Towards a phylogenetic reappraisal of Parmulariaceae and Asterinaceae (Dothideomycetes)

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Key words

Asterinales
eptype
Neotropical fungi
taxonomic novelties
type species

Abstract Members of the Asterinaceae and Parmulariaceae are obligate biotrophic fungi with a pantropical distribution that grow in direct association with living plant tissues and produce external ascomata and bitunicate ascospores. These fungi are poorly known, with limited information about their taxonomic position in the Dothideomycetes. Much of what is known is conjectural and based on observation of morphological characters. An assessment of the phylogenetic position of the Asterinaceae and Parmulariaceae is provided based on a phylogenetic analysis of the nrDNA operon (ITS) and the large subunit rDNA (LSU) sequence data obtained from fresh material of selected species collected in Brazil. Three key species were included and epitypified, namely Asterina melanostomatis, which is the type species for the type genus of the Asterinaceae; Prillieuxinax bacchandinicola (Asterinaceae); and Parmularia styracis, which is the type species for the type genus of the Parmulariaceae. An LSU rDNA phylogenetic analysis was performed indicating the correct phylogenetic placement of the Asterinales within the Dothideomycetes. From this initial analysis it is clear that the Parmulariaceae as currently circumscribed is polyphylectic, and that the Asterinaceae and Parmulariaceae are related, which justifies the maintenance of the order Asterinales. Asterotexis cucumbereae is recognised as distinct from other Dothideomycetes and placed in the newly proposed family and order (Asterotecixaeae, Asterotexales), while the higher order polyphyly of Incyclus angularis remains unresolved. Additionally, Lembosia abaxialis is introduced as a novel species and the phylogenetic placement of the genera Batistinula and Prillieuxinax is clarified.

INTRODUCTION

The Parmulariaceae (Ascomycota) was informally proposed by Müller & von Arx (1962) to accommodate plant parasitic fungi with superficial, dimidiate shape-associated or crust-like, pulvinate stromata, strongly flattened ascomata that open by irregular disintegration, or by lateral to radial, or ring-like splits. The externally visible stromata usually originate from internal hyphae or internal hypostroma (von Arx & Müller 1975). Asci in this family are ovoid to clavate, with fissitunicate or rostrate dehiscence with a hamathecium composed of pseudoparaphyses. Ascospores of members of this family are hyaline or brown, usually septate and, with or without a mucilaginous sheath. Asexual morphs of fungi in this group are poorly known. The family was formally described by Barr (1979). A more detailed account of the Parmulariaceae was provided in the monograph published by Inácio & Cannon (2008).

The Parmulariaceae together with families of foliicolous ascomycetes such as Asterinaceae and Autographaceae, has traditionally been treated as a group with uncertain placement (incertae sedis) in the Dothideomycetes (Hyde et al. 2013). The Parmulariaceae differs from the supposedly closely related Asterinaceae, by having an apical stroma formed by several layers of pigmented cells, and a basal hypostroma formed by fungal hyphae, as well as by the absence of appressoria (Inácio et al. 2012a, Honganan et al. 2014). Superficial hyphae are absent in species of Parmulariaceae with the exception of Antonioniomyces, Aulacostroma, Mintera and Symphaeophyma, although commonly found in the Asterinaceae (Inácio et al. 2012a). The taxonomic value of this feature was considered an artificial criterion for distinguishing the two families (von Arx & Müller 1975). Nevertheless as a matter of convenience, this morphological feature is still widely used to recognise whether a taxon belongs to one family or the other. The hypothesis of affinity between these two families has never been tested with modern molecular tools.

Lévêillé (1845) described eight species in two genera, Asterina and Lembosia. In 1899, Asterina was included in Microthyriaceae and the family was divided into two subfamilies, Asterinae and Microthyriinae, based on the presence or absence of superficial mycelium (Theissen 1913a, b). Subsequently, the family Asterinaceae was described and 18 genera were included (Hansford 1946).

Currently the Asterinaceae includes species that are either epiphytic or obligate biotrophs. Fungi in this family have dimidiate ascomata that open irregularly at maturity by means of stelar, longitudinal or irregular slits. Ascomata contain bitunicate asci, which are globose to oval or cylindrical. Colonies are formed on the surface of leaves or green stems of plants. When present, superficial mycelium is composed of hyphae that have opposite, alternate or irregular branches with uni- or...
bi-cellular appressoria that are either alternate, unilateral or a mixture of these forms and with shapes that vary between oval, ampulliform, lobate or variable. Haustoria are present in many genera (von Arx & Müller 1975, Eriksson 1981, Bezerra 2004, Hofmann et al. 2010, Hofmann & Piepenbring 2011, Hosagoudar 2012).

Recent studies have shown that morphological features alone are not a reliable basis for a natural classification that reflects true phylogenetic relationships. Some examples are found at the generic level in taxa such as Cladosporium, Microcylindrospora, Phaeoanellula, Radulidium, Ramichloridium and Septoria, among others (Arzanlou et al. 2007, Schubert et al. 2007, Frank et al. 2010, Quaedvlieg et al. 2013) and at the family level in Botryosphaeriaceae and Teratosphaeriaceae (Slippers et al. 2013, Quaedvlieg et al. 2014). Delimitation and affiliation of both the Asterinaceae and Parmulariaceae and the genera they contain have relied entirely on morphological features such as ascospore septation, hamathecium reaction to iodine, presence and shape of internal stomata, plectenchyma texture and colour, ascomata and ascus dehiscence.

Morphological features are often combined with conjectured host specificity. However, the host specificity of fungi in these families has never been experimentally tested (Hofmann et al. 2010). The Asterinaceae and Parmulariaceae were regarded as probably polyphyletic both by Inácio & Cannon (2008) and Hongsanan et al. (2014), respectively. Practical difficulties related to DNA extraction from old herbarium material and difficulties with recollection of type specimens have hampered a reappraisal of these two families.

Inácio & Cannon (2008) included 35 genera as members of the Parmulariaceae, while Lumbsch & Huhndorf (2010) recognised 34 genera, with the inclusion of Hemisphaera and exclusion of Apoa and Parmularia. The latest publication mentioning this family (Hyde et al. 2013) added Antoniomyces and excluded this four genera (Coccomothodis, Dothidasteroma, Englerodothis and Perischizon) from the Parmulariaceae based on the shape of the ascomata, reducing the total number to 31 genera. Now, with the addition of the recently described genus Rhagadolobiopsis (Guatimosim et al. 2014a), the Parmulariaceae include 32 genera and 114 synonyms (Inácio & Cannon 2008, Lumbsch & Huhndorf 2010, Inácio et al. 2012b, Hyde et al. 2013, Guatimosim et al. 2014a, b).

Lumbsch & Huhndorf (2010) included 38 genera in the Asterinaceae but, more recently, Hongsanan et al. (2014) revised the Asterinaceae, and recognised only 17 genera and 42 synonyms as belonging to the family. These revisions were mostly based on morphological observations, and were not substantiated by molecular data.

Molecular phylogenetic studies of the Parmulariaceae are difficult because of their biotrophic nature as well as the difficulties involved in DNA extraction from herbarium specimens. The pioneering study of the phylogenetic placement of Asterinaceae (Hofmann et al. 2010) and recent successful DNA extraction from the Meliolales (Pinho et al. 2012, 2014), shows that phylogenetic approaches can be applied to obligate biotrophs, even when only old herbarium material is available.

The aim of this study was to assess the phylogenetic placement of the Asterinaceae and Parmulariaceae based on the study of newly collected epitype materials of Parmularia stryacis (the type species of Parmulariaceae), Asterina melanostomatis (the type species of Asterinaceae) and Phyllleuxia baccharidincola (Asterinaceae). Asterotexis cucurbitacearum, formerly placed in the Asterinaceae, was re-examined and found to represent a separate family, described here as new. Additionally, a new species of Lembosia is introduced and the phylogenetic placement of B. gallesiae and P. baccharidincola is elucidated.

**MATERIALS AND METHODS**

**Sample collection and morphology**

Leaf samples bearing black fungal colonies were collected in Brazil in different biomes between 2009 and 2014. These were dried in a plant press and later examined under a stereo microscope. Freehand sections of fungal colonies were prepared and fungal structures mounted in clear lactic acid, lactophenol, lacto-fuchsin, and/or Melzer’s reagent. When necessary, sections were made using a Microm HM 520 freezing microtome. Observations were made with a Zeiss V20 Discovery stereo microscope and with a Zeiss Axio Imager 2 light microscope using differential interference contrast (DIC) illumination and an MRC5 camera and ZEN imaging software. Representative specimens were deposited at the herbarium of the Universidade Federal de Viçosa (VIC) and CBS Herbarium (CBS H).

**Scanning electron microscopy**

Samples of dried material containing fungal structures were mounted on stubs with double-sided adhesive tape and gold-coated using a Balzer’s FDU 010 sputter coater. A Carl-Zeiss Model LEO VP 1430 scanning electron microscope (SEM) was used to analyse and generate images from the samples.

**DNA isolation**

Leaves harbouring fertile ascomata were examined under a stereo-microscope to check for possible contamination by other fungi, including yeasts. The leaves were then soaked in sterile water for 1 h in order to hydrate and remove the ascomata. Thirty fertile ascomata were removed from the leaves with a sterile fine pointed needle, and placed into a microcentrifuge tube (1.5 mL). Total genomic DNA was extracted by using Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, USA) following the manufacturer’s instructions and the steps described by Pinho et al. (2012).

**PCR amplification**

The LSU region of each fungus included in the study was sequenced with the primers LR0R + LR5 (Vilgalys & Hester 1990). For the Parmulariaceae, two additional loci, including the internal transcribed spacer regions and intervening 5.8S rDNA (ITS) and the translation elongation factor 1-alpha (EF-1α) were amplified and sequenced with the primer pairs ITS1-F (Gardes & Bruns 1993) + ITS4 (White et al. 1990), EF2-Fd (Groenewald et al. 2013) or EF1-728F (Carbone & Kohn 1999) + EF-2 (O’Donnell et al. 1998). PCR amplifications were performed in a total volume of 12.5 μL solution containing 10–20 ng of template DNA, 1× PCR buffer, 0.63 μL DMSO (99.9 %), 1.5 mM MgCl2, 0.5 μM of each primer, 0.25 mM of each dNTP, 1.0 U BioTag DNA polymerase (Bioline GmbH Luckenwalde, Germany). PCR conditions for ITS and LSU were set as follows: an initial denaturation temperature of 95 °C for 5 min, followed by 35 cycles of denaturation temperature of 95 °C for 30 s, primer annealing at 52 °C for 30 s, primer extension at 72 °C for 1 min and a final extension step at 72 °C for 1 min. PCR conditions for EF-1α were set as follows: an initial denaturation temperature of 94 °C for 5 min, followed by 45 cycles of denaturation temperature of 94 °C for 45 s, primer annealing at 52 °C for 30 s, primer extension at 72 °C for 90 s and a final extension step at 72 °C for 6 min.

**DNA sequencing and phylogenetic inference**

PCR amplicons of the regions targeted in this study served as templates for DNA sequencing reactions with the BigDye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems Life Technologies, Carlsbad, CA, USA) following the protocol of the manufacturer. DNA sequencing reactions used the same
Fig. 1 A Bayesian 50 % majority rule tree based on a full length LSU alignment, containing all strains generated in this study. Bayesian posterior probabilities support values for the respective nodes are displayed in the tree. The tree was rooted to Saccharomyces cerevisiae. The scale bar indicates 0.08 expected changes per site. New sequence data are in bold.
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ccons were purified through Sephadex® G-50 Superfine columns (Sigma Aldrich, St. Louis, MO) in MultiScreen HIV plates (Millipore, Bellerica, MA). Purified sequence reactions were run on an ABI Prism 3730xl DNA Sequencer (Life Technologies, Carlsbad, CA, USA).

DNA sequence data were analysed in MEGA (Molecular Evolutionary Genetics Analysis) v. 6.0 (Tamura et al. 2013). Consensus sequences were generated and imported into MEGA for initial alignment and the construction of sequence datasets. Sequences obtained from Schoch et al. (2009), TreeBASE study S10245, and from GenBank (www.ncbi.nlm.nih.gov) and the novel sequences generated on this study were aligned using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/index.html; Katoh et al. 2002) and manually improved in MEGA as indicated.

Phylogenetic analysis

Appropriate gene models were selected using MrModeltest v. 2.3 (Nylander 2004) and applied to the gene partition. Based on the results of MrModeltest, a Bayesian phylogenetic analy
sis was performed with MrBayes v. 3.1.2 applying a general time-reversible (GTR+I+G) substitution model with inverse gamma rates and dirichlet base frequencies and a heating para
ter set at 0.01. Saccharomyces cerevisiae DAOI 216365 (JN938921) served as outgroup for the phylogenetic analyses. Posterior probabilities were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.2.1 (Ronquist et al. 2012). Six simultaneous Markov chains were run for 10 000 000 generations and trees were sampled every 100th generation and 10 000 trees were obtained. The first 2 000 trees, repre
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RESULTS

Taxonomy

Parmulariaceae M.E. Barr, Mycologica 71: 944. 1979

Type species. Parmularia styracis Lév., Ann. Sci. Nat., Bot. 5: 286. 1846.

This family includes fungi forming foliicolous or lichenicolous, superficial, dark brown to black colonies. Haustoria coralloid, hyaline, numerous in each host-cell. Ascomata solitary to
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spores can be distinguished, truncated at the base, subcylindrical; mature asci thick-walled (particularly in the upper portion), cylindric-clavate to clavate, 47–81 × 9–18 µm, non-amyloid, 6–8-spored, biseriate (with colourless hyaline ascospores) or unordered but becoming uniseriate at maturity (the stage containing pale brown ascospores), dehiscence through a large apical fracture in the outer wall, with the inner layer extending through it. Ascospores ellipsoidal to clavate, mostly hyaline to pale brown, thin-walled, verrucose, 1-septate, constricted at the septum, the upper cell broader and rounded, and the lower cell tapering towards a rounded end, 14–20 × 5–7 µm, smooth. 

*Asexual morph* unknown.

**Fig. 2 Parmularia styracis** VIC 42447. a. Living leaves of *Styrax ferrugineus* with epiphyllous colonies; b, c. detail of the mature colony, opening by radiating fissures; d. vertical section showing entirely superficial ascoma with fertile locules; e, f. detail of the fertile locules; g, h. hyphal columns which connect the colony with the host tissue; i. horizontal section showing the detail of a tuft of internal mycelium that ruptures the cuticle and produce the initial stages of the ascostromata; j. detail of the fertile locule with fully developed asci and pseudoparaphyses; k, l. asci; m–t. ascospores. — Scale bars: d = 100 µm; e, f = 50 µm; g−m = 10 µm.

**Type material.** **Brazil.** Planaltina, on living leaves of *Styrax*, Clauseen, 1846 (PCCI, holotype); on living leaves of *Styrax ferrugineus*, vicinities of the Estação Ecológica de Águas Emendadas, Cerrado biome, 16 Apr. 2013, M. Silva & O.L. Pereira (VIC 42447 = CBS H-22026, epitype designated here, MBT200333).

**Additional materials examined.** **Brazil.** Planaltina, on living leaves of *Styrax ferrugineus*, vicinities of the Estação Ecológica de Águas Emendadas, Cerrado biome, 18 Apr. 2013, M. Silva & O.L. Pereira, VIC 42450 = CBS H-22025; Minas Gerais, Capitólio, Furnas, on living leaves of *S. ferrugineus*, S20°38’54.5”W46°13’36.8”, 9 Nov. 2012, R.W. Barreto, VIC 42587 = CBS H-22027.
Notes — The ontogeny of ascomata of *P. styracis* resembles that recently described for the genus *Rhogadoluobiopias*, in that mature ascostromata are produced from several ascostromatal primordia that coalesce to form a multiloculate structure (Guatimosim et al. 2014a) (Fig. 2b, c). In contrast, *Parmularia* produces a column of internal mycelium in the centre of the colony that ruptures through the cuticle (Fig. 2). When the ascomatal disk is removed, the hyphal columns are limited to the central portion of the area below the colony (Fig. 2g, h).

### Asterinaceae

**Hansf., Mycol. Pap. 15: 188. 1946**

**Type species.** *Asterina melastomatis* Lév., Ann. Sci. Nat., Bot. 3: 59. 1845.

Follicolous, epiphytic, obligately biotrophic. **Sexual morph:** External mycelium usually with or without appressoria, opposite, alternate or irregular branches, blackened. Appressoria uni- or bi-cellular, lateral and/or intercalary, and opposite, alternate or alternate and opposite, oval, ampulliform, lobate or variable, brown to dark brown, with penetration peg piercing through cuticle and invading the epidermic cells or on top of guard cells, forming stomatopodia. **Haustration** present in various genera. **Ascomata** dimidiate, superficial, growing on the surface of plant leaves or stems, circular, elongate or linear, dehiscence non-ostiolate, opening by radiating star-like, longitudinal or irregular slits. **Scutellum** radiate, composed of isodiametric to cylindrical cells, with straight to dichotomously branched hyphae. **Hypostroma** (internal stroma or internal hyphae) present in some members. **Pseudoparaphyses** present or not, cylindrical, septate, branched or unbranched, hyaline to yellowish. **Asci** fissitunicate, upright and parallel, globose, ovoid or cylindrical, 4–8-spored, usually lacking a stalk, hyaline. **Ascospores** ellipsoidal, occasionally cylindrical, 2–6-celled, yellowish to brown (mostly brown when mature), walls smooth or with capitate ornamentation. **Setae** present or not on the ascomata and/or mycelium. **Asexual morph** hyphomycetous or coelomycetous states with pycnothyria. **Conidiophores** solitary, unbranched, brown. **Conidiogenous cells** monoblastic or proliferating percurrently, hyaline or brown. **Conidia** ovoid, cylindrical, conical or staurosporous, brown (von Arx & Müller 1975, Eriksson 1981, Bezerra 2004, Hofmann et al. 2010, Hofmann & Pienaar 2011, Hosagoudar 2012, Hyde et al. 2013, Hongsanan et al. 2014).

**Asterina melastomatis** Lév., Ann. Sci. Nat., Bot. 3: 59. 1845.

— **Fig. 3**

### Notes

Colonies epiphyllous, irregular to circular, single to confluent, black, 2–6 mm diam. **External mycelium** straight to flexuous, branching alternate to unilaterial, rarely opposed, pale brown to brown, septate, hyphal cells cylindrical, 4–5 μm diam, smooth. **Appressoria** numerous, entire, sessile, straight to angular, rarely crooked, rectangular to long-ovate, unicellular, alternate to unilaterial, never opposed, 6–7.5 × 7–8 μm, brown, penetration peg in middle part of appressorial cell. **Ascomata** thyriothecia, dimidiate, superficial, developing below external mycelium, circular, single to confluent, in small clusters, fringed at margins, 165–220 μm diam, dark brown to blackish, opening by a central star-shaped fissure. **Pseudoparaphyses** cylindrical, septate, unbranched, hyaline to yellowish. **Scutellum** radiate, composed of isodiametric to cylindrical cells. **Asci** bitunicate, ovoid to slightly clavate, 8-spored, 47.5–57.5 × 27.5–30 μm, hyaline. **Ascospores** 2-celled, cylindrical, straight, constricted at the septum, hyaline initially, pale brown to brown at maturity, smooth, 19.5–21 × 9.5–11 μm. **Asexual morph** absent.

**Type material.** **BRAZIL**, locality unknown, on living leaves of *Miconia* sp., date unknown, Guillemin, (herbarium specimen not preserved); Minas Gerais, Lavras Novas, on living leaves of *Miconia* sp., on the track of the Cachoeira das Três Quedas, S20°28’39.63” W43°29’42.27”, 26 Oct. 2013, A.L. Firmino (VIC 42822, neotype designated here MBT200348). — **French Guiana**, Cayene, on leaves of *Melastomataceae*, Nov. 1800, Leprieur (herb., Montagne 1133, Crypt. Guyan. 582); PC0084477. Referred to by Hongsanan et al. (2014) as a neotype designated by Theissen (1912 – actually 1913), but that author only referred to species being represented by that collection.

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**Fig. 3** *Asterina melastomatis* VIC 42822. a. Living leaves of *Miconia* sp. with epiphyllous colonies; b. colony with open thyriothecia and external mycelium; c. appressoria cylindrical to long-ovate, unicellular; d. asci ovoid to slightly clavate; e. ascospores hyaline, becoming pale brown to brown at maturity. — Scale bars = 10 μm.
**Fig. 4** *Asterina chrysophylli* VIC 42823. a. Living leaves of *Henrietta succosa* with epiphyllous colonies; b, c. SEM images: b. thyriothecium opened by a central star-shaped fissure; c. ascospore oblong, smooth, constricted at the septum; d. appressoria straight, globose to pyriform, unicellular; e. asci globose to ovoid; f. ascospores hyaline, becoming brown at maturity. — Scale bars = 10 μm.

**Fig. 5** *Batistinula gallesiae* VIC 42514. a. Living leaves of *Caesalpinia echinata* with epiphyllous colonies; b–d, f, g. SEM images: b. colony with open thyriothecia and external mycelium; c. thyriothecium opened by a central star-shaped fissure; d. appressoria straight, lobate, cylindrical, unicellular; e. asci ovoid, showing immature ascospores; f. ascospores oblong, with ends broadly rounded, constricted at the septum; g. conidia of *Triposporium* (asexual morph) and erect conidiophore; h. ascospores with lobate appressoria. — Scale bars: b = 100 μm; c, d, f, g = 20 μm; e, h = 10 μm.
Asterina chrysophylli Henne., Hedwigia 48: 12. 1908. — Fig. 4
Colonies epiphyllous, irregular to circular, solitary to confluent, black 0.5–6 mm diam. External mycelium straight to slightly flexuous, branching irregularly, pale brown to brown, septate, hyphal cells cylindrical, 4.5–5 μm diam, smooth. Appressoria numerous, entire, sessile, straight, globose to pyriform, unicellular, alternate to unilateral, never opposed, 7.5–9.5 x 11–12.5 μm, brown, penetration peg in the middle portion of the appressorial cell. Ascomata superficial, thyrithecioid, bitunicate, globose to ovate, 8-spored, 52.5–57.5 × 32.5–35 μm, hyaline, smooth. Ascospores oblongoglum, straight to slightly curved, 2-celled, hyaline, becoming brown at maturity, smooth. Asexual morph absent.

Material examined. BRASIL, Espírito Santo, Sooretama, Reserva Natural Vale, on living leaves of Henrietta succosa, 19 June 2012, A.L. Firmino, VIC 42823.

Batistinula gallesiae Arx, Publicações Inst. Micol. Univ. Recife 287: 6. 1960. — Fig. 5
Colonies amphigenous, irregular to circular, solitary becoming confluent, black, 1–7 mm diam. External mycelium straight, branching alternate, unilateral or opposite, pale brown to brown, septate, composed of cylindrical hyphal cells, 4.5–5 μm diam, smooth. Appressoria numerous, sessile, straight, cylindrical, 2–3 lobe, 9.5–15 x 9.5–14 μm, unicellular, alternate or unilateral, never opposed, brown, penetration peg centrally on the appressorial cell. Ascomata thyrithecioid, bitunicate, superficial, developed below external mycelium, circular, fringed at margins, 152–213 μm diam, opening by a central star-shaped fissure, dark brown to black. Scutellum radiate, composed of somewhat isodiametric to cylindrical cells, straight. Ascii bitunicate, globose to ovoid, 8-spored, 52.5–57.5 x 32.5–47.5 μm, brown, septate, composed of isodiametric to cylindrical cells, straight. Ascospores fusiform, straight to slightly curved, 2-celled, hyaline, becoming brown at maturity, smooth. Asexual morph absent.

Type material. BRASIL, Rio de Janeiro, Bosque da Barra, Barra da Tijuca, on living leaves of Miconia jucunda, 22 Mar. 2014, R.W. Barreto (VIC 42825, holotype).

Notes — Twelve species of Lembosia have been recorded on Melastomataceae (Montagne 1855, 1856, Henning 1904, Theissen 1913c, Arnaud 1918, Petrak & Ciferri 1930, Petrak & Sydow 1931, Song & Hosagoudar 2003, Hosagoudar & Appaiah 2005, Hosagoudar 2012, Farr & Rossman 2014). All three of these have been reported from Brazil, namely, L. catervaria, L. melanostomatum and L. miconicola. All are distinct from L. abaxialis (Table 2).

Based on morphological characters, L. domingensis shows similarities with L. abaxialis, but differs by epiphyllous colonies, few, sparse, entire and conic appressoria, hysterothecia that are Y- or X-shaped, with scarce, smaller ascii, and slightly clavate

Table 2 Morphological characteristics of Lembosia spp. from Melastomataceae.

| Taxon | Appressoria (μm) | Ascomata (μm) | Asci (μm) | Ascospores (μm) |
|-------|-----------------|---------------|-----------|-----------------|
| Lembosia abaxialis | 7–10 x 10–10.5 | 340–550 x 160–250 | 52.5–57.5 x 25–37.5 | 25–29 x 12.5–15 |
| Lembosia catervaria | 6–8 diam | 500–700 x 70–100 | 40 x 70 | 30–38 x 15–19 |
| Lembosia domingensis | 5–6 x 7–9 | 300–800 x 150–250 | 40–52 x 28–35 | 25–33 x 11–15 |
| Lembosia gigantea | 12–17 x 9 | 784–1064 x 302–504 | 84–96 x 33–41 | 26–29 x 14 |
| Lembosia melanostomatum | 14 x 9 | 784 x 336 | 55–72 x 41–48 | 26–29 x 12 |
| Lembosia melanocyclus | 6–8 diam | 700 x 250 | 70–96 x 42–52 | 35–40 x 16–20 |
| Lembosia memecylce | – | 200–450 x 120–150 | 35–55 x 26–35 | 20–23 x 8–10 |
| Lembosia memecylcecola | 4–12 x 6–8 | 294–882 x 176–300 | up to 45 diam | 22–26 x 11–13 |
| Lembosia miconica-prasinae | 7 wide | 470–860 x 313–448 | 69–84 x 33–43 | 24–29 x 12 |
| Lembosia miconicola | – | 500–800 high | 22 x 11.5 | 23–28 x 11–13 |
| Lembosia rollickiae | 5–7 wide | 300–350 x 100 | 50–60 x 30 | 24–26 x 10–11 |
| Lembosia ryanii | 7–17 x 5 | 235–425 x 145–168 | 36–46 x 21–31 | 20–21 x 9–12 |
| Lembosia sclerolobii | – | up to 1000 x 140–180 | 35–50 x 30–40 | 17–23 x 6–9 |

1 Montagne (1855), Montagne (1856), Henning (1904), Theissen (1913c), Arnaud (1918), Petrak & Ciferri (1930), Petrak & Sydow (1931), Song & Hosagoudar (2003), Hosagoudar & Appaiah (2005), Hosagoudar (2012).
2 This publication.

Additional material examined. BRASIL, Espírito Santo, Sooretama, Reserva Natural Vale, on living leaves of Caesalpinia echinata, S19‘19’03.28” W40°05’42.10”, 15 July 2012, A.L. Firmino, D.B. Pinho & O.L. Pereira VIC 42514.

Notes — Batistinula gallesiae was originally described from living leaves of Gallesia gorazemae (Phytolaccaceae) in the state of Pernambuco (Brazil). The present collection was from living leaves of Caesalpinia echinata (Fabaceae) collected in the state of Espírito Santo (Brazil). This specimen has the same morphological and biometric characteristics of the type. Caesalpinia echinata is a new host of B. gallesiae and the genus remains monotypic, with distribution restricted to Brazil.

Lembosia abaxialis Firmino & R.W. Barreto, sp. nov. — MycoBank MB812000; Fig. 6

Etymology. Name derived from the observation that colonies of this taxon are only formed abaxially.

Colonies hypophyllous, irregular to circular, solitary to confluent, black, 2–6 mm diam. External mycelium straight to flexuous, branching irregularly, septate, composed of cylindrical hyphal cells, 3–5 μm diam, brown, smooth. Appressoria numerous, entire to irregularly lobate, sessile, straight to angular, 7–10 x 10–10.5 μm, unicellular, unilateral to alternate, never opposed, brown, penetration peg centrally on the appressorial cell. Ascomata hysterothecioid, superficial, developed below external mycelium, mostly linear, rarely Y-shaped, solitary to grouped, fringed at margins, 340–550 x 160–250 μm, dark brown to black, opening by longitudinal fissures. Scutellum radiated, composed of isodiametric to cylindrical cells, straight. Ascii bitunicate, slightly clavate, 52.5–57.5 x 25–37.5 μm, 8-spored, hyaline. Pseudoparaphyses cylindrical, septate, unbranched, hyaline. Ascospores oblong to cylindrical, 25–29 x 12.5–15 μm, 2-celled, constricted at the septum, hyaline, becoming pale brown to brown at maturity, smooth. Asexual morph absent.

Type material. BRASIL, Rio de Janeiro, Bosque da Barra, Barra da Tijuca, on living leaves of Miconia jucunda, 22 Mar. 2014, R.W. Barreto (VIC 42825, holotype).

Notes — Twelve species of Lembosia have been recorded on Melastomataceae (Montagne 1855, 1856, Henning 1904, Theissen 1913c, Arnaud 1918, Petrak & Ciferri 1930, Petrak & Sydow 1931, Song & Hosagoudar 2003, Hosagoudar & Appaiah 2005, Hosagoudar 2012, Farr & Rossman 2014). All three of these have been reported from Brazil, namely, L. catervaria, L. melanostomatum and L. miconicola. All are distinct from L. abaxialis (Table 2).
ascospores (Petrak & Ciferri 1930). Additionally, *L. catervaria* differs from *L. abaxialis* by epiphyllous colonies, thicker hyphae, smaller appressoria, longer and narrower hysterothecia, wider asci and larger ascospores (Montagne 1855). *Lembosia melastomatum* differs from *L. abaxialis* by epiphyllous colonies, smaller appressoria, larger asci and ascospores (Montagne 1856). Finally, *L. miconiicola* differs from *L. abaxialis*, by epiphyllous colonies, larger hysterothecia and smaller asci (Arnaud 1918). *Lembosia abaxialis* is the first asterinaceous fungus reported on *Miconia jucunda* (Melastomataceae).

**Prillieuxina baccharidincola** (Rehm) Petr., Sydowia 4: 536. 1950. — Fig. 7

Basionym. *Lembosia drimydis* var. *baccharidincola* Rehm, Ann. Mycol. 5: 532. 1907.

≡ *Echidnodes baccharidincola* (Rehm) Theiss. & Syd., Ann. Mycol. 15: 422. 1926.

Colonies epiphyllous, irregular to circular, solitary becoming confluent, black, 1–6.5 mm diam. *External mycelium* straight to flexuous, branching irregularly, septate, hyphal cells cylindrical, 3–4 μm diam, pale brown, smooth. *Appressoria* absent. 

*Ascomata* thyriothecoid, single to confluent, superficial, developed below external mycelium, circular to ellipsoid, 102–160 μm diam, dark brown to blackish, opening by a central star-shaped fissure. *Asci* bitunicate, ovoid to subclavate, 37.5–50 × 20–30 μm, 8-spored, hyaline. *Ascospores* cylindrical to oblong, straight, 15–22 × 9–11.5 μm, base and apex broadly rounded, 2-celled, constricted at the septum, brown, smooth. *Asexual morph* absent.

Type materials. **Brazil**, São Paulo, on living leaves of *Baccharis* sp., unknown date, A. Usteri 8 (Z+ZT, syntype, here designated lectotype MBT200871); São Paulo, on living leaves of *Baccharis* sp., 5 July 1907, Usteri 41 (Z+ZT, syntype); ibid., 24 July 1907, Usteri 5 (Z+ZT, syntype); Minas Gerais, Nova Lima, on living leaves of *Baccharis* sp., 18 July 2012, O.L. Pereira (VIC 42817, epitype designated here MBT200345).

Additional material examined. **Brazil**, Minas Gerais, Lavras Novas, on living leaves of *Baccharis* sp., 10 Sept. 2012, A.L. Firmino, VIC 42818.

**Asterotexiales** Firmino, O.L. Pereira & Crous, ord. nov. — MycoBank MB812001

Type family. *Asterotexiaceae* Firmino, O.L. Pereira & Crous, fam. nov.

Description as for the constituent family *Asterotexiaceae* (see below).

Notes — Representative sequences of the major orders in the Dothideomycetes support *Asterotexiales* as a separate entity (Fig. 1). Within *Asterotexiales*, two lineages can be defined, one that contains the *Asterotexiaceae*, and another that contains *I. angularis*, which is maintained as *incertae sedis* at the family level. The type species of *Inocyclus* needs to be recollected and its phylogenetic position resolved.

**Asterotexiaceae** Firmino, O.L. Pereira & Crous, fam. nov. — MycoBank MB812002

Type genus. *Asterotexis* Arx, Fungus 28: 6. 1958.

Type species. *Asterotexis cucurbitacearum* (Rehm) Arx (as ‘cucurbitarum’), Fungus 28: 6. 1958.

Foliar pathogens, asterinaceae-like, obligately biotrophic, colonies irregular to star-shaped, solitary to confluent, sometimes extending along the veins, dark brown to black. *External mycelium* growing through ascomatal cavity towards the host epidermis, connecting the neighbouring ascomata, septate, hyaline (unlike members of *Asterinaceae*), smooth. *Appressoria* formed underneath the ascomata, solitary or forming in small clusters, globose, cone-shaped, ovoid to elongate, brown, with a central, hyaline penetration peg. *Ascomata* superficial, scutellate, dimidiate, brown to blackish. *Scutellum* formed by radially arranged rows of cells, opening by numerous irregular fissures, smooth. *Asci* bitunicate, fissitunicate, clavate to cylindrical, 8-spored, hyaline, numerous, parallel, vertically oriented within ascomata. *Ascospores* ellipsoidal to slipper-shaped, unequally 2-celled, slightly constricted at the septum, upper cell subglobose, lower cell smaller, subcylindrical to subcuneate, hyaline to slightly yellowish (unlike members of the *Asterinaceae*), smooth. *Asexual morph* unknown.

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Fig. 6  *Lembosia abaxialis* VIC 42825. a. Living leaves of *Miconia jucunda* with hypophyllous colonies; b. colony with open hysterothecia and external mycelium; c. appressoria straight to angular, entire to irregularly lobate, unicellular; d. asci ovoid to slightly clavate; e. ascospores hyaline becoming pale brown to brown at maturity. — Scale bars: b = 20 μm; c–e = 10 μm.
**Fig. 7** *Prillieuxina baccharidincola* VIC 42817. a. Living leaves of *Baccharis* sp. with epiphyllous colonies; b. SEM image; thyriothecium opened by a central star-shaped fissure; c. vertical section of the ascoma; d. asci ovoid to subclavate showing pseudoparaphyses; e. ascospores hyaline becoming pale brown to brown at maturity. — Scale bars = 20 μm.

**Fig. 8** *Asterotexis cucurbitacearum* VIC 42814. a, b. Symptoms on leaves of *Cucurbita pepo*: a. adaxial side; b. abaxial side, showing the hypophyllous colonies; c. external mycelium hyaline, connecting the ascomata in formation; d. immature ascomata in formation; e. fertile locules exposed on irregular fissures; f, g. vertical section of the ascomata, showing the appressoria with a central hyaline penetration peg, covered by the mature ascomata; h. vertical section of a fully developed ascoma, showing parallel and vertically orientated asci; i. asci; j. ascospores. — Scale bars: c–i = 10 μm; j = 5 μm.
**Asterotexis cucurbitacearum** (Rehm) Arx, Fungus 28: 6. 1958. — Fig. 8

Basionym. Dothidella cucurbitacearum Rehm, Hedwigia 36: 376. 1897.  \[ Rhagadobolium cucurbitacearum (Rehm) Thieiss. & Syd., Ann. Mycol. 12: 275.1914. \]

Colonies hypophyllous, irregular to star-shaped, solitary to confluent, sometimes extending along the veins, dark brown to black, 1–3 mm. *External mycelium* growing through ascomatal cavity towards the host epidermis, connecting the neighbouring ascomata, 3–4 μm diam, hyaline, septate, smooth. *Appressoria* formed underneath the ascomata, solitary or forming small groups, globose, cone-shaped, ovoid to elongate, 8–10 x 5–7 μm, brown, with a hyaline central penetration peg. *Ascomata* superficial, solitary to confluent, sometimes growing to surround the basis of individual trichomes of the host, scutellate, dimidiate, circular to irregular, 1–3 mm diam, upper cells irregularly shaped and thin-walled, brown to black. *Scutellum* formed by radially arranged rows of cells, opening by numerous irregular fissures, pale brown, smooth. A. scutellum bitunicate, fissitunicate, clavate to cylindrical, 40–45 x 9.5–12.5 μm, 8-spored, numerous, parallel, vertically orientated within ascomata, hyaline, smooth. Ascospores ellipsoid to slipper-shaped, 10–14 x 4–5 μm, unequally 2-celled, slightly constricted at the septum, upper cell subglobose, lower cell smaller, subcylindrical to subcuneate, hyaline to slightly yellowish, smooth. Asexual morph unknown.

Type materials. \[ BRASIL, Blumenau, on living leaves of Cucurbita pepo, May 1887, E. Ule 1415 (S F47805 syntype, here designated lectotype MBT200872); Rio de Janeiro, on living leaves of Cucurbita pepo, May 1887, E. Ule 676 (S F7565, syntype); Bahia, Igrapiúna, Reserva Ecológica Michelin, on living leaves of Cucurbita pepo, 15 July 2010, O.L. Pereira & A.L. Firmino, S13°49’17.90” W39°10’16.31” (VIC 42814, epitype designated here MBT200349). \]

Notes — *Asterotexis cucurbitacearum* has been recorded on living leaves of *Cayaponia americana* in the Dominican Republic and West Indies; on *Cucurbita moschata* in Venezuela and West Indies; on *Cucurbita pepo* in Brazil, Panama, Trinidad & Tobago and West Indies; on *Cucurbita* sp. in Brazil and Grenada; on *Gurania* sp. in the Dominican Republic; on *Trichosanthes* sp. in the Dominican Republic and on *Sechium edule* in Costa Rica; *Asterotexis quercina* has been recorded on *Quercus glauca* in Nepal (Guerrero et al. 2011, Farr & Rossman 2014).

**INCERTAE SEDIS**

*Inocyclus angularis* Guatimosim & R.W. Barreto, IMA Fungus 5: 52. 2014. — MB805976

Description and illustrations — Guatimosim et al. (2014b).

Materials examined. \[ BRASIL, Rio de Janeiro, Nova Friburgo, Mury, Sitio Coloniai, on living leaves of *Pleopeltis astrolepis*, 30 Mar. 2013, R.W. Barreto VIC 39747, holotype; CBS H-22028, isotype; ibid., 8 June 2013, R.W. Barreto VIC 39748, CBS H-22029, Rio de Janeiro, Nova Friburgo, Riograndina, Fazenda Barreto, on living leaves of *P. astrolepis*, 9 June 2013, R.W. Barreto VIC 39749, CBS H-22030. \]

Notes — Although *I. angularis* is not the type species of the genus *Inocyclus*, it is presently the only species from which DNA is available. A fresh collection of the type species, *I. psychotriae*, is required to clarify the correct placement of this genus.

**DISCUSSION**

The order *Asterinales* was included within *Dothideomycetes* based on the SSU and LSU analyses of five species of *Asterina* and a related asexual morph (Hofmann et al. 2010). In recent years, *Asterinales* was thought to comprise the families *Asterinaceae*, *Parmulariaceae* and *Aulographaceae* (Wu et al. 2011, Hyde et al. 2013). Recently, Hongsanan et al. (2014) provided a reassessment of the order. Based on LSU maximum likelihood and Bayesian analysis, and, despite the absence of molecular data for the *Parmulariaceae*, the authors concluded that only *Asterinaceae* should be included within *Asterinales*.

In the present study, we provide a robust molecular dataset that includes the type species of *Asterina*, as well as three other genera of *Asterinaceae*, the type species of the *Parmulariaceae* and a genus formerly assigned to the *Parmulariaceae*. The resulting LSU rDNA tree (Fig. 1) agrees in general with recent multigene analysis of the *Dothideomycetes* (Schoch et al. 2009) and demonstrated that the *Asterinales* comprises both *Asterinaceae* and *Parmulariaceae* as proposed by Barr & Huhndorf (2001), clustering with *Phaeotrichiaceae* and *Venturaceae*.

A second analysis (available in TreeBASE), was done aiming at verifying if the former molecular studies involving species of *Asterina* and *Lemobisia* (Hofmann et al. 2010, Hongsanan et al. 2014) correctly assigned the taxa included to the *Asterinaceae*. Based on these studies we conclude that these taxa, although considered by the authors as representative of species in the *Asterinales*, are in fact misplaced, and should be treated as *incertae sedis*, since they do not group with *A. melastomatis* – the type species of this family. The *Asterinaceae*, including the genera *Asterina*, *Batistinula*, *Lemobisia* and *Phylleucoma* may, therefore, be polyphyletic, requiring a thorough reassessment. Nevertheless, it is important to note that all studies performed until now (Hofmann et al. 2010, Hongsanan et al. 2014), used relatively short LSU sequences (c. 490 bp) that may not provide the necessary resolution needed.

*Asterotexis cucurbitacearum* was initially classified in the *Parmulariaceae* (Theissen & Sydow 1914) and then transferred to *Asterinaceae* (Inácio & Cannon 2008, Kirk et al. 2008, Guerrero et al. 2011). This species is clearly not a member of the *Asterinaceae* (contradictory to what was shown by Hongsanan et al. 2014) and is transferred here to the newly proposed family *Asterotexiaceae*. This new family grouped (Fig. 1) with *Inocyclus angularis* (originally described as a member of the *Parmulariaceae*).

Nuclear DNA of *P. styracis*, the type species of the *Parmulariaceae* was isolated and studied for the first time here. DNA was successfully isolated from *I. angularis*, allowing a preliminary assessment of the *Parmulariaceae*. Although involving only two taxa, the finding that *I. angularis* does not group with the type of *Parmulariaceae*, confirm that the *Parmulariaceae* is polyphyletic (Inácio & Cannon 2008, Hongsanan et al. 2014). The status of *I. angularis* within the genus *Inocyclus* requires confirmation, ideally with a molecular assessment of the type species of *Inocyclus*.

The molecular phylogenetic analysis presented here clearly indicates that both the *Parmulariaceae* and *Asterinaceae* are polyphyletic. Only the epitypification of the taxa in these and other families of thyrnesobiliod ascomycetes, followed by molecular phylogenetic analysis will resolve their taxonomic placement and produce a more natural classification for these neglected tropical fungi.

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