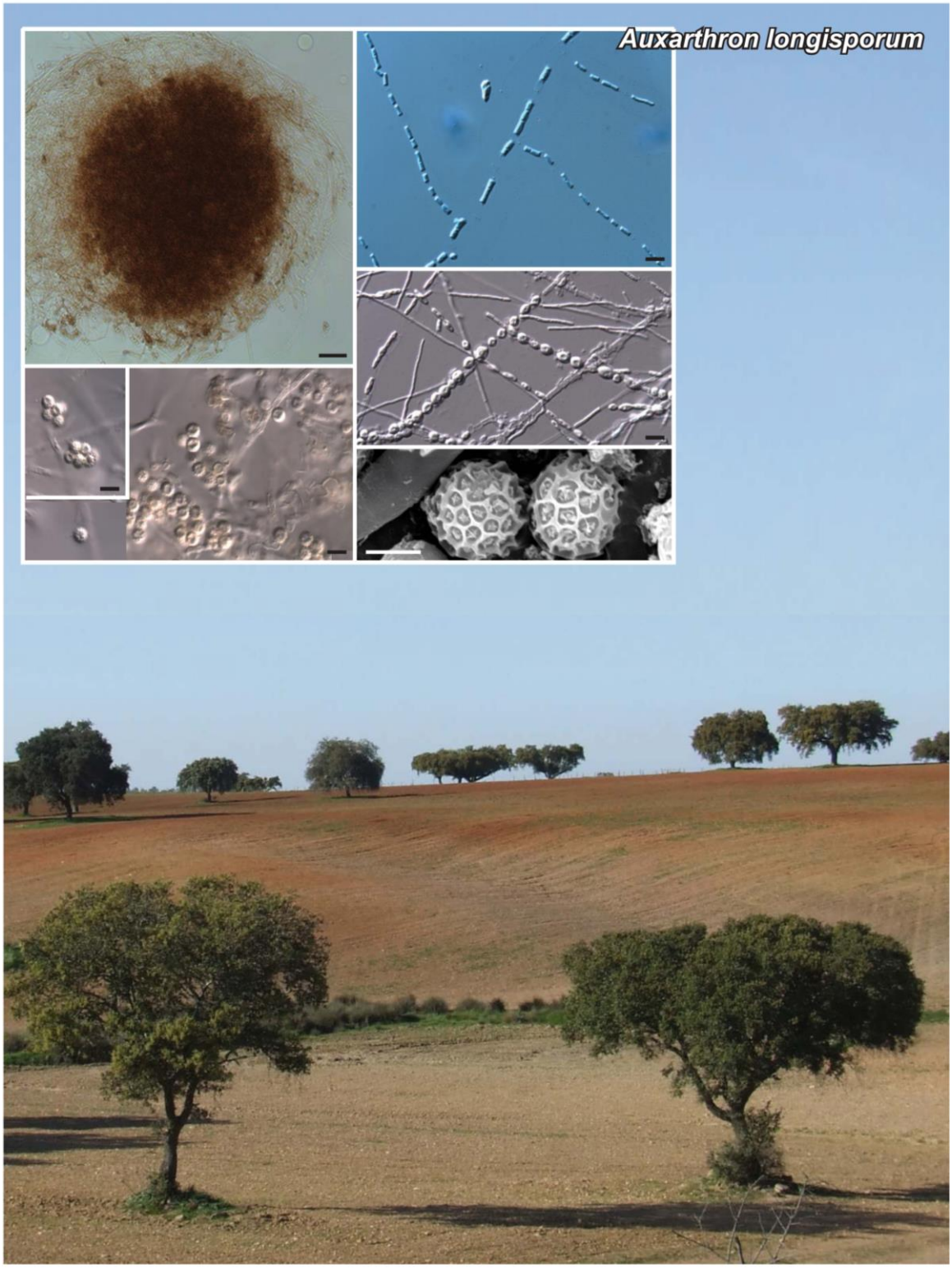


### 4.3.2. *Auxarthron longisporum*

Stchigel AM, Marin-Felix Y, Guarro J, Cano-Lira JF

Fungal Planet description sheets, Persoonia 2013; 31: 266–267



Fungal Planet 198 – 26 November 2013

# ***Auxarthron longisporum*** Stchigel, Y. Marín, Guarro & Cano, *sp. nov.*

*Etymology*. *longus-* and *-sporarum* (L.), referring to the long arthroconida.

*Mycelium* composed of hyaline, branched, septate, smooth- and thick-walled hyphae, 1–4 µm wide. *Ascomata* superficial, scattered or aggregated, globose, 170–450 µm diam, initially white, soon becoming orange-brown to reddish brown; peridial hyphae pale yellow to orange-brown, thick-walled, tuberculate, septate, 1.5–2.5 µm wide, branched and anastomosed to form a loosely reticulate network. *Asci* 8-spored, globose, 9–11 × 7.5–10 µm, evanescent. *Ascospores* subhyaline to golden-yellow or reddish brown, spherical, 3.5–4.5 µm, regularly reticulate, with small polygonal meshes, and narrow and conspicuous ridges. *Conidia* entero-arthric, aseptate, cylindrical, barrel-shaped or irregularly shaped, hyaline, 4–24 × 1–5.5 µm, formed from broad primary hyphae and from narrow, flexuose lateral branches, which sometimes are slightly curved. *Chlamydospores* present in mycelium, spherical to pyriform, 3.5–10 µm.

Culture characteristics — Colonies on oatmeal agar attaining 50–55 mm diam after 15 d at 25 °C, velvety and slightly cottony, margins arachnoid, yellowish white to light yellow (M. 3A3–3A5) (Kornerup & Wanscher 1984); reverse yellowish white to pale yellow (4A2–A3). Colonies on potato dextrose agar attaining 41–47 mm diam after 15 d at 25 °C, cottony to slightly granulate, white to pale yellow (3A1–3A3); reverse pale yellow to light yellow (4A3–4A5). Minimum and maximum temperature of growth: 5 and 30 °C, respectively. Ascomata are not produced at 30 °C. Optimal ascomatal production at 25 °C.

*Typus*. Portugal, Beja, Castro Verde, from forest soil, 28 Dec. 1996, coll. *A.M. Stchigel*, *J*, *Guarro & S.K. Abdullah*, isol. *A.M. Stchigel* (holotype CBS H-21352, cultures ex-type CBS 135817 = FMR 12768, ITS sequence GenBank HG326873, LSU sequence GenBank HG326874, MycoBank MB804882).

Notes — Hitherto, the genus *Auxarthron* encompassed 18 species. Some phylogenetic studies placed this genus into the family *Onygenaceae* (Sigler et al. 2002, Sugiyama et. al. 2002). To date, species of this genus has not been considered as human pathogens. However, Hubka et al. (2013) recently described *Auxarthron ostraviense* associated with a confirmed case of onychomycosis. A Blast search of the LSU sequence of our isolate showed a high degree of similarity (95 %) with those of *Auxarthron californiense* (AF038352), *Malbranchea gypsea* (AB359425) and *Malbranchea floccifor-mis* (AB359421). A Blast search using the ITS sequence of our isolate showed a 92 % similarity with those of *Auxarthron chlamydosporum* (AJ426458) and *A. concentricum* (AJ271428). Morphologically, the species most similar to *A. longisporum* are *A. chlamydosporum*, due to the production of chlamydospores and tuberculate peridial hyphae, and *A. concentricum*, with similar ascospore ornamentation (under the scanning microscope). However, *A. longisporum* can be distinguished from *A. concentricum* by the presence of ascospores with smaller polygonal meshes in the former, and from *A. chlamydosporum* because the arthroconidia are twice as long in *A. longisporum* than in *A. chlamydosporum*. On the other hand, *A. longisporum* is not able to grow above 30 °C, while *A. chlamydosporum* and *A. concentricum* grow at this temperature.

*Colour illustrations*. Castro Verde, Beja, Portugal; ascoma, arthroconidia, chlamydospores, asci and ascospores. Scale bars: ascoma = 50 µm; arthroconidia, chlamydospores, asci and ascospores = 5 µm; ascospore (SEM) = 2 µm.

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## 4.3.3. *Emmonsiellopsis*, a new genus related to the thermally dimorphic fungi of the family Ajellomycetaceae

Marin-Felix Y, Stchigel AM, Cano-Lira JF, Sanchis M, Mayayo E, Guarro J

Mycoses, in press

Original article

# *Emmonsiellopsis*, a new genus related to the thermally dimorphic fungi of the family Ajellomycetaceae

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## Summary

Two interesting fungi were isolated from fluvial sediments collected in the North of Spain. They were morphologically related to the thermally dimorphic fungi of the family Ajellomycetaceae, but the analysis of the internal transcribed spacer region of the rDNA, and the domains D1 and D2 of the 28S rRNA gene sequences confirmed that they were different from all the species described in that family. They were accommodated in the new genus *Emmonsiellopsis* as *E. coralliformis* sp. nov. and *E. terrestris* sp. nov. The two species are distinguished mainly by the maximum temperature of growth (up to 33 °C for *E. coralliformis* and to 42 °C for *E. terrestris*), the dendritic mycelium of *E. coralliformis* and the conidial ornamentation (verrucose in *E. coralliformis* and spinulose in *E. terrestris*). In addition, the phylogenetic data demonstrated that *Ajellomyces griseus* also represents a new genus within the Ajellomycetaceae, namely *Helicocarpus*. This new genus is easily distinguished by the lack of asexual morph, the production of brownish gymnothecial ascomata and oblate to lenticular, sparingly pitted ascospores. The proposal of both new genera was confirmed by the analysis of actin gene sequences.

**Key words:** Ajellomycetaceae, *Emmonsiellopsis*, *Helicocarpus*, phylogeny, taxonomy, dimorphic fungi.

## Introduction

The thermally dimorphic fungi are a group of systemic pathogens that develop a mycelial morph in the environment, such as soil, but that grow as a yeast-like morph at body temperature (35–37 °C) and are responsible for severe infections.[1] Currently, apart from the genera *Coccidioides* and *Sporothrix* and the species *Talaromyces marneffei*, the thermally dimorphic fungi are included in the family Ajellomycetaceae (Onygenales, Eurotiomycetes, Ascomycota). Most of these fungi produce endemic mycoses, being represented by the genera *Blastomyces*, *Emmonsia*, *Histoplasma*, *Paracoccidioides* and *Lacazia*.[2,3] The only sexual morph found for some members of this family, although only in nature (and consequently not developing in infected tissues of the host), is *Ajellomyces*. The species of *Ajellomyces* are heterothallic and characterised by globose to stellate gymnothecial ascomata ornamented with spirally twisted appendages, and minute, oblate or subglobose to globose, finely spinulose or roughened ascospores. The asexual morphs are characterised by one-celled, smooth-walled "aleuroconidia" (blastoconidia arising singly, laterally on a hyphae) in *Paracoccidioides*, smooth to echinulate in *Emmonsia* and *Blastomyces*, and rough or warty in *Histoplasma*.[4–6] Although some taxonomic studies have been conducted in the order Onygenales in recent years,[7,8] those concerning the family Ajellomycetaceae are very rare. The new species *Paracoccidioides lutzii* and *Blastomyces gilchristii* were recently proposed to accommodate several strains that had previously been identified as *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*, respectively, based on molecular data.[9,10] During a survey on fluvial sediments in Spain, two

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different fungi, morphologically related to *Emmonsia*, were isolated in pure culture. These isolates were phenotypically and molecularly characterised and proposed here as new species. In addition, based on a phylogenetic study of the species of Ajellomycetaceae, the taxonomy of that family has been reviewed.

## Materials and methods

### Sediment sampling and fungal isolation

We collected numerous fluvial sediments from Girona, Spain, at the location of Empuriabrava (42.23, 3.12) and Estartit (42.03, 3.19). For the isolation of the ascomycetes present in these samples we followed a previously described protocol using the "actidione (cycloheximide) submerged particle plating technique".[11] Briefly, approximately 2 g of sediment was plated onto three Petri dishes of 9 cm diameter, and melted Sabouraud glucose agar (SGA: glucose, 20 g; peptone, 10 g; agar-agar, 15 g; tap water, 1 l) was added with L-chloramphenicol (100 mg $l^{-1}$) and cycloheximide (2 g $l^{-1}$) at 50–55 °C, mixed by hand and incubated at room temperature, alternating 12-h intervals of darkness and fluorescent light. Portions of the colonies of the fungi considered of interest were transferred using a sterile needle to 5 cm diameter Petri dishes containing oatmeal agar (OA: oatmeal flakes, 30 g; agar-agar, 20 g; tap water, 1 l). The cultures were incubated at 15, 25 and 35 °C.

### Phenotypic study

Two of the isolates, CBS 137499 and CBS 137500, belonging to the Ajellomycetaceae, and the morphologically related strain *Emmonsia parva* CBS 273.77 were grown for up to 30 days on phytone yeast extract agar (PYE: papaic digest of soy bean meal, 10 g; yeast extract, 5 g; dextrose, 40 g; streptomycin, 0.03 g; chloramphenicol, 0.05 g; agar-agar, 17 g; tap water, 1 l) and potato dextrose agar (PDA; Pronadisa, Madrid, Spain) at 5, 15, 25, 28, 30, 33, 35, 37, 40, 42 and 44 °C, for testing growth rates. Colour notations in parentheses are from Kornerup & Wanscher.[12] To test the development of the yeast-like morph, the isolates were grown for 10 days on brain and heart infusion agar (BHIA; Difco, Sparks, MD, USA) and BHIA supplemented with 5% sheep blood (MAIM, Barcelona, Spain) and tryptic soy agar supplemented with 5% sheep blood (TSA + 5% sheep blood; Difco, Madrid, Spain) at 33, 35, 37, 40 and 42 °C. Tolerance to cycloheximide was evaluated by measuring growth rates on Mycosel agar (MYC: papaic digest of soybean meal, 10 g; dextrose, 10 g; cycloheximide, 0.4 g; chloramphenicol, 0.05 g; agar-agar, 15.5 g; tap water, 1 l), as well as the response on bromocresol purple-milk solids-glucose agar (BCP-MS-G: skim milk powder, 40 g; glucose, 20 g; agar-agar, 15 g; bromocresol purple solution [bromocresol purple, 0.16 g; ethanol 95% solution, 10 ml] 1 ml; tap water, 1 l).[13] Production of urease was determined on Christensen's urea agar (Merck, Darmstadt, Germany) and the lipase activity on Tween 80 opacity test medium (TOTM: bacteriological peptone, 10 g; NaCl, 5 g; $CaCl_2$, 0.1 g; Tween 80, 5 ml; agar-agar, 15 g; tap water, 1 l). The keratinolytic capability was evaluated by culturing the fungus on blonde, sterile, prepubertal child's hair on tap water agar (TWA: agar-agar, 20 g; tap water, 1 l) in a 9 cm diameter Petri dish, incubating for 4 weeks at 25 °C in darkness, and examining the hairs under a light-field microscope by mounting them on a drop of 20% KOH.[14]

### In vivo study

The potential virulence of the fungi was determined in a murine model. The isolates CBS 137499 and CBS 137500 were subcultured on PDA plates at 25 °C for 30 days. Inocula were prepared by flooding the fungal growth with saline solution and filtering through sterile gauze to remove clumps of conidia and hyphae. The resulting suspensions were adjusted to the desired concentration by haemocytometer count and viability was confirmed by serial plating on PDA.

Male OF1 mice, 4 weeks old, weighing 30 g (Charles River, Criffa S.A., Barcelona, Spain) were used. Animals were housed in standard boxes with free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Groups of five animals were immunosuppressed by an intraperitoneal (i.p.) injection of 200 mg $kg^{-1}$ of cyclophosphamide 2 days prior to infection and then every 5 days. Mice were challenged i.p. with $1 \times 10^4$, $1 \times 10^5$ and $1 \times 10^6$ CFU of each of the two isolates in 0.2 ml of sterile saline.

Virulence was evaluated by survival and histopathological studies. For the survival study, groups of five mice were established and were checked daily for 30 days after challenge. For histopathology examination, groups of five mice were established and on day 30 post infection the animals were killed and the livers, kidneys and lungs were aseptically removed in order to observe the progress of the infection. Later, half of each organ was fixed with 10% buffered formalin. Samples were dehydrated, paraffin embedded, and

sliced into 2-µm sections, which were coloured with haematoxylin–eosin (H-E), periodic acid-Schiff, and Grocott methenamine silver stainings.

### Phylogenetic study

The DNA of the isolates CBS 137499 and CBS 137500, and the strains *Pseudeurotium ovale* FMR 13600, *Emmonsia parva* CBS 273.33 and *Ajellomyces griseus* CBS 128.88 (culture ex-type) was extracted and purified directly from the fungal colonies according to the Fast DNA Kit protocol (MP Biomedicals, Solon, Ohio). The internal transcribed spacer (ITS) region of the nuclear rDNA was amplified with the primer pair ITS5 and ITS4,[15] the D1 and D2 domains of the 28S rRNA (D1/D2) gene were amplified with the primer pair NL1–NL4,[16] and a fragment of actin gene (*ACT*) was amplified with the primers Act1 and Act4R.[17] The sequences of these amplicons were obtained using the protocol of the Taq Dye-Deoxy Terminator Cycle Sequencing Kit. PCR products were purified and sequenced at Macrogen Europe (Amsterdam, the Netherlands) with a 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA) and the consensus sequences were obtained using SeqMan (version 7.0.0; DNASTAR, Madison, WI, USA). Our sequences, together with sequences retrieved from GenBank and NBRC database, were aligned using MEGA v. 6.06 (Tamura, Stecher, Peterson, Filipski and Kumar, 2013), which was also used to carry out the maximum likelihood (ML) phylogenetic analysis. This analysis was based on the combined data set (ITS and D1/D2) of our isolates, type species and reference strains included in the family Ajellomycetaceae (with the exception of *Lacazia loboi*), selected members of the orders Arachnomycetales, Ascosphaerales, Eurotiales, Onygenales and Spiromastixales, and a strain of *Pseudeurotium ovale* (FMR 13600) and the type strain of *Pseudogymnoascus destructans* (Leotiomycetes) as outgroup. To corroborate the possibility of combining the loci for the phylogenetic analysis, the phylogeny from each one was obtained and compared, and given that no incongruence was observed, they were combined. The ML phylogeny was constructed using the Tamura-Nei model with gamma distribution and invariant sites, with the partial deletion of gaps option. Maximum parsimony (MP) was analysed using the PAUP* version 4.0b10 software (Sinauer Associates, Inc. Publishers, Sunderland, MA, USA) and Bayesian inference (BI) using MrBayes v. 3.2 (Ronquist, Huelsenbeck and Teslenko, 2011), following the parameters detailed in previous studies.[18,19] The sequences generated in this study were deposited in GenBank. Accession numbers for the sequences of the ITS, D1/D2 and *ACT* loci of CBS 137500 are KP101584, KP101585 and KP686189, and those of CBS 137499 are KP101582, KP101583 and KP686188 respectively. The accession number for the sequence of the *ACT* locus of CBS 273.77 is KP686187, for the ITS and *ACT* loci of the ex-type strain of *Ajellomyces griseus* is KP686191 and KP686190, and for the ITS and D1/D2 loci of *Pseudeurotium ovale* FMR 13600 is KP686192 and KP686193 respectively. The alignment used in the phylogenetic analyses can be found in TreeBASE (www.treebase.org) under the accession number S17054.

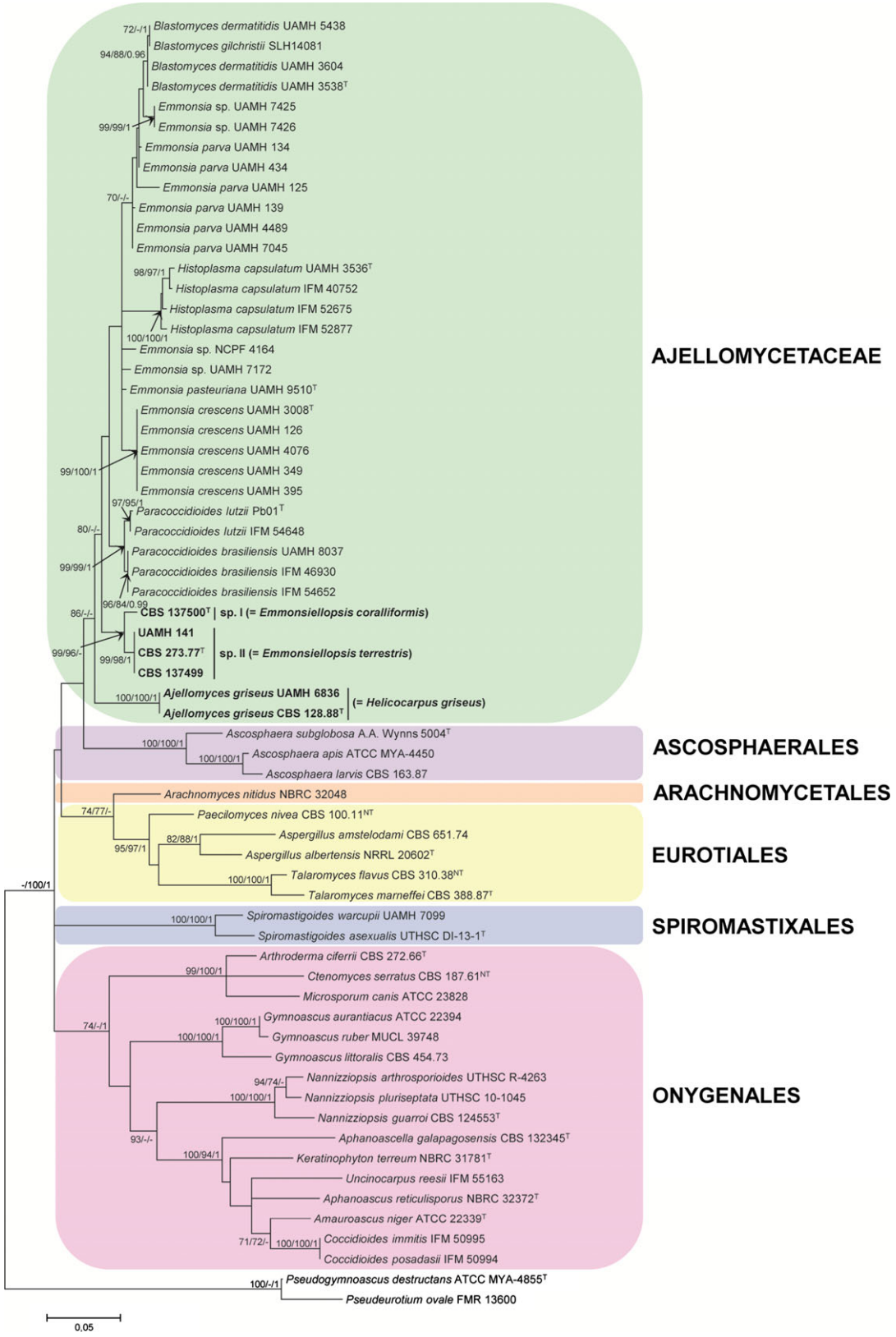
## Results

### Field collections

Among the numerous fungi recovered from the study of the fluvial sediments, the strains CBS 137499 and CBS 137500 were included in the phylogenetic study because they showed typical morphological features of *Emmonsia*-related taxa.

### Molecular analysis

The lengths of the amplicons used in the combined data set of the first phylogenetic study were 360 bp (ITS) and 456 bp (D1/D2). The length of the final combined alignment was 816 bp, from which 288 bp were parsimony informative. Figure 1 shows the ML-tree inferred from the combined data set. Our two isolates were located in a well-supported subclade, with a ML-bootstrap support (ML-bs) of 99%, MP-bootstrap support (MP-bs) of 96% and less than 0.95 bayesian posterior probability (pp), within a main clade (86% ML-bs / -% MP-bs / - pp) composed of all the members of the Ajellomycetaceae included in the study, i.e. reference strains of *Ajellomyces griseus*, *Blastomyces dermatitidis*, *Blastomyces gilchristii*, *Emmonsia crescens*, *Emmonsia parva*, *Emmonsia pasteuriana*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*, and some strains identified as *Emmonsia* spp. Strain CBS 137500 formed a terminal branch (species I) and strain CBS 137499 clustered (99% ML-bs / 98% MP-bs / 1 pp) with two strains UAMH 141 and CBS 273.77 previously identified as *Emmonsia parva* and representing a new species (species II). While the genera *Histoplasma* (100% ML-bs / 100% MP-bs / 1 pp) and *Paracoccidioides* (99% ML-bs / 99% MP-bs / 1 pp) constituted two separated well-supported subclades, this phylogenetic study was unable to resolve the circumscription and boundaries of the genera *Blastomyces* and *Emmonsia*. The two isolates



of *Ajellomyces griseus* included in the study (the type strain and a reference strain) were placed at considerable genetic distance from the other members of the family, showing that they are a different lineage. The comparison of *ACT* nucleotide sequences of the members of Ajellomycetaceae included in this study, the total length of the alignment being 835 bp, revealed that *Blastomyces dermatitidis* was the most closely related to the isolates of species I (85% identity) and species II (83% identity). The similarity between the *ACT* sequences of the two isolates of the species II was 99%, and between the isolates of species I and II it was 92%. The identity between the type strain of *Ajellomyces griseus* and the type species of *Ajellomyces*, *A. dermatitidis* (*Blastomyces dermatitidis*) was 86%.

### Morphology

The two isolates of the species II (CBS 273.77 and CBS 137499) produced similar morphology, i.e. smooth-walled and spinulose blastoconidia that are mostly sessile, but also on short or long, sometimes inflated, pedicels, while the isolate of species I (CBS 137500) also formed blastoconidia that are mostly sessile or on pedicels but the conidia were smooth-walled and verrucose, and the long, inflated pedicels were much less abundant than in species II. Species I also produced a characteristic dendritic mycelium.

### Physiology

On BCP-MS-G, the isolates of species I and II showed acidity and casein hydrolysis. All the isolates were tolerant to cycloheximide but not keratinolytic. Also, all of them produced urease on Christensen's urea agar and lipase on TOTM. Neither yeast-like morphs nor adiaspores were observed in BHIA, BHIA + 5% sheep blood or TSA + 5% sheep blood for all strains. The maximum growth temperature was 33 °C for the isolate of species I and 42 °C for species II.

### In vivo study

The histopathological study did not show the production of any type of lesion nor yeast-like morph or other invasive structure in any of the organs studied.

The molecular results demonstrated that species I and II represent two species of a genus different from those previously known in Ajellomycetaceae, therefore they are proposed as new in the next section. It was also proven that *Ajellomyces griseus* corresponds to a new genus within the family Ajellomycetaceae, which agrees with morphological data as it is the only species of Ajellomycetaceae that does not show any type of asexual morph or a yeast-like morph either.

**Taxonomy**

***Emmonsiellopsis*** Y. Marín, Stchigel, Guarro & Cano, *gen. nov.* — MycoBank MB 811334.

Etymology: referring to the morphological similarity with *Emmonsia*.

Diagnosis: Characterised by its smooth-walled and verrucose or spinulose conidia, born sessile or on pedicels, rarely septate and intercalary, and the lack of sexual morph, yeast-like phase and adiaspores.

Hyphae septate, branched, hyaline, smooth- and thin- to thick-walled, sometimes contorted to dendritic. Conidia blastic, hyaline, globose to ovoid or clavate, smooth-walled and verrucose or spinulose, thick-walled, rarely 1-septate, sessile, born laterally or terminally on the hyphae, or on pedicels, solitary or in chains of two conidia, rarely intercalary. Pedicels hyaline, short or long, smooth- and thin-walled, inflated at the middle or at the apex. Chlamydospores, sexual morph and yeast-like morph absent (Figs. 2 and Fig. 3).

Type species: *Emmonsiellopsis terrestris* Y. Marín, Stchigel, Guarro & Cano.

***Emmonsiellopsis coralliformis*** Y. Marín, Stchigel, Guarro & Cano, *sp. nov.* — MycoBank MB 811335.

Etymology: the epithet refers to the coral-shape of the hyphae.

Diagnosis: Characterised by its maximum temperature of growth of 33 °C, dendritic mycelium and smooth-walled and verrucose conidia.

Colonies on PYE at 28 °C attaining a diameter of 26–31 mm after 28 days, orange grey to brownish grey (M. 5B2 to 5D2), velvety, cerebriform, pulvinate, margins regular; reverse pale orange to greyish orange (M. 5A3 to 5B3). Hyphae septate, branched, hyaline, smooth- and thin- to thick-walled, sinuose, contorted to dendritic, 1–5 µm wide. Conidia blastic, hyaline,

**Figure 1** Maximum likelihood (ML) tree obtained from the combined DNA sequence data from two loci (ITS and D1/D2) of our isolates, type species and reference strains included in the family Ajellomycetaceae, with the exception of *Lacazia loboi,* and members of all the families and orders of the subclass Eurotiomycetidae. The type strain of *Pseudogymnoascus destructans* and one strain of *Pseudeurotium ovale* were used as outgroup. ML and maximum parsimony (MP) bootstrap support values ≥70 and Bayesian posterior probability scores ≥0.95 are indicated along branches. New taxa are indicated in boldface and type and neotype strains of the different species with $^{T}$ and $^{NT}$ respectively.

globose to ovoid, smooth-walled and verrucose, thick-walled, rarely 1-septate, 3–11 × 2–7 µm, sessile, borne laterally or terminally on the hyphae, on pedicels, solitary or in chains of two conidia, rarely intercalary. Pedicels hyaline, short, rarely long, smooth- and thin-walled, rarely inflated at the middle or at the apex, 1–3 µm wide. Chlamydospores, sexual morph and yeast-like phase not observed (Fig. 2a–i).

Colonies on PDA at 25 °C attaining a diameter of 25–28 mm after 28 days, pale yellow to olive brown (M. 4A3 to 4D3) and white at the margins, velvety, cerebriform, margins regular; reverse pale yellow to olive brown (M. 4A3 to 4D3).

Minimum and maximum temperatures of growth 15 and 33 °C respectively. Tolerant to cycloheximide (colonies reaching a diam of 25–26 mm on MYC after 28 days at 28 °C), urease positive, non-keratinolytic, and showing acidity and casein hydrolysis on BCP-MS-G, lipase positive.

*Specimens examined:*

Spain, Girona, Empuriabrava (42.23, 3.12), fluvial sediment, 09-VI-1991, col. P. Vidal, holotype CBS H-21624, cultures ex-type CBS 137500, FMR 4024.

***Emmonsiellopsis terrestris*** Y. Marín, Stchigel, Guarro & Cano, *sp. nov.* — MycoBank MB 811598.

Etymology: the epithet refers to the origin of the ex-type strain.

Diagnosis: Characterised by its maximum growth temperature of 42 °C, the long and usually inflated pedicels and smooth-walled and spinulose conidia.

Colonies on PYE at 28 °C attaining a diameter of 66–75 mm after 28 days, greyish orange to greyish brown (M. 6B3 to 6D3), velvety to woolly, irregularly folded, margins regular; reverse light orange to dark orange (M. 5A5 to 5A8). Hyphae septate, branched, hyaline, smooth- and thin-walled, 1–5 µm wide. Conidia blastic, hyaline, globose to clavate or pyriform, smooth-walled and spinulose, thick-walled, rarely 1-septate, 3–8(–10) × 2.5–5.5 µm, sessile, borne laterally or, less frequently, terminally on the hyphae, on pedicels, solitary, rarely in chains of two conidia, rarely intercalary. Pedicels hyaline, short or long, smooth- and thin-walled, sometimes inflated at the middle or at the apex, 1–3.5 µm wide. Chlamydospores, sexual morph and yeast-like phase absent (Fig. 3a–g).

Colonies on PDA at 25 °C attaining a diameter of 61–70 mm after 28 days, orange white to pale orange (M. 5A2 to 5A3), velvety to slightly woolly, slightly irregularly folded, margins regular; reverse yellowish white to pale yellow (M. 4A2 to 4A3).

Minimum and maximum growth temperatures of 20 and 42 °C respectively. Tolerant to cycloheximide (colonies reaching a diam of 52–55 mm on MYC after 28 days at 28 °C), urease test positive, non-keratinolytic, and showing acidity and hydrolysis of casein on BCP-MS-G depending on the strain, lipase positive.

Specimens examined:

USA, Kansas, Phillips County, ex soil, VI-1977, col. C.W. Emmons, holotype CBS H-22118, cultures ex-type CBS 273.77, FMR 13882, UAMH 2304; Spain, Girona, Estartit (42.03, 3.19), ex fluvial sediment, 08-XI-1990, col. P. Vidal, living cultures CBS 137499, FMR 4023.

***Helicocarpus*** Y. Marín, Guarro, Cano & Stchigel, *gen. nov.* — MycoBank MB 811275.

Etymology: referring to the spirally twisted appendages of the ascomata.

Diagnosis: Characterised by its gymnothecial ascomata with spirally twisted appendages, lenticular to oblate and sparingly pitted ascospores, and its lack of asexual morph and yeast-like phase.

Ascomata gymnothecial, pale greyish brown, globose; peridium composed of irregularly branched, contorted hyphae with spirally twisted appendages; appendages thick- and smooth-walled, septate, arising from the central part of the ascoma, usually coiled. Asci 8-spored, pyriform to ellipsoidal, evanescent. Ascospores lenticular to oblate, hyaline, smooth-walled under light-field microscope, sparingly pitted along the equatorial zone, under scanning electron microscope, without germ pores.

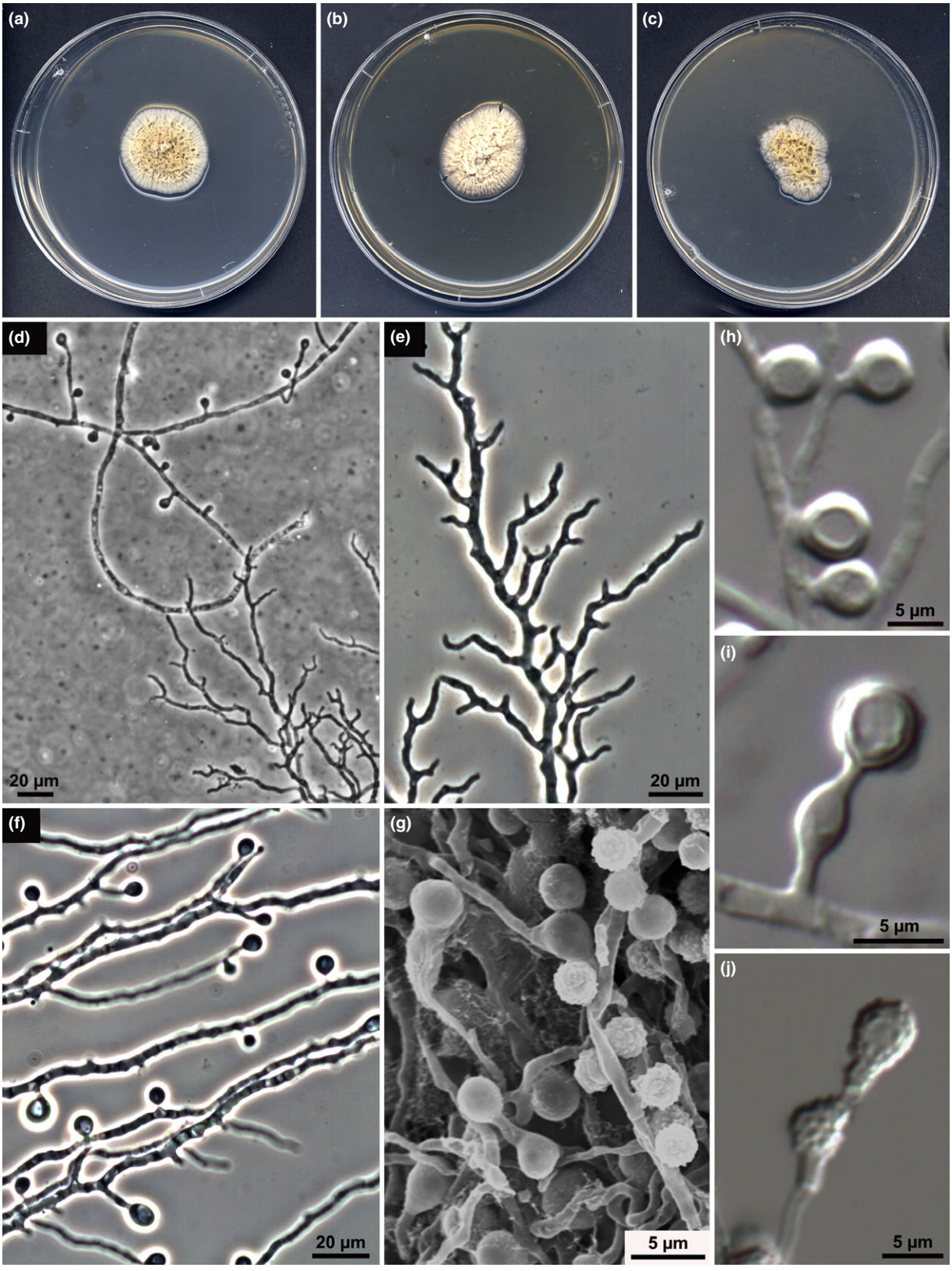
Type species: *Helicocarpus griseus* (Currah & Locq.-Lin.) Y. Marín, Guarro, Cano & Stchigel.

***Helicocarpus griseus*** (Currah & Locq.-Lin.) Y. Marín, Guarro, Cano & Stchigel, comb. nov. — MycoBank MB 811337.

*Basionym*. *Spiromastix grisea* Currah & Locq.-Lin., Can. J. Bot. 66: 1135. 1988.

≡ *Ajellomyces griseus* (Currah & Locq.-Lin.) Unter. & J.A. Scott [as 'grisea'], in Untereiner, Scott, Naveau, Currah & Bachewich, Stud. Mycol. 47: 33. 2002.

**Figure 2** *Emmonsiellopsis coralliformis* sp. nov. CBS 137500. (a) Colony on PDA after 28 days at 25 °C (surface). (b) Colony on PYE after 28 days at 28 °C (surface). (c) Colony on Mycosel after 28 days at 28 °C (surface). (d) Globose to ovoid conidia borne sessile and on pedicels. (e) Contorted to dendritic hyphae. (f) Sessile conidia and conidia bornig on pedicels. (g) Smooth-walled and verrucose conidia borne sessile and on pedicels. (h) Sessile conidia. (i) Conidium on an inflated pedicel. (j) Verrucose intercalary conidia.



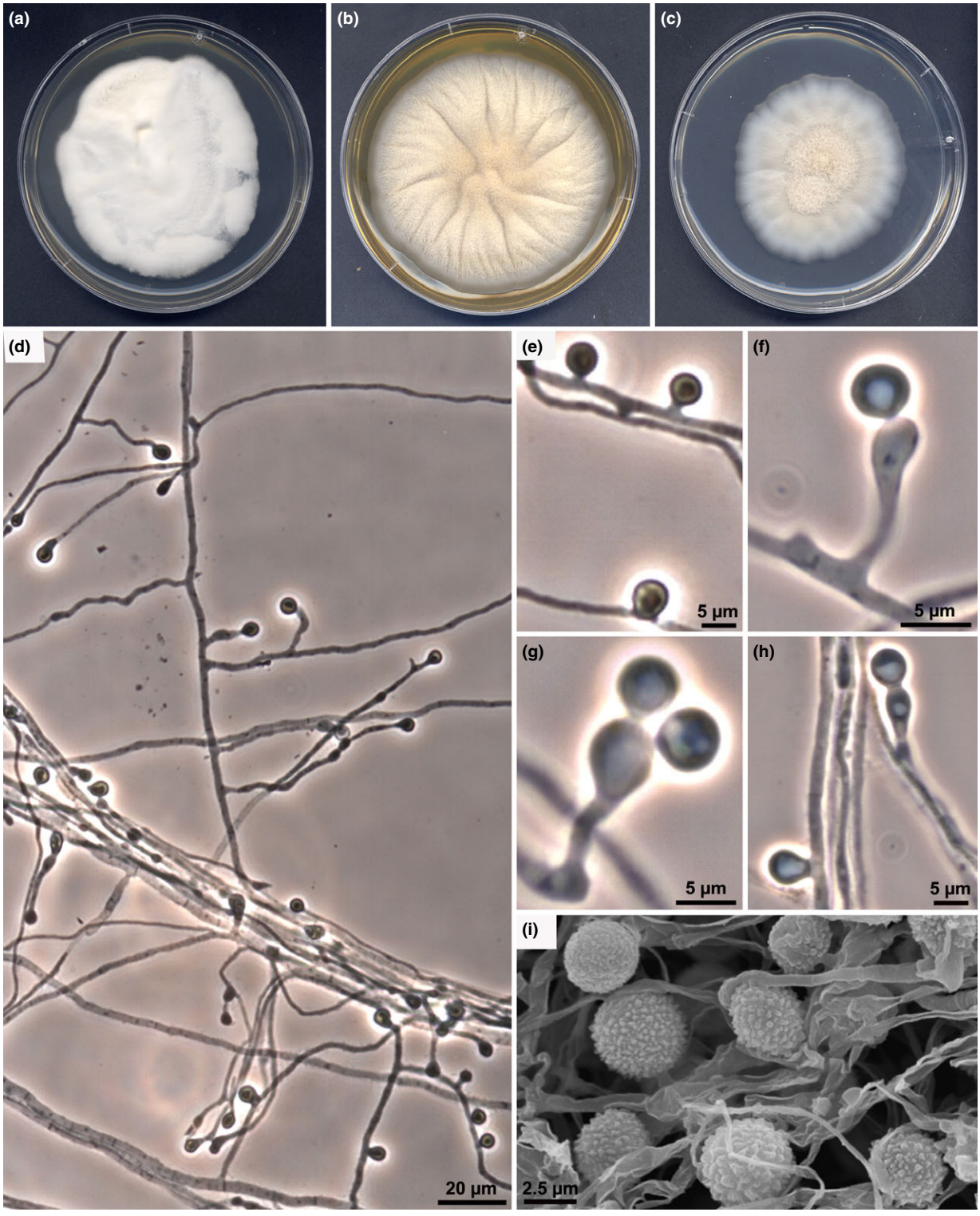



**Figure 3** *Emmonsiellopsis terrestris* sp. nov. CBS 273.77. (a) Colony on PDA after 28 days at 25 °C (surface). (b) Colony on PYE after 28 days at 28 °C (surface). (c) Colony on Mycosel after 28 days at 28 °C (surface). (d) Smooth-walled, globose to ovoid conidia borne sessile or on pedicels. (e) Sessile conidia. (f, g) Conidia on inflated pedicels. (h) Sessile conidium and conidium borning on inflated pedicel. (i) spinulose conidia.

≡ *Spiromastigoides grisea* (Currah & Locq.-Lin.) Doweld, Index Fungorum 30: 1. 2013.

## Discussion

On the basis of newly described species belonging to the Ajellomycetaceae, the taxonomy of the family has been revised. Our phylogenetic studies based on the analysis of the combined data set of ITS and D1/D2 sequences show that our isolates belong to a new genus, *Emmonsiellopsis*, closely related to the pathogenic, thermally dimorphic fungi of the family Ajellomycetaceae. In fact, two of the isolates of this new genus, CBS 273.77 and UAMH 141, had been identified previously in different studies as *Emmonsia parva.*[20,21] However, the two species of *Emmonsiellopsis* can be differentiated from the other members of this family for a series of outstanding features, mainly the absence of thermal dimorphism. *Emmonsiellopsis* is morphologically similar to *Blastomyces* and *Emmonsia*. The three genera are characterised by whitish colonies, and globose to piriform aleurioconidia, sessile or on pedicels, and usually solitary. However, *Blastomyces* on nutritionally rich media at 37 °C, or infecting host tissues, produces yeast-like structures with double-contoured refractile walls and a broad base. The conidia of *Emmonsia crescens* and *E. parva* on blood agar at 37 °C may inflate and become transformed into adiaspores. On the other hand, *Emmonsia pasteuriana* on BHIA with blood at 37 °C produces yeast-like structures with thin walls and polar budding on a narrow base. The production of the yeast-like morph or adiaspores was not observed in our fungi even in the different culture media traditionally used to obtain this and was not observed in the *in vivo* study either. This appears to contradict a previous study that has shown the ability of UAMH 141 to produce adiaspores larger than *Emmonsia parva.*[20]

*Emmonsiellopsis* can produce septate and intercalary conidia, though only scarcely. The only genus of the Ajellomycetaceae that also presents septate and intercalary conidia, even more frequently than in *Emmonsiellopsis,* is *Paracoccidioides*. Furthermore, *Paracoccidioides* differs by the production of only smooth-walled conidia and yeast-like structures when it infects a host. *Histoplasma* is easily distinguishable from *Emmonsiellopsis* and other Ajellomycetaceae by its production of tuberculate macroconidia and microconidia with more or less roughened walls.

The main differences between the two species of *Emmonsiellopsis* are the maximum temperature of growth (33 °C for *E. coralliformis* and 42 °C for *E. terrestris*), the presence of contorted to dendritic mycelium in *E. coralliformis* and the conidial ornamentation (verrucose in *E. coralliformis* and spinulose in *E. terrestris*). Apart from those, long and inflated pedicels are more common in *E. terrestris* than in *E. coralliformis*.

The new genus *Helicocarpus* is introduced to accommodate *Ajellomyces griseus*, which in the present phylogenetic study is located very far away from the other fungi tested. It was originally described as a species of *Spiromastix*[22] but later transferred to the *Ajellomyces* because it was grouped with the type species of *Ajellomyces*, *A. dermatitidis.*[23] A phylogenetic study of the genus *Spiromastix,* recently renamed as *Spiromastigoides*,[24] confirmed the correct placement of *A. griseus* into the family Ajellomycetaceae.[8] The morphology of this species agrees with the molecular results because it does not produce asexual morphs, unlike the other species of Ajellomycetaceae, or a yeast-like morph. *Helicocarpus griseus* also differs from the other species of *Ajellomyces* by its bigger, lenticular to oblate and pitted ascospores, whereas in the other species they are minute, globose to subglobose, finely spinulose or roughened.[6]

Although *Blastomyces* and *Emmonsia* are considered separate genera because of some phenotypic differences and their different infecting morphs, our phylogenetic study has failed to delimit and separate them. The main phenotypic differences are that cycloheximide does not inhibit *Blastomyces dermatitidis*, whose conidia are mostly solitary and sessile, or scarcely produced on non-swollen / slightly swollen conidiophores, whereas *Emmonsia* spp. are more or less sensitive to the cycloheximide and their conidia are produced on swollen conidiophores and frequently in short chains; however, the sexual morphs produced by both taxa are almost identical. The recent new species *Blastomyces gilchristii* grouped in the same well-supported clade as the type strain of *B. dermatitidis*, showing a nucleotide identity of almost 100%, suggesting that the proposal as a new taxon could be wrong. Although seven genomic loci were sequenced to propose the new species *B. gilchristii*, the overall genetic diversity from *B. dermatitidis* was only 1.29%,[10] and considering that there are no phenotypic differences between both species, we do not accept *B. gilchristii* as a valid taxon.

Several phylogenetic studies have also placed the species *Lacazia loboi* in the family Ajellomycetaceae.[25–27] The monotypic genus *Lacazia* is phylogenetically close to *Paracoccidioides*. *Lacazia loboi* produces lobomycosis, a chronic cutaneous and subcutaneous infection in mammals.[28,29] This species is only known at its parasitic stage.

In our phylogenetic study, the family Ajellomycetaceae constituted an independent lineage, clearly

separate from the other orders of the class Eurotiomycetes. This agrees with previous phylogenies also based on ribosomal genes, where the placement of Ajellomycetaceae was independent from Onygenales.[8,23,30,31] However, further phylogenetic studies including more genes must be carried out to verify whether or not this family constitutes a new order. Although the new fungi in this study are apparently not pathogenic to mammals, the likelihood of finding isolates that are morphologically compatible with the dimorphic fungi of the Ajellomycetaceae as saprobes in soil and river sediments, must be treated with caution considering their potential for causing severe infections.

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## Conflict of interest

None.

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UNIVERSITAT ROVIRA I VIRGILI
SOIL ASCOMYCETES FROM DIFFERENT GEOGRAPHICAL REGIONS.
Yasmina Marín Félix
Dipòsit Legal: T 996-2015

# 4.4. SORDARIALES

## 4.4. SORDARIALES

The order Sordariales (Hawksworth and Eriksson, 1986) is taxonomically diverse. Depending on the authors, it had consisted of 7 to 14 families, until Huhndorf et al. (2004) carried out a phylogenetic study and restricted it to three: i.e. Chaetomiaceae, Lasiosphaeriaceae and Sordariaceae. Figure 27 shows the phylogenetic tree based on D1–D3 sequences of our isolates belonging to the Sordariales, which have been distributed in these three families. The length of the alignments used was 827 bp (D1–D3), of which 169 bp were parsimony informative.

Figure 27 shows that the families Chaetomiaceae and Lasiosphaeriaceae did not form monophyletic clades. This was first demonstrated by Huhndorf et al. (2004), who decided to refer to both families as complexes to emphasize their paraphyletic nature. Further studies on more genes should be made in order to delimit both families accurately. The problematical situation within these families also includes lots of genera not well-delimited, being most of them of polyphyletic nature. Miller and Huhndorf (2005) also carried out a phylogenetic study using the D1–D3, *BT2* and *RPB2* sequences of many members of the Sordariales, mostly from the family Lasiosphaeriaceae. They observed that the morphology of the ascospore was an extremely homoplastic taxonomic character and could not be used to predict phylogenetic relationships, but the nature of the ascomata wall could be used to delimit certain genera and clades. Subsequently, Cai et al. (2005) reached the same conclusion, considering the ascomatal wall a better predictor of phylogeny. On the basis of these criteria, they redefined the genus *Schizothecium* (Lasiosphaeriaceae) after performing a phylogenetic study using D1–D3, ITS and *BT2* sequences.

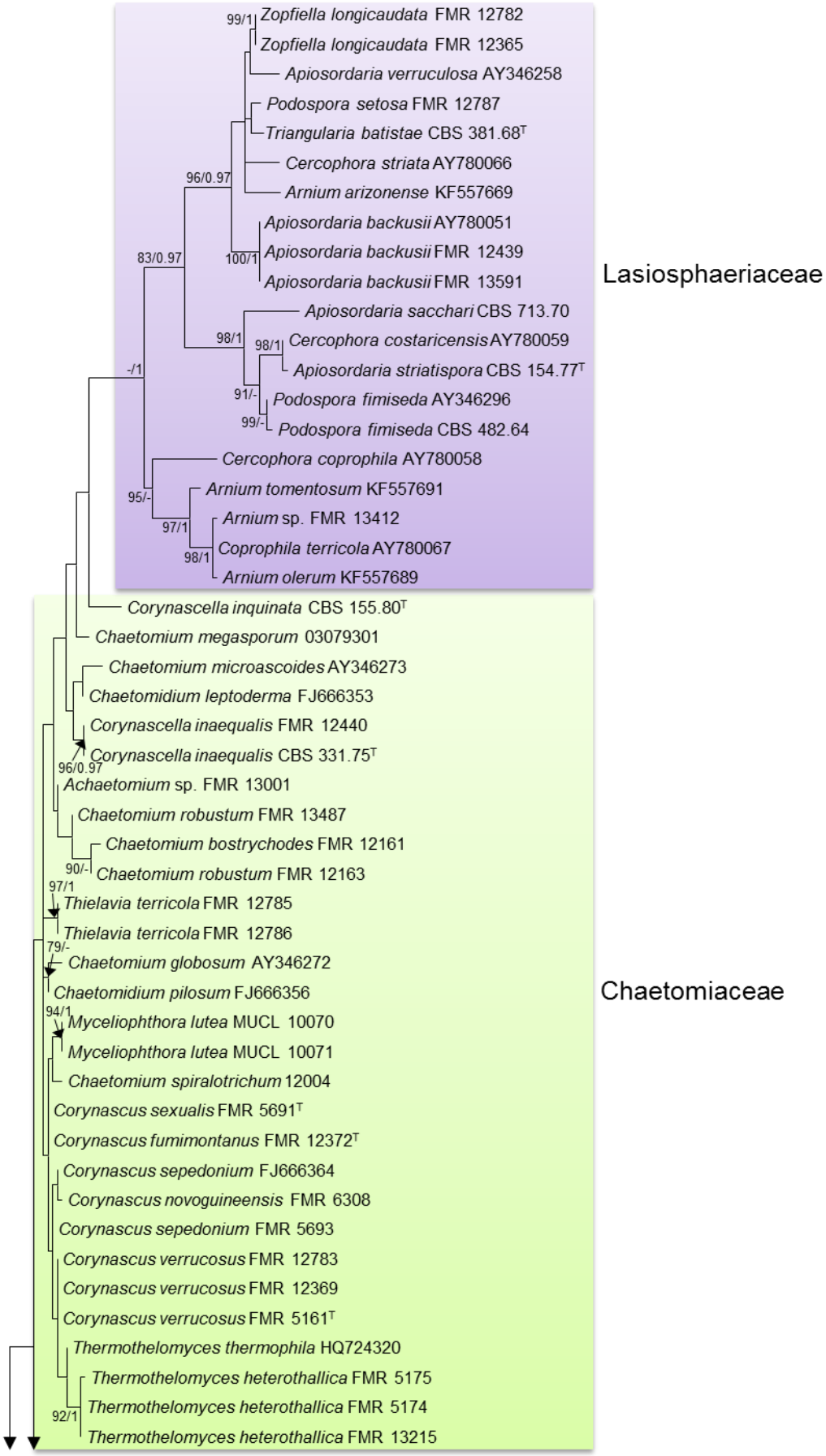
Since most of the genera of the Lasiosphaeriaceae are artificial and taxonomically unsustainable, we carried out a phylogenetic study to clarify their correct placement as well as to determine their boundaries, using a large number of fungi isolated by us and also studying several type and reference strains. The main results were that *Bombardia* and *Jugulospora* were redefined, the new genera *Navicularispora, Rinaldiella* and *Rhypophila*, and the new species *Naviculispora citrispora, Rinaldiella pentagonospora* introduced (section 4.4.1 and 4.4.2), and a new species of *Diplogelasinospora* (*Diplogelasinospora moalensis*) described (section 4.4.3). Moreover, the new genus *Rhypophila* and the new combination *Rhypophila cochleariformis*, *Rhypophila Rhypophila decipiens*, *Rhypophila myriaspora* and *Rhypophila pleiospora*, were proposed.

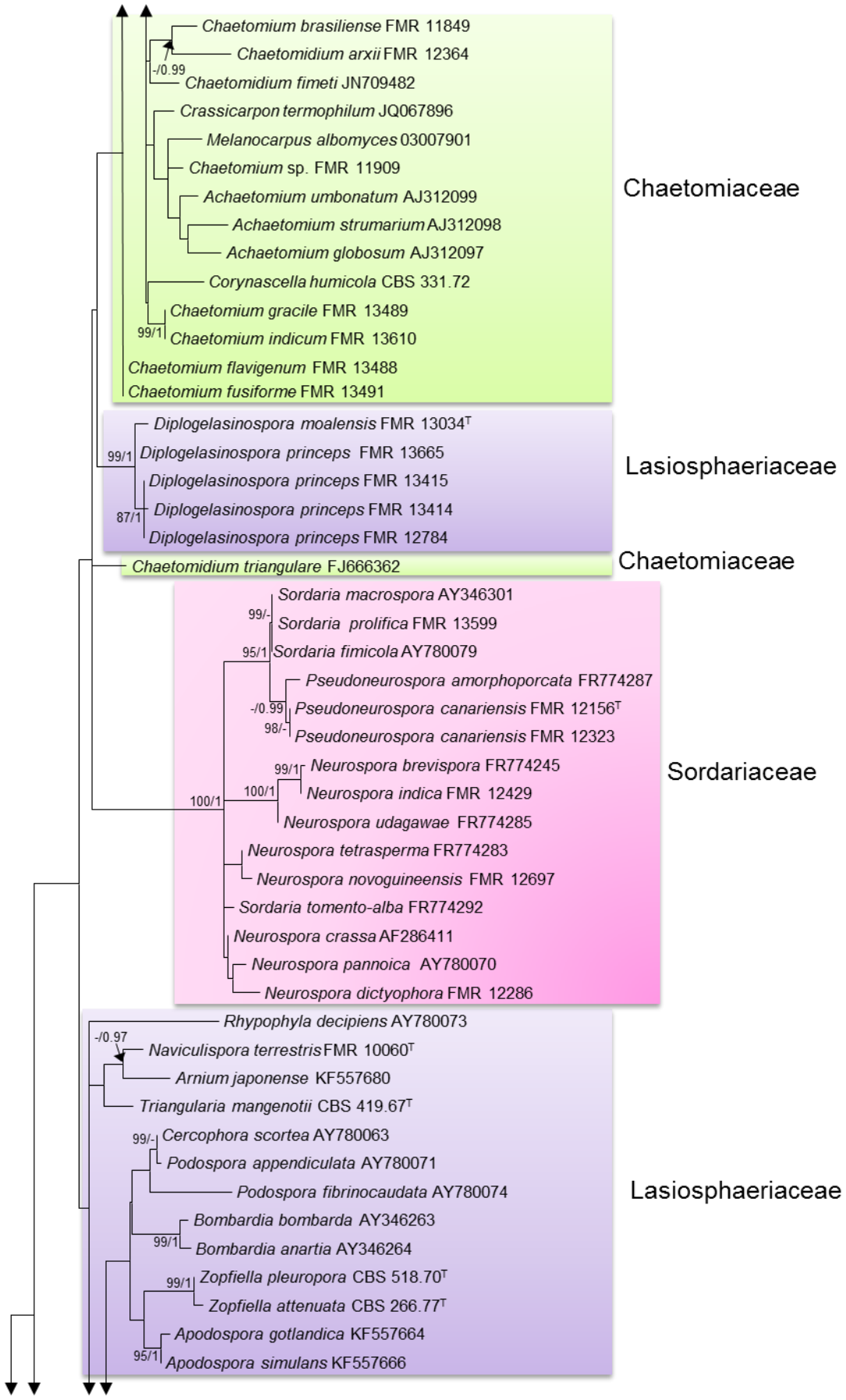
In the Sordariaceace, ascospore ornamentation used to be one of the characteristics most commonly used for generic delimitation, although the introduction of

molecular tools and phylogenetic analysis demonstrated that this criterion is not always taxonomically useful (Dettman et al., 2001, García et al. 2004). In 2004, and despite the differences in ascospore ornamentation, García et al. tranferred the species included in *Gelasinospora* to *Neurospora* on the basis of a phylogenetic study using D1–D2 sequences. They also introduced the genus *Pseudoneurospora* to accommodate the species *Pseudoneurospora amorphoporcata*, previously classified in *Gelasinospora*. The re-circumscription of the genus *Neurospora* and the position of the new genus were corroborated by Nygren et al. (2011) in a phylogenetic study using seven nuclear loci. One of our isolates located in this family belongs to *Pseudoneurospora*, which until this point had been regarded as a monotypic genus. Therefore, *Pseudoneurospora canariensis* sp. nov. was described and illustrated (section 4.4.4).

The phylogenetic tree also shows that most of the genera included in Chaetomiaceae are polyphyletic (i.e. *Achaetomium, Chaetomium, Chaetomidium, Corynascella* and *Thielavia*). Thus, these genera should be subjected in the future to a deep phenotypic and phylogenetic study. Our study based on ITS, *EF1* and *RPB2* (section 4.4.5) showed that the species traditionally included in *Myceliophthora* could be separated into four monophyletic clades, each of which represents a genus with different phenotypical and physiological features. Therefore, we have restricted *Myceliophthora* to the type species, re-established *Corynascus* and introduced the genera *Crassicarpon* and *Thermothelomyces* to accommodate five species previously belonging to *Myceliophthora.*

The large number (77 of 171) of isolates belonging to the Sordariales obtained during the development of this thesis demonstrates that the “activation” techniques used to recover ascomycetes in pure culture were highly selective for recovering of such taxa, as was reported in previous studies (Stchigel, 2000; Rodríguez, 2003; García, 2005).


Zopfiella longicaudata FMR 12782
Zopfiella longicaudata FMR 12365
Apiosordaria verruculosa AY346258
Podospora setosa FMR 12787
Triangularia batistae CBS 381.68T
Cercophora striata AY780066
Arnium arizonense KF557669
Apiosordaria backusii AY780051
Apiosordaria backusii FMR 12439
Apiosordaria backusii FMR 13591
Apiosordaria sacchari CBS 713.70
Cercophora costaricensis AY780059
Apiosordaria striatispora CBS 154.77T
Podospora fimiseda AY346296
Podospora fimiseda CBS 482.64
Cercophora coprophila AY780058
Arnium tomentosum KF557691
Arnium sp. FMR 13412
Coprophila terricola AY780067
Arnium olerum KF557689
99/1
96/0.97
83/0.97
100/1
98/1
98/1
-/1
91/-
99/-
95/-
97/1
98/1
Lasiosphaeriaceae
Corynascella inquinata CBS 155.80T
Chaetomium megasporum 03079301
Chaetomium microascoides AY346273
Chaetomidium leptoderma FJ666353
Corynascella inaequalis FMR 12440
Corynascella inaequalis CBS 331.75T
Achaetomium sp. FMR 13001
Chaetomium robustum FMR 13487
Chaetomium bostrychodes FMR 12161
Chaetomium robustum FMR 12163
Thielavia terricola FMR 12785
Thielavia terricola FMR 12786
Chaetomium globosum AY346272
Chaetomidium pilosum FJ666356
Myceliophthora lutea MUCL 10070
Myceliophthora lutea MUCL 10071
Chaetomium spiralotrichum 12004
Corynascus sexualis FMR 5691T
Corynascus fumimontanus FMR 12372T
Corynascus sepedonium FJ666364
Corynascus novoguineensis FMR 6308
Corynascus sepedonium FMR 5693
Corynascus verrucosus FMR 12783
Corynascus verrucosus FMR 12369
Corynascus verrucosus FMR 5161T
Thermothelomyces thermophila HQ724320
Thermothelomyces heterothallica FMR 5175
Thermothelomyces heterothallica FMR 5174
Thermothelomyces heterothallica FMR 13215
96/0.97
90/-
97/1
79/-
94/1
92/1
Chaetomiaceae



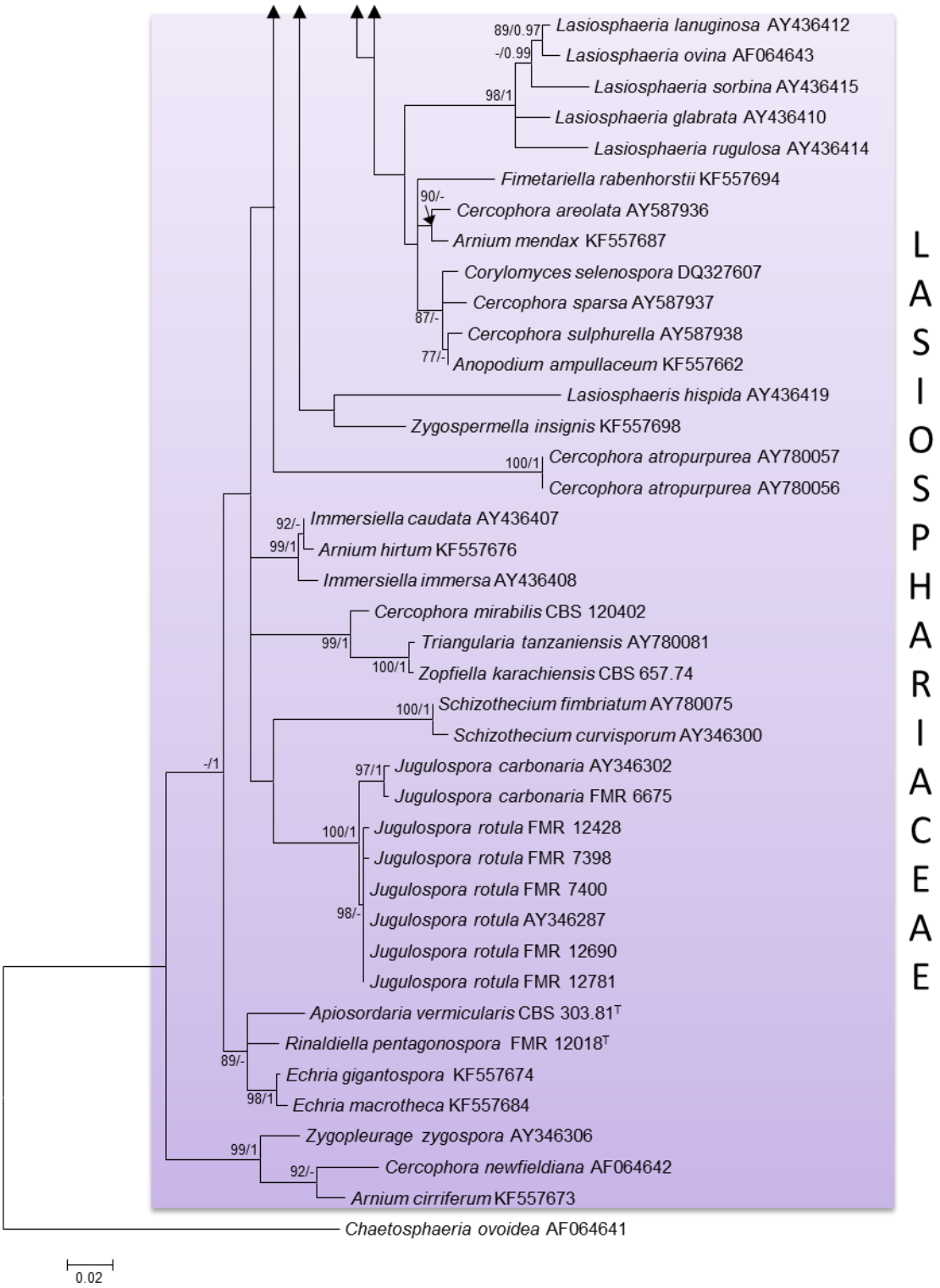
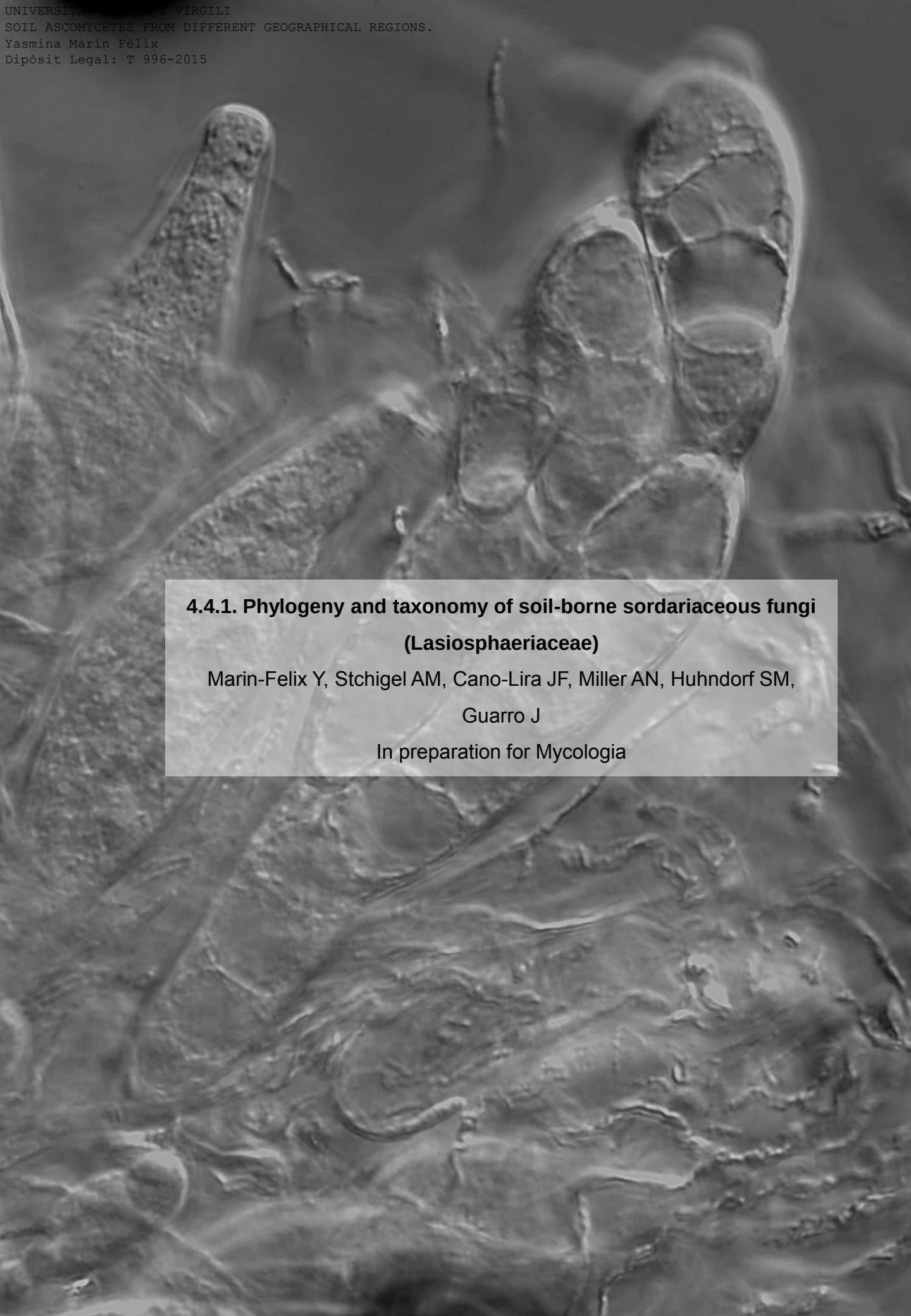



**Fig. 27** Maximum-likelihood (ML) tree obtained from D1–D3 sequences of our isolates and type and reference strains of members of the Sordariales obtained by us and retrieved from GenBank and NBRC database. *Chaetosphaeria ovoidea* was used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Branch lengths are proportional to distance. Type strains of the different species are indicated with $^{T}$.



## 4.4.1. Phylogeny and taxonomy of soil-borne sordariaceous fungi (Lasiosphaeriaceae)

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In preparation for Mycologia

Taxonomy of Lasiosphaeriaceae

**Phylogeny and taxonomy of soil-borne sordariaceous fungi (Lasiosphaeriaceae)**

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***Abstract***: The polyphyletic family Lasiosphaeriaceae (order Sordariales, class Sordariomycetes, phylum Ascomycota) comprises approximately 30 genera with paraphysate ascomata, asci with apical differentiations, and mostly two-celled ascospores, composed of a dark apical cell and a hyaline basal one, frequently with mucilaginous appendages. The delimitation of the genera of this family has been based on ascospore morphology, but recent molecular studies have demonstrated that the largest genera are

polyphyletic and that ascomatal wall morphology is better indicator of phylogenetic relationships. We carried out a phylogenetic analysis based on partial nuc 28S rDNA, β-tubulin (*BT2*) and ribosomal polymerase II subunit 2 (RPB2) genes sequences of fresh soil isolates and reference strains of species of Lasiosphaeriaceae, from different geographic regions, for a better delimitation of the members of this family. According to our results, we redefine the genera *Jugulospora* and *Bombardia* and propose the new genera *Naviculispora* and *Rhypophila.*

***Keywords*:** Ascomycota, Lasiosphaeriaceae, Sordariales, soil.

## INTRODUCTION

The family Lasiosphaeriaceae, erected in 1932 by Nannfeldt, is the largest and most diverse family of Sordariales (phylum Ascomycota). It comprises usually coprophilous, plant debris inhabitant and soil-borne species that develop ascomata paraphysate with different types of peridia. The asci are cylindrical or clavate, unitunicate and non-amyloid but usually with an apical apparatus, and the ascospores are mostly two-celled with an apical dark cell and a hyaline basal cell, generally smooth-walled, with germ pores and mostly with mucilaginous appendages (Lunqvist 1972). The boundaries of this family have not been well defined and its relationships particularly with Sordariaceae, considered as its synonym by some authors, are unclear (Munk 1953, 1958; Dennis 1960, 1968). Lundqvist (1972) distinguished the family Sordariaceae by absence of paraphyses and by having one-celled ascospores often with gelatinous sheaths, but never with mucilaginous appendages. This author performed the most comprehensive review of these fungi, based on morphological criteria, being *Arnium*, *Cercophora*, *Podospora* and *Zopfiella* the largest genera of the family. However, recent molecular studies have

demonstrated that the family and most of its genera are polyphyletic (Huhndorf et al. 2004, Miller and Huhndorf 2004, 2005, Cai et al. 2005, Chang et al. 2010, Kruys et al. 2015) and hence the taxonomy of Lasiosphaeriaceae has become obsolete. Some lasiosphaeriaceous genera, such as *Lasiosphaeria* and *Schyzothecium,* has been satisfactorily revised in recent years, mainly by using DNA sequence analysis (Miller and Huhndorf 2004, Cai et al. 2005) but the delimitation of many others is still confusing. The genus *Zopfiella* currently comprises 21 species characterized by usually non-ostiolate, membranaceous to coriaceous ascomata, more or less clavate asci and two-celled ascospores with a dark and smooth-walled upper cell, and a hyaline or pale brown lower cell (Guarro et al. 1991). *Triangularia* and *Apiosordaria* also show two-celled ascospores but the former can be distinguished by a conical upper cell, triangular in lateral view, and a triangular or hemispherical lower cell, while that *Apiosordaria* shows ascospores with a pitted or spiny upper cell (Guarro and Cano 1988, Guarro et al. 2012). *Arnium* comprises species with particular peridia which can be from membranaceous and light coloured to coriaceous or carbonaceous and opaque, its ascospores being dark, 1-celled or with a transverse septum, and often with gelatinous appendages or sheats (Lundqvist 1972, Kruys et al. 2015). Two large and polymorphic genera that share some morphological features are *Cercophora* and *Podospora*. They can be distinguished by their immature ascospores, which in *Podospora* have a septum already in the first stages of development while the upper cell swells and becomes pigmented, while in *Cercophora* the septation, swelling and pigmentation of the ascospores occur at a later stages of their development. *Podospora* was revised by Mirza and Cain (1969) who accepted 64 species while Lundqvist (1972) accepted near 80 species in *Cercophora*. More recently, Chang et al. (2010) demonstrated that that both genera were polyphyletic.

Some recent molecular studies have demonstrated that the traditional circumscription of most of the genera of Lasiosphaeriaceae are artificial and unsustainable, being the ascospores morphology an extremely homoplastic character not useful to predict phylogenetic relationships (Miller and Huhndorf 2004, 2005). The appreciation about that the morphology of the ascospores is not always useful as a taxonomic criterion to separate genera has also been reported in the *Sordariales*, e.g. the genus *Gelasinospora* was assimilated to *Neurospora* despite of the different pattern in the ascospore ornamentation (Dettman et al. 2001; García et al. 2004). By contrast, Miller and Huhndorf (2005) demonstrated that in Sordariales the structure of the ascomata wall is clearly more useful for genera delimitation than ascospore morphology.

In an effort to contribute to the more natural definition of the taxa included in Lasiosphaeriaceae we have carried out a phylogenetic study based on the sequences of 28S nrDNA and fragments of *BT2* and *RPB2* genes of a significant set of reference and fresh strains of species of ascomycetes isolated from soil belonging to this family.

## MATERIALS AND METHODS

*Soil sampling and fungal isolation.* — Soil samples were collected in Great Smoky Mountains National Park, an International Biosphere Reserve of USA, in Gwalior, India, in different locations of Spain, and in Abra del Infiernillo in Tafí del Valle, Argentina. For the isolation of soil-borne ascomycetes we followed a previously described procedure (Stchigel et al. 2000) to activate dormant spores by using thermal shock at 60 ºC, and chemical agents, i.e. 5 % v/v acetic acid and 2 % w/v phenol. Fungal colonies were examined under a stereomicroscope and sexual structures were transferred to Petri dishes containing oatmeal agar (OA: oatmeal flakes, 30 g; agar-agar, 20 g; distilled water, 1 L) using a sterile needle, being incubated at 15, 25 and 35 ºC.

*Phenotypic study* — For cultural characterization, the isolates were grown for up to 30 d on OA, potato-carrot agar (PCA; grated potatoes, 20 g; grated carrot, 20 g; agar-agar, 20 g; L-chloramphenicol, 100 mg; distilled water, 1 L) and potato dextrose agar (PDA; Pronadisa, Madrid, Spain) at 5, 10, 15, 25, 30, 35 and 40 C. Color notations in parentheses are from Kornerup and Wanscher (1984). Fertile fungal structures were mounted and measured on water and on lactic acid. Photomicrographs were obtained with a Zeiss Axio Imager M1 light field microscope. The scanning electron microscope techniques used were described previously by Figueras and Guarro (1988) and micrographs were taken with a Jeol JSM 840 at 15 keV.

*Molecular study* — DNA of the fungal isolates (Table I) was extracted and purified directly from colonies according to the Fast DNA Kit protocol (MP Biomedicals, Solon, Ohio). The amplification of the D1−D3 domains of the 28S large subunit (D1–D3) of the nuc rRNA genes and fragments of *BT2* and *RPB2* genes were performed according to Vilgalys and Hester (1990) (D1–D3) and Miller and Huhndorf (2005) (*BT2* and *RPB2*). The sequences of these amplicons were obtained using the protocol of the Taq Dye-Deoxy Terminator Cycle Sequencing Kit. PCR products were purified and sequenced at Macrogen Europe (Amsterdam, The Netherlands) with a 3730XL DNA analyzer (Applied Biosystems). Consensus sequences were obtained using SeqMan (version 7.0.0; DNASTAR, Madison, WI, USA) and the sequences were aligned using Clustal X (version 2.0) (Larkin et al. 2007) followed by manual adjustments with a text editor. Two phylogenetic analyses were carried out based on the domains D1–D2 of the 28S gene (D1–D2) sequences and on the combination of the three loci sequences (D1–D3, *BT2* and *RPB2*), of our isolates belonging to the Lasiosphaeriaceae and selected members of the families Lasiosphaeriaceae, Sordariaceae and Chaetomiaceae, and *Camarops amorpha* and *Chaetosphaeria ovoidea* as outgroups, using MEGA v. 6.06 (Tamura et al. 2013).

Maximum Likelihood (ML) method using the Tamura-Nei and the General Time Reversible models were carried out for the phylogenetic analysis of D1–D2 and of the combined dataset, respectively, both with a gamma distribution, invariable sites and the pair-wise deletion of gaps option. The robustness of branches was assessed by bootstrap analysis with 1000 replicates. Bayesian inference (BI) was carried out using MrBayes v. 3.1 according to Alvarez et al. (2010). The sequences generated in this study are deposited in GenBank (Table I) and the alignments used in the phylogenetic analyses are deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S17160).

## RESULTS

The length of the D1–D2 alignment was of 468 bp, 148 bp of them being parsimony informative. The lengths of the individual alignments used in the combined dataset were 814 bp (D1–D3), 554 bp (*BT2*) and 797 bp (*RPB2*), and the final total alignment was 2165 bp, 757 bp of which were parsimony informative. In the D1–D2 and in the combined dataset trees (Figs. 1 and 2), the members of the family Lasiosphaeriaceae were grouped in three main clades (Lasiosphaeriaceae I, II and III); other well suppoted clades represented the members of the families Chaetomiaceae and Sordariaceae, and of the genus *Diplogelasinospora.* The genera *Apiosordaria*, *Arnium*, *Cercophora*, *Podospora*, *Triangularia* and *Zopfiella* revealed to be polyphyletic with their members scattered in different clades of the three groups of Lasiosphaeriaceae. By contrast the genera *Apodospora*, *Cuspidatispora, Diffractella, Diplogelasinospora, Echria, Fimetariella, Lasiosphaeria*, *Lasiosphaeris, Rinaldiella, Schizothecium* and *Zygospermella* constituted monophyletic clades in both phylogenetic trees. Despite in the D1–D2 tree the species of *Immersiella* grouped with *Arnium hirtum*, the species of that genus were placed in a monophyletic terminal clade in the tree based on the combined dataset. *Immersiella* was

only well-delimitated in a monophyletic terminal clade in the tree based on the combined dataset, whereas in the D1–D2 tree the species of this genus grouped with *Arnium hirtum*.

In the D1–D2 tree, our isolate CBS 137295 (species I) was located in the Lasiophaeriaceae I clade, constituting a well-supported subclade (73% bs/ 0.99 pp) together with *Arnium caballinum, Arnium japonense, Arnium leporinum,* and *Cercophora aquatica*. However, species I constituted a terminal branch with a nucleotide identity lower than 95% with respect to the other species of that subclade. In the combined dataset tree, species I also grouped with *A. japonense* but with no significant support and at a considerable phylogenetic distance among them. Species I was easily distinguished from the other members of Lasiosphaeriaceae by its ascospores with a navicular and septate upper cell and a pale brown, thick-walled, non collapsing lower cell. Sequences of the species *Bombardia bombarda* and *Bombardioidea anartia*, constituted in both tree a well supported subclade within the Lasiosphaeriaceae I clade with a nucleotide identity between both species higher than 98%. Both genera share a particular fruiting-body, i.e. ostiolate, multi-layered, with the two outer layers stromatic, which is unique in the family. In the D1–D2 tree, also within the Lasiosphaeriaceae I, the species *Podospora cochleariformis, Podospora decipiens* and *Podospora pleiospora,* located in the section *Rhypophila* by Lundqvist (1972), formed a well-supported clade (genus I) (94% bs/ 1 pp). These species share some characteristic features not present in other species of *Podospora* such as an ascomatal neck with elongate tubercles at the base, asci usually with more than 8 ascospores and ascospores with a lower cell usually longer than the upper cell.

In both trees, Lasiosphaeriaceae II grouped several species of *Cladorrhinum*, which were characterized by only the presence of an asexual morph consisting of conidiogenous cells with lateral phialidic openings that produce one-celled conidia in slimy masses, together

with some species of *Apiosordaria*, *Triangularia, Podospora* and *Cercophora* which produce a *Cladorrhinum*-like asexual morph too. *Apiosordaria vermicularis* is the only lasiosphaeriaceous species studied that constitutes the exception among the species producing a *Cladorrhinum*-like asexual morph, because it was placed in both trees into the clade Lasiosphaeriaceae III constituting a terminal independent branch.

In both trees, the clade Lasiosphaeriaceae III included a well-supported subclade that grouped a reference strain of *Jugulospora rotula*, a reference strain of *Strattonia carbonaria*, the type strains of *Apiosordaria antarctica*, *Apiosordaria globosa* and *Apiosordaria hispanica*, and an isolate that based on morphological features (ascospores with a warted upper cell with longitudinal ridges) we identified as *Rhexosporium terrestre*. All these species produces similar ascomata (semi-transparent, with dark brown papillate necks) clavate and early septate ascospores with the upper cell warted or finely granulate.

Based on the molecular and morphological results mentioned above, we propose the new genus *Naviculispora* to accommodate our isolate CBS 137295 (species I), the synonymy of *Bombardioidea* with *Bombardia*, the redefinition of *Jugulospora* and the raising of *Podospora* section *Rhypophila* (genus I) to a genus level.

***Taxonomy***

***Bombardia*** (Fr.) P. Karst. — MycoBank MB616

*Type species: Bombardia fasciculata* Fr., Bidr. Känn. Finl. Nat. Folk 23: 20. 1873.

*Basionym: Sphaeria* subgen. *Bombardia* Fr., Summa vegetabilium Scandinaviae 2: 389. 1849.

= *Bombardioidea* C. Moreau ex N. Lundq., Symb. bot. upsal. 20: 274. 1972.

*Ascomata* ostiolate, clavate to fusiform, tough, multi-layered; peridium black, carbonaceous; two outer layers stromatic. Paraphyses filiform, septate. Asci 4 or 8-spored, clavate or cylindrical, very long-stipitate, with a thickened apical ring.

Ascospores one- or two-celled, with a single apical germ pore when two-celled, or with one germ pore at each end with several smaller pores surrounding them or scattered all over when one-celled; gelatinous sheath usually present in the species producing one-celled ascospores.

*Bombardia anartia* (J.C. Krug & J.A. Scott) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB 812138

*Basionym*: *Bombardioidea anartia* J.C. Krug & J.A. Scott, Can. J. Bot. 72: 1303. 1994.

*Bombardia bombardioides* (Auersw.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB 812139

*Basionym: Sordaria bombardioides* Auersw., in Niessl, Verh. nat. Ver. Brünn 10: 189. 1872.

≡ *Hypocopra bombardioides* (Auersw.) Sacc., Syll. fung. (Abellini) 1: 243. 1882.

≡ *Bombardioidea bombardioides* (Auersw.) C. Moreau ex C. Moreau, in Lundqvist, Symb. bot. upsal. 20: 277. 1972.

*Bombardia serignanensis* (Fabre) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB 812141

*Basionym*: *Hypocopra serignanensis* Fabre, Annls Sci. Nat., Bot. 9: 77. 1879.

≡ *Bombardioidea serignanensis* (Fabre) N. Lundq., Symb. bot. upsal. 20: 284. 1972.

*Bombardia stercoris* (DC.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB 812142

*Basionym*. *Sphaeria stercoris* DC., in Lamarck & de Candolle, Fl. franç., Edn 3 (Paris) 2: 294. 1805.

≡ *Sporormia stercoris* (DC.) Pirotta, Monogr. Spororm.: no. 1. 1878.

≡ *Bombardioidea stercoris* (DC.) N. Lundq., Symb. bot. upsal. 20: 281. 1972.

= Sordaria maxima Niessl, Verh. nat. Ver. Brünn: 38. 1870.

≡ *Hypocopra maxima* (Niessl) Sacc., Syll. fung. (Abellini) 1: 245. 1882.

***Jugulospora*** N. Lundq., Symb. bot. upsal. 20(no. 1): 256. 1972, emend. Fig. 3

= Rhexosporium Udagawa & Furuya, *Trans. Mycol. Soc. Japan* 18: 302. 1977.

*Type species: Jugulospora rotula* (Cooke) N. Lundq.

*Ascomata* ostiolate, covered with pale brown, flexuous, septate, thick-walled hairs; neck dark brown to black, composed of papillate cells disposed around the ostiole; peridium membranaceous, semi-transparent, 3–9-layered; outer peridial cells isodiametric. *Paraphyses* filiform. *Asci* 8-spored, cylindrical, with a thin apical ring. *Ascospores* at first one-celled, hyaline, clavate, early septate; upper cell brown, navicular, globose or obovoid, warted or finely granulated, sometimes with warts arranged forming ridges or large spots, with an apical germ pore; lower cell hyaline, conical to cylindrical, collapsing, smooth-walled to slightly warted. *Asexual morph* absent or present, *conidia* hyaline to pale brown, almost smooth-walled, ovate to elongate, produced laterally or terminally on undifferentiated hyphae, solitary.

*Jugulospora carbonaria* (W. Phillips & Plowr.) Y. Marín, Stchigel, Guarro, A.N. Mill. & Huhndorf, comb. nov. Fig. 3c, d, m, n

MycoBank MB 812137

*Basionym: Sphaeria carbonaria* W. Phillips & Plowr., Grevillea 2(no. 22): 188. 1874.

≡ *Podospora carbonaria* (W. Phillips & Plowr.) Niessl, Hedwigia 22: 156. 1883.

≡ *Psilosphaeria carbonaria* (W. Phillips & Plowr.) Cooke & Plowr., Grevillea 7(no. 43): 85. 1879.

≡ *Sordaria carbonaria* (W. Phillips & Plowr.) Sacc., Syll. fung. (Abellini) 1: 233. 1882.

≡ *Strattonia carbonaria* (W. Phillips & Plowr.) N. Lundq., Symb. bot. upsal. 20(no. 1): 269 (1972)

≡ *Zopfiella carbonaria* (W. Phillips & Plowr.) Arx, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 76(3): 291. 1973.

= *Apiosordaria antarctica* Stchigel & Guarro, in Stchigel, Guarro & Mac Cormack, Mycologia 95: 1219. 2003.

*Jugulospora rotula* (Cooke) N. Lundq., emend. Fig. 3a, b, e–l

*Basionym: Sphaeria rotula* Cooke, Handb. Brit. Fungi 2: no. 2598. 1871.

= *Apiosordaria globosa* Dania García, Stchigel & Guarro, Mycologia 95: 137. 2003.

= *Apiosordaria hispanica* Dania García, Stchigel & Guarro, Mycologia 95: 134. 2003.

= *Rhexosporium terrestre* Udagawa & Furuya, Trans. Mycol. Soc. Japan 18: 303. 1977.

Ascomata scattered to aggregated, superficial or immersed, pyriform, pale brown to brown, semi-transparent, ostiolate, 350–770 x 200–540 µm, covered with pale brown, wide near the base, septate hyphae-like hairs of 1–5 µm diam; neck brown to dark-brown, cylindrical to conical, papillate, 78–280 µm long, 90–250 µm wide; peridium membranaceous, 3–9-layered, 15–45 µm thick, brownish-orange to brown; outer layers with *textura angularis* to *textura intricata*; inner layers with *textura epidermoidea*. *Paraphyses* and *periphyses* filiform-ventricose, up to 2 µm in diam. *Asci* 8-spored, cylindrical, evanescent, 146–250 x 14–28 µm, stipitate, with a thin apical ring. *Ascospores* at first hyaline, one-celled and clavate, becoming transversely septate and two celled; upper cell dark brown, obovoid to globose, truncate at the base, ornamented with warts arranged uniformly or forming longitudinal ridges or large spots, 18–29 x 12–27 µm, with an apical to lateral germ pore of 0.5–3 µm; lower cell hyaline, conical, smooth-walled to slightly warted, 1–6 µm, collapsing; gelatinous cauda absent. *Asexual morph* absent or present, c*onidia* hyaline to pale-coloured, almost smooth-walled, ovate to elongate, 2–6 x 1.5–2.5 µm, produced laterally or terminally on undifferentiated hyphae, solitary.

Specimens examined: USA, NORTH CAROLINA, Great Smoky Mountains National Park, from soil, 8-VIII-2008, M. Calduch, A.N. Miller & A.M. Stchigel, culture FMR

12690; USA, TENNESSEE, Great Smoky Mountains National Park, from soil, 9-VIII-2008, M. Calduch, A.N. Miller & A.M. Stchigel, culture FMR 12781; SPAIN, TARRAGONA, Els Gorgs de la Febró, from soil, 25-IX-1996, A.M. Stchigel & M. Calduch, culture CBS 110112, ex-type strain of *Apiosordaria hispanica*; SPAIN, TARRAGONA, Els Gorgs de la Febró, from soil, 25-IX-1996, A.M. Stchigel & M. Calduch, culture CBS 110113, ex-type strain of *Apiosordaria globosa*; ANTARCTICA, King George Island, Jubany Argentinian base, from soil, 11-XI-1996, W. Mac Cormack, culture IMI 381338, ex-type strain of *Apiosordaria antarctica*; JAPAN, from burned soil, Y. Horie, culture ATCC 34567.

***Naviculispora*** Stchigel, Y. Marín, Cano & Guarro, gen. nov. Fig. 4

MycoBank MB 812135

*Type species: Naviculispora terrestris* Stchigel, Cano, Y. Marín & Guarro.

*Etymology*. From Latin *navicularibus*-, navicular, and -*spora*, spore, referring to the shape of the ascospores.

*Diagnosis:* This genus is distinguished by the production of ascospores with a dark navicular brown upper cell, and a pale brown to brown thick-walled lower cell which does not collapse with the age, and holoblastic conidia with a small truncate base, sessile, or less commonly on sympodially proliferating conidiophores.

*Ascomata* scattered to aggregated, superficial and immersed, pyriform, ostiolate, pale brown to brown, covered with brown, septate hyphae-like hairs, with a short neck; neck brown to dark-brown, cylindrical to conical, papillate; peridium membranaceous of *textura angularis*. *Asci* 8-spored, cylindrical, evanescent, short-stipitate, with a small apical ring, ascospores uniseriate to biseriate. *Ascospores* at first hyaline, one-celled and clavate, becoming transversely septate and two celled; upper cell dark brown, navicular,

truncate at the base, septate, with a subapical to lateral germ pore; lower cell thick-walled, pale brown to brown, cylindric-conical, not collapsing with the age; gelatinous cauda absent. *Asexual morph* present, *conidia* holoblastic, hyaline to subhyaline, ellipsoidal to obovoid or clavate, with a small truncate base, sessile, or less commonly on sympodially proliferating conidiophores.

*Naviculispora terrestris* Stchigel, Y. Marín, Cano, & Guarro, sp. nov. Fig. 4

MycoBank MB 812136

*Typification*. ARGENTINA: Tucumán province, Tafí del Valle, -26.8667, -65.6833, from soil, 17-V-2000, *A. M. Stchigel, J. Cano, J. Guarro.* (**holotype** CBS H-21595). **Ex-type culture** CBS 137295, FMR 10060).

*Etymology*. Referring to the source where the species has been isolated.

*Mycelium* composed of subhyaline to brown, septate, smooth-walled, branched hyphae, 1.5–4 µm wide. *Ascomata* scattered to aggregated, superficial or immersed, pyriform, pale brown to brown, ostiolate, 210–410 x 160–380 µm, covered with brown, septate hyphae-like hairs, with a short neck, occasionally with 2 necks; neck brown to dark-brown, cylindrical to conical, papillate, 35–70 µm long, 62.5–105 µm wide; peridium membranaceous, *textura angularis*, 10–20 µm thick, composed of up to 10 layers of flattened cells of 2–10 µm in diam. *Asci* 8-spored, cylindrical, evanescent, 100–150 x 15–20 µm, short-stipitate, with small apical ring, ascospores arranged uniseriately to biseriately. *Ascospores* at first hyaline, one-celled, clavate, becoming transversely septate and two-celled; upper cell dark brown, navicular, 20–29 x 9–13.5 µm, truncate at the base, septate, with a subapical to lateral germ pore of 0.5–1 µm; lower cell thick-walled, pale brown, cylindric-conical, 10–14(–17) x (2–)3–4 µm, not collapsing; lacking of gelatinous cauda. *Asexual morph* present, *conidia* holoblastic, 3–8 x 2–3 µm, hyaline to

subhyaline, ellipsoidal to ovoid or clavate, with a small truncate base, sessile, produced laterally and terminally on the hyphae, or less commonly on sympodially proliferating conidiophores.

Culture characteristics — Colonies on PCA attaining a diam. of 40–47 mm in 14 d at 25 ºC, velvety, slightly lobulate, margins fringed, radially zonate, greyish-brown (M 5D3 to 5E3) and brown to dark brown (6E3 to 6F3) at centre; reverse greyish-yellow to olive brown (4B4 to 4D4). Colonies on OA attaining a diam. of 35–38 mm in 14 d at 25 ºC, velvety, margins regular to slightly arachnoid, grey to greyish-brown (M 8F1 to 8F3); reverse grey to greyish brown (M 8F1 to 8F3). Ascomata produced after at least two months. Maximum and minimum temperatures of growth, 5 and 30 ºC, respectively. Optimal temperature 25 ºC.

***Rhypophila*** Y. Marín, A.N. Mill., Guarro & Huhndorf, *gen. nov.* Fig. 5

MycoBank MB 812130

*Type species: Rhypophila myriospora* (P. Crouan & H. Crouan) Y. Marín, A.N. Mill., Guarro & Huhndorf.

*Etymology*. Due to the section *Rhypophila* of the genus *Podospora* (Lundqvist 1972) where these species were included.

*Diagnosis*: Ascomata ostiolate, with a neck showing elongated tubercles at the base, asci normally containing more than 8 ascospores, and ascospores with a lower cell as long as, or longer than, the upper cell.

*Ascomata* scattered or aggregated, semi-immersed or superficial, pyriform, ostiolate, glabrous or covered with flexous or stiff hairs; neck long, conical or cylindrical, with blackish, obtuse, straight or curved, elongate tubercles at the base; peridium membranaceous, semi-transparent, yellowish to light brown. *Paraphyses* present or

absent. *Asci* 8 to 128-spored, clavate, long or short stipitate; apical ring absent or indistinct, ascospores biseriate or irregularly arranged. *Ascospores* at first hyaline, one-celled, cylindrical or spatuliform, later swelling above, clavate, becoming transversely uniseptate; upper cell ellipsoidal to ellipsoidal-fusiform, dark brown, with an apical or subapical germ pore; lower cell cylindrical to cylindrical-obclavate, hyaline, same length or longer than upper cell, frequently collapsing; several small secondary appendages at the base of the lower cell; apical gelatinous cauda absent or present, fibrillate or lamellate. *Asexual morph* absent.

Key to species of *Rhypophila*

1. Asci 8-spored.....*R. decipiens*

1. Asci 16 to 128-spored.....2

2. Asci 128-spored; upper and lower cells shorter than 20 µm; absence of cauda and gelatinous appendages.....*R. cochleariformis*

2. Asci 16 to 32-spored; upper and lower cells longer than 20 µm; fibrillate upper cauda and gelatinous appendages in the lower cell.....3

3. Asci 16 or 32-spored; upper cell 23–34 x 14–19 µm.....*R. myriaspora*

3. Asci 64-spored; upper cell 25–37 x 18–23 µm.....*R. pleiospora*

*Rhypophila cochleariformis* (Cailleux) Y. Marín, A.N. Mill., Guarro & Huhndorf, comb. nov.

MycoBank MB 812131

*Basionym: Podospora cochleariformis* Cailleux, Cahiers de La Maboké 7: 100. 1969.

*Rhypophila decipiens* (G. Winter ex Fuckel) Y. Marín, A.N. Mill., Guarro & Huhndorf, comb. nov. Fig. 5e, g–i

MycoBank MB 812132

*Basionym: Sordaria decipiens* G. Winter, Abh. naturforsch. Ges. Halle 13: 28. 1873.

≡ *Podospora decipiens* (G. Winter ex Fuckel) Niessl, Hedwigia 22: 156. 1883.

≡ *Pleurage decipiens* (G. Winter) Kuntze, Revis. gen. pl. (Leipzig) 3(2): 505. 1898.

*Rhypophila myriospora* (P. Crouan & H. Crouan) Y. Marín, A.N. Mill., Guarro & Huhndorf, comb. nov. Fig. 5b, d, k

MycoBank MB 812133

*Basionym*. *Sordaria myriospora* P. Crouan & H. Crouan, Florule Finistère (Paris): 22. 1867.

≡ *Ryparobius myriosporus* (P. Crouan & H. Crouan) Boud., Boud., Annls Sci. Nat., Bot., sér. 5 10: 240. 1869.

≡ *Philocopra myriospora* (P. Crouan & H. Crouan) Sacc., Syll. fung. (Abellini) 1: 251. 1882.

≡ *Podospora myriospora* (P. Crouan & H. Crouan) Niessl, Hedwigia 22: 156. 1883.

≡ *Ascophanus myriosporus* (P. Crouan & H. Crouan) Quél., Enchir. fung. (Paris): 296. 1886.

*Rhypophila pleiospora* (G. Winter) Y. Marín, A.N. Mill., Guarro & Huhndorf, comb. nov. Fig. 5a, c, f, j

MycoBank MB 812134

*Basionym*. *Sordaria pleiospora* G. Winter, Abh. naturforsch. Ges. Halle 13: 13. 1873.

≡ *Philocopra pleiospora* (G. Winter) Sacc., Syll. fung. (Abellini) 1: 249. 1882.

≡ *Podospora pleiospora* (G. Winter) Niessl, Hedwigia 22: 156. 1883.

= *Podospora decipiens* var. *pleiospora* (G. Winter) Chenant., Bull. Soc. mycol. Fr. 35: 114. 1919.

## DISCUSSION

We performed a phylogenetic study based on 28S, *BT2* and *RPB2* sequences of members of the family Lasiosphaeriaceae in order to contribute to a more natural classification of

this family. Up to now, the taxonomy of this fungal group is mainly based, with the exception of some recent phylogenetic approaches (Miller and Huhndorf 2004, Cai et al. 2005, Kruys et al. 2015), on morphological criteria that has been demonstrated to be unsustainable. Our data corroborated that Lasiosphaeriaceae is paraphyletic as was demonstrated in previous phylogenetic studies (Huhndorf et al. 2004, Miller and Huhndorf 2005, Kruys et al. 2015), being divided in three main clades (Lasiosphaeriaceae I, II and III).

The study also confirmed that several large genera were clearly polyphyletic, their members being scattered in the three mentioned clades. The clade corresponding to Lasiosphaeriaceae II, grouped all the species, included in the study, that only produce asexual morphs in the genus *Cladorrhinum,* i.e. *Cladorrhinum flexuosum, Cladorrhinum foecundissimum, Cladorrhinum microsclerotigenum, Cladorrhinum phialophoroides* and *Cladorrhinum samala*, and species that produced both, ascomata and *Cladorrhinum*-like asexual morphs. The fact that this particular type of conidiogenesis is also present in the members of this clade among the Ascomycota suggests that members of clade Lasiosphaeriaceae II could represent a new family characterized by the presence of this particular asexual morph. The placement of members of Lasiosphaeriaceae producing a *Cladorrhinum*-like asexual morph together to *Cladorrhinum* spp. was previously observed by Madrid et al. (2011) in a phylogenetic study of that genus. However, there are few taxa in Lasiosphaeriaceae II that produce a holoblastic asexual morph different from *Cladorrhinum*, e.g. *Cercophora terricola*, *Podospora austro-america* and *Podospora pauciseta*. Therefore, further phylogenetic studies will be needed in order to corroborate this hypothesis. The only species that shows *Cladorrhinum* asexual morph and did not group in Lasiosphaeriaceae II is *Apiosordaria vermicularis,* that additionally produces an holoblastic asexual morph. This species constitutes an independent lineage

and considering that shows a combination of morphological features unique in Ascomycota i.e. the presence of two types of asexual morphs, one of them with probably phylogenetic significance (*Cladorrhinum*), probably it is represent a new genus.

The new monotypic genus *Naviculispora* is characterized by the particular morphology of the ascospore with presence of a septum in the upper cell. Such feature can be also present in other members of Lasiosphaeriaceae; however, *Naviculispora* can be easily distinguished from such taxa by ascospores with brownish, thick-walled lower cells that do not collapse with the age. The most phylogenetically related species is *Arnium caballinum.* This latter species produces 1-celled ascospores, although Lundqvist (1972) mentioned also the occasional presence of a septum. The species with ascospores with a septate upper cell included in this study were dispersed in different clades which demonstrated the poor taxonomic value of this feature. For instance while *Zopfiella tabulata* (the type species of the genus) was located in a well-supported clade of Lasiosphaeriaceae I together with *Cercophora sulphurella*, *Corylomyces selenospora*, *Jobellisia rhynchostoma* and *Podospora dydima* all them with septate ascospore upper cell, another species of *Zopfiella* with the same characteristic, *Z. pleuropora*, was nested with species of other genera without such feature such as *Cercophora sparsa* and *Anopodium ampullaceum*.. *Zopfiella pleuropora* grouped with *Zopfiella attenuata* in a well-supported subclade in both phylogenetic studies. Both differ from the other species having ascospores with septate upper cell by its non-ostiolate ascomata. *Cercophora areolata* and *Cercophora ambigua*, which grouped together in a well-supported clade in Lasiosphaeriaceae I, also present a septate upper cell; however, both species are easily distinguished by their ascomata with an areolate peridium breaking in polyhedral plates when crushes. On the basis of phenotypic and genotypic data, both species could represent a new genus which would demonstrate that the ascoma wall morphology is a

good predictor of phylogenetic relationships, as was previously demonstrated by Miller and Huhndorf (2005). Apart from the species mentioned above, *Cercophora newfieldiana*, *Cercophora sordarioides* and occasionally *Cercophora atropurpurea*, included in Lasiosphaeriaceae III clade, show also a septum in the upper ascospore cell. The two latter species were located in independent terminal branches whereas *C. newfieldiana* was grouped with other lasiosphaeriaceous species without a septum in the upper cell. These three species can be easily distinguished from the new genus *Naviculispora* by the evanescent lower cell in *C. newfieldiana* and *C. sordarioides*, and by the 3–5-septate lower cell in *C. atropurpurea.*

Further evidence about the relevance of the ascomata structure as a phylogenetically informative taxonomic character has been observed in the close molecular relationships between *Bombardioidea* and *Bombardia*, that show a very similar ascoma wall structure (stromatic) and that are proposed here as synonyms. When Lundqvist proposed the genus *Bombardioidea* (Lundqvist, 1972) already mentioned the similarity of the ascomata among both genera, but based on the ascospore morphology of such genera that are two-celled in *Bombardia* and one-celled in *Bombardioidea*, considered both genera as different. The ascomata of *Bombardia* are composed of two stromatic outer layers, being the most internal of which fibrous and cartilaginous (Lundqvist 1972). This kind of peridium is unique in Lasiosphaeriaceae. Other species of the family such as *Arnium ontariensis*, *Cercophora albicollis, Cercophora costaricensis*, *Cercophora elephantina, Cercophora palmicola, Cercophora scortea, Podospora appendiculata, Podospora fimiseda* and *Podospora perplexens* also have a pseudo-bombardioid peridium with a similar structure, showing also a gelatinized wall layer. However, important differences exist between bombardioid and pseudo-bombardioid ascomata since the latter are non-stromatic and the gelatinized wall layer is composed of interwoven hyphae (Miller 2003).

Moreover, the species with pseudo-bombardioid ascomata lack a carbonaceous elongated base of the ascomata which is present in *Bombardia* (Lundqvist, 1972). Only four of the above mentioned species were included in the present study, i.e. *C. costaricensis, C. scortea, P. appendiculata* and *P. fimiseda* which were nested in different clades, *C. scortea* and *P. appendiculata* in Lasiosphaeriaceae I, and *C. costariciensis* and *P. fimiseda* in Lasiosphaeriaceae II. This suggests that this sort of ascoma could have evolved independently at least two times in the evolutionary history of the family. Because *Podospora fimiseda* is the type species of *Podospora*, we considered the possibility of reducing *Podospora* to only those species with pseudo-bombardioid peridium. However, the fact that some species without pseudo-bombardioid such as *Apiosordaria sacchari* and *Apiosordaria striatispora* were nested in the same clade that *P. fimiseda* and *C. costariciensis*, makes this hypothesis, at least based on the available data, inconsistent. Consequently, further studies are needed to delimitate more correctly the complex genus *Podospora*.

The genus *Jugulospora* was introduced by Lundqvist (1972) to accommodate *Sphaeria rotula* based on its verrucose ascospores, with anastomosing warts arranged in large spots. The molecular data demonstrated that *A. globosa*, *A. hispanica* and *R. terrestre*, with also warted ascospores, belonged to *J. rotula*, being synonymized therein. *Rhexosporium* was proposed as a new genus by Udagawa and Furuya (1977) due to the presence of the longitudinal ridges in the upper cell of the ascospores, despite these authors cited a similar ascospore ornamentation in *Apiosordaria* and *Jugulospora* spp. Observing the pictures of the ascospores under SEM, we noticed that such ridges were really a linear arrangement of warts and, then, not differing much respect to the ornamentation of those of *J. rotula*. In the present study, *J. rotula* was emended to incorporate all the morphological features observed in the species synonymized. Based on

the early ascospore septation, the negative staining of the apical ring of the asci with cotton blue and the presence of paraphysis, Lundqvist (1972) highlighted the close similarity of *Jugulospora* with *Strattonia carbonaria*. Our study confirmed the similarity between such fungi already demonstrated in previous phylogenetic studies (Miller and Huhndorf 2005, Kruys et al. 2015). Therefore, we transferred *Strattonia carbonaria* to *Jugulospora* as *J. carbonaria*. Although the name *Strattonia* is older than *Jugulospora*, the type species of that genus, *S. tetraspora*, has an ascomatal neck with rigid, cylindrical, septate, long hairs and ascospores with a gelatinous sheath, features which don't match with those of *Jugulospora*. Hence, the genus *Strattonia* should be reduced to those species with themorphology of the type species. Both species of *Jugulospora* are easily distinguished among them by the ornamentation of the ascospores, being finely granulated in *J. carbonaria* and warted in *J. rotula*. *Apiosordaria antarctica* is synonymized with *Jugulospora carbonaria*, which shows common morphological features. The only difference among them is the length of the neck, which was described as short in *J. carbonaria* and reaching to 400 µm in length in *A. antarctica*.

*Rhypophila* spp. are characterized by the production of ostiolate ascomata with dark elongate tubercles at the base of a long neck and asci usually with more than 8 ascospores, and ascospores with lower cell at least as long as the upper one. The species grouped in this genus were formerly classified in *Podospora* but in a separate section called *Rhypophila* (Lundqvist 1972). Similar asci are seen in *Schizothecium*, but this genus is distinguished by swollen agglutinated hairs or prominent protruding peridial cells all over the ascomata, and the lower cell of the ascospores is usually persistent and not so long as in *Rhypophila*. *Rhypophila myriospora* was not included in the phylogenetic study since there are not sequences of the loci used of this species. However, it is considered a species of this new genus due to the morphological features

and the molecular study based on ITS region and *GPD* gene carried out by Chang et al. (2010) which corroborated its location in the clade that grouped the other species of *Rhypophyla*. Although *R. decipiens* and *R. pleiospora* did not show molecular variability in the D1–D2 tree, these were considered as different species also on the basis of the molecular data shown by Chang et al. (2010).

Currently, the phylogenetic relationships of the most important genera of Lasiosphaeriaceae remain unclear and an important effort, trying to study more strains representing the different taxa included in this family, is required to solve this problem. Important caveats for the progress of the studies on this field are the absence of ex type cultures for sequencing and the fact that numerous sequences available from coprophilous species are obtained directly from fungi growing in their natural substrates. Herbivores dung are usually colonized by a huge number of species and it is difficult to ascertain if the amplified DNA belong really to the suspected fungus.

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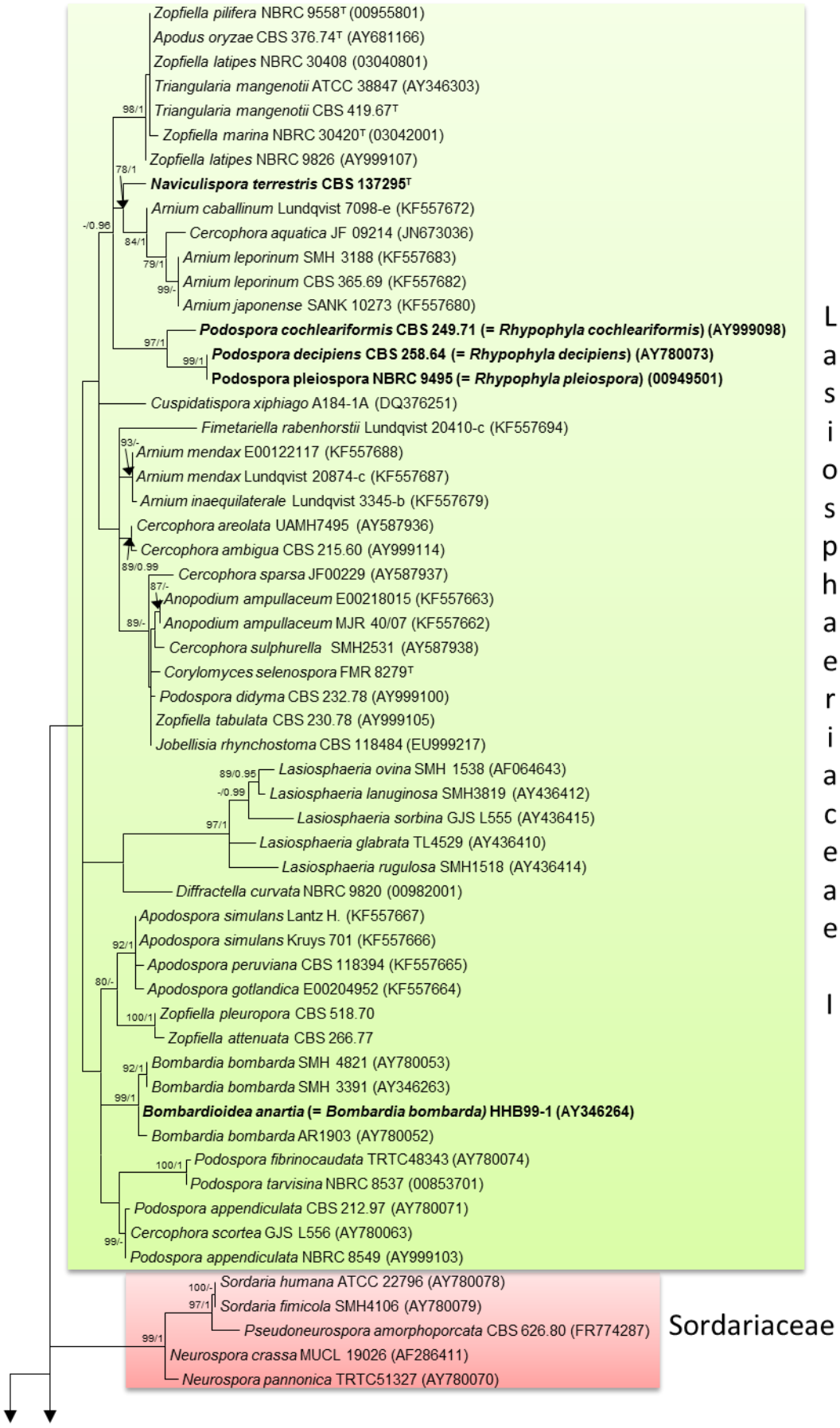
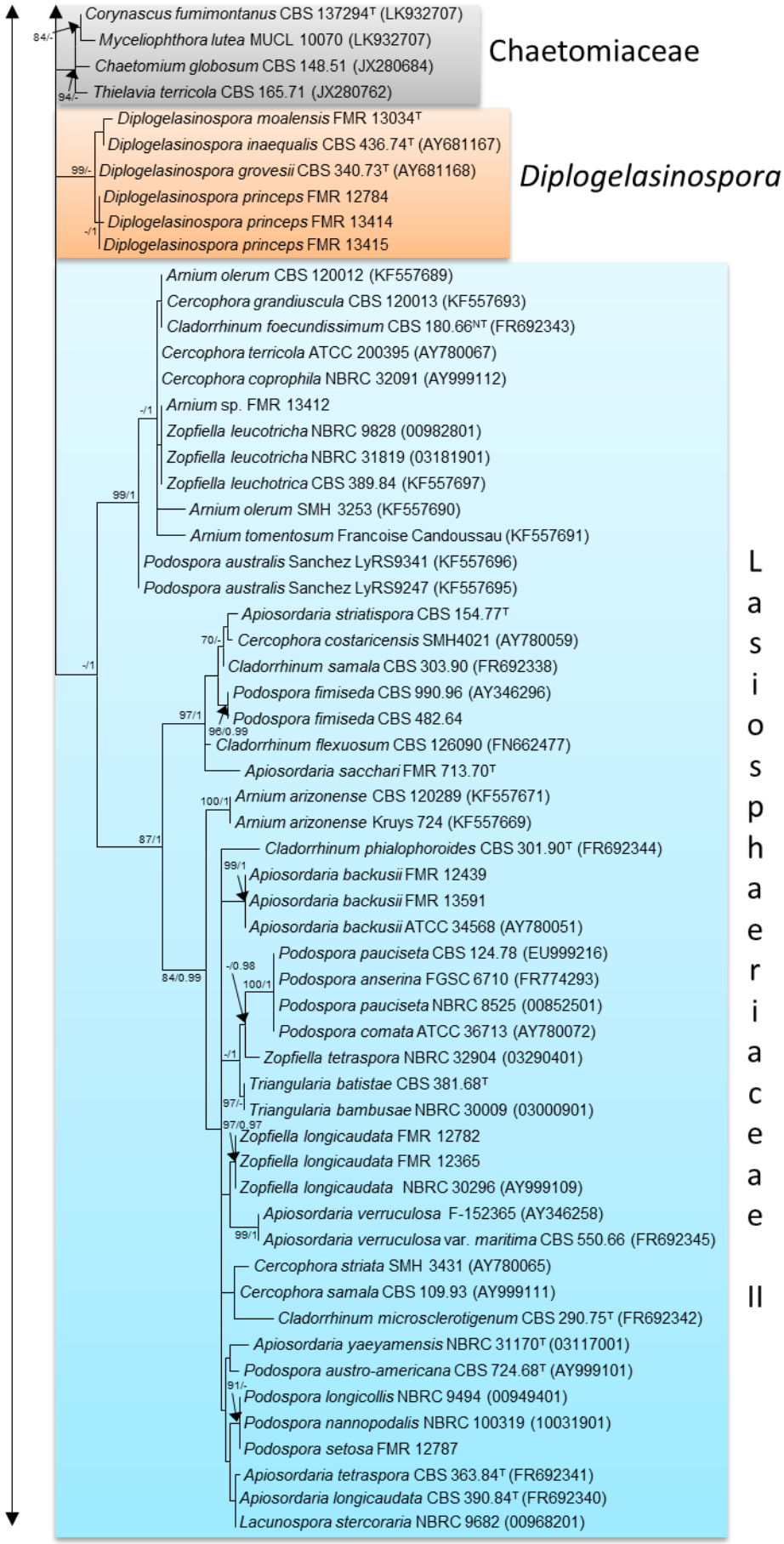
**Table 1** Isolates and reference strains of the order Sordariales included in this study

| Taxa | Strain | Source | GenBank accession # | | |
|---|---|---|---|---|---|
| | | | LSU | BT | RPB2 |
| *Anopodium ampullaceum* | MJR 40/07 | GenBank | KF557662 | KF557701 | - |
| *Anopodium ampullaceum* | E00218015 | GenBank | KF557663 | KF557702 | - |
| *Apiosordaria verruculosa* | F-152365 | GenBank | AY346258 | AY780086 | AY780150 |
| *Apiosordaria backusii* | ATCC 34568 | GenBank | AY780051 | AY780085 | AY780149 |
| *Apiosordaria backusii* | FMR 12439 | Soil, Tennessee, Great Smoky Mountains National Park | **KP981423** | **KP981550** | **KP981605** |
| *Apiosordaria backusii* | FMR 13591 | Soil, Spain, Tarragona, Els Gorgs de la Febró | **KP981424** | **KP981551** | **KP981606** |
| *Apiosordaria sacchari* | CBS 713.70 | Root, Jamaica, Janswood Estates | **KP981425** | **KP981552** | **KP981607** |
| *Apiosordaria striatispora* | CBS 154.77$^{T}$ | Soil, Thailand, Sukhotai | **KP981426** | **KP981553** | **KP981608** |
| *Apiosordaria vermicularis* | CBS 303.81$^{T}$ | Soil, Hong Kong, Campus of University of Hong Kong | **KP981427** | **KP981554** | **KP981609** |
| *Apodospora gotlandica* | E00204952 | GenBank | KF557664 | KF557703 | - |
| *Apodospora simulans* | Kruys 701 | GenBank | KF557666 | KF557704 | - |
| *Apodospora simulans* | | GenBank | KF557667 | KF557705 | - |
| *Arnium arizonense* | Santesson 18211-c | GenBank | KF557668 | KF557706 | - |
| *Arnium arizonense* | Kruys 724 | GenBank | KF557669 | KF557707 | - |
| *Arnium arizonense* | E00204509 | GenBank | KF557670 | KF557708 | - |
| *Arnium cirriferum* | CBS 120041 | GenBank | KF557673 | KF557709 | - |
| *Arnium hirtum* | E00204950 | GenBank | KF557675 | KF557711 | - |
| *Arnium hirtum* | E00204487 | GenBank | KF557676 | KF557712 | - |
| *Arnium japonense* | SANK 10273 | GenBank | KF557680 | KF557713 | - |
| *Arnium mendax* | Lundqvist 20874-c | GenBank | KF557687 | KF557716 | - |
| *Arnium mendax* | E00122117 | GenBank | KF557688 | KF557717 | - |
| *Arnium olerum* | CBS 120012 | GenBank | KF557689 | KF557718 | - |
| *Arnium* sp. | FMR 13412 | Soil, India, Gualior | **KP981428** | **KP981555** | **KP981610** |
| *Arnium tomentosum* | | GenBank | KF557691 | KF557720 | - |
| *Bombardia anartia* (=*Bombardioidea anartia*) | HHB99-1 | GenBank | AY346264 | AY780092 | AY780155 |
| *Bombardia bombarda* | AR1903 | GenBank | AY780052 | AY780089 | AY780152 |
| *Bombardia bombarda* | SMH 3391 | GenBank | AY346263 | AY780090 | AY780153 |
| *Bombardia bombarda* | SMH 4821 | GenBank | AY780053 | AY780091 | AY780154 |
| *Camarops amorpha* | SMH 1450 | GenBank | AY780054 | AY780093 | AY780156 |
| *Cercophora areolata* | UAMH 7495 | GenBank | AY587936 | AY600252 | AY600275 |
| *Cercophora atropurpurea* | SMH 2961 | GenBank | AY780056 | AY780099 | - |

| | | | | | |
|---|---|---|---|---|---|
| *Cercophora atropurpurea* | SMH 3073 | GenBank | AY780057 | AY780100 | AY780160 |
| *Cercophora coprophila* | SMH 3794 | GenBank | AY780058 | AY780102 | AY780162 |
| *Cercophora costaricensis* | SMH 4021 | GenBank | AY780059 | AY780103 | AY780163 |
| *Cercophora mirabilis* | CBS 120402 | Wallaby dung, Australia, Victoria, Eucalyptus forest near Healesville | **KP981429** | **KP981556** | **KP981611** |
| *Cercophora newfieldiana* | SMH 2622 | GenBank | AF064642 | AF466019 | AY780166 |
| *Cercophora newfieldiana* | SMH 3303 | GenBank | AY780062 | AY780106 | AY780167 |
| *Cercophora scortea* | GJS L556 | GenBank | AY780063 | AY780107 | AY780168 |
| *Cercophora striata* | SMH 3431 | GenBank | AY780065 | AY780108 | AY780169 |
| *Cercophora sparsa* | JF 00229 | GenBank | AY587937 | AY600253 | - |
| *Cercophora sulphurella* | SMH 2531 | GenBank | AY587938 | AY600254 | AY600276 |
| *Cercophora terricola* | ATCC 200395 | GenBank | AY780067 | AY780109 | AY780170 |
| *Chaetomium fimeti* | CBS 168.71 | GenBank | FJ666358 | FJ666374 | - |
| *Chaetomium globosum* | SMH 4214b | GenBank | AY346272 | AY780110 | - |
| *Chaetomium microascoides* | F-153395 | GenBank | AY346273 | AY780111 | AY780171 |
| *Chaetosphaeria ovoidea* | SMH 2605 | GenBank | AF064641 | AF466057 | AY780173 |
| *Corylomyces selenosporus* | CBS 113930$^{T}$ | Dry fruti, France, Saint Pé de Bigorre | DQ327607 | **KP981557** | **KP981612** |
| *Corynascus sepedonium* | FMR 9123 | GenBank | FJ666364 | FJ666380 | - |
| *Diplogelasinospora moalensis* | FMR 13034$^{T}$ (CBS 136018$^{T}$) | Soil, Spain, Principado de Asturia, Cangas del Narcea, Veiga de Rengos, Moal | **KP981430** | **KP981558** | **KP981613** |
| *Diplogelasinospora princeps* | FMR 13414 | Soil, Tennessee, Great Smoky Mountains National Park | **KP981431** | **KP981559** | **KP981614** |
| *Diplogelasinospora princeps* | FMR 13415 | Soil, Tennessee, Great Smoky Mountains National Park | **KP981432** | **KP981560** | **KP981615** |
| *Echria gigantospora* | F77-1 | GenBank | KF557674 | KF557710 | - |
| *Echria macrotheca* | Lundqvist 2311 | GenBank | KF557684 | KF557715 | - |
| *Fimetariella rabenhorstii* | Lundqvist 20410-c | GenBank | KF557694 | KF557721 | - |
| *Immersiella caudata* | SMH 3298 | GenBank | AY436407 | AY780101 | AY780161 |
| *Immersiella immersa* | SMH 4104 | GenBank | AY436403 | AY780123 | AY780181 |
| *Jugulospora carbonaria* | ATCC 34567 | GenBank | AY346302 | AY780141 | AY780196 |
| *Jugulospora carbonaria* | IMI 381338 | Soil, Antarctica, King George Island, Jubany Argentinian base | **KP981433** | **KP981561** | **KP981616** |
| *Jugulospora rotula* | ATCC 38359 | GenBank | AY346287 | AY780120 | AY780178 |
| *Jugulospora rotula* (≡*Apiosordaria hispanica*) | CBS 110112 | Soil, Spain, Tarragona, Gorgs de la Febró | **KP981434** | **KP981562** | **KP981617** |
| *Jugulospora rotula* (≡*Apiosordaria globosa*) | CBS 110113 | Soil, Spain, Tarragona, Gorgs de la Febró | **KP981435** | **KP981563** | **KP981618** |
| *Jugulospora rotula* (≡*Rhexosporium terrestre*) | FMR 12428 | Soil, Tennessee, Great Smoky Mountains National Park | **KP981436** | **KP981564** | **KP981619** |
| *Jugulospora rotula* | FMR 12690 | Soil, North Carolina, Great Smoky Mountains National Park | **KP981437** | **KP981565** | **KP981620** |

| | | | | | |
|---|---|---|---|---|---|
| *Jugulospora rotula* | FMR 12781 | Soil, Tennessee, Great Smoky Mountains National Park | **KP981438** | **KP981566** | **KP981621** |
| *Lasiosphaeria glabrata* | TL 4529 | GenBank | AY436410 | AY600255 | AY600277 |
| *Lasiosphaeria lanuginosa* | SMH 3819 | GenBank | AY436412 | AY600262 | AY600283 |
| *Lasiosphaeria ovina* | SMH 1538 | GenBank | AF064643 | AF466046 | AY600287 |
| *Lasiosphaeria rugulosa* | SMH 1518 | GenBank | AY436414 | AY600272 | AY600294 |
| *Lasiosphaeria sorbina* | GJS L555 | GenBank | AY436415 | AY600273 | AY600295 |
| *Lasiosphaeris hirsuta* | SMH 1543 | GenBank | AY436417 | AY780121 | AY780179 |
| *Lasiosphaeris hispida* | SMH 3336 | GenBank | AY436419 | AY780122 | AY780180 |
| *Naviculispora terrestris* | CBS 137295$^{T}$ (=FMR 10060$^{T}$) | Soil, Argentina, Tucumán province, Tafí del Valle | **KP981439** | **KP981567** | **KP981622** |
| *Neurospora crassa* | MUCL 19026 | GenBank | AF286411 | M13630 | AF107789 |
| *Neurospora pannoica* | TRTC 51327 | GenBank | AY780070 | AY780126 | AY780185 |
| *Neurospora tetrasperma* | ATCC 96230 | GenBank | AY346280 | AY780117 | AY780176 |
| *Podospora appendiculata* | CBS 212.97 | GenBank | AY780071 | AY780129 | AY780186 |
| *Podospora fibrinocaudata* | TRTC 48343 | GenBank | AY780074 | AY780131 | AY780188 |
| *Podospora fimiseda* | CBS 990.96 | GenBank | AY346296 | AY780133 | AY780190 |
| *Podospora fimiseda* | CBS 482.64 | Dung of cow, Switzerland, Kt. Aargau, Ober-Erlinsbach, Barmelweid | **KP981440** | **KP981568** | **KP981623** |
| *Podospora setosa* | FMR 12787 | Spain, Gran Canaria | **KP981441** | **KP981569** | **KP981624** |
| *Rinaldiella pentagonospora* | CBS 132344$^{T}$ | Contaminated human lesion, USA, Georgia, Dahlonega | **KP981442** | **KP981570** | **KP981625** |
| *Rypophyla decipiens* (≡*Podospora decipiens*) | CBS 258.64 | GenBank | AY780073 | AY780130 | AY780187 |
| *Schizothecium curvisporum* | ATCC 36709 | GenBank | AY346300 | AY780136 | AY780192 |
| *Schizothecium fimbriatum* | CBS 144.54 | GenBank | AY780075 | AY780132 | AY780189 |
| *Sordaria fimicola* | SMH 4106 | GenBank | AY780079 | AY780138 | AY780194 |
| *Sordaria macrospora* | Buck s.n. | GenBank | AY346301 | AY780140 | AY780195 |
| *Triangularia batistae* | CBS 381.68$^{T}$ | Soil, Brazil | **KP981443** | **KP981577** | **KP981626** |
| *Triangularia mangenotii* | CBS 419.67$^{T}$ | Leaf, France, near Bordeaux | **KP981444** | **KP981571** | **KP981627** |
| *Triangularia tanzaniensis* | TRTC 51981$^{T}$ | GenBank | AY780081 | AY780143 | AY780197 |
| *Zopfiella attenuata* | CBS 266.77$^{T}$ | Soil, Japan | **KP981445** | **KP981572** | **KP981628** |
| *Zopfiella karachiensis* | CBS 657.74 | Arid soil, Egypt, Western Desert, Kharga Oasis | **KP981447** | **KP981478** | **KP981630** |
| *Zopfiella longicaudata* | FMR 12365 | Soil, Tennessee, Great Smoky Mountains National Park | **KP981448** | **KP981474** | **KP981631** |
| *Zopfiella longicaudata* | FMR 12782 | Soil, Spain, Gran Canaria | **KP981449** | **KP981475** | **KP981632** |
| *Zopfiella pleuropora* | CBS 518.70$^{T}$ | Dung of deer, Ontario, Haliburton Co., S of Dorset | **KP981450** | **KP981476** | **KP981633** |
| *Zygopleurage zygospora* | SMH 4219 | GenBank | AY346306 | AY780147 | - |
| *Zygospermella insignis* | Lundqvist 2444 | GenBank | KF557698 | KF557722 | - |
| *Zygospermella insignis* | E00204312 | GenBank | KF557699 | KF557723 | - |


Zopfiella pilifera NBRC 9558T (00955801)
Apodus oryzae CBS 376.74T (AY681166)
Zopfiella latipes NBRC 30408 (03040801)
Triangularia mangenotii ATCC 38847 (AY346303)
Triangularia mangenotii CBS 419.67T
Zopfiella marina NBRC 30420T (03042001)
Zopfiella latipes NBRC 9826 (AY999107)
Naviculispora terrestris CBS 137295T
Arnium caballinum Lundqvist 7098-e (KF557672)
Cercophora aquatica JF 09214 (JN673036)
Arnium leporinum SMH 3188 (KF557683)
Arnium leporinum CBS 365.69 (KF557682)
Arnium japonense SANK 10273 (KF557680)
Podospora cochleariformis CBS 249.71 (= Rhypophyla cochleariformis) (AY999098)
Podospora decipiens CBS 258.64 (= Rhypophyla decipiens) (AY780073)
Podospora pleiospora NBRC 9495 (= Rhypophyla pleiospora) (00949501)
Cuspidatispora xiphiago A184-1A (DQ376251)
Fimetariella rabenhorstii Lundqvist 20410-c (KF557694)
Arnium mendax E00122117 (KF557688)
Arnium mendax Lundqvist 20874-c (KF557687)
Arnium inaequilaterale Lundqvist 3345-b (KF557679)
Cercophora areolata UAMH7495 (AY587936)
Cercophora ambigua CBS 215.60 (AY999114)
Cercophora sparsa JF00229 (AY587937)
Anopodium ampullaceum E00218015 (KF557663)
Anopodium ampullaceum MJR 40/07 (KF557662)
Cercophora sulphurella SMH2531 (AY587938)
Corylomyces selenospora FMR 8279T
Podospora didyma CBS 232.78 (AY999100)
Zopfiella tabulata CBS 230.78 (AY999105)
Jobellisia rhynchostoma CBS 118484 (EU999217)
Lasiosphaeria ovina SMH 1538 (AF064643)
Lasiosphaeria lanuginosa SMH3819 (AY436412)
Lasiosphaeria sorbina GJS L555 (AY436415)
Lasiosphaeria glabrata TL4529 (AY436410)
Lasiosphaeria rugulosa SMH1518 (AY436414)
Diffractella curvata NBRC 9820 (00982001)
Apodospora simulans Lantz H. (KF557667)
Apodospora simulans Kruys 701 (KF557666)
Apodospora peruviana CBS 118394 (KF557665)
Apodospora gotlandica E00204952 (KF557664)
Zopfiella pleuropora CBS 518.70
Zopfiella attenuata CBS 266.77
Bombardia bombarda SMH 4821 (AY780053)
Bombardia bombarda SMH 3391 (AY346263)
Bombardioidea anartia (= Bombardia bombarda) HHB99-1 (AY346264)
Bombardia bombarda AR1903 (AY780052)
Podospora fibrinocaudata TRTC48343 (AY780074)
Podospora tarvisina NBRC 8537 (00853701)
Podospora appendiculata CBS 212.97 (AY780071)
Cercophora scortea GJS L556 (AY780063)
Podospora appendiculata NBRC 8549 (AY999103)
Sordaria humana ATCC 22796 (AY780078)
Sordaria fimicola SMH4106 (AY780079)
Pseudoneurospora amorphoporcata CBS 626.80 (FR774287)
Neurospora crassa MUCL 19026 (AF286411)
Neurospora pannonica TRTC51327 (AY780070)
Lasiosphaeriaceae I
Sordariaceae



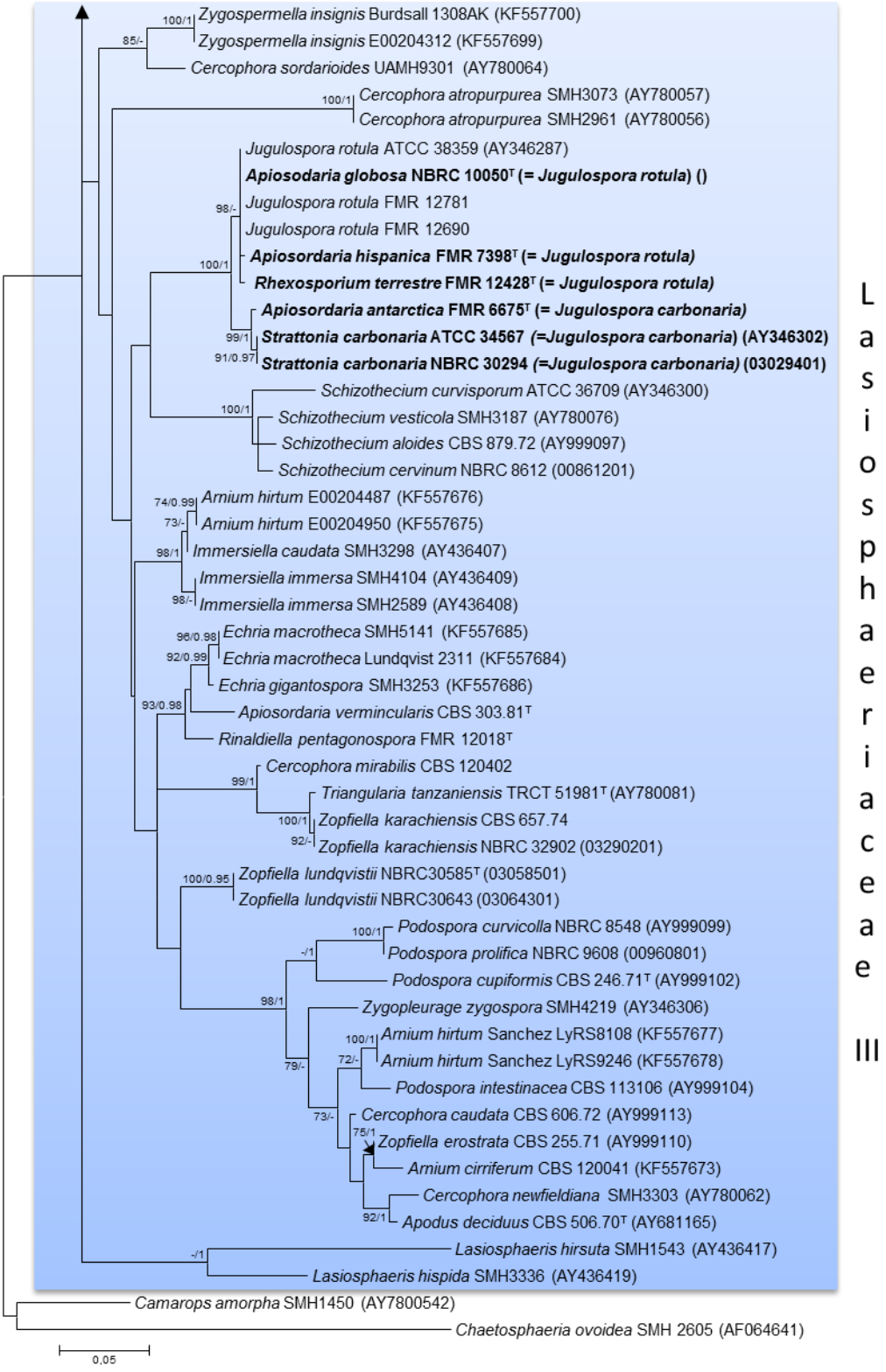
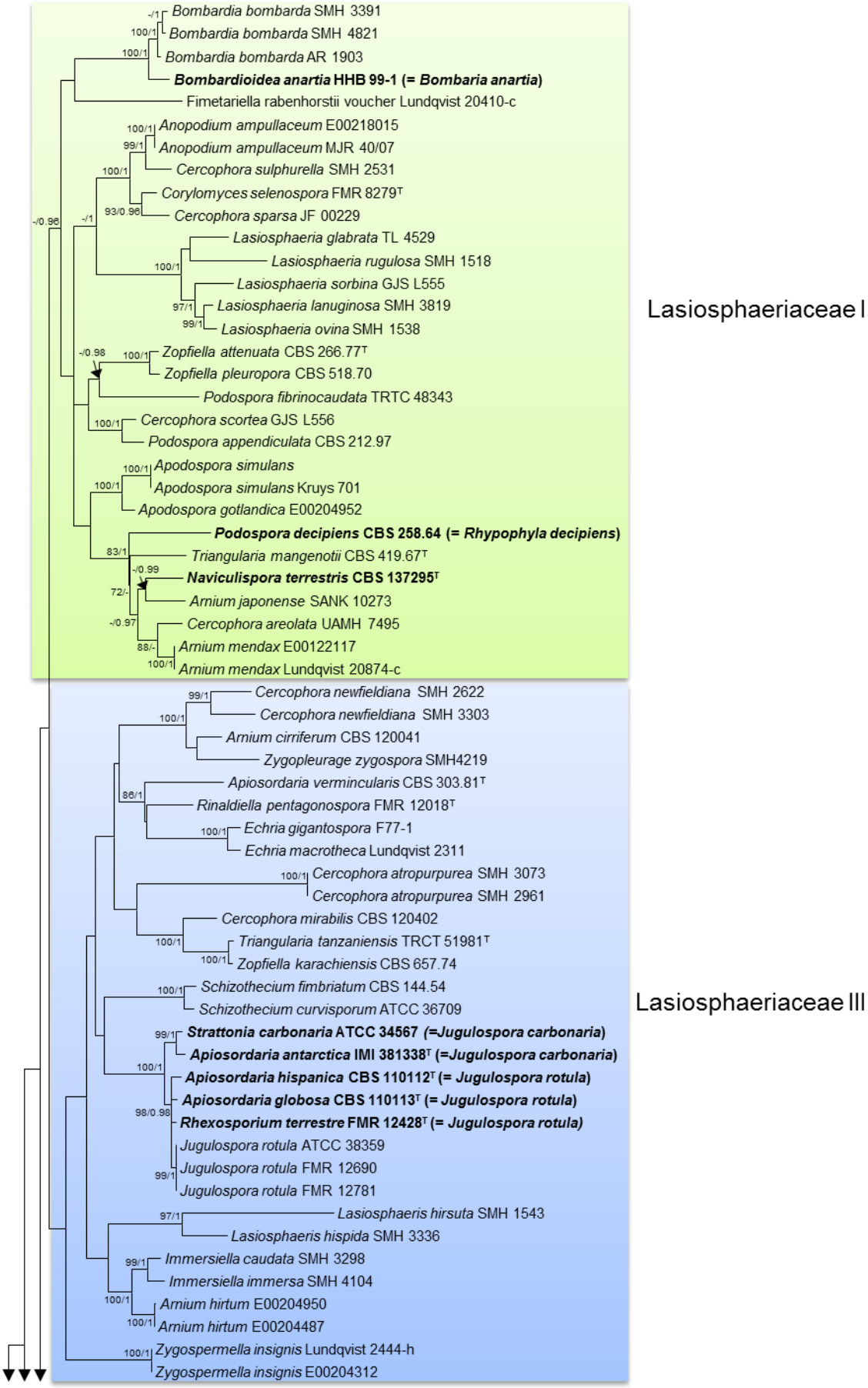



Fig. 1 Maximum-likelihood (ML) tree obtained from D1−D2 sequences of our isolates and selected strains belonging to the families Chaetomiaceae, Lasiosphaeriaceae and Sordariaceae. *Camarops amorpha* and *Chaetosphaeria ovoidea* were used as outgroup. Bootstrap support values ≥70 and Bayesian posterior probability scores ≥0.95 are indicated along branches. Type and neotype strains of the different species are indicated with $^{T}$ and $^{NT}$, respectively.



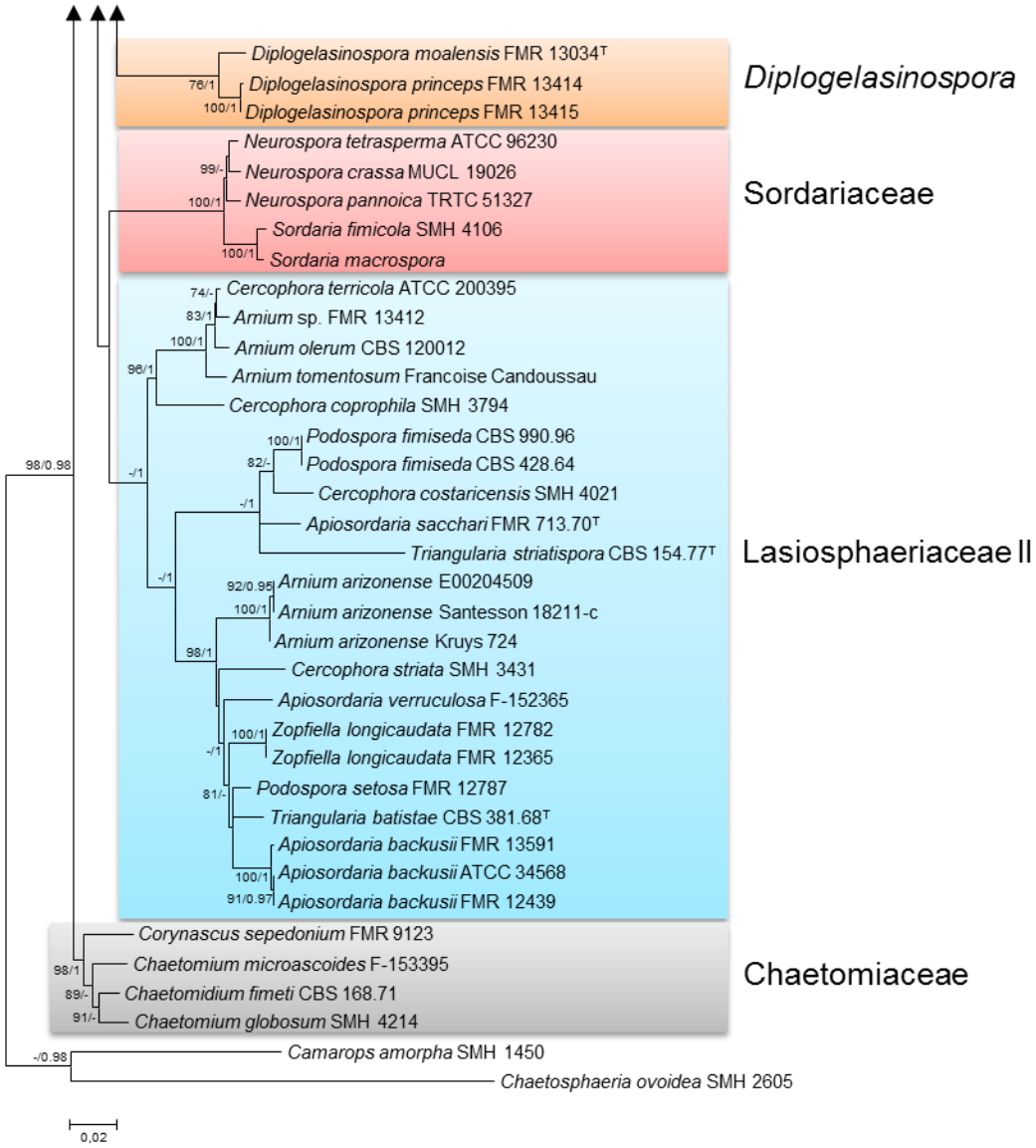



Fig. 2 Maximum-likelihood (ML) tree obtained from the combined DNA sequence data from three loci (D1−D3, BT2 and RPB2) of our isolates and selected strains belonging to the families Chaetomiaceae, Lasiosphaeriaceae and Sordariaceae. *Camarops amorpha* and *Chaetosphaeria ovoidea* were used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Branch lengths are proportional to distance. Type strains of the different species are indicated with $^{T}$.

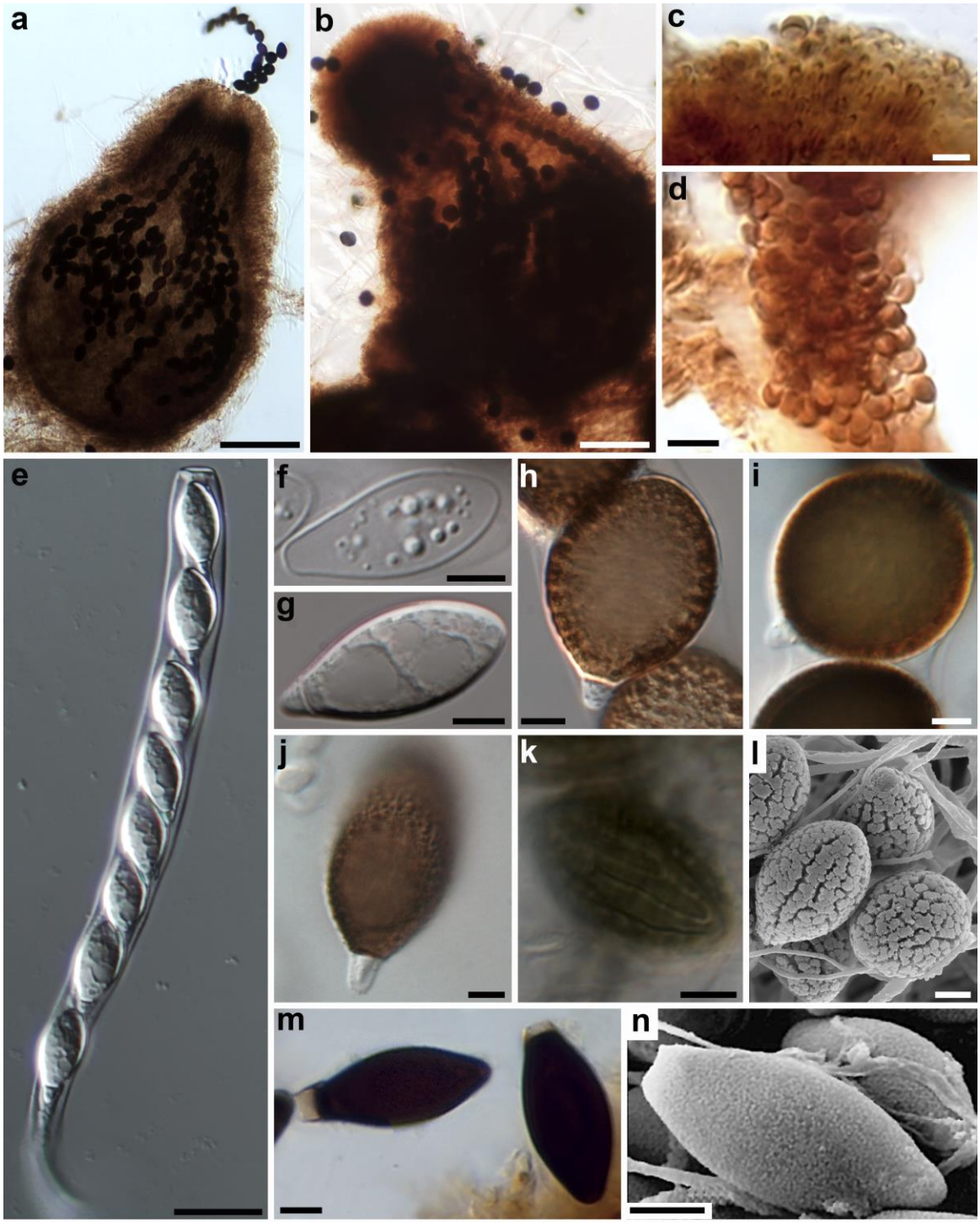



Fig. 3 Morphology of *Jugulospora*. Ascoma. a. *Jugulospora rotula* FMR 12428. b. *Jugulospora rotula* FMR 12690. Detail of the papillate neck. c, d. *Jugulospora carbonaria* ATCC 34657. Ascus. e. *Jugulospora rotula* FMR 12428. Immatured ascospores. f, g. *Jugulospora rotula* FMR 12428. Different ascospore morphology of *Jugulospora rotula*. h. CBS 12690. i. CBS 110113. j. CBS 110112. k, l. FMR 12428. Ascospores of *Jugulospora carbonaria*. m. ATCC 34567. n. IMI 381338. Bars: a, b = 100 µm. c, e = 20 µm. d = 10 µm. e = 15 µm. f–n = 5 µm.

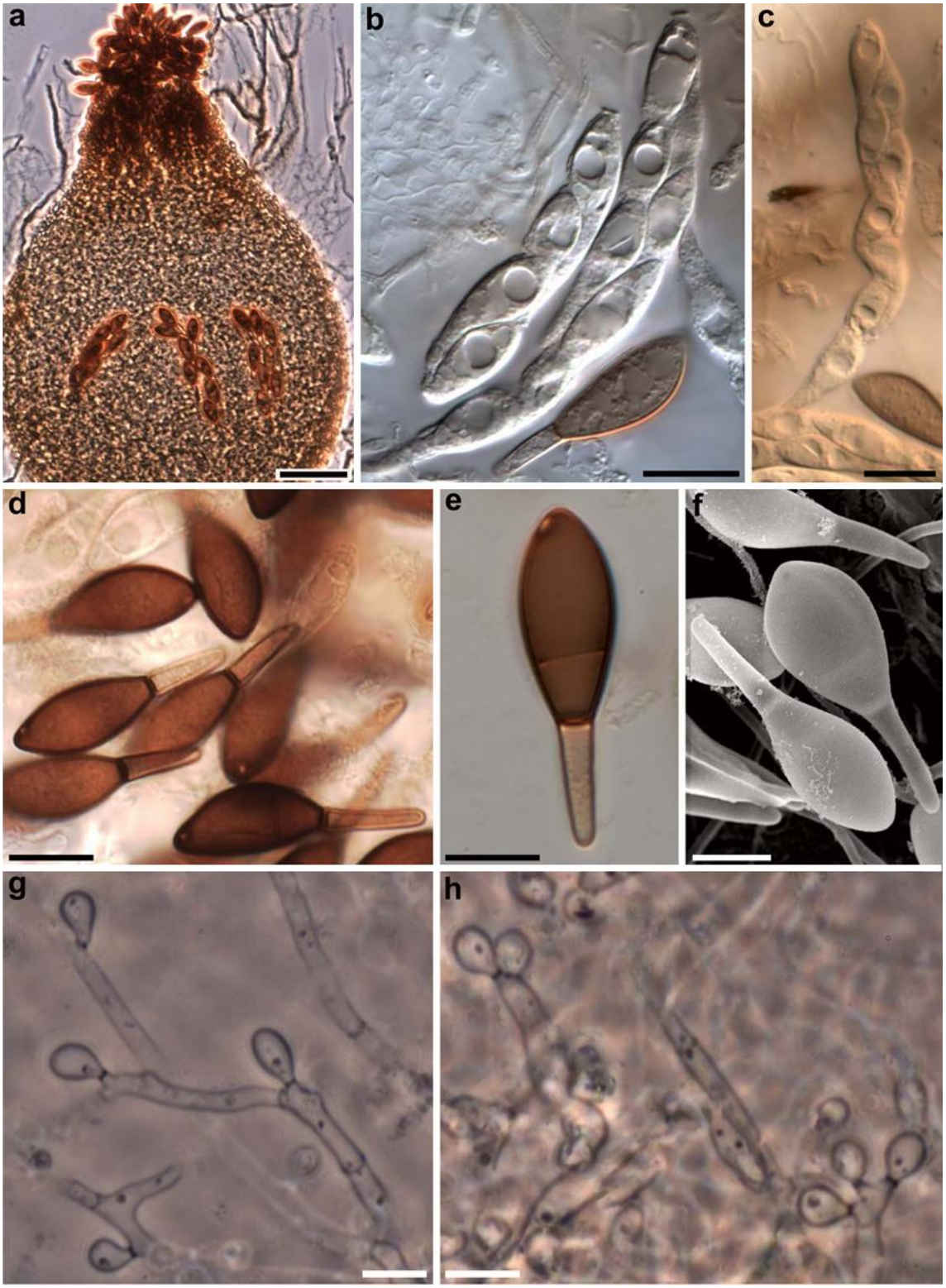



Fig. 4 *Naviculispora terrestris* CBS 137295$^{T}$. a. Ascomata. b, c. asci. d, e, f. ascospores. g. conidia sessile. h. conidia borning on sympodially proliferating conidiophores. Bars: a = 100 µm. b, c = 20 µm. d = 15 µm. e, f = 10 µm. g, h = 5 µm.

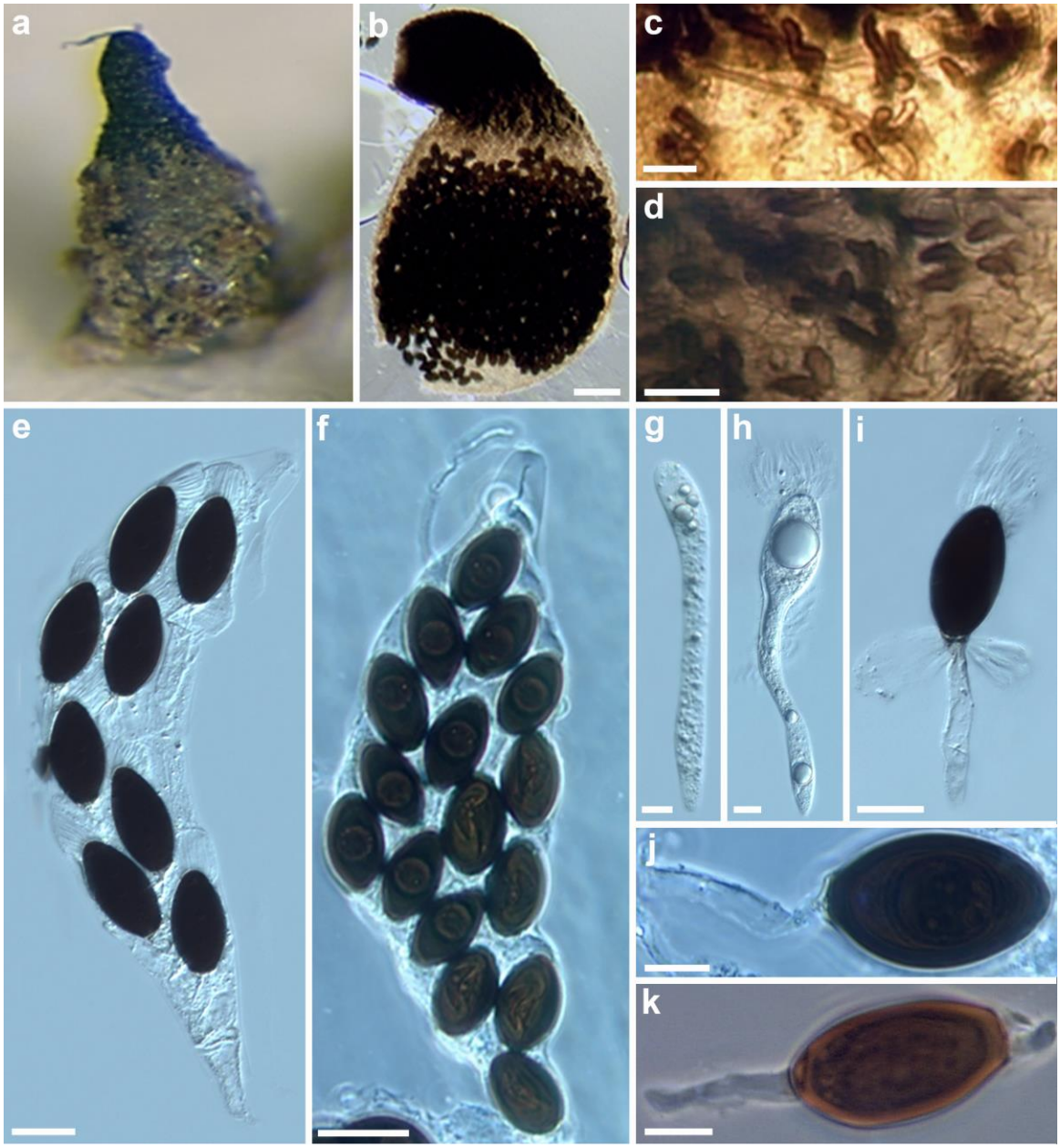
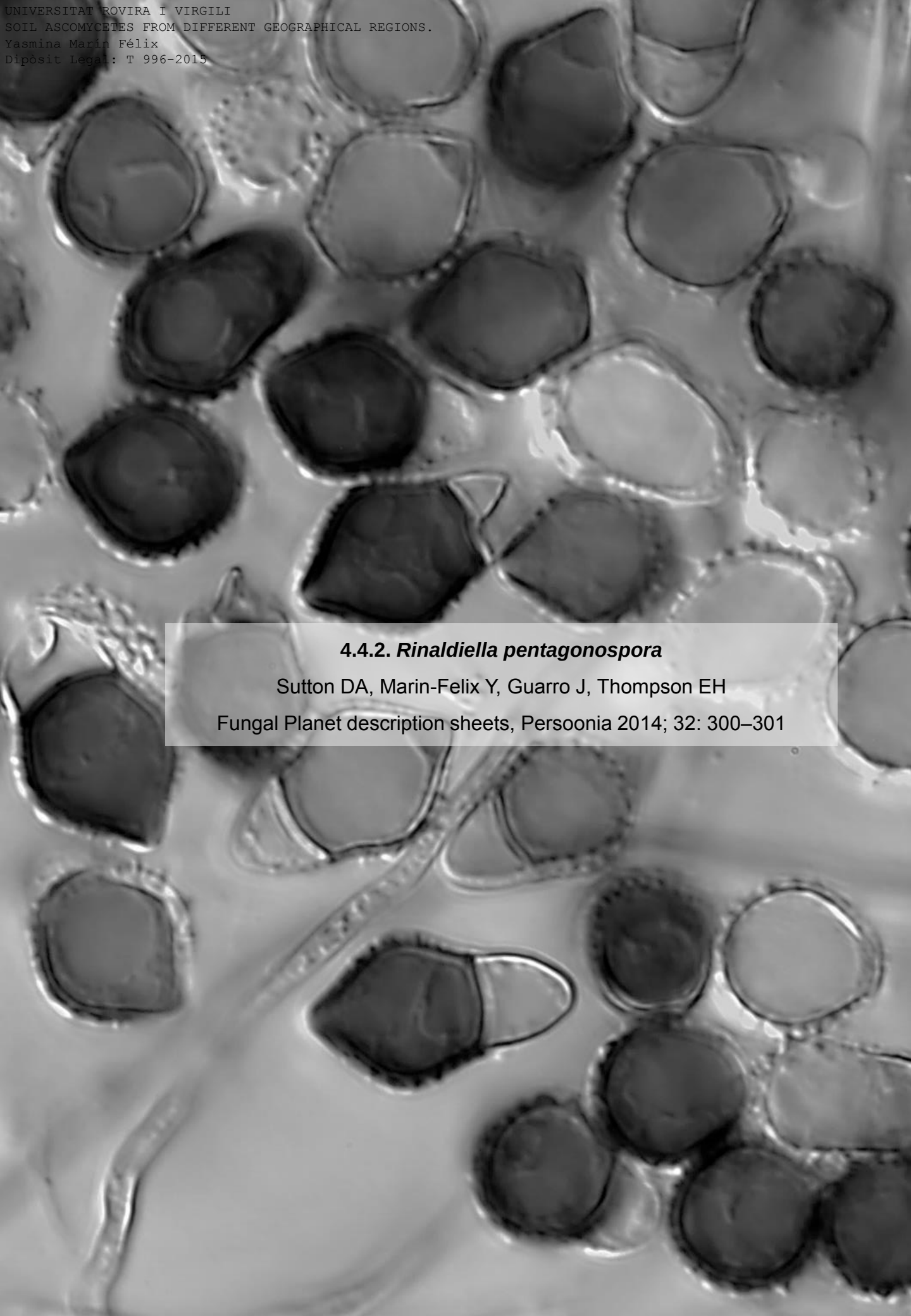



Fig. 5 Morphology of *Rhypophila*. Ascoma. a. *Rhypophila pleiospora* LyRS9223.1. b. *Rhypophila myriospora* CBS 115804. Elongated tubercles at the neck. c. *Rhypophila pleiospora* LyRS9223.1. d. *Rhypophila myriospora* CBS 115804. Ascus. e. *Rhypophila decipiens* LyRS8109.2. f. *Rhypophila pleiospora* LyRS9223.1. Immatured ascospores. g, h. *Rhypophila decipiens* LyRS8109.2. Different ascospore morphology. i. *Rhypophila decipiens* LyRS8109.2. j. *Rhypophila pleiospora* LyRS9223.1. k. *Rhypophila myriospora* CBS 115804. Bars: b = 100 µm. c, d = 15 µm. e, f = 25 µm. g, h, j, k = 10 µm. i = 20 µm.



## 4.4.2. *Rinaldiella pentagonospora*

Sutton DA, Marin-Felix Y, Guarro J, Thompson EH

Fungal Planet description sheets, Persoonia 2014; 32: 300–301

Fungal Planet 279 – 10 June 2014

## ***Rinaldiella*** D.A. Sutton, Y. Marín, Guarro & E.H. Thomps., *gen. nov.*

*Etymology*. Named in honour of the eminent medical mycologist Michael G. Rinaldi.

*Ascomata* immersed, ostiolate, pyriform to subglobose, dark brown to black, covered with hyphal-like hairs, with a conspicuous conical neck. *Peridium* membranaceous, translucent, brown to yellowish brown, *textura epidermoidea*. *Asci* 8-spored, fasciculate, clavate to cylindrical, without apical ring, short stipitate, early evanescent. *Paraphyses* and periphyses hyaline, filiform, septate. *Ascospores* biseriate to uniseriate, clavate, hyaline, and aseptate when young, finally becoming transversely 1-septate; upper cell polygonal, 5-angled in side view, truncate at the base and with a slightly acuminate apex, brown, thick-walled, warted, with an apical germ pore; lower cell subhyaline, conical and slightly warted.

*Type species*. *Rinaldiella pentagonospora*.
MycoBank MB807137.

## ***Rinaldiella pentagonospora*** D.A. Sutton, Y. Marín, Guarro & E.H. Thomps., *sp. nov.*

*Etymology*. Named after the shape of the upper cell of the ascospore.

*Mycelium* composed of brown to pale olive brown, septate, branched, smooth-walled hyphae, 1–6 µm diam. *Ascomata* immersed, ostiolate, pyriform to subglobose, dark brown to black, 180–300 × 160–230 µm, covered with long, yellowish brown, septate hypha-like hairs, 2–3 µm diam, with a conspicuous conical neck; neck 20–40 µm long, 50–90 µm wide at the base, with brown, papillate cells around the ostiole. *Peridium* membranaceous, translucent, 4–6-layered, 7–11 µm thick, brown to yellowish brown *textura epidermoidea*. *Asci* 8-spored, fasciculate, clavate to cylindrical, 50–71 × 6–12 µm, without apical ring, short stipitate, early evanescent. *Paraphyses* and *periphyses* hyaline, filiform, septate, 0.5–2 µm wide. *Ascospores* biseriate to uniseriate, clavate, hyaline, and aseptate when young, finally becoming transversely 1-septate; upper cell polygonal, 5-angled in side view, truncate at the base and with a slightly acuminate apex, brown, thick-walled, 9–10 × 7–8 µm, ornamented with rounded warts of 0.5–1 µm diam, with an apical germ pore, 0.5–1 µm diam; lower cell subhyaline, conical and slightly warted, 3–5 µm long.

Culture characteristics — Colonies on PDA growing slowly, reaching 5–8 mm diam after 30 d at 22–25 °C, velutinose, elevated, folded, zonate and slightly radiate, dark green; ascomata produced after 2 mo; reverse dark green.

*Typus*. USA, Georgia, Dahlonega, from a contaminated human lesion, May 2011, *J.L. Robertson & D.R. Hospenthal* (holotype CBS H-20903, cultures ex-type UTHSC 11-1352 = FMR 12018; LSU sequence GenBank KC702790, ITS sequence GenBank KC702789, MycoBank MB564228).

Notes — This fungus was found contaminating a lesion in a man probably acquired when he struck his hand against a tree. Morphologically, *Rinaldiella* resembles *Apiosordaria* (*Sordariales*, *Ascomycota*). However, the genetic distances between the present species and the members of that genus, in our unpublished DNA sequence database, are too long to be considered the same genus, and are closer to other genera of the same order. Currently, *Apiosordaria* comprises 24 saprobic species (Guarro et al. 2012) usually isolated from soil or herbivore dung. The similarity of D1/D2 and ITS sequences of our fungus with the type species of *Apiosordaria*, *A. verruculosa* and the ex-type strains of *A. striatispora* and *A. yaeyamensis*, which are the most closely related species morphologically, is 92.69, 90.92 and 92.09 %; and 80.11, 78.11 and 80.28 %, respectively. Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the D1/D2 sequence is *Immersiella immersa* (GenBank AY436409 and AY436408; Identities = 583/604 (97 %), Gaps 2/604 (0 %)); and using ITS, no sequences with significant identities were found. *Rinaldiella* is very different morphologically from *Immersiella*, the latter producing cylindrical, sigmoid or geniculate and 1-celled ascospores (Miller & Huhndorf 2004).

*Colour illustrations*. Dahlonega, Georgia (Photo credit: Jack Anthony); ascomata, ascus and ascospores. Scale bars = 50 µm, 10 µm, 2.5 µm.

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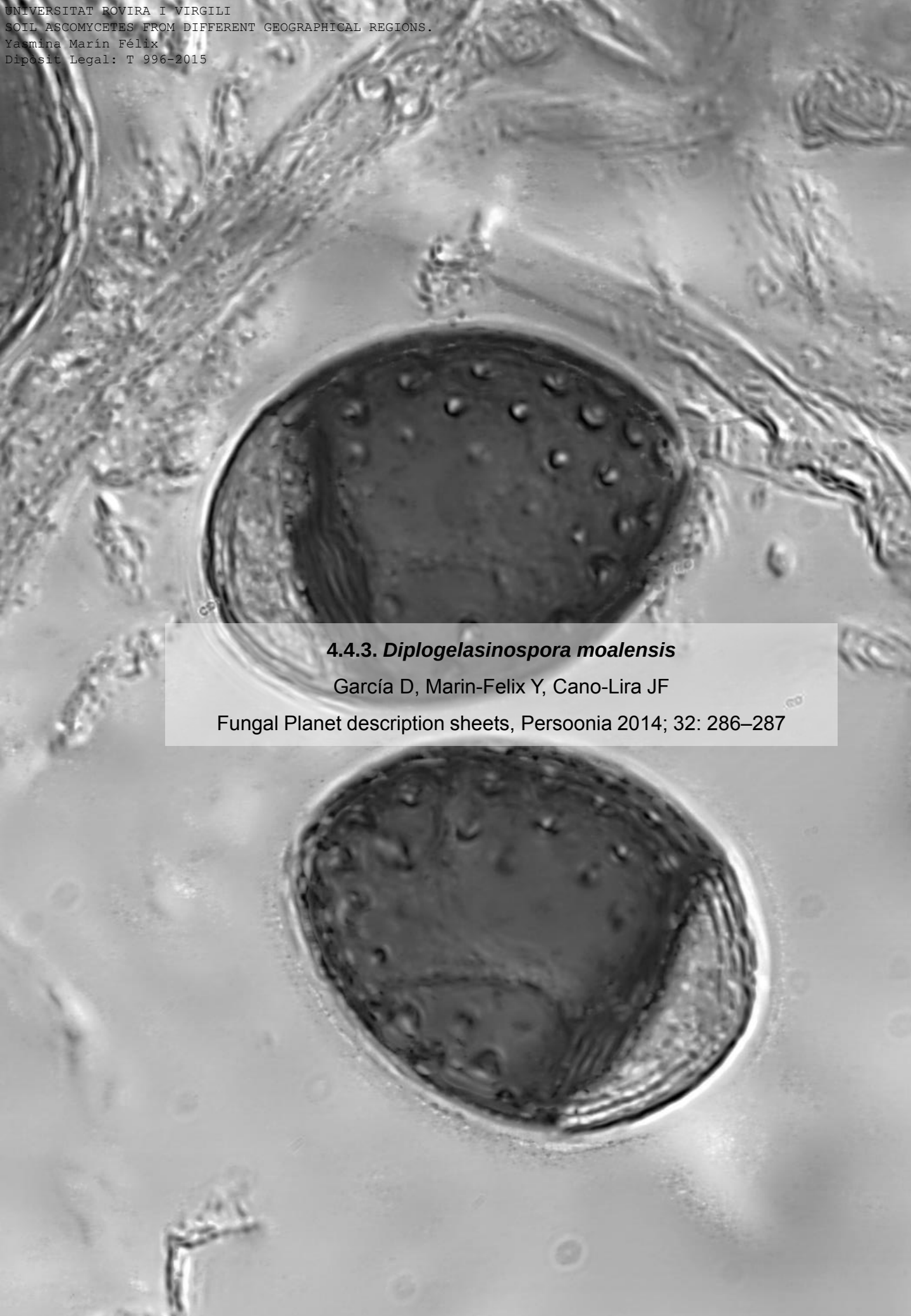


### 4.4.3. *Diplogelasinospora moalensis*

García D, Marin-Felix Y, Cano-Lira JF

Fungal Planet description sheets, Persoonia 2014; 32: 286–287

Fungal Planet 272 – 10 June 2014

# *Diplogelasinospora moalensis* Dania García, Y. Marín & Cano, *sp. nov.*

*Etymology*. Named after woodland Moal, surrounding the village where the soil samples were collected.

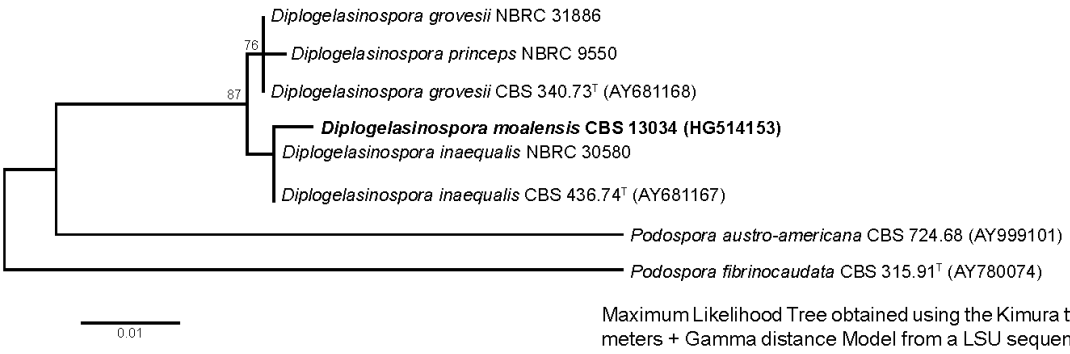
*Mycelium* consisting of branched, septate, smooth, 2–5 µm diam hyphae. *Ascomata* cleistothecial, superficial to immersed, scattered to grouped, at first yellowish brown, becoming black at maturity, 170–310 µm diam, hairy, covered by hyphae-like, setae. *Setae* flexuous, slightly rugose- and thick-walled, and yellowish brown to brown, up to 500 µm long, measuring 2–7 µm at base. *Peridium* membranaceous to slightly carbonaceous, brown to black and opaque, 3–4-layered, 2–5 µm thick, outer layer *textura intricata* composed of brown, thick-walled hypha-like cells which cover a second layer with *textura cephalothecoid* cells; inner layers hyaline, cells of *textura angularis*. *Asci* 8-spored, soon evanescent, lacking a distinct apical ring, at first cylindrical, becoming cylindrical-clavate, short stipitate, 150–180 × 14–22 µm. *Paraphyses* abundant, cylindrical to moniliform, septate and sometimes constricted at the septa, 3–13 µm diam. *Ascospores* at first aseptate and hyaline, irregularly uniseriate, ellipsoidal, becoming transversely septate without constriction at the septum, which is near the upper third of the ascospore; one cell becoming dark brown to black, whereas the other remaining hyaline or less frequently pale yellowish brown and usually collapsing at maturity. Ascospores (18–)20–27 µm in length, dark cell 16–19 × 15–18 µm; both cells showing ornamented walls with circular inwardly projecting pits showing an endodentate endosporium; germ pore inconspicuous, subapical to laterally disposed at dark cell, 1–2 µm diam.

Culture characteristics — Colonies on Czapek agar and oatmeal agar attaining 80 mm diam after 14 d at 25 °C; velvety to cottony olive-brown (4E8); reverse dark brown (8F4) (Kornerup & Wanscher 1984). Minimum and maximum temperature of growth: 10 and 45 °C, respectively. Production of initial ascomata were detected at 25, 30 and 35 °C after 14 d, mature ascomata were observed after 21 d at the same temperatures.

*Typus*. Spain, Principado de Asturia, Cangas del Narcea, Veiga de Rengos, Moal, 14 Aug. 2012, *G. Sisó-Boix & D. Garcia* (holotype CBS H-21382, cultures ex-type FMR 13034 = CBS 136018; ITS sequence GenBank HG514152, LSU sequence GenBank HG514153, MycoBank MB804992).

Notes — The genus *Diplogelasinospora* was erected to accommodate *D. princeps* characterised by non-ostiolate ascomata and 2-cell ascospores with pitted walls (Udawaga & Horie 1972). Although the ex-type strain of *D. princeps* was isolated from flax seed, the other strains of this species had been isolated from soil, the same source where the other species of the genus were collected. Currently, the genus includes two other species, namely *D. grovesii* and *D. inaequalis* (Udagawa et al. 1973). The morphological characters used for species delimitation are the total length of the ascospores, the septum position and the ornamentation of the ascospore wall (Udawaga & Horie 1972, Udagawa et al. 1973). *Diplogelasinospora moalensis* has a septum in the upper third of the ascospores, as observed in *D. inaequalis*; however, the latter has smaller ascospores (up to 22 µm) and the ornamentation pattern is similar to *D. princeps* and *D. grovesii*, i.e. reticulated wall formed by circular to elongate pits (up to 3 µm in *D. princeps*). On the other hand, *D. moalensis* has finely ornamented ascospores showing small, inward pits. Both ornamentation patterns are similar to those found in *Gelasinospora* (syn. *Neurospora*); however, Cai et al. (2006) confirmed that *Diplogelasinospora* is phylogenetically unrelated to *Gelasinospora* and showed that *Diplogelasinospora* is more related to *Lasiosphaeriaceae* than *Sordariaceae*.

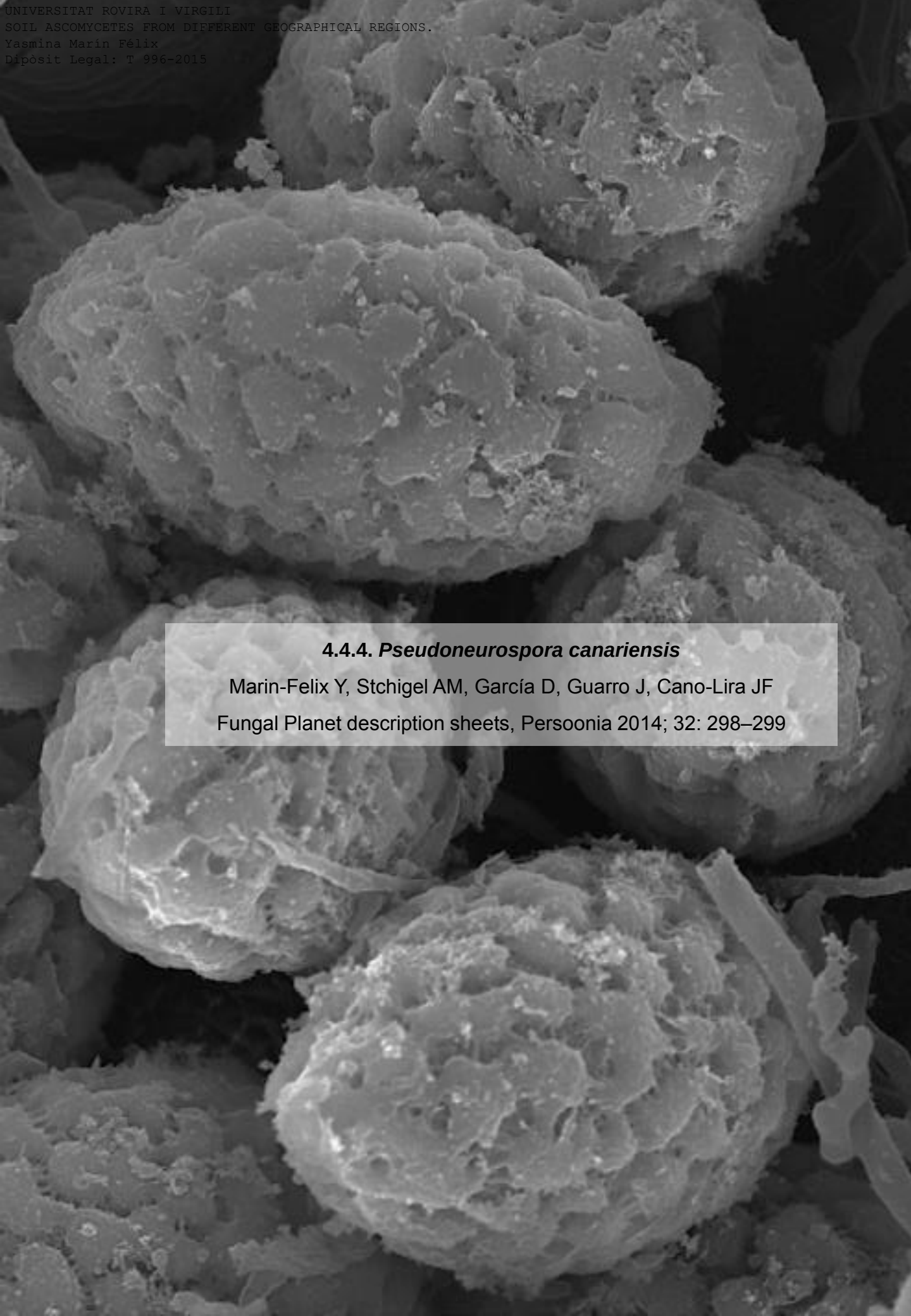
A Blast search using the ITS sequence of our isolate showed a 98 % and 96 % similarity with those of *D. inaequalis* (AY681201) and *D. grovesii* (NR_077164), respectively. The LSU tree corroborated that *D. moalensis* is a new taxon in the genus, more closely related to *D. inaequalis* (NBRC 30580 and CBS 436.74) (TreeBASE ID 14810). The two branches formed suggest that the septal position is an informative character in this group, since the species with a median septum, such as *D. princeps* (NBRC 9550) and *D. grovesii* (CBS 340.73 and NBRC 31886) form a separate lineage, whereas those species with the septum displaced in the upper third generate another group. Although only a tentative hypothesis should currently be suggested, the inclusion of more isolates and new taxa may possibly help to solve the phylogenetic position of the genus and elucidate the real value of these morphological characters.




Maximum Likelihood Tree obtained using the Kimura two Parameters + Gamma distance Model from a LSU sequence alignment generated with MUSCLE in MEGA v. 5.2 (Tamura et al. 2011). The bootstrap support values above 70 % from 1 000 replicates are shown at the nodes. Bar represents number of substitutions per site. The species described here is printed in **bold** face. *Podospora austro-americana* and *Podospora fibrinocaudata* were used as outgroup. T = Ex-type strain.

*Colour illustrations*. Moal forest, Asturias, Spain; *Diplogelasinospora moalensis* growing on Czapeck and OAT, asci and ascospores. Scale bars = 10 µm

Dania García, Yasmina Marin-Felix & José F. Cano-Lira, Mycology Unit, Medical School, IISPV, University Rovira i Virgili Reus, Spain; e-mail: dania.garcias@urv.cat, yasmina.marin@urv.cat & jose.cano@urv.cat



**4.4.4. *Pseudoneurospora canariensis***

Marin-Felix Y, Stchigel AM, García D, Guarro J, Cano-Lira JF

Fungal Planet description sheets, Persoonia 2014; 32: 298–299

Fungal Planet 278 – 10 June 2014

# *Pseudoneurospora canariensis* Y. Marín, Stchigel, Dania García, Guarro & Cano, *sp. nov.*

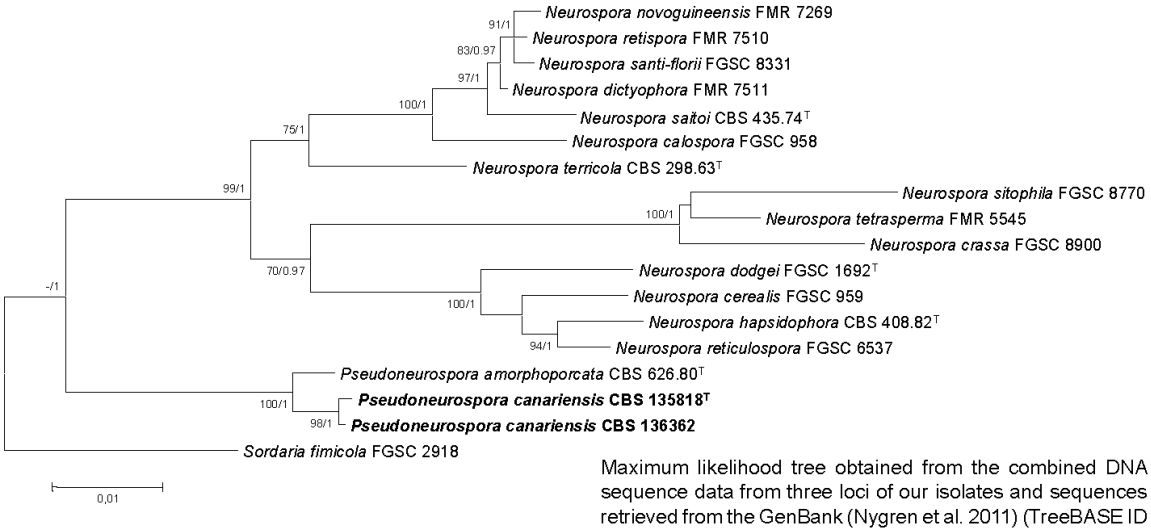
*Etymology*. *Canariensis* = from Canary Islands, Spain; referring to the geographical origin of the fungus.

*Mycelium* composed of subhyaline to brown, branched, septate, smooth-walled, thin- to thick-walled hyphae, 1.5–9 µm wide. *Ascomata* non-ostiolate, superficial to immersed, scattered to grouped, at first yellowish brown and translucent, becoming dark yellowish brown to black at maturity, 120–220 µm diam, covered by moniliform hyphae, up to 17 µm wide; setae scarce, sinuose, smooth- and thick-walled, septate, brown, up to 150 µm long, 3–8 µm at the base, apex rounded; peridium 3–4-layered, 5–15 µm thick, brown, of *textura angularis* to *globulosa*, composed of brown, thick-walled, flattened cells, measuring 13–35 × 12–23 µm; ascomatal initials arising as side branches from mycelium, involving neighbouring hyphae, coiled and contorted. *Paraphyses* absent. *Asci* 8-spored, clavate, short-stipitate, 200–300 × 20–25 µm, without apical structures, soon evanescent. *Ascospores* uniseriate, aseptate, ellipsoidal, (30–)33–36 × (18–)22–25 µm, at first with a hyaline, smooth-walled exosporium and a pigmented, echinulate endosporium, becoming dark brown to black at maturity, opaque, strongly verrucose to tuberculate, with a slightly protruding apical germ pore, 1.5–2.5 µm diam.

Culture characteristics — Colonies on potato carrot agar attaining a diameter of more than 80 mm in 14 d at 25 °C, velvety to powdery, brown (5E5–5F5) (Kornerup & Wanscher 1984); reverse dark brown (8F4), black at the margins. Colonies on oatmeal agar attaining 52–61 mm diam in 14 d at 25 °C, velvety to granular, brown (5E5–5F5); reverse of the same colour than the surface. The fungus does not grow below 5 °C nor above 40 °C. Optimal ascomatal production occurred between 15 °C and 25 °C.

*Typus*. Spain, Gran Canaria, Pico de Osorio, from forest soil, 10 Aug. 2009, coll. *M. Calduch & A.M. Stchigel*, isol. *Y. Marín* (holotype CBS H-21353, cultures ex-type CBS 135818 = FMR 12156; other strain CBS 136362 = FMR 12323; LSU sequences of CBS 135818 and CBS 136362 GenBank HG326871 and HG326872, respectively; Tub sequences of CBS 135818 and CBS 136362 GenBank HG423208 and HG423209, respectively; and tef-1 sequences of CBS 135818 and CBS 136362 GenBank HG326875 and HG326876, respectively; MycoBank MB804881).

Notes — Hitherto, *Pseudoneurospora* has been a monotypic genus (García et al. 2004). The molecular analyses carried out by Nygren et al. (2011), based on seven gene regions including fragments of the β-tubulin (Tub), translation elongation factor 1-α (tef-1), protein kinase C (pkc), 28S rDNA, mitogen-activated protein kinase-2 (mak-2), nonidentical kinase-1 (nik-1) and hypothetical protein-coding (NCU02332) genes, corroborated *Pseudoneurospora* as a different genus to *Neurospora*. Our phylogenetic tree obtained by sequencing of 28S rDNA, Tub and tef-1 loci corroborated our fungus as representing a new species of *Pseudoneurospora*. *Pseudoneurospora canariensis* can be distinguished from *Pseudoneurospora amorphoporcata*, the type species of the genus, by the production of small, non-ostiolate ascomata (pyriform and ostiolate in *P. amorphoporcata*), the absence of an apical ring in the asci (present in those of *P. amorphoporcata*) and by the presence of a single apical germ pore on the ascospores (one at each end in *P. amorphoporcata*).




Maximum likelihood tree obtained from the combined DNA sequence data from three loci of our isolates and sequences retrieved from the GenBank (Nygren et al. 2011) (TreeBASE ID 14380). In the tree, branch lengths are proportional to distance. Bootstrap support values ≥ 70/Bayesian posterior probability scores ≥ 0.95 are indicated on the nodes. *Sordaria fimicola* (FGSC2918) was used as outgroup. Ex-type strains of the different species are indicated with T. Strains of the new species proposed in this study are indicated in **bold**.

*Colour illustrations*. Pico de Osorio, Gran Canaria, Spain. Ascomata, ascus and ascospores. Scale bars (top) = 50 µm, (bottom) = 10 µm.

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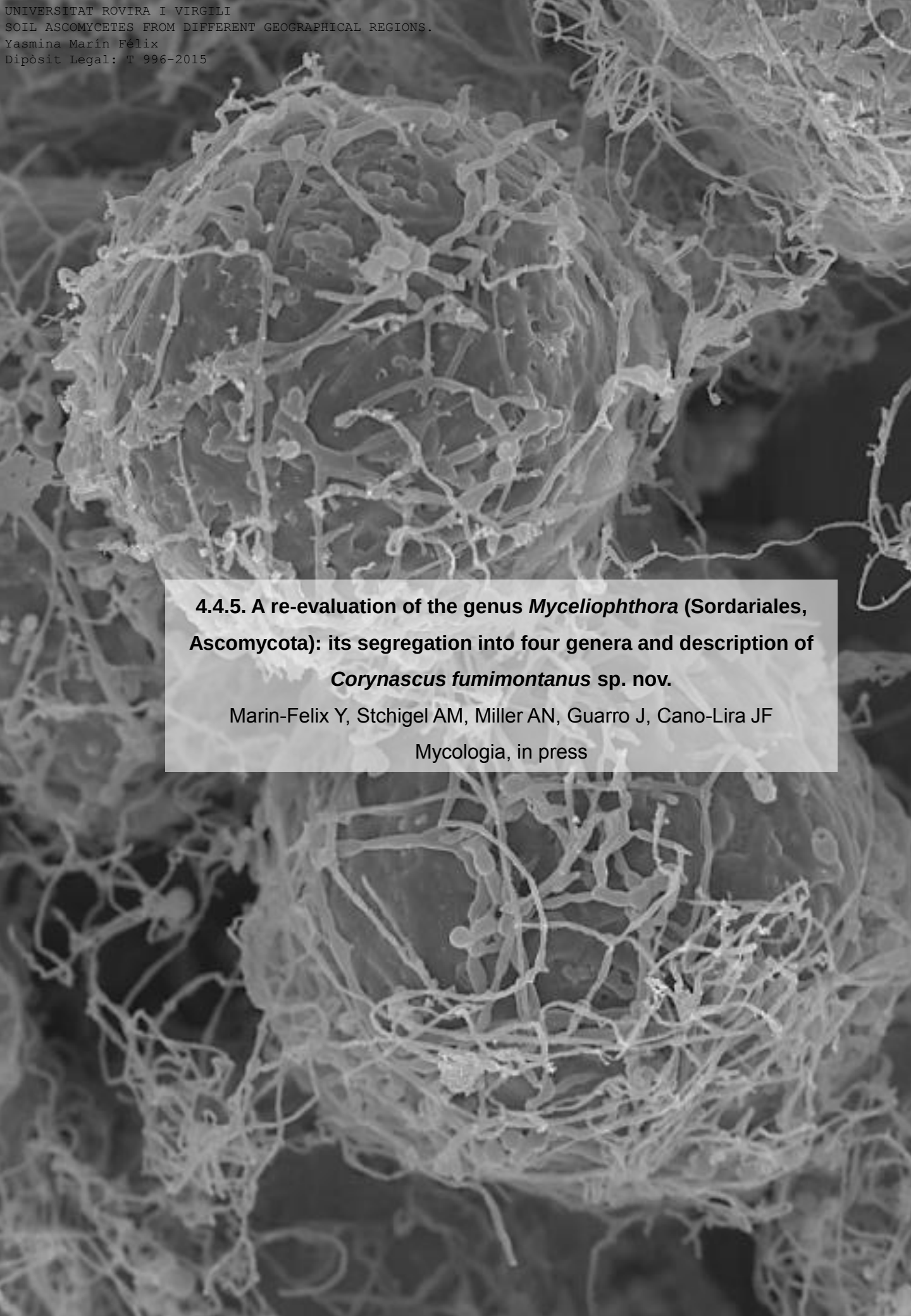

**4.4.5. A re-evaluation of the genus *Myceliophthora* (Sordariales, Ascomycota): its segregation into four genera and description of *Corynascus fumimontanus* sp. nov.**

Marin-Felix Y, Stchigel AM, Miller AN, Guarro J, Cano-Lira JF

Mycologia, in press

In Press at Mycologia, preliminary version published on February 6, 2015 as doi:10.3852/14-228

Short title: Segregation genus *Myceliophthora*

A re-evaluation of the genus *Myceliophthora* (Sordariales, Ascomycota): its segregation into four genera and description of *Corynascus fumimontanus* sp. nov.

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***Abstract:*** Based on a number of isolates of *Myceliophthora* (Chaetomiaceae, Sordariales, Ascomycota) recently isolated from soil samples collected in USA, the taxonomy of the genus was re-evaluated through phylogenetic analyses of sequences from the nuc rDNA internal transcribed spacer region and genes for the second largest subunit of RNA polymerase II and translation elongation factor 1□. Members of *Myceliophthora* were split into four monophyletic clades strongly supported by molecular and phenotypic data. Such clades correspond with *Myceliophthora*, now restricted only to the type species of the genus *Corynascus*, which is re-established with five species, the new monotypic genus *Crassicarpon* and also the new genus *Thermothelomyces* (comprising four species). *Myceliophthora lutea* is mesophilic and a permanently asexual morph compared to the members of the other three mentioned

genera, which also are able to sexually reproduce morphs with experimentally proven links to their asexual morphs. The asexual morph of *M. lutea* is characterized by broadly ellipsoidal, smooth-walled conidia with a wide, truncate base. *Crassicarpon thermophilum* is thermophilic and heterothallic and produces spherical to cuneiform, smooth-walled conidia and cleistothecial ascomata of smooth-walled, angular cells and ascospores with a germ pore at each end. *Corynascus* spp. are homothallic and mesophilic and produce spherical, mostly ornamented conidia and cleistothecial ascomata with textura epidermoidea composed of ornamented wall cells, and ascospores with one germ pore at each end. *Thermothelomyces* spp. are thermophilic, heterothallic and characterized by similar ascomata and conidia as *Corynascus* spp., but its ascospores exhibit only a single germ pore. A dichotomous key to distinguish *Myceliophthora* from the other mentioned genera are provided, as well as dichotomous keys to identify the species of *Corynascus* and *Thermothelomyces*. A new species, namely *Corynascus fumimontanus*, characterized by verrucose ascomatal wall cells and irregularly shaped ascospores, is described and illustrated.

***Key words:*** Chaetomiaceae, *Crassicarpon*, Pezizomycotina, soilborne fungi, *Thermothelomyces*

## INTRODUCTION

*Myceliophthora* spp. (Chaetomiaceae, Sordariales) traditionally were characterized by the production of one-celled, subhyaline to reddish brown, smooth-walled to verrucose, globose to pyriform blastoconidia, sessile or arising on swollen protrusions from the vegetative hyphae, solitary or in short chains, and show a narrow basal scar due to their rhexolytic dehiscence (Oorschot 1980). Mycelia and conidia of *Myceliopthora* spp. are mostly hyaline or nearly so, with the only exception of the conidia of *Myceliophthora hinnulea*, which become dark brown with age, and the

mycelium of *Myceliopthora vellerea*, which is pale brown. The conidiogenesis in *Myceliophthora* spp. is similar among them, producing holoblastic conidia, sessile (frequently named as aleuroconidia) or on micronematous to semimicronematous conidiophores, mostly solitary but also grouped in short chains of 2–4 conidia. Sessile holoblastic conidia are also present in other members of the family Chaetomiaceae, that is *Thielavia arenaria*, *Thielavia microspora* and *Thielavia subthermophila* (Mouchacca 1973). *Myceliophthora* spp. are mostly found in soil but they also have been reported on compost used for growing mushrooms (Costantin and Matruchot 1894), some species being parasites of mushrooms (Costantine 1892) and rarely infecting human (Hoog et al. 2000).

The genus *Myceliophthora* was erected by Costantin (1892) to accommodate the mycoparasitic fungus *Myceliophthora lutea*, characterized by pyriform to globose conidia born terminally or laterally on aerial hyphae, sometimes with a basal short pedicel, and occasionally producing an additional apical conidium. Later three new species were added to the genus, they are *Myceliophthora sulphurea* Goddard (Goddard 1913), *Myceliophthora fusca* Doyer (Doyer 1927) and *Myceliophthora inflata* Burnside (Burnside 1928). van Oorschot (1977, 1980) revised the genus and transferred three additional species, *Myceliophthora fergusii* (Klopotek) Oorschot and *Myceliophthora thermophila* (Apinis) Oorschot from *Chrysosporium* and *Myceliophthora vellerea* (Sacc. & Speg.) Oorschot from *Sporotrichum*. The same author excluded *M. fusca*, *M. inflata* and *M. sulphurea* from the genus. *Myceliophthora fusca* was thought to be identical to *Ptychogaster rubescens* Boud., the anamorph of the basidiomycete *Punctularia atropurpurascens* (Berk. & Br.) Petch; *M. inflata* was synonymized with *Taifanglania inflata* (Burnside) Z.Q. Liang, Y.F. Han & H.L. Chu; and *M. sulphurea* was found indistinguishable from *Chrysosporium merdarium* (Ehrenb.) J.W. Carmich.

More recently *Myceliophthora hinnulea* Awao & Udagawa (Awao and Udagawa 1983) was described.

The sexual morphs of *Myceliophthora* have been included in several genera belonging to different orders and even classes, *Arthroderma* (*Arthroderma tuberculatum* Kuehn; order Onygenales; class Eurotiomycetes), *Corynascus* (*Corynascus* spp.; order Sordariales; class Sordariomycetes) and *Ctenomyces* (*Ctenomyces serratus* Eidam; order Onygenales; class Eurotiomycetes) (Oorschot 1980, Guarro et al. 1985, Stchigel et al. 2000). Some of these sexual morphs from heterothallic species have been obtained in vitro by crossing sexually compatible strains, as is the case for *Arthroderma tuberculatum*, *Myceliopthora thermophila*/*Corynascus heterothallicus* and *Myceliophthora fergusii*/*Corynascus thermophilus* (Klopotek 1974, 1976). On the other hand, crossings of isolates of *Myceliophthora gutulata*, *Myceliophthora hinnulea* and *Myceliophthora lutea* have never been reported to produce their sexual stage. The homothallic species of *Corynascus* produced ascomata in monospore cultures on several culture media (von Arx et al. 1984; Oorschot 1980). *Myceliophthora* spp. linked with their sexual morph have never been treated as a *Myceliophthora*-like asexual stage.

The genus *Corynascus* was proposed by von Arx in 1973 based on two species of *Thielavia* (i.e. *Thielavia novoguineensis* Udagawa & Y. Horie and *Thielavia sepedonium* C.W. Emmons) that possess ascospores with two germ pores, one at each end, as opposed to species of *Thielavia* that have only a single germ pore. Three additional species of *Thielavia* also were transferred under the same criteria to *Corynascus*, (i.e. *Thielavia heterothallica* Klopotek, *Thielavia setosa* Dade and *Thielavia thermophila* Fergus & Sinden Klopotek [Klopotek 1974, von Arx 1975, von Arx et al. 1984]. In 1978 *Corynascus setosus* moved to *Chaetomidium* (Lodha 1978). In

the current century, three new species have been included in *Corynascus* (i.e. *Corynascus sexualis* Stchigel, Cano & Guarro, *Corynascus similis* Stchigel, Cano & Guarro and *Corynascus verrucosus* Stchigel, Cano & Guarro [Stchigel et al. 2000]).

In a recent phylogenetic study (van den Brink et al. 2012), *Corynascus* spp. grouped together with the type species of *Myceliophthora* (*M. lutea*), and based on the current fungal nomenclature (McNeill et al. 2012), the name *Myceliophthora* was chosen while *Corynascus* was considered a synonym. In the same study *Myceliophthora vellerea* was placed far from *M. lutea*, clustering with *C. serratus* and *A. tuberculatum* in a different and phylogenetically distant clade (family Arthrodermataceae), being therefore excluded from *Myceliophthora*. *Myceliophthora* is restricted currently to those species belonging to the family Chaetomiaceae (Sordariales), characterized by the production of cleistothecial ascomata with an ascomata wall of textura epidermoidea, unitunicate asci and one-celled, ellipsoidal or broadly fusiform, brownish ascospores, usually with a distinct germ pore at each end (Stchigel et al. 2000, Guarro et al. 2012). Zhang et al. (2014) described the new species *Myceliophthora guttulata* Y. Zhang & L. Cai, from a soil sample in China. The following 11 species of *Myceliophthora* currently are accepted (i.e. the already mentioned *M. fergusii*, *M. guttulata*, *M. heterothallica*, *M. hinnulea*, *M. lutea*, *M. thermophila*, in addition to *M. novoguineensis* [Udagawa & Y. Horie] van den Brink & Samson, *M. sepedonium* [C.W. Emmons] van den Brink & Samson, *M. sexualis* [Stchigel, Cano & Guarro] van den Brink & Samson, *M. similis* [Stchigel, Cano & Guarro] van den Brink & Samson, and *M. verrucosa* [Stchigel, Cano & Guarro] van den Brink & Samson [van den Brink et al. 2012, Zhang et al. 2014]).

During a survey on soilborne ascomycetes from Great Smoky Mountains National Park (USA), several fungi belonging to *Myceliophthora* were isolated. Becausee some of these isolates could not be properly identified, a phylogenetic and

phenotypic study was conducted to better define the boundaries between *Myceliophthora* and related genera, resulting in the proposal of two new genera and one new species.

## MATERIALS AND METHODS

*Soil sampling and isolation of fungi.*—Soil samples were collected in Aug 2008 in Great Smoky Mountains National Park (35.60, −83.52), USA, located in Tennessee and North Carolina and containing more than 2100 square kilometres. This area is mainly composed of cove hardwood, hemlock, northern hardwood, pine-oak and spruce-fir forests (Whittaker 1956) and includes more than 1570 species of vascular plants of which 130 are native trees (Sharkey 2001). To carry out the isolation of soilborne ascomycetes we followed a previously described protocol for activation of the dormant ascospores using acetic acid (Stchigel et al. 2001). Fungal structures of those specimens that developed in the primary cultures were examined under the stereomicroscope and transferred with a sterile needle to Petri dishes containing oatmeal agar (OA; oatmeal flakes, 30 g; agar-agar, 20 g; tap water, 1 L), and incubated at 15, 25 and 35 C.

*Phenotypic study.*—Fungal isolates were grown on OA, 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) and potato dextrose agar (PDA; Pronadisa, Madrid, Spain) at 5, 15, 25, 30, 35, 40, 45 and 50 C. Color notations in parentheses in the species descriptions are from Kornerup and Wanscher (1984). Fertile fungal structures were mounted and measured in lactic acid. Photomicrographs were obtained with a Zeiss Axio Imager M1 light microscope. The scanning electron microscope (SEM) techniques used were described by Figueras and Guarro (1988). SEM micrographs were taken with a Jeol JSM 840 at 15 keV.

*Phylogenetic studies.*—DNA of the isolates was extracted and purified directly from fungal colonies according to the Fast DNA Kit protocol (MP Biomedicals, Solon, Ohio). The amplification of the internal transcribed spacer region (ITS) of the nuc rDNA (ITS1-5.8S-ITS2) and partial segments of the translation elongation factor 1-□ (*EF*1) and RNA polymerase II (*RPB*2) loci was performed for all isolates, according to Cano et al. (2004) (ITS) and Houbraken et al. (2007) (*RPB*2 and *EF*1). The sequences of these amplicons were obtained with the protocol of the Taq Dye-Deoxy Terminator Cycle Sequencing Kit, and PCR products were purified and sequenced by Macrogen Europe (Amsterdam, the Netherlands) with a 3730XL DNA analyzer (Applied Biosystems). Consensus sequences were obtained with SeqMan

(7.0.0; DNASTAR, Madison, Wisconsin), and the sequences were aligned with Clustal X 2.0 (Larkin et al. 2007) followed by manual adjustments with a text editor. Sequences retrieved from GenBank and included in these analyses are provided (TABLE I). The phylogenetic analyses was carried out with MEGA 5.21 of the combined dataset (ITS, *RPB*2, *EF*1) of our isolates, the type and reference strains of the accepted species of *Myceliophthora*, the type strain of *Corynascella inaequalis* and one strain of *Thielavia terricola*, *Chaetomidium arxii* and *Chaetomium globosum*, using the type strain of *Hypocrea aurantefussa* and a strain of *Nectria pseudotrichia* as outgroups, (Tamura et al. 2011). The combined dataset was tested for incongruence with the partition homogeneity test (PHT) as implemented in PAUP* (Swofford 2002). Maximum likelihood (ML) analysis was conducted on the dataset using the Tamura-Nei model, with gamma distribution and the pairwise deletion of gaps option. The robustness of branches was assessed by bootstrap analysis with 1000 replicates. Bayesian inference (BI) was carried out with MrBayes 3.1 following the parameters detailed in Alvarez et al. (2010). The sequences generated in this study are deposited in GenBank, and the alignments used in the phylogenetic analyses are deposited in TreeBASE: (www.treebase.org, accessionURL: http://purl.org/phylo/treebase/phylows/study/TB2:S16736).

## RESULTS

The individual alignments used in the combined dataset were 473 bp (ITS), 634 bp (*EF1*) and 499 bp (*RPB2*), and the final total alignment was 1606 bp, 361 bp of which were parsimony informative. Because the result of the partition homogeneity test showed that the dataset for the three loci were congruent ($P = 0.508$), they were combined into a single dataset. ML analysis produced a single tree (FIG. 1). Three of our recently collected American isolates (CBS 137294, CBS 135878, CBS 137791) grouped in a main clade (71% bs and less than 0.95 bayesian posterior probability [pp]) with the type strains of *M. lutea*, *M. novoguineensis*, *M. sepedonium*, *M. sexualis* and *M. similis*. This clade was divided into two sister clades. The first one included the type strain and other strains of *M. lutea* (100% bs/1 pp), which were characterized by holoblastic, pyriform to globose, thick- and smooth-walled hyaline conidia, broadly truncate at the base, sometimes with a pedicel, borne terminally or laterally on aerial

hyphae (FIG. 2). *Myceliophthora lutea* is mesophilic, with an optimal growth at 30–35 C. The second sister clade (99% bs/1 pp) grouped species that previously were included in *Corynascus*, including the type species of this genus. For this reason we think *Corynascus* should be re-established. Within the *Corynascus* spp. sister clade our isolate CBS 137294 formed a terminal branch, although at significant distance, together with *M. sexualis* (100% bs/1 pp). This isolate had both asexual and sexual morphs, the latter being characterized by cleistothecial ascomata with an ascomatal wall of textura epidermoidea composed of verrucose cells, and irregularly-shaped ascospores with a germ pore at each end. Its conidia were globose, yellowish and verrucose (FIG. 3). The optimal growth of this fungus was at 35–40 C. This combination of features does not match any known species. Its most closely related species, *M. sexualis*, can be differentiated by the absence of an asexual morph and ascomata composed of verrucose cells and limoniform ascospores. The type strains of *M. novoguineensis*, *M. sepedonium*, *M. sexualis*, *M. similis* and *M. verrucosa*, all grouped in the same clade, being characterized by their homothallism, in contrast with the members of the other clades, and by the production of cleistothecial ascomata of textura epidermoidea and ornamented (mostly reticulate) ascomata wall cells, and brown ascospores with a distinct germ pore at each end (FIG. 4). The asexual morph was observed in all these species with the exception of *M. sexualis*, as was reported by Stchigel et al. (2000), and was characterized by holoblastic, spherical or nearly so, hyaline to pale yellow conidia with an ornamented cell wall, except for *M. novoguineensis*, which produced smooth-walled conidia, sessile or on short protrusions, sometimes also on swollen, sometimes catenate, conidiogenous cells (FIG. 5). These species were mesophilic with an optimal growth at 25–40 C. The type strains of *M. sepedonium* and *M. similis* grouped together in the same clade with a nucleotide identity over 99%. Morphologically both species

were distinguished only by the shape of the ascospores and the position of the germ pores (i.e. irregularly shaped ascospores with two subapical germ pores in *M. similis*) and broadly fusiform ascospores with two apical germ pores in *M. sepedonium*.

The other species of *Myceliophthora* (i.e. *M. fergusii*, *M. guttulata*, *M. heterothallica*, *M. hinnulea*, *M. thermophile*) were located in two distinct, well-supported sister clades, each of them representing a new genus. The first contains the type strains of *M. guttulata*, *M. heterothallica*, *M. hinnulea* and *M. thermophila* (98% bs/1 pp), and the other includes *M. fergusii* (100% bs/1 pp). The members of both clades were thermophilic, with an optimal growth at 40–45 C. The species in the first clade produced holoblastic, subglobose or obovoid to ellipsoidal conidia truncate at the base, brown, thick-walled and ornamented, with the exception of *M. guttulata* that produces hyaline, smooth-walled and guttulate conidia on terminally and laterally on hyphae (sessile), or on ampulliform to clavate polyblastic conidiogenous cells, sometimes with a short or long basal pedicel (FIG. 2). Only *M. heterothallica* was capable of producing sexual morphs in culture after mating sexually compatible strains. They were dark cleistothecial ascomata with an ascomatal wall of textura epidermoidea, producing ellipsoid to ovoid ascospores with a terminal germ pore. The strains of *M. fergusii* produced holoblastic, hyaline to yellow in mass, thick- and smooth-walled conidia, sessile or in swollen conidiogenous cells, arising singly or in chains of up to five conidia (FIG. 2). This fungus was also heterothallic, producing cleistothecial ascomata with a thick-walled ascomatal wall of textura angularis and ellipsoidal ascospores, pinkish when young but becoming dark brown, with a germ pore at each end.

The nucleotide identities in the combined dataset among the type strains of *M. fergusii*, *M. lutea*, *M. sepedonium* and *M. thermophila* were ≤ 93%.

TAXONOMY

Based on the molecular and phenotypic results mentioned above, we propose the revalidation of *Corynascus* as a genus distinct from *Myceliophthora*, and the new genera *Thermothelomyces* and *Crassicarpon*. To accommodate the isolate CBS 137294, we propose the new species *C. fumimontanus.*

KEY TO THE GENERA *CORYNASCUS*, *CRASSICARPON*, *MYCELIOPHTHORA* AND *THERMOTHELOMYCES*

1. No growth at 50 C.……...2

1. Growth at 50 C.....……3

2. Sexual morph present in culture.....……..……..*Corynascus*

2. Sexual morph absent in culture..……..……*Myceliophthora*

3. Conidia hyaline, spherical to cuneiform, smooth-walled……..*Crassicarpon*

3. Conidia brown, subglobose or obovoid to ellipsoidal; ornamentated or, rarely, smooth……...……..…..… *Thermothelomyces*

***Corynascus*** Arx, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 76: 295. 1973. FIGS. 3, 4, 5

*Type species*: *Corynascus sepedonium* (C.W. Emmons) Arx, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 76: 292. 1973.

Notes. *Corynascus* is characterized by its mesophilic habit, having an ascomata wall of textura epidermoidea composed of reticulate or verrucose cells, ascospores with a germ pore at each end and yellowish conidia usually verrucose or echinulate to tuberculate, rarely smooth.

*Corynascus fumimontanus* Y. Marín, Stchigel, Cano & A.N. Mill., sp. nov. FIGS. 3; 4a, f; 5a–d

MycoBank MB809486

*Typification*. USA. TENNESSEE: Great Smoky Mountains National Park, Cosby Creek trail, 35.78, −83.22, from forest soil, 01-VIII-2008, *A.N. Miller*, *M. Calduch*, *A.M.*

*Stchigel*. (**holotype** CBS H-21594). **Isotypes** FMR 12372, ILLS 71950. Ex-type cultures FMR 12372, CBS 137294.

*Etymology:* From the Latin *fumi*-, smoky, and -*montanus*, mountains, referring to the name of the national park where the fungus was isolated.

*Diagnosis*: This species is characterized by verrucose ascomata wall cells, mostly irregularly shaped ascospores, greenish brown when young, and conidia sessile, intercalary or on swollen conidiogenous cells.

Mycelium composed of hyaline to pale yellow, branched, anastomosing, septate, smooth-walled hyphae of 1–2 µm diam. Colonies on PCA attaining 72–75 mm diam in 14 d at 35 C, light yellow with olive patches, olive gray at center, flattened, powdery to granular due to the production of conidia and ascomata, margins fimbriate; reverse pale yellow to light yellow, with olive patches. Ascomata superficial, globose, cleistothecial, brown to dark brown, 50–110 µm diam, ascomata wall of textura epidermoidea, composed of 1–3 layers of irregularly shaped, verrucose, golden brown to brown cells, covered by hyphae anastomosing with the ascomata wall cells. Paraphyses absent. Asci eight-spored, subglobose to broadly ellipsoidal, 24–31 × 15×22 µm, thin-walled, short-stipitate, evanescent. Ascospores one-celled, broadly fusiform to irregularly shaped, 13–17 × 7–9 µm, hyaline to greenish brown when young becoming brown, thick- and smooth-walled, with a conspicuous subterminal to terminal germ pore at each end. Conidiophores micronematous, 1–2.5 µm wide and up to 26 µm long, or semimacronematous, flask-shaped, 5–10 × 3–8 µm. Conidia holoblastic, globose to subglobose, 6–10 µm diam, subhyaline to pale yellow, thick-walled, verrucose, sessile or on swollen conidiogenous cells, and holothallic when intercalary, morphologically similar to the holoblastic ones.

Colonies on PDA attaining 73–75 mm diam in 14 d at 35 C, yellowish white to pale yellow, velvety to powdery, radially folded, umbilicate, lobulate, margins regular; reverse pale yellow to light yellow. Ascomata absent. The minimum and maximum temperature of growth are 15 and 45 C respectively. Optimal temperature 35–40 C.

*Corynascus novoguineensis* (Udagawa & Y. Horie) Arx, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 76: 295. 1973. Figs. 4b, g, h; 5e͂h

*Basionym: Thielavia novoguineensis* Udagawa & Y. Horie, Bull. natn. Sci. Mus., Tokyo 15: 191. 1972.

≡ *Myceliophthora novoguineensis* (Udagawa & Y. Horie) van de Brink & Samson, in Brink, Samson, Hagen, Boekhout & Vries, Fungal Divers. 52: 206. 2012.

*Notes. Corynascus novoguineensis* is characterized by slightly verrucose ascomata wall cells, pinkish ascospores when young, and smooth-walled conidia. In the original description the immature ascospores were described as greenish brown (Udagawa and Horie 1972).

*Corynascus sepedonium* (C.W. Emmons) Arx, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 76: 292. 1973. Figs. 4c, i; 5i͂l

*Basionym: Thielavia sepedonium* C.W. Emmons, Bull. Torrey bot. Club 59: 417. 1932

≡ *Chaetomidium sepedonium* (C.W. Emmons) Lodha, in Subramanian (Ed.), Taxonomy of Fungi (Proc. int. Symp. Madras, 1973), Pt 1: 248. 1978.

≡ *Myceliophthora sepedonium* (C.W. Emmons) van den Brink & Samson, in Brink, Samson, Hagen, Boekhout & Vries, Fungal Divers. 52: 206. 2012.

= *Thielavia sepedonium* var. *minor* B.S. Mehrotra & Bhattacharjee, Antonie van Leeuwenhoek 32: 391. 1966.

= *Myceliophthora similis* (Stchigel, Cano & Guarro) van de Brink & Samson, in Brink, Samson, Hagen, Boekhout & Vries, Fungal Divers. 52: 206. 2012.

Ascomata superficial, globose, cleistothecial, brown to dark brown, 50–110 µm diam, glabrous, ascomata wall of textura epidermoidea, composed of 1–3 layers of irregularly shaped, reticulate, golden-brown to brown cells. Paraphyses absent. Asci eight-spored,

subglobose to broadly ellipsoidal, 26–40 × 20–31 µm, thin-walled, short-stipitate, evanescent. Ascospores one-celled, ellipsoidal to broadly fusiform or navicular in lateral view, 11–23 × 6.5–13 µm, hyaline becoming brown when mature, thick- and smooth-walled, with a conspicuous subterminal to terminal germ pore at each end. Conidiophores micronematous or semimacronematous. Conidia holoblastic, globose to subglobose, 6–12 µm diam, subhyaline to pale yellow, thick-walled, finely echinulate to tuberculate, sessile or on swollen conidiogenous cells.

*Notes*. *Corynascus sepedonium* is characterized by reticulate ascomata wall cells and echinulate to tuberculate conidia. The description is from the protolog with slight modifications based on the study of the type strain of *C. similis* (IMI 378521).

*Corynascus sexualis* Stchigel, Cano & Guarro, in Stchigel, Sagués, Cano & Guarro, Mycol. Res. 104: 880. 2000. Fig. 4d, j

≡ *Myceliophthora sexualis* (Stchigel, Cano & Guarro) van de Brink & Samson, in Brink, Samson, Hagen, Boekhout & Vries, Fungal Divers. 52: 206. 2012.

*Notes*. *Corynascus sexualis* differs from the other species of the genus by the lack of asexual morph and its lemon-shaped ascospores.

*Corynascus verrucosus* Stchigel, Cano & Guarro, in Stchigel, Sagués, Cano & Guarro, Mycol. Res. 104: 884. 2000. Figs.; 4e, k; 5m–p

≡ *Myceliophthora verrucosa* (Stchigel, Cano & Guarro) van de Brink & Samson, in Brink, Samson, Hagen, Boekhout & Vries, Fungal Divers. 52: 206. 2012.

*Notes*. *Corynascus verrucosus* is characterized by verruciform dark brown projections from the ascomata wall, and broadly fusiform ascospores with a subterminal germ pore at each end.

KEY TO THE SPECIES OF *CORYNASCUS*

1. Asexual morph absent; ascospores limoniform…….*C. sexualis*

1. Asexual morph present; ascospores irregularly shaped, ellipsoidal or fusiform...……2

2. Conidia smooth-walled, or nearly so; ascospores pinkish when young……...……..……..*C. novoguineensis*

2. Conidia verrucose or tuberculate; ascospores greenish or brownish when young.……3

3. Ascomata wall cells with verrucose projections; ascospores irregularly shaped……...……*C. fumimontanus*

3. Ascomata wall cells reticulated……...…….4

4. Ascomata glabrous; ascospores ellipsoidal to broadly fusiform...…...*C. sepedonium*

4. Ascomata with short, brown verruciform projections on entire ascomata wall; ascospores broadly fusiform..…..……..……..……*C. verrucosus*

**Crassicarpon** Y. Marín, Stchigel, Guarro & Cano, gen. nov. FIG. 2a–g

MycoBank MB809487

*Type species: Crassicarpon thermophilum* (Fergus & Sinden) Y. Marín, Stchigel, Guarro & Cano.

*Etymology:* From the Greek *Crassum*- and -*karpos*, referring to the thick ascomatal wall.

*Diagnosis:* Characterized by its thermophilic habit, blackish ascomata with a thick wall of textura angularis, broadly ellipsoidal ascospores with a germ pore at each end, and hyaline, smooth-walled conidia, yellow in mass.

Ascomata superficial or immersed, globose, cleistothecial, dark brown to black, glabrous, ascomatal wall thick, of textura angularis, composed of an outer layer of thick-walled swollen cells, and an inner layer of flattened cells. Asci 4–6-spored, broadly clavate, thin-walled, stalked, evanescent. Paraphyses absent. Ascospores one-celled, broadly ellipsoidal, first hyaline, becoming pink and finally dark brown, smooth- and thick-walled, with a germ pore at each end. Conidiophores micronematous or semimacronematous. Conidia holoblastic, hyaline to yellow in mass with the age, spherical to cuneiform, variable in size, thick- and smooth-walled, sessile or produced

in swollen conidiogenous cells, sometimes with short pedicels; secondary apical conidia may be produced. Heterothallic. Thermophilic.

**Crassicarpon thermophilum** (Fergus & Sinden) Y. Marín, Stchigel, Guarro & Cano, comb. nov. FIG. 2a–g

MycoBank MB809488

*Basionym: Thielavia thermophila* Fergus & Sinden, Can. J. Bot. 47: 1635. 1969.

≡ *Corynascus thermophilus* (Fergus & Sinden) Klopotek, Arch. Mikrobiol. 98: 366. 1974.

≡ *Chaetomidium thermophilum* (Fergus & Sinden) Lodha, in Subramanian (Ed.), Taxonomy of Fungi (Proc. int. Symp. Madras, 1973), Pt 1: 248. 1978.

= *Myceliophthora fergusii* (Klopotek) Oorschot, Persoonia 9: 406. 1977.

≡ *Chrysosporium fergusii* Klopotek, Arch. Mikrobiol. 98: 366. 1974.

*Notes.* We decided to use the epithet *thermophilum* instead *fergusii*, which had been chosen by van den Brink et al. (2012) for this taxon because *Thielavia thermophila* was the first morph described.

*Myceliophthora* Costantin, C. r. hebd. Séanc. Acad. Sci., Paris 114: 849. 1892. FIG. 2h–k

*Type species*. *Myceliophthora lutea* Costantin, C. r. hebd. Séanc. Acad. Sci., Paris 114: 2. 1892. Fig. 2h̄k

*Notes. Myceliophthora* is characterized by its mesophilic habit, hyaline and smooth-walled conidia and the lack of sexual morph.

**Thermothelomyces** Y. Marín, Stchigel, Guarro & Cano, gen. nov. FIGS. 2l–o

MycoBank MB809489

*Type species: Thermothelomyces thermophila* (Apinis) Y. Marín, Stchigel, Guarro & Cano.

*Etymology*. From the Greek *thermos*-, hot, *thelo*-, love, and -*myces*, fungi, referring to the thermophilic habit of the fungus.

*Diagnosis:* Characterized by its thermophilic habit, ascomata with a wall of textura epidermoidea, ellipsoidal ascospores with a single apical germ pore, and hyaline or pale brown conidia, mostly ornamented.

Ascomata immersed to sub-immersed, globose, cleistothecial, black, glabrous, ascomata wall thin, of textura epidermoidea. Asci eight-spored, ellipsoidal, thin-walled, stalked, evanescent. Paraphyses absent. Ascospores ellipsoidal, occasionally irregularly shaped, first hyaline, dark brown to black, thick- and smooth-walled, with one germ pore. Conidiophores micronematous or semimacronematous. Conidia holoblastic, hyaline or pale brown, subglobose, ellipsoidal or obovoid to pyriform, thick-walled, conspicuously verrucose-spinulose or tuberculate, rarely smooth-walled and guttulate, producing terminally or laterally on hyphae, sometimes with short or long pedicels, or on swollen conidiogenous cells in a number of 1–4; occasionally a secondary apical conidium is produced. Heterothallic. Thermophilic.

**Thermothelomyces guttulata** (Y. Zhang & L. Cai) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB 809490

*Basionym: Myceliophthora guttulata* Y. Zhan & L. Cai, Mycol Progress 13: 165. 2014.

*Notes. Thermothelomyces guttulata* is distinguished from the other species by its hyaline, smooth-walled and guttulate conidia.

**Thermothelomyces heterothallica** (von Klopotek) Y. Marín, Stchigel, Guarro & Cano, comb. nov. FIGS. 2l–o

MycoBank MB809491

*Basionym: Thielavia heterothallica* von Klopotek, Arch. Mikrobiol. 107: 223. 1976.

≡ *Corynascus heterothallicus* (von Klopotek) von Arx, Dreyfuss & Müller, Persoonia 12: 174. 1984.

≡ *Myceliophthora heterothallica* (von Klopotek) van den Brink & Samson, in Brink, Samson, Hagen, Boekhout & Vries, Fungal Divers. 52: 206. 2012.

*Notes.* This species is characterized by pale orange-brown, long ellipsoidal, tuberculate conidia and the production of ascomata after mating. The conidia previously were described as hyaline (Klopotek 1974, 1976; van Oorschot 1977).

**Thermothelomyces hinnulea** (Awao & Udagawa) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB809492

*Basionym: Myceliophthora hinnulea* Awao & Udagawa, Mycotaxon 16: 436. 1983.

*Notes.* This species is characterized by yellowish brown to brown, subglobose to ovate, conspicuously verrucose-spinulose conidia.

**Thermothelomyces thermophila** (Apinis) Y. Marín, Stchigel, Guarro & Cano, comb. nov.

MycoBank MB809493

*Basionym: Sporotrichum thermophilum* Apinis, Nova Hedwigia 5: 74. 1963.

≡ *Chrysosporium thermophilum* (Apinis) Klopotek, Arch. Mikrobiol. 98: 366. 1974.

≡ *Myceliophthora thermophila* (Apinis) Oorschot, Persoonia 9: 403. 1977.

*Notes*. The asexual morph of this species is similar to those of *T. heterothallica* but *T. thermophila* does not produce a sexual morph after mating.

KEY TO THE SPECIES OF *THERMOTHELOMYCES*

1. Conidia smooth-walled and guttulate, (3.8–)4.8–7.2 × 3–5 µm…....…….*T. guttulata*

1. Conidia with an ornamented surface.……2

2. Conidia 7–12 × 5–10 µm…...……..…….…….*T. hinnulea*

2. Conidia 4.5–11 × 3–4.5 µm....……..…...…….*T. heterothallica/T. thermophila*[*]

[*] *Thermothelomyces heterothallica* produces ascomata after mating.

DISCUSSION

Based on recent molecular studies that demonstrated that *M. lutea*, the type species of *Myceliophthora*, clustered with some members of the family Chaetomiaceae, that genus

was restricted to the species of such family (van den Brink et al. 2012, Zhang et al. 2014); consequently *M. vellerea* (now renamed *Ctenomyces vellereus* [Sacc. & Speg.] P.M. Kirk) and *Myceliopthora* anamorph of *Arthroderma tuberculatum* were transferred to the family Arthrodermataceae, where they were phylogenetically located (van den Brink et al. 2012, Kirk 2014).

In previous phylogenies (van den Brink et al. 2012, 2013) the species of *Myceliophthora* spp. grouped with a confidence value of below 50%, being divided into two main clades (each one composed of one or two terminal clades depending on the nuclear loci employed in the phylogenetic inference) according to their mesophilic and thermophilic habit. However in our study, in agreement with Zhang et al. (2014), *Myceliophthora* spp. formed a well-supported clade, but the genetic distances among the terminal clades (below of 93% similarity) are such that they should be treated as separate genera. Consequently we proposed to split *Myceliophthora* into four genera, revalidating *Corynascus* and erecting two new genera: *Crassicarpon* and *Thermothelomyces*. Our proposal is also supported by phenotypic data (e.g. *Crassicarpon thermophilum* presents dark, thick-walled ascomata with a wall of textura angularis composed of nonornamented ascomata wall cells, while the *Corynascus* spp. and *Thermothelomyces* spp. are characterized by the production of many pale, thin-walled ascomata with a wall of textura epidermoidea composed of ornamented ascomata wall cells. The type of ascomata wall previously had been used successfully in the delimitation of genera in Lasiosphaeriaceae (Miller and Huhndorf 2004, 2005; Cai et al. 2005). The number of germ pores in the ascospores is also a distinctive feature because *Corynascus* spp. and *Crassicarpon thermophilum* have one at each end, whereas *Thermothelomyces heterothallica* presents only one. The conidia are produced by similar ontogenetic processes in all four genera but also show morphological

differences: both *Myceliophthora lutea* and *Crassicarpon thermophilum* produce hyaline, smooth-walled conidia, but while in the first taxon they are pyriform to globose and produced singly (or rarely in chains up to two conidia), in the second one they are spherical to cuneiform and in chains of up to five conidia. *Corynascus* spp. and *Thermothelomyces* spp. produce mostly ornamented, yellowish conidia, even though *Thermothelomyces* spp. present more complex conidiophores, with ovoid to clavate conidia with a truncate base, whereas in *Corynascus* spp. they are spherical.

*Corynascus fumimontanus* sp. nov. is easy to distinguish morphologically from the rest of the species of the genus *Corynascus* by its verrucose ascomata wall cells (reticulate in the other species) and its irregularly shaped ascospores. Finally the synonymy of *C. similis* with *C. sepedonium* was proposed due to the high nucleotide identity and the minor morphological differences among them.

ACKNOWLEDGMENTS

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## LEGENDS

FIG. 1. Maximum-likelihood (ML) tree obtained from the combined DNA sequence data from three loci (ITS, *EF1* and *RPB2*) of our isolates, selected strains previously included in the genus *Myceliophthora*, the type strain of *Corynascella inaequalis* and one strain of *Thielavia terricola*, *Chaetomidium arxii* and *Chaetomium globosum*. The type strain of *Hypocrea aurantefussa* and a reference strain of *Nectria pseudotrichia* were used as outgroup. Bootstrap support values ≥ 70/Bayesian posterior probability scores ≥ 0.95 are indicated along branches. Branch lengths are proportional to distance. Type strains of the different species are indicated with $^{T}$.

FIG. 2. Asexual morph of the genera *Crassicarpon*, *Myceliophthora* and *Thermothelomyces*. a–g. *Crassicarpon thermophilum* CBS 406.69. a, b. Conidiophores; c–g. conidia. h–k. *Myceliophthora lutea*

MUCL 10070. h–k. Conidiophores bearing conidia. l–o. *Thermothelomyces heterothallica* CBS 137789; l–n. Conidiophores bearing terminal and lateral conidia; o. conidium (SEM). Bars: a, l = 10 µm; b, c, h–k, m, n = 5 µm; d–g, o = 2.5 µm.

FIG. 3. *Corynascus fumimontanus* CBS 137294. a. Ascomata. b. Irregular network of distorted hyphae on the ascomatal wall. c. Detail of the ascomatal wall. d. Asci. e, f. Ascospores. g. Sessile conidia. h. Conidia on short inflated protusion. i. Intercalary conidia. j. Conidia (SEM). Bars: a = 50 µm; b, c, f–j = 5 µm; d = 15 µm; e = 10 µm.

FIG. 4. Sexual morphs of the species of *Corynascus*. *Corynascus fumimontanus* CBS 137294. a. Detail of ascomata wall. f. Ascospores. *Corynascus novoguineensis* NBRC 9556. b. Detail of ascomata wall; g. Ascus and immature pinkish ascospores; h. Ascospores. *Corynascus sepedonium* IMI 378521. c. Detail of ascomata wall; i. Ascospores. *Corynascus sexualis* IMI 378520. d. Detail of ascomata wall. j. Ascospores. *Corynascus verrucosus* CBS 137791. e. Detail of ascomata wall. k. Ascospores. Bars: a–k = 5 µm.

FIG. 5. Asexual morphs of the species of *Corynascus*. a–d. *Corynascus fumimontanus* CBS 137294. a. Sessile conidium; b. Conidia on short inflated protrusion; c. sessile and intercalary conidia; d. conida (SEM). e–h. *Corynascus novoguineensis* NBRC 9556. e. Sessile conidia; f. conidia on short inflated protrusion; g. conidiophores; h. conidium (SEM). i–l. *Corynascus sepedonium* IMI 378521. i. Sessile conidia; j. conidia on short inflated protrusion; k. conidiophore bearing terminal conidium (SEM); l. conidia (SEM). m–p. *Corynascus verrucosus* FMR 12369 (= CBS 137791). m. Sessile conidia; n. conidia on short inflated protrusion; o. conidiophores bearing terminal conidia; p. conida (SEM). Bars: a–c, e–g, i–k, m–o = 5 µm; d, h, l, p = 2.5 µm.

## FOOTNOTES

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TABLE I. Isolates and reference strains of the genus *Myceliophthora* and related genera included in this study

| Taxa | Strain | Source | GenBank accession numbers | | |
|---|---|---|---|---|---|
| | | | ITS | EF1 | RPB2 |
| *Chaetomidium arxii* | FMR 12364 | Soil, Gran Canaria, Spain | KP204014[a] | KP204012[a] | KP204013[a] |
| *Chaetomium globosum* | CBS 148.51 | Man, Greifswald, Germany | GU563374 | KC485028 | NT_165981 |
| *Corynascella inaequalis* | CBS 331.75[T] | Soil, Kirovograd, Ukraine | KP204017[a] | KP204015[a] | KP204016[a] |
| *Corynascus fumimontanus* | CBS 137294[T] (=FMR 12372[T]) | Soil, Great Smoky Mountains National Park, Tennessee | LK932694[a] | LK932719[a] | LK932733[a] |
| *Corynascus novoguineensis* | CBS 359.72[T] | Soil, Papua New Guinea | HQ871762 | HQ871733 | HQ871838 |
| | NBRC 9556 | Soil, unknown location | LK932698[a] | LK932716[a] | LK932731[a] |
| *Corynascus sepedonium* | CBS 111.69[T] | Soil, Uttar Pradesh, India | HQ871751 | HQ871734 | HQ871827 |
| | CBS 632.67 | Unknown source, Russia | HQ871759 | HQ871744 | HQ871830 |
| | IMI 378521 | Soil, Ajmer, India (ex-type strain of *Corynascus similis*) | AJ224201 | LK932715[a] | LK932730[a] |
| *Corynascus verrucosus* | IMI 378522[T] | Soil, Quilmes, Argentina | AJ224203 | LK932723[a] | LK932726[a] |
| | CBS 137791 (=FMR 12369) | Soil, Great Smoky Mountains National Park, Tennessee | LK932699[a] | LK932717[a] | LK932732[a] |
| | CBS 135878 (=FMR 12783) | Soil, Great Smoky Mountains National Park, Tennessee | LK932695[a] | LK932718[a] | LK932734[a] |
| *Corynascus sexualis* | IMI 378520[T] | Soil, Jaipur, India | AJ224202 | LK932714[a] | LK932729[a] |
| *Crassicarpon thermophilum* | CBS 406.69[T] | Mushroom compost, Pennsylvania, USA; MT − | HQ871794 | HQ871732 | HQ871715 |
| | CBS 405.69 | Mushroom compost, Pennsylvania, USA; MT + | HQ871793 | HQ871731 | HQ871714 |
| *Hypocrea aurantefussa* | CBS 119284[T] | Partly decorticated branches on ground, Weins, Austria | FJ860728 | FJ860613 | FJ860520 |
| *Myceliophthora lutea* | CBS 145.77[T] | Hay, UK | HQ871775 | HQ871722 | HQ871816 |
| | MUCL 10070 | Unknown source, Natick, USA | LK932701[a] | LK932710[a] | LK932724[a] |
| | MUCL 10071 | Unknown | LK932702[a] | LK932711[a] | LK932725[a] |
| *Nectria pseudotrichia* | CBS 641.83 | Wood, Edo Tachira, Venezuela | HM534899 | HM534878 | HM534889 |
| *Thermothelomyces guttulata* | CGMCC 3.15185[T] | Soil, China | KC352943 | KC352946 | KC352949 |
| | CGMCC 3.15186 | Soil, China | KC352944 | KC352947 | KC352950 |
| *Thermothelomyces heterothallica* | CBS 202.75[T] | Garden soil, Germany | HQ871771 | HQ871710 | HQ871798 |
| | CBS 203.75 | Soil, Indiana, USA | HQ871772 | HQ871711 | HQ871800 |
| | CBS 137789 (=FMR 13215) | Soil, Great Smoky Mountains National Park, Tennessee | LK932697[a] | LK932721[a] | LK932736[a] |
| | FMR 5174 | Soil, Spain | LK932692[a] | LK932712[a] | LK932727[a] |
| | FMR 5175 | Soil, Spain | LK932693[a] | LK932713[a] | LK932728[a] |
| *Thermothelomyces hinnulea* | CBS 597.83[T] | Cultivated soil, Japan | HQ871791 | HQ871719 | HQ871813 |

| | | | | | |
|---|---|---|---|---|---|
| | CBS 544.82 | Soil, New Zealand | HQ871790 | HQ871718 | HQ871812 |
| *Thermothelomyces thermophila* | CBS 117.65[T] | Dry pasture soil, UK | HQ871764 | HQ871705 | HQ871803 |
| | CBS 381.97 | Man, HIV positive patient, unknown location | HQ871766 | HQ871707 | HQ871805 |
| *Thielavia terricola* | FMR 12786 | Soil, Gran Canaria, Spain | LK932696[a] | LK932720[a] | LK932735[a] |

[T] Type strains. [a] Sequences derived from this work. CBS = Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CGMCC = China General Microbial Culture Collection, China; FMR = Facultat de Medicina, Reus, Spain; IMI = International Mycological Institute, England; MUCL = Belgian Co-ordinated Collections of Micro-organisms, Belgium; NBRC = Biological Resource Center, Chiba, Japan.

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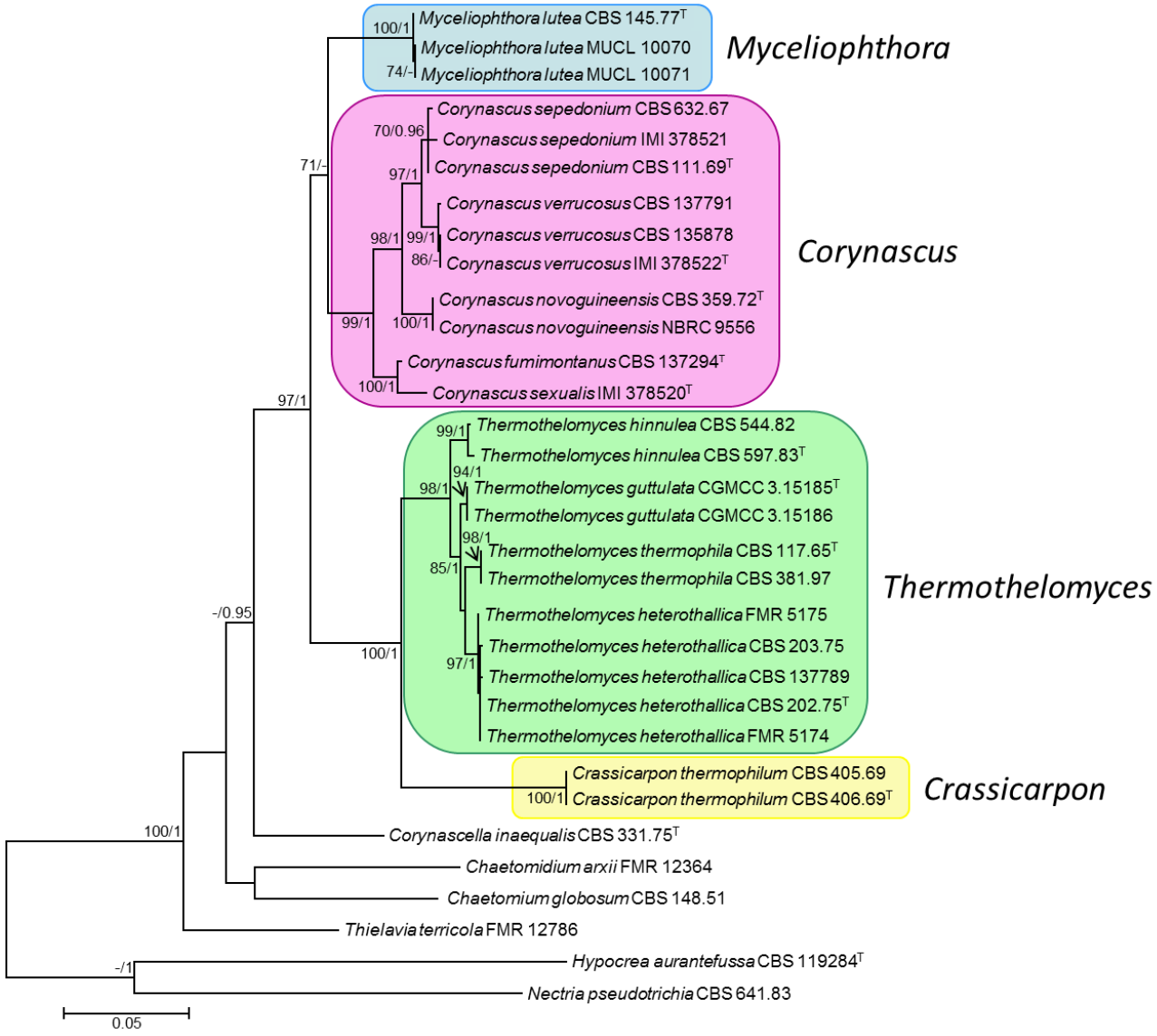
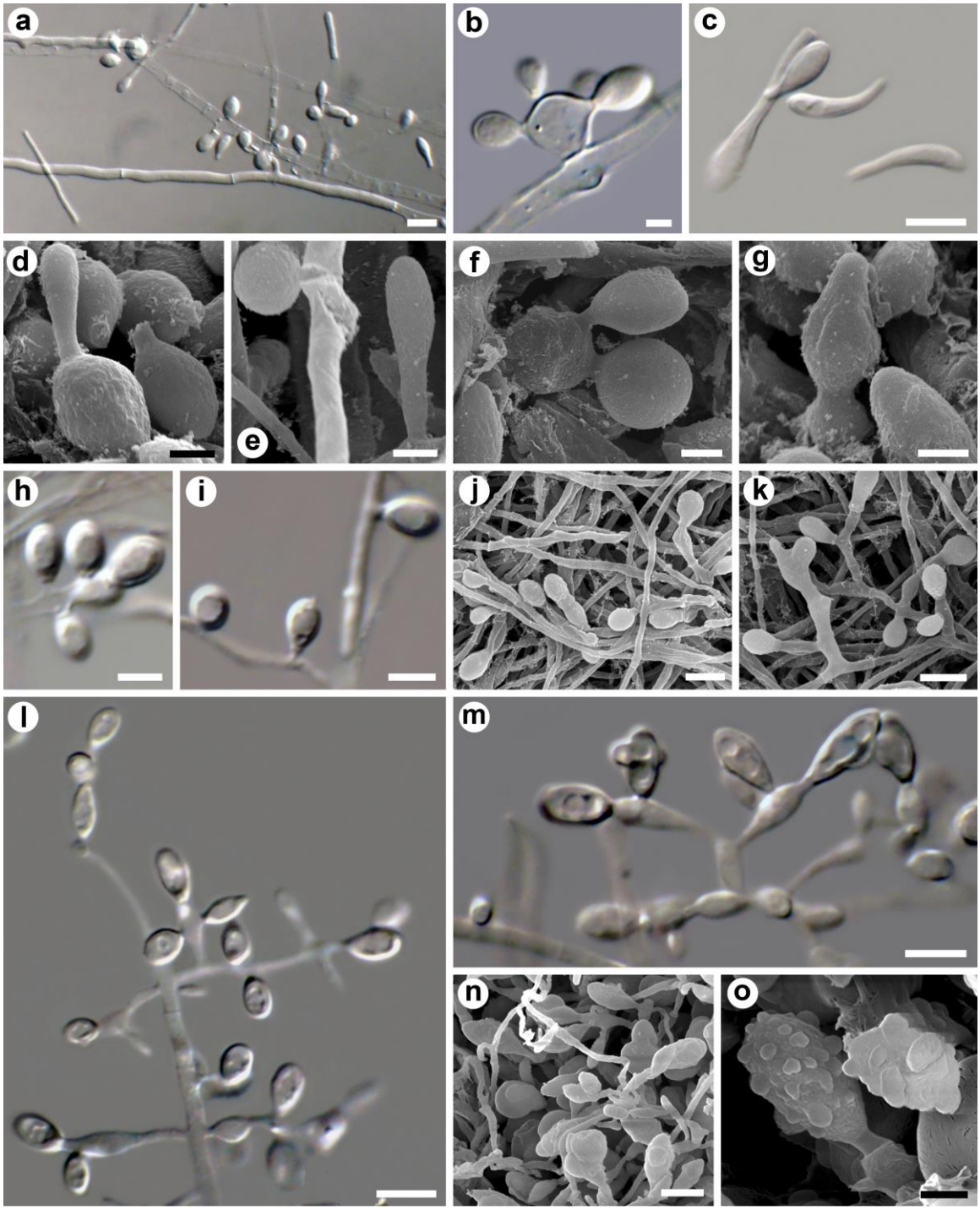
SOIL ASCOMYCETES FROM DIFFERENT GEOGRAPHICAL REGIONS.
Yasmina Marín Félix
Dipòsit Legal: T 996-2015

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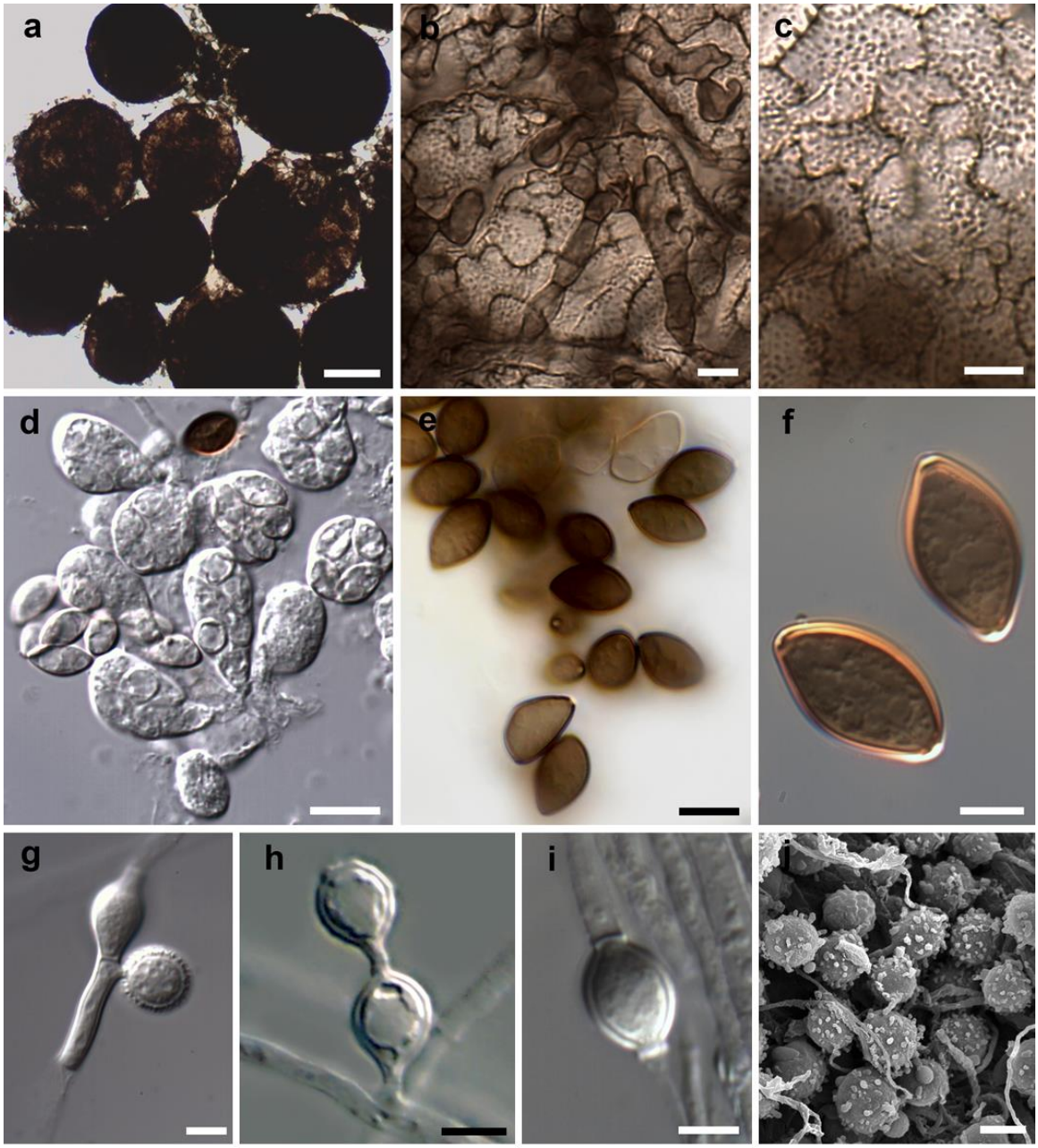
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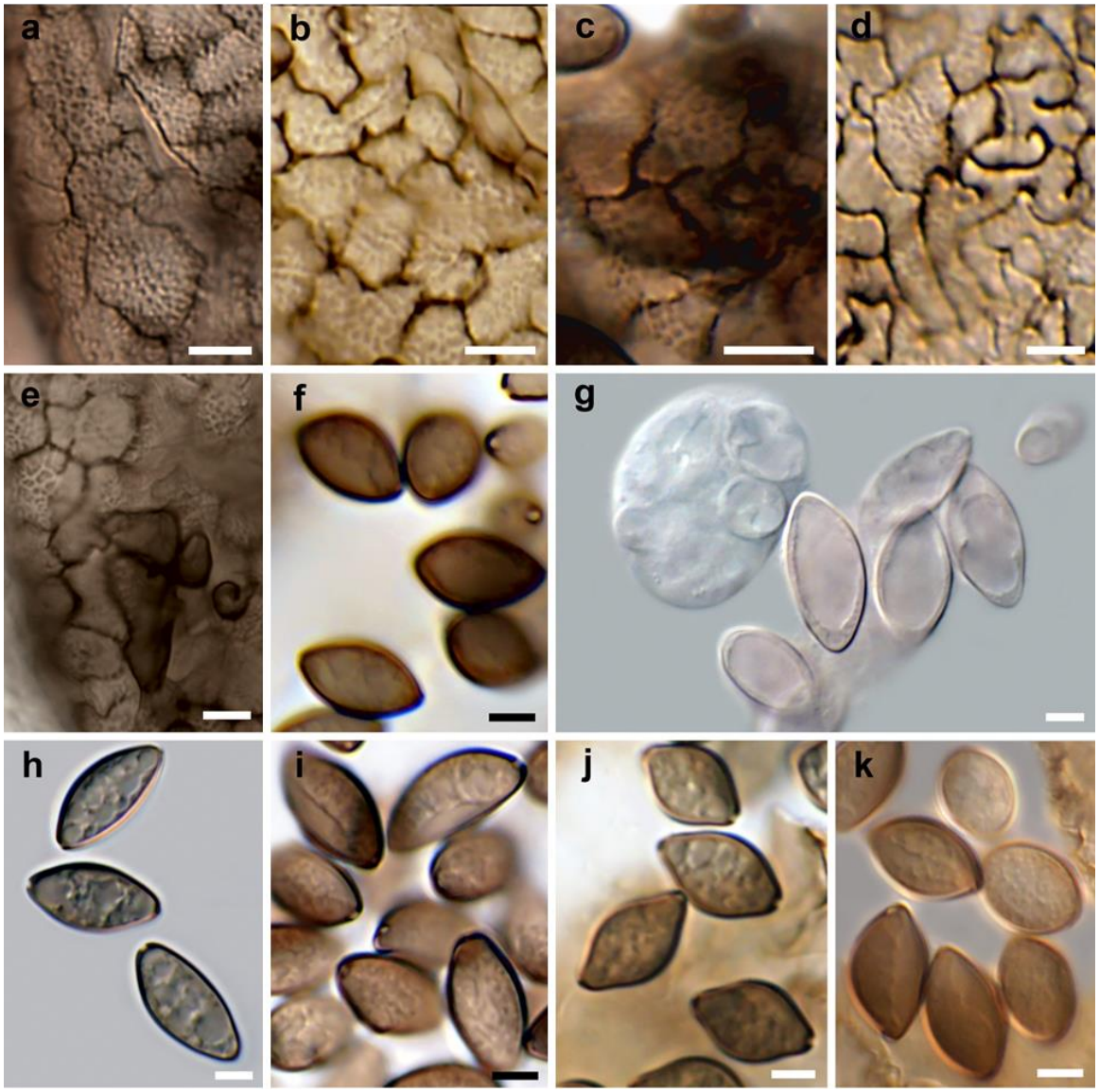
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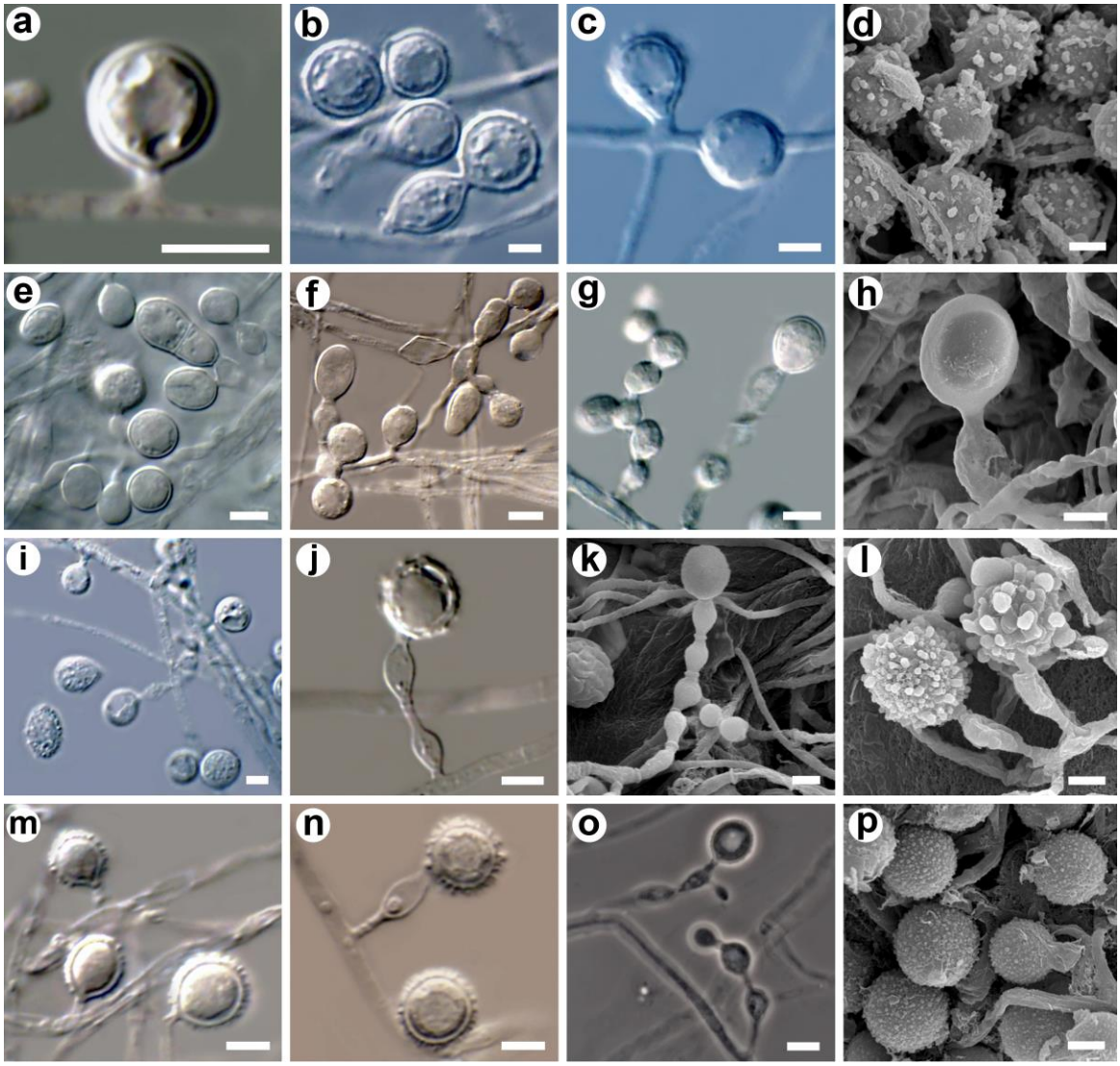
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# 4.5. OTHER ORDERS

## 4.5. OTHER TAXA

Figure 28 shows the tree based on ITS and D1–D3 sequences of the taxa belonging to the class Leotiomycetes. The lengths of the individual alignments used in the combined data set were 395 bp and 785 bp, respectively. The total alignment length was 1180 bp, of which 172 bp were parsimony informative.

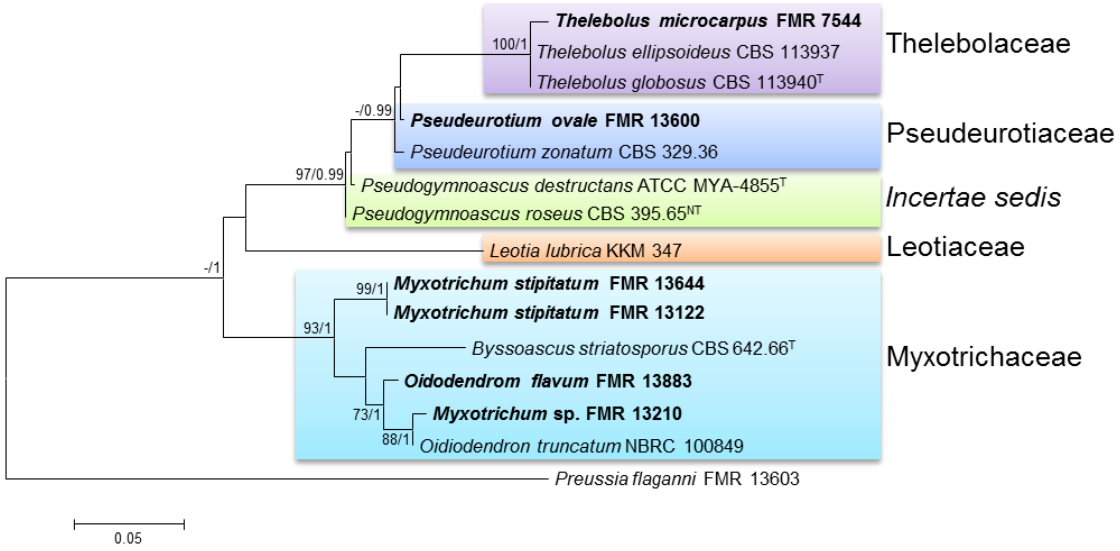



**Fig. 28** Maximum-likelihood (ML) tree obtained from the ITS and D1–D3 sequence data of our isolates and selected strains of the Leotiomycetes. An isolate of *Preussia flaganni* was used as outgroup. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated along branches. Branch lengths are proportional to distance. Type strains of the different species are indicated with $^{T}$.

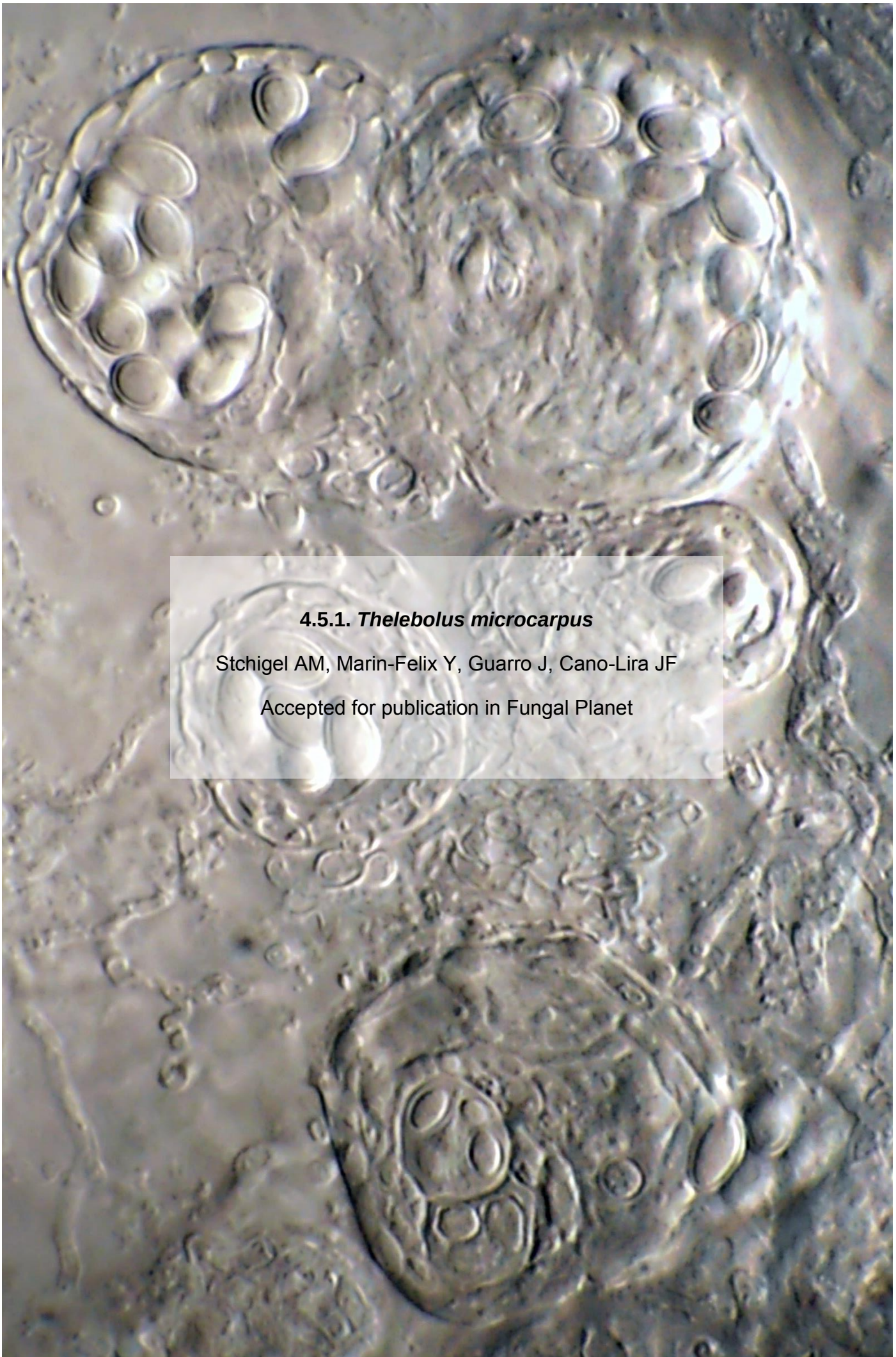
Our isolates belonging to the class Leotiomycetes (indicated in bold) were located in the clades that represented the families Thelebolaceae (100% bs / 1 pp), Myxotrichaceae (93% bs / 1 pp) and Pseudeurotiaceae.

We isolated a few fungi morphologically identified as *Myxotrichum* (sexual morph) / *Oidodendron* (asexual morph). The family Myxotrichaceae has traditionally been classified in the order Onygenales because of its gymnothecial ascomata, unicellular ascospores and arthroconidial anamorph (Currah, 1985). However, it has always been regarded as atypical because of its more elaborate dendritic arthroconidia and fusoid to navicular, striated ascospores (oblate, globose or allantoid and smooth or pitted in the families of the Onygenales). Subsequently, morphological and molecular studies demonstrated that this family belonged to the class Leotiomycetes, being related to the inoperculate discomycetes (Currah, 1994; Sugiyama et al., 1999; Wang et al., 2006a). Another family recently included in the class Leotiomycetes is the Pseudeurotiaceae, and, like the Myxotrichaceae, is also placed as *incertae sedis* (Wang et al., 2006b).

However, further studies are required to clarify the assignment of both families to the correct order.

The genus *Pseudogymnoascus* had been located in Myxotrichaceae but was transferred to Pseudeurotiaceae based on morphology and phylogeny (Sogonov et al., 2005; Wang et al., 2006b). However, we did not obtain a well-supported clade corresponding to this family that corroborate the location of this genus in Pseudeurotiaceae.

The family Thelebolaceae is also located in the Leotiomycetes. We isolated a fungus belonging to this family which represented a new species of the genus *Thelebolus* (*Thelebolus microcarpus*; section 4.5.1).



## 4.5.1. *Thelebolus microcarpus*

Stchigel AM, Marin-Felix Y, Guarro J, Cano-Lira JF

Accepted for publication in Fungal Planet


Thelebolus nanus
EL INFIERNILLO
3042 m. SOBRE el NIVEL del MAR
VISITE
AMAICHA DEL VALLE 28
RUINAS DE QUILMES 46

***Thelebolus microcarpus*** Stchigel, Y. Marín, Guarro & Cano, *sp. nov.*

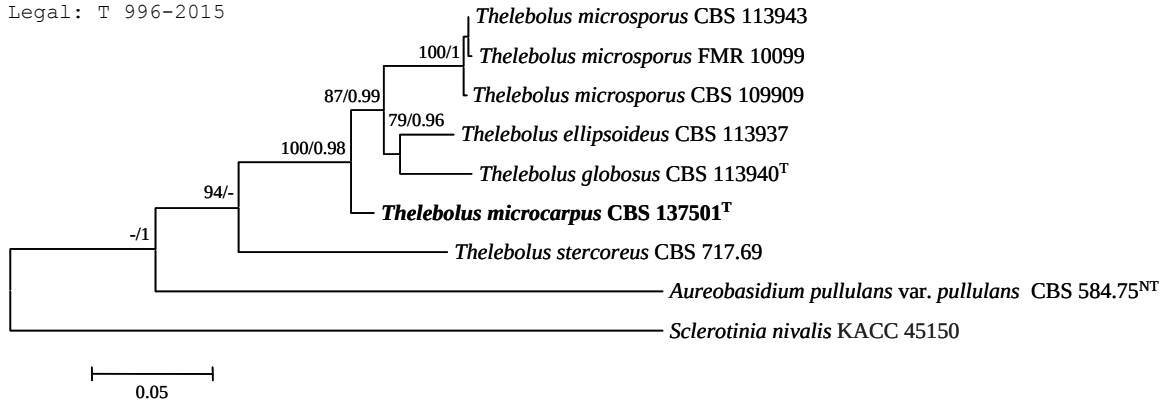
*Etymology*. Due to the small (μικρο-; Greek) size of the sexual body (-καρποσ; Greek).

*Mycelium* composed of hyaline to pale yellow, branched, septate, smooth-walled, irregularly swollen hyphae, 1–7 μm wide. *Ascomata* globose to subglobose, non-ostiolate and not becoming apothecioid with age, 18–70 μm diam, superficial to immersed, scattered to grouped, in clusters up to 5 ascomata, each arising on a single hypha of 2–4 μm diam, colourless to pale amber, glabrous. *Hymenium* absent or inconspicuous. *Peridium* of *textura angularis* up to 5 μm thick, composed of 2–3 layers of hyaline to pale yellow, thick-walled cells of 3–10 μm diam. *Paraphyses* absent. *Asci* 8-spored, 1–5 per ascoma, subglobose to broadly ellipsoidal, thin-walled, evanescent, 12–17 × 10–15 μm. *Ascospores* irregularly disposed inside the ascus, one-celled, subhyaline to pale yellow, thick- and smooth-walled, ellipsoid, 5–9 × 3–4 μm.

Culture characteristics — Colonies on OA attaining 30–36 mm diam in 14 d at 15 ºC, velvety, margins fimbriate to arachnoid, colourless to yellowish white (M. 4A2; Kornerup & Wanscher 1984); reverse yellowish white to pale yellow (4A2 to 4A3). Colonies on PCA attaining 30–31 mm diam in 14 d at 15 ºC, velvety, margins fimbriate to arachnoid, white; reverse of the same colour than the surface. The fungus does not grow below 5 ºC, nor above 30 ºC. Optimal ascomata production at 15 ºC.

*Typus.* ARGENTINA, Tucumán, Tafí el Valle, Abra del Infiernillo, from soil, 15 May 2000, coll. *A.M. Stchigel, J.F. Cano-Lira & J. Guarro*, isol. *A.M. Stchigel* (holotype CBS H-21625, cultures ex-type CBS 137501 = FMR 7544; ITS and LSU sequences GenBank LN609269, BT2 sequence GenBank LN609270; MycoBank MB810286).

Notes — The genus *Thelebolus* (*Thelebolales*) was introduced in 1790 by Tode to erect the new species *T. stercoreus*. Despite more species being included in the genus, de Hoog et al. (2005) carried out a phylogenetic study based on SSU, ITS and β-tubulin (*BT2*) sequences, accepting as valid only *T. microsporus* and *T. stercoreus*, and proposing *T. ellipsoideus* and *T. globosus* as new species. Based on a phylogenetic tree of ITS and *BT2* sequences we have confirmed the isolate CBS 137501 as a new species of *Thelebolus*, namely *T. microcarpus*. The most morphologically related species are *T. ellipsoideus* and *T. globosus*, the main differences being the absence of an asexual morph (hyphozyma-like in *T. ellipsoideus* and in *T. globosus*) and the *textura angularis* of the ascomatal peridium of *T. microcarpus* (*textura globulosa* in *T. ellipsoideus* and *T. globosus*). The main difference between *T. microcarpus* and *T. microsporus* is that the later produces ascomata that open in the meso- or the telo-hymenial phase (often becoming apothecial), while in *T. microcarpus* the ascoma remains closed until its disintegration. *Thelebolus microcarpus* can easily be distinguished from *T. stercoreus* based on its smaller (18–70 μm diam) ascomata (40–220 µm diam in *T. stercoreus*) and the fewer (–8) ascospores per ascus (from 32 to over 2000 in *T. stercoreus*).




A maximum likelihood tree was obtained from the ITS and β-tubulin (*BT2*) sequences of our isolates and other sequences retrieved from the GenBank (de Hoog et al. 2005) (TreeBase Submission ID. 16400). In the tree, branch lengths are proportional to distance. Bootstrap support values ≥70/Bayesian posterior probability scores ≥0.95 are indicated on the nodes. A strain derived from the neotype of *Aureobasidium pullulans* var. *pullulans* and a strain of *Sclerotinia nivalis* were used as outgroup. Ex-type and ex-neotype strains of the different species are indicated with $^{T}$ and $^{NT}$, respectively. The new species proposed in this study is indicated in **bold**.

***Colour illustrations.*** Abra del Infiernillo, Tucumán, Argentina. Ascomata, ascus and ascospores. Scale bars = 5 µm.

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# 5. CONCLUSIONS

# 5. CONCLUSIONS

1. During this doctoral thesis 140 soil samples from six different countries (Argentina, Chile, Colombia, Costa Rica, Spain and USA) have been processed, and a total of 171 species of ascomycetes have been isolated and characterized.

2. A total of 18 new taxa have been characterized and fully described. These comprise the new genera *Aphanoascella*, *Medusitheca*, *Naviculispora*, *Emmonsiellopsis* and *Rinaldiella*, and the following new species:

*Aphanoascella galapagosensis*
*Aspergillus posadasensis*
*Auxarthron longisporum*
*Corynascus fumimontanus*
*Diplogelasinospora moalensis*
*Emmonsiellopsis coralliformis*
*Emmonsiellopsis terrestris*
*Leiothecium cristatum*
*Medusitheca citrispora*
*Naviculispora terrestris*
*Pseudoneurospora canariensis*
*Rinaldiella pentagonospora*
*Thelebolus microcarpus*

3. A phylogenetic study of the genus *Aphanoascus* (order Onygenales), based on the analysis of the ITS and D1–D2 rDNA sequences, showed that this taxon was composed of two separate, monophyletic groups. Therefore, the genus *Keratinophyton* has been re-established, as well as *Keratinophyton terreum* and *Keratinophyton durum*. The following four combinations have been proposed:

*Keratinophyton hispanicum* (≡*Aphanoascus hispanicus*)
*Keratinophyton multiporum* (≡*Aphanoascus multiporus*)
*Keratinophyton punsolae* (≡*Aphanoascus punsolae*)
*Keratinophyton saturnoideum* (≡*Aphanoascus saturnoideus*)

4. Using a polyphasic approach with morphological, physiological and molecular data from ITS, *EF*1 and *RPB*2 nucleotide sequences, the genus *Myceliophthora* (order Sordariales) has been divided into four monophyletic clades, each one of which represents a different genus. Accordingly, the genus *Corynascus* has been re-established, as have the species:

*Corynascus novoguineensis*
*Corynascus sepedonium*
*Corynascus sexualis*
*Corynascus verrucosus*

The new genera *Crassicarpon* and *Thermothelomyces* have been introduced, being proposed the following five new combinations:

*Crassicarpon thermophilum* (≡*Myceliophthora fergusii*)
*Thermothelomyces guttulata* (≡*Myceliophthora guttulata*)
*Thermothelomyces heterothallica* (≡*Myceliophthora heterothallica*)
*Thermothelomyces hinnulea* (≡*Myceliophthora hinnulea*)
*Thermothelomyces thermophila* (≡*Myceliophthora thermophila*)

*Corynascus similis* has been synonymized to *Corynascus sepedonium*, and the description of this species has been amended to incorporate novel morphological features.

5. On the basis of morphological and molecular data of SSU, ITS, D1–D3, *Act* and *EF*1 nucleotide sequences of taxa belonging to the order Melanosporales, the genus *Melanospora* has been amended and *Microthecium* has been re-established, as well as all the following species:

*Microthecium beatonii*
*Microthecium ciliatum*
*Microthecium compressum*
*Microthecium episphaerium*
*Microthecium geoporae*
*Microthecium hypomyces*
*Microthecium levitum*
*Microthecium perplexum*
*Microthecium retisporum*
*Microthecium zobelii*

The following new combinations have been proposed:

*Melanospora mycoparasitica* (≡*Sphaerodes mycoparasitica*)
*Microthecium africanum* (≡*Persiciospora africana*)
*Microthecium brevirostratum* (≡*Melanospora brevirostrata)*
*Microthecium brevirostre* (≡*Melanospora brevirostris*)
*Microthecium fallax* (≡ *Melanospora fallax*)
*Microthecium japonicum* (≡ *Pteridiosperma japonica*)
*Microthecium lenticulare* (≡ *Pteridiosperma lenticulare*)
*Microthecium marchicum* (≡ *Sphaeroderma marchicum*)
*Microthecium masonii* (≡ *Persiciospora masonii*)
*Microthecium micropertusum* (≡ *Sphaerodes micropertusa*)

*Microthecium fayodii* (≡ *Melanospora fayodii*)
*Microthecium fimbriatum* (≡*Melanospora fimbriata*)
*Microthecium fimicola* (≡*Melanospora fimicola*)
*Microthecium foveolatum* (≡ *Pteridiosperma foveolatum*)
*Microthecium fusisporum* (≡*Melanospora fusispora*)
*Microthecium internum* (≡ *Melanospora interna*)
*Microthecium moreaui* (≡ *Persiciospora moreaui*)
*Microthecium nectrioides* (≡ *Melanospora nectrioides*)
*Microthecium pegleri* (≡ *Melanospora pegleri*)
*Microthecium quadrangulatum* (≡*Sphaerodes quadrangularis*),
*Microthecium sepedonioides* (≡*Papulaspora sepedonioides*)
*Microthecium tenuissimum* (≡ *Sphaerodes tenuissima*)

The genera *Annulispora* and *Pseudomicrothecium* have also been proposed as new members of the order Melanosporales, as well as the new combinations:

*Annulispora ellipsospora* (≡*Sphaerodes ellipsospora*)
*Annulispora singaporensis* (≡*Sphaerodes singaporensis*)
*Pseudomicrothecium subterraneum* (≡ *Melanospora subterranea*)

6. Within the family Lasiosphaeriaceae (order Sordariales), the new genera *Rhypophila* have been proposed on the basis of morphological and molecular data derived from the analysis of D1–D3, *BT*2 and *RPB*2 sequences, as well as the new combinations:

*Rhypophila cochleariformis* (≡*Cercophora cochleariformis*)
*Rhypophila decipiens* (≡*Cercophora decipiens*)
*Rhypophila myriospora* (≡*Cercophora myriospora*)
*Rhypophila pleiospora* (≡*Cercophora pleiospora*)

The genera *Bombardia* and *Jugulospora* have been redefined, proposing the new combinations:

*Bombardia anartia* (≡*Bombardioidea anartia*)
*Bombardia bombardioides* (≡*Bombardioidea bombardioides*)
*Bombardia serignanensis* (≡*Bombardioidea serignanensis*)
*Bombardia stercoris* (≡*Bombardioidea stercoris*)
*Jugulospora carbonaria* (≡*Strattonia carbonaria*)

The genus *Rhexosporium* has been synonymyzed with *Jugulospora. Apiosordaria globosa, Apiosordaria hispanica* and *Rhexosporium terrestre* have been considered synonym with *Jugulospora rotula,* and *Apiosordaria antarctica* with *Jugulospora carbonaria*.

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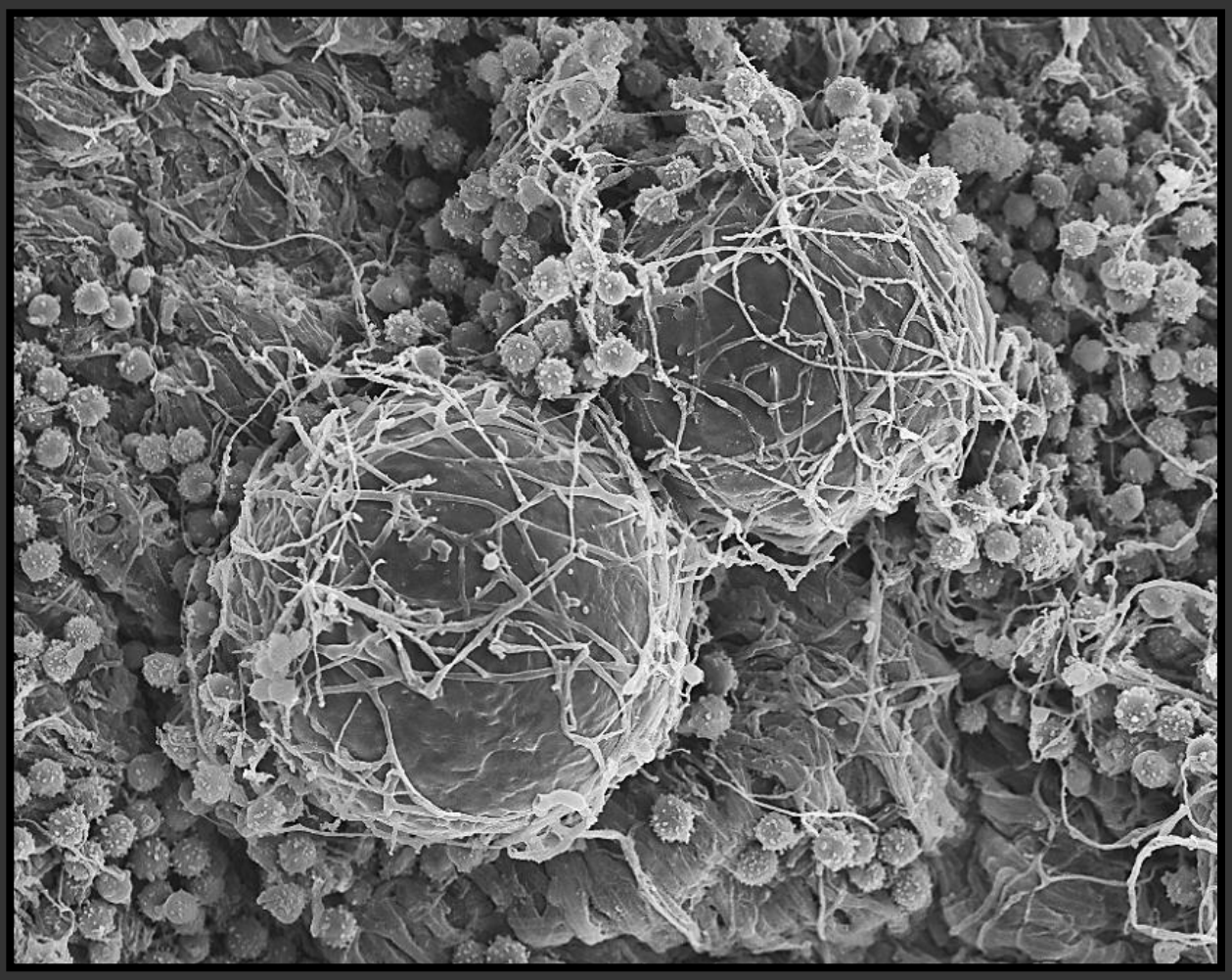

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