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Standard Paper

The identity, ecology and distribution of *Polypyrenula* (Ascomycota: Dothideomycetes): a new member of *Trypetheliaceae* revealed by molecular and anatomical data

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Abstract

New collections are reported of the monospecific genus *Polypyrenula*, an apparently extinct and doubtfully lichenized fungus, typically classified in the *Pyrenulaceae*. Anatomical studies reveal that it is facultatively lichenized. The structure of its hamathecium suggests affinities with Dothideomycetes rather than Eurotiomycetes. Molecular analysis using nuLSU and mtSSU markers demonstrates for the first time its inclusion in *Trypetheliaceae*, outside the core genera as part of the early diverging lineages in this family. The known distribution of *Polypyrenula* is extended to Mexico and South America, new information on its phorophyte associations is provided, and the name *Polypyrenula sexlocularis* is reinstated as the correct name for this species.

Key words: *albissima*, Bolivia, lichen, Mexico, seasonally dry tropical forest, *sexlocularis*

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Introduction

Recent studies in the family *Pyrenulaceae* have revealed that systematic revision at the genus level is necessary. For instance, the genus *Pyrenula* Ach. (Acharius 1814), with the largest number of species in the family, is not monophyletic. Other genera currently accepted in *Pyrenulaceae* for which sequences are available, namely *Anthracothecium*, *Lithothelium* and *Pyrgillus*, are nested within *Pyrenula* s. lat. (Gueidan *et al.* 2008, 2016; Aptroot 2012; Weerakoon *et al.* 2012). Several genera are included in the family based on phenotype features but without any molecular data, such as *Clypeopyrenis*, *Distopyrenis*, *Pyrenowilmsia* and *Sulcopyrenula* (Lücking *et al.* 2017). In addition, the monospecific genus *Polypyrenula* D. Hawksw. (Hawksworth 1985) had been placed provisionally in *Pyrenulaceae* (Hawksworth 1983, 1985; Harris 1995; Lumbsch & Huhndorf 2010).

Thus far, *Polypyrenula* was known with certainty only from the type collection of *Microthelia sexlocularis* Müll. Arg. This consists of two small pieces of bark collected by Fée almost 200 years ago from a medicinal plant brought to Europe from the Caribbean, identified as *Croton cascarilla* (L.) L. An earlier collection from the same substratum and originally identified by Fée (1825) as *Verrucaria epidermidis* var. *albissima* was also considered to represent this taxon (Hawksworth 1983; Aptroot 1991). Clements (1909) first established the generic name *Polythelis* for *Microthelia sexlocularis*, thereby generating an illegitimate later homonym of *Polythelis* Arthur, established only three years earlier for an unrelated rust fungus. This was probably in the course of Clements's work on his book and therefore Clements was presumably unaware of this pre-existing name. Hawksworth (1985) subsequently introduced the replacement name *Polypyrenula* and the combination *P. sexlocularis* (Müll. Arg.) D. Hawksw. Aptroot (1991) reasoned that the earlier epithet *albissima* applied to this taxon and proposed the combination *P. albissima* (A. Massal.) Aptroot, which was based on a misconception as explained below.

Regarding the ecology and distribution of *P. sexlocularis*, Hawksworth (1983) argued that the reported phorophyte name, *Croton cascarilla*, was a synonym of *C. eluteria*, a species presumed to be restricted to the Bahamas, but that the name *C. cascarilla* had generally been misapplied to *C. linearis*, an apparently more widespread species. The taxonomy of these plants remains unresolved (Webster 1993; Van Ee & Berry 2010) but this group of species is characteristic for the Caribbean. Given that most tropical crustose lichens have little to no phorophyte specificity (Cáceres *et al.* 2007; Rosabal *et al.*

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2013), it is unlikely that *P. sexlocularis* is restricted to a single phorophyte species, as suggested by Hawksworth (1983), or even a single genus, although a distribution restricted to the Caribbean may be conceivable. Aptroot (1991) assumed a rather narrow distribution, considering the species possibly extinct. Potential phorophyte specificity also depends on whether the taxon is lichenized or not: if lichenized, there should be no strict specificity but rather a preference for certain bark types depending on structure and pH, among other characters; if non-lichenized, specificity depends on the source of carbohydrates for the fungus (i.e. whether saprotrophic or even parasitic). Previous studies considered *Polypyrenula* to be doubtfully lichenized (Aptroot 1991; Gueidan *et al.* 2016), although Hawksworth (1983) suggested it might be associated with *Trentepohlia*.

Polypyrenula sexlocularis is unique in having ascospores with a pronounced basal euseptum (formed from the septal plate, the septal material is deposited starting from the centre towards the periphery, more or less parallel to the separating membrane), followed by 3–4 distosepta (lacking a septal plate and formed from the endospore, the septal material is deposited from the periphery to the centre, usually more densely near the edges, resulting in asymmetrical thickness). However, due to the poor state of the type collection, the apothecial anatomy of this taxon has not been studied in detail (Hawksworth 1983). The unusual disposition of the ascospore septa, together with the poorly conserved nature of the type collection and some misconceptions about the underlying material, has provoked several nomenclatural changes over the years. At least two different epithets (*albissima*, *sexlocularis*) in six different genera (*Verrucaria*, *Sagedia*, *Pyrenula*, *Microthelia*, *Polythelis*, *Polypyrenula*) have been applied to this taxon, most of them incorrectly (see below).

Often the type of ascospore septation is diagnostic at the genus, family or even order level for many lineages of lichenized and non-lichenized Ascomycota (Hawksworth 1983; Aptroot 2012; Sweetwood 2012). Families such as *Pyrenulaceae* and *Trypetheliaceae* have species with both eusepta and distosepta but the eusepta are reduced instead of pronounced (Aptroot 1991; Aptroot *et al.* 2008; Sweetwood *et al.* 2012). The ascospores of *P. sexlocularis* resemble some species of *Splanchnonema* (*Pleosporales*: *Pleomassariaceae*) that have a pronounced submedial euseptum in addition to distosepta (Barr 1982).

The discovery of several new collections of *Polypyrenula* from Mexico and Bolivia is reported here, greatly extending the range of this species and allowing us to study its characteristics in detail. Observations of the hamathecium structure indicate it is more likely that *Polypyrenula* belongs to *Trypetheliaceae* in the Dothideomycetes than to *Pyrenulaceae* in the Eurotiomycetes. Unpublished results from this study were included in the current classification of *Polypyrenula* in *Trypetheliaceae* (Lücking *et al.* 2017). In this paper we present original morphological and molecular evidence for the inclusion of *Polypyrenula* in *Trypetheliaceae*, and provide a new understanding of its nomenclature, distribution and ecology.

Materials and Methods

Anatomical studies

Specimens from Mexico were studied at Oregon State University, USA using standard techniques with an Olympus SZ61 dissecting microscope and an Olympus BX41 compound microscope, both connected to a NIKON D5300 digital camera. Specimens from Bolivia were studied at the Universidade Federal Mato de Grosso do Sul, Brazil. Sections were mounted and measured in tap water, and KOH and IKI reagents were used when necessary at 10% and 0.3%, respectively, following Bungartz (2002). Lichen products were identified using spot tests (Hale 1979) and thin-layer chromatography (TLC), with solvent system C, following established methods (Culberson & Ammann 1979; Culberson & Johnson 1982; Orange *et al.* 2010).

Taxon sampling

Two samples of *Polypyrenula sexlocularis* collected from Mexico were used to generate new sequences for each of the following markers: nuclear ribosomal internal transcribed spacer region (ITS), a fragment of nuclear large subunit rDNA (nuLSU) and a fragment of the mitochondrial small subunit rDNA (mtSSU). Initial BLAST searches showed members of *Trypetheliaceae* to be the closest relatives. Based on this, two analyses were performed. Firstly, we included our nuLSU sequences in the analysis of Wijayawardene *et al.* (2014) to place the new sequences within Dothideomycetes and test their position relative to *Trypetheliaceae* (see Supplementary Material Fig. S1, available online). Secondly, we performed an analysis within the framework of the most recent phylogeny of *Trypetheliaceae* (Nelsen *et al.* 2014; Hyde *et al.* 2016; Lücking *et al.* 2016), with an emphasis on the basal lineages of the family. We included a total of 171 sequences, 85 of mtSSU and 86 of nuLSU, for 82 ingroup species, including representatives from all of the genera of *Trypetheliaceae* currently published in GenBank (Supplementary Material Table S1, available online). *Cladosporium cladosporioides* and relatives were selected as outgroup following Nelsen *et al.* (2014) and Lücking *et al.* (2016).

DNA extraction, PCR amplification, and sequencing

Total DNA was isolated from the new collections using the Sigma-Aldrich REDExtract-N-Amp Plant PCR Kit (St. Louis, Missouri, USA) following the manufacturer's instructions, except only two ascomata per sample were used in 15 µl of extraction buffer followed by 15 µl of dilution buffer. The whole ITS and portions of mtSSU and nuLSU were amplified and sequenced using the following primers: ITS1F/ITS4 (White *et al.* 1990; Gardes & Bruns 1993), mrSSU1/mrSSU3R (Zoller *et al.* 1999) and AL2R/LR6 (Vilgalys & Hester 1990; Mangold *et al.* 2008) respectively.

Each 10 µl PCR reaction consisted of 5 µl R4775 Sigma-Aldrich REDExtract-n-Amp PCR Ready Mix, 0.5 µl of each primer (10 µM), 3 µl water, and 1 µl undiluted DNA. The PCR cycling conditions for ITS were: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 45 s and 72 °C for 105 s, followed by 72 °C for 5 min. The PCR cycling conditions for mtSSU and nuLSU were: 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 53 °C (for mtSSU) or 57 °C (for nuLSU) for 1 min and 72 °C for 105 s, followed by 72 °C for 10 min. Then 2 µl of each PCR product was visualized on 1.5% TBA agarose gel stained with GelRed (Biotium). Samples showing single bands were cleaned directly from PCR products with ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA). If double bands were visible, the PCR product was gel-extracted and cleaned with GELase (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's instructions.

Samples were sequenced at Eurofins MWG Operon LLC (Louisville, KY, USA). Each 12 µl reaction consisted of 2.4 µl of

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primer (at 10 µM), and either 2 µl undiluted PCR product cleaned with ExoSAP-IT and 7.6 µl water or 2.4 µl of primer (at 10 µM) and 9.6 µl DNA cleaned with GELase.

Phylogenetic analysis

New sequences were edited in Geneious 8.1.9 (Kearse *et al.* 2012). All sequences of mtSSU and nuLSU were aligned independently using the multiple sequence alignment algorithm MAFFT 7 (Katoh & Standley 2013). Ambiguously aligned columns were removed using trimAl 1.2 (Capella-Gutierrez *et al.* 2009) on the automated 1 settings. Single locus analyses were performed to visually test for topological incongruence. A maximum likelihood (ML) analysis of all genes partitioned by locus was performed in the RAxML-HPC BlackBox 8.2.10 (Stamatakis 2014), with 552 bootstrapping replicates as automatically determined by RAxML using a saturation criterion. In addition, a Bayesian analysis was performed in MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001), with two independent runs of two million generations each, resampling every 1000 trees, 25% burn-in, and heated chains of 0.2. Both analyses were carried out with the GTR GAMMA model and run on the Cipres Gateway server (Miller *et al.* 2010). The final Bayesian tree was plotted using Geneious and edited in Photoshop CS6.

Results

Phylogenetic analysis

The new sequences generated in this study comprise two of ITS, two of mtSSU and two of nuLSU, all from Mexican material. The combined data set consisted of 82 ingroup species (Supplementary Material Table S1, available online) and 838 unambiguously aligned characters (357 from mtSSU and 481 from nuLSU); ITS was not included in the analysis due to the lack of sufficient reference sequences for the other taxa. The final topology for the *Trypetheliaceae* (Fig. 1) was consistent with previous studies (Nelsen *et al.* 2014; Lücking *et al.* 2016).

Our analysis showed that *Polypyrenula sexlocularis* belongs in the *Trypetheliaceae*, in the basal clade that also includes species of *Bogoriella*, *Constrictolumina*, *Julella* and *Novomicrothelia*. *Polypyrenula* and *Alloarthopyrenia italica* formed an unsupported sister clade but the relationships of *Polypyrenula* with the other lineages in this basal portion of the tree remain unresolved.

Taxonomy

Polypyrenula sexlocularis (Müll. Arg.) D. Hawksw.

Bull. Br. Mus. Nat. Hist., Bot. **14**, 165 (1985).—*Microthelia sexlocularis* Müll. Arg., *Mém. Soc. Phys. Hist. Nat. Genève* **30**(3), 38 (1888); *Polythelis sexlocularis* (Müll. Arg.) Clem., *Gen. Fung.*, 173 (1909).—*Polypyrenula albissima* Aptroot, *Biblioth. Lichenol.* **44**, 102 (1991); as '(A. Massalongo) Aptroot' [nom. illeg., see below]; type: [Caribbean], unknown locality, on [medicinal] bark of *Croton cascarilla* brought to Europe, s. dat., *Fée* s. n. (G—holotype!).

Non *Verrucaria epidermidis* var. *albissima* Fée ex Müller, *Mém. Soc. Phys. Hist. Nat. Genève* **30**(3), 39 (1888); type: '*Habitat in America supra epidermidem Crotonis cascarillae (Linn.)*', (not seen); = *Pyrenula* sp.?

Non *Verrucaria epidermidis* var. *albissima* Ach., *K. Vetensk-Acad. Nya Handl.* **30**, 149 (1809).—*Sagedia albissima* (Ach.) A. Massal., Ric. Auton. Lich. Crost. (Verona), 161 (1852).—*Pyrenula albissima* (Ach.) Trevis., Spighe Paglie, 18 (1853); type: Sweden, unknown locality, on birch bark [BM-ACH 281-282(3); lectotype *fide* Aguirre-Hudson, *Bull. Br. Mus. Nat. Hist., Bot.* **21**, 106 (1991); H-ACH 0774A—isolectotype]; = *Leptorhaphis epidermidis* (Ach.) Th. Fr.

(Fig. 2A–H)

Thallus ecorticate, endoperidermal, thin, whitish grey to brownish, without pseudocyphellae, black hypothallus sometimes present at contact points with other lichens. *Photobiont* trentepohlioid; however, not always present.

Ascomata perithecioid, solitary, erumpent from the substratum, sometimes partly covered by bark cells, 0.20–0.35 mm wide; usually with a well-defined involucrellum and then 0.35–0.55(–0.65) mm in total. *Ostiole* apical, brownish black, up to 0.06 mm wide. *Wall* without crystals, proper exciple apically and laterally carbonized but basally only reddish brown. *Hamathecium* not inspersed, IKI−, pseudoparaphyses branched and anastomosing, 0.5–1.4 µm diam., no septation seen in water at ×400, embedded in a gelatinous matrix. *Asci* bitunicate, subcylindrical, tholus not amyloid, ocular chamber wide and rounded, (6–)8-spored, 62–70 × 15 µm, not seen after discharge. *Ascospores* biseriate in the asci, elongate-ellipsoid, with rounded ends, reddish brown (greyish in KOH) but basal cell frequently paler, 20–30(–35) × 5.8–8.7 µm (means = 24.58, 7.29; standard deviations = 3.1, 0.65; $n = 45$), 1(–2) pronounced and transverse basal euseptum that may constrict the cell, 3–5 transversal distosepta; basal euseptum forming first, followed by the distosepta, then later the pigmentation, endospore thick up to 1.3 µm, lumina rounded to angular but not astrothelioid, spore wall smooth, gelatinous sheath not seen.

Pycnidia not observed.

Chemistry. KOH−, UV−. No substances detected by TLC.

Remarks. Most of the ascospores observed in the material had a pronounced basal euseptum, although it was common to see ascospores from the same ascoma with the euseptum reduced (Fig. 2H). Two ascospores of different thalli had two pronounced basal eusepta instead of one (Fig. 2D). Previously, Hawksworth (1983) found 4–6 spores per ascus but our samples agreed with Müller's (1888) description in having (6–)8 spores per ascus.

Over the centuries, the nomenclature of this taxon has suffered a series of misconceptions that began with Fée (1825) misapplying the name *Verrucaria epidermidis* var. *albissima* Ach., originally established for a temperate European taxon (Acharius 1809), to tropical material. As so often occurs, subsequent authors, such as Müller (1888) and Zahlbruckner (1922), considered Fée's misidentification a separately established name and, consequently, a later homonym, '*Verrucaria epidermidis* var. *albissima* Fée', which is not only incorrect, since Fée clearly referred to Acharius's name (and hence its type), but also inadmissible by the *Code*. According to ICN Art. 48.1, the case 'When an author adopts an existing name but definitely excludes its type, a later homonym that must be attributed solely to that author is considered to have been published' refers to very specific instances but not to misapplied names (i.e. misidentifications). Fée (1825) simply believed that his tropical material was conspecific with the type of *Verrucaria epidermidis* var. *albissima* Ach.: 'Habitat in America supra epidermidem *Crotonis cascarillae*

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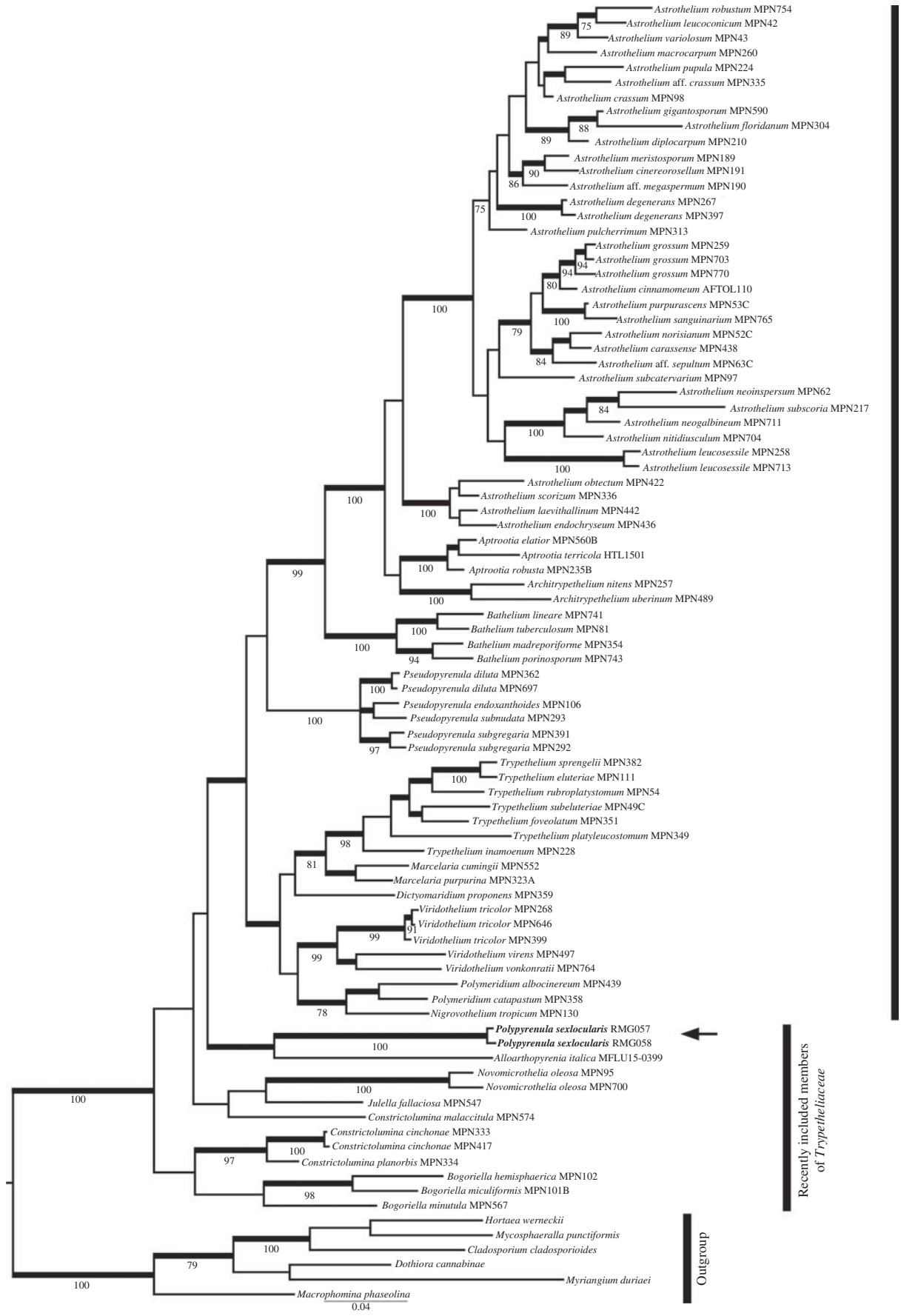

Fig. 1. Phylogeny of the family *Trypetheliaceae* based on a Bayesian analysis of the markers mtSSU and nuLSU. Support values are shown as numbers if maximum likelihood bootstrap values are ≥ 75 and as bold branches if Bayesian posterior probabilities are ≥ 0.95. Bold names and arrow show the position of *Polypyrenula sexlocularis*. The alphanumeric codes following the species names represent the DNA associated with the voucher specimens and are derived from GenBank. Full details of each species are given in Supplementary Material Table S1 (available online).

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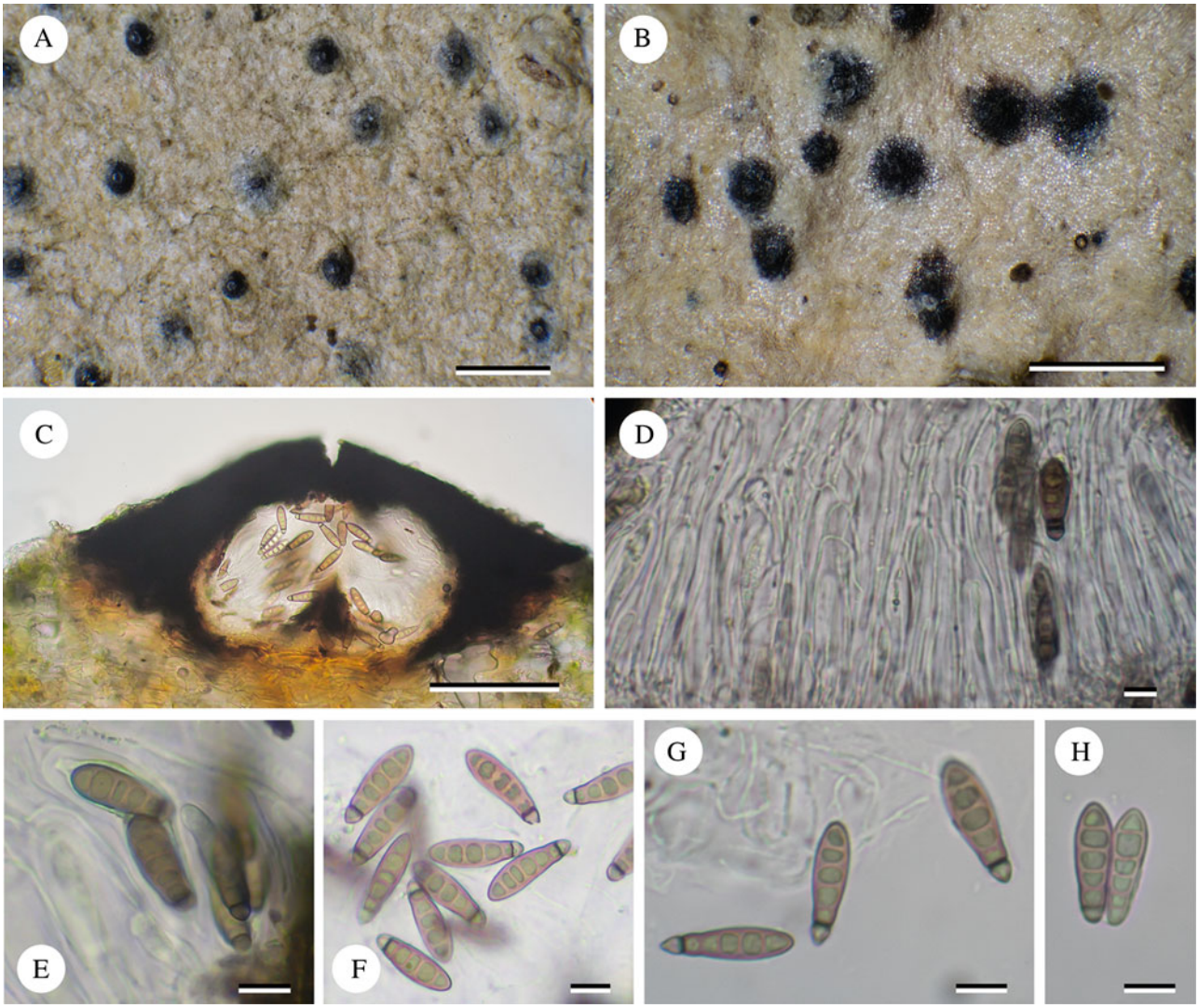

Fig. 2. *Polypyrenula sexlocularis*. A & B, thallus; C, section of ascoma showing Trentepohlioid algal cells; D, hamathecium showing anastomosing pseudoparaphyses and ascospore with two eusepta; E, ascus; F & G, ascospores; H, ascospores, with reduced septum (right) and with pronounced euseptum (left). Collection numbers: A, C, E–G, *Miranda* 2736; B & D, *Miranda* 1791; H, *Miranda* 2539. Scales: A & B = 1 mm; C = 100 μm; D–H = 10 μm. In colour online.

(Linn.), nec non [as well as] in Europa supra epidermidem destructam variarum arborum … Cette variété est commune sur la cascarille' (Fée 1825: 84).

Müller's (1888) and Zahlbruckner's (1922) error was based on Massalongo's combination of the infraspecific epithet *albissima* into *Sagedia*, as *Sagedia albissima* (Massalongo 1852), which was deemed the legitimate replacement name for the presumed homonym and served as a basis for the combination of this epithet into *Pyrenula*, as '*P. albissima* (A. Massal.) Trevis.' (Trevisan 1853) and subsequently into *Polypyrenula*, as '*P. albissima* (A. Massal.) Aptroot' (Aptroot 1991). ICN Art. 58.1 does not apply for two reasons. Firstly, the presumed later homonym '*Verrucaria epidermidis* var. *albissima* Fée' does not exist when the work of Fée (1825, 1837) is consulted. The first to establish this name was Müller (1888) and much later this name was again quoted by Zahlbruckner (1922). Hence there was no case of illegitimacy on the basis of which Art. 58.1 could be invoked for the combinations given by Massalongo (1852) and Trevisan (1853), both published long before Müller (1888). One must consider the name '*Verrucaria epidermidis* var. *albissima* Fée' to have been validly established by Müller (1888) and ascribed to Fée, but then to be cited as *Verrucaria epidermidis* var. *albissima* Fée ex Müller (ICN Art. 46. 3), with a publication date identical to that of *Microthelia sexlocularis* Müll. Arg., which therefore takes priority at the rank of species. Secondly, Massalongo (1852) did not quote the name '*Verrucaria epidermidis* var. *albissima* Fée' but cited the basionym of his combination as *Verrucaria epidermidis* var. *albissima* Ach., including its type and leaving no doubt as to the application of his combination to the taxon including the type of Acharius's name (ICN Art. 7.3). For the same reason, the presumed combination '*Pyrenula albissima* (A. Massal.) Trevis.' is neither valid nor a replacement name (as '*P. albissima* Trevis.'), since Trevisan (1853) provided a mechanical combination and did not exclude the name *Verrucaria epidermidis* var. *albissima* Ach. from synonymy; it must be cited as *P. albissima* (Ach.) Trevis. and applies to the taxon typified by the type of *Verrucaria epidermidis* var. *albissima* Ach. (ICN Art. 7.3).

Aptroot (1991) proposed the combination '*Polypyrenula albissima* (A. Massal.) Aptroot', which must be treated as a replacement name under ICN Art. 58.1, as *Polypyrenula albissima*

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Aptroot, but not based on Massalongo (1852) as Acharius's type was excluded, but instead on the notion that Müller (1888) first established the illegitimate later homonym *Verrucaria epidermidis* var. *albissima* Fée ex Müller. Since Aptroot (1991) did not cite the correct replaced synonym (i.e. *Verrucaria epidermidis* var. *albissima* Fée ex Müller (1888)) but cited the name erroneously from Fée's (1825) earlier publication, the name *Polypyrenula albissima* Aptroot is invalid as a replacement name according to ICN Art. 41.8(c). The type of *Microthelia sexlocularis* was part of the original material of the name *Verrucaria cascarillae* Fée (1837), and Fée (1837) clearly indicated that the material previously identified by him as *Verrucaria epidermidis* var. *albissima* was a different specimen, although he considered it possibly conspecific with *V. cascarillae*. Also, Müller (1888) clearly referred to that different specimen as the basis for the name *Verrucaria epidermidis* var. *albissima* Fée established by him. Hence, according to ICN 58.1, Aptroot's name is to be treated as '… the name of a new taxon with a different type …' and is consequently validly published. It is, however, illegitimate since Aptroot (1991) cited the name *Microthelia sexlocularis* Müll. Arg. in synonymy, a name that should have been adopted (ICN Art. 52.1), as it has priority over *Verrucaria epidermidis* var. *albissima* Fée ex Müller.

While the temperate *Verrucaria epidermidis* var. *albissima* Ach. has been established as a synonym of *Leptorhaphis epidermidis* (Ach.) Th. Fr. (Harris 1973; Aguirre-Hudson 1991), the taxonomic status of Fée's tropical material originally identified as *V. epidermidis* var. *albissima* and later illustrated by Massalongo (1852, fig. 316) remains unresolved. The spores were illustrated as fusiform with pointed ends, with three distosepta but without a euseptum, and more or less brown in colour, and the hamathecium was shown as mostly unbranched and lacking anastomoses. These characters point to a species of *Pyrenula* with an ecorticate, white thallus, such as the widespread *P. microcarpa* Müll. Arg. Indeed, Trevisan (1853) recombined *albissima* into *Pyrenula* based on Massalongo's (1852) description.

Müller (1888) found that the type material of Fée's *Verrucaria cascarillae* was a mix of no less than seven species, three corresponding to existing names at the time: *Pseudopyrenula diluta* (Fée) Müll. Arg., *Arthopyrenia cinchonae* (Ach.) Müll. Arg. (now *Constrictolumina cinchonae* (Ach.) Lücking *et al.*) and *Pyrenula guayaci* (Fée) Müll. Arg. (now *Parapyrenis guayaci* (Fée) Aptroot). The other four were established as new species: *Microthelia dominans* Müll. Arg., *Arthopyrenia feeana* Müll. Arg. (now *Anisomeridium feeanum* (Müll. Arg.) R. C. Harris), *Porina cascarillae* Müll. Arg. and *Microthelia sexlocularis* Müll. Arg., the last providing the valid epithet for the species studied here. Müller (1888) considered the material identified by Fée (1825) as *V. epidermidis* var. *albissima* to represent pycnidia of *M. sexlocularis*, which contrasts with Massalongo's (1852) depiction of asci and ascospores for the same material.

Distribution and ecology. *Polypyrenula sexlocularis* was so far known with certainty only from the type collection, stated as growing on *Croton cascarilla* (Fée 1837). As outlined above, the substratum indicates a likely origin in the Caribbean, where *Croton cascarilla* and other species of this complex (*C. eluteria*, *C. linearis*) are widespread (Webster 1993; Van Ee & Berry 2010). However, new collections were found along the Pacific Coast of Mexico and in Bolivia, suggesting that this species is widespread in the Neotropics and probably overlooked, since its morphology is rather nondescript and corresponds to that of several other genera that are often under-collected due to their rather uniform phenotype, such as *Anisomeridium*, *Bogoriella*, *Constrictolumina*, *Polymeridium* and *Pseudopyrenula*.

All new samples were associated to some degree with dry areas, especially with seasonally dry tropical forests, which corresponds with the wide distribution of this biome in the Caribbean. This ecosystem typically consists of more than 50% deciduous trees, has an extended dry season of three to eight months, a mean annual precipitation of 400–2000 mm, mean annual temperature above 25 °C, and an elevation from sea level to 2000 m (Trejo & Dirzo 2000; Sánchez-Azofeifa *et al.* 2005; Portillo-Quintero & Sánchez-Azofeifa 2010). Most of this ecosystem in the Neotropics is found in Mexico, followed by Bolivia, with important areas in the West Indies and similar forest-types such as Caatinga in Brazil (Pennington *et al.* 2006; Portillo-Quintero & Sánchez-Azofeifa 2010; DRYFLOR *et al.* 2016).

In Mexico, *Polypyrenula sexlocularis* is a rare species mostly found in secondary forests, with only one out of seven samples originating from mature forest. It was associated with the following phorophytes: *Apoplanesia paniculata*, *Caesalpinia caladenia*, *Gliricidia sepium*, *Heliocarpus pallidus* and *Leucaena lanceolata*. Most samples were found at elevations below 340 m, but one Bolivian sample (*M. Kukwa* 11367) was found at 1500 m.

Specimens examined. **Mexico:** *Jalisco*: La Huerta, Chamela Biological Station (CBS), 300 m W of Tejón Trail, 600 m, 19° 30′11″N, 105°2′52″W, mature seasonally dry tropical forest, elev. 44 m, 2010, *Miranda* 1791 (MEXU); surrounding areas of CBS: Ejido Santa Cruz, 19°35′57″N, 105°2′55″W, secondary seasonally dry tropical forest, elev. 118 m, 2010, *Miranda* 2736 (MEXU); *ibid.*, very disturbed seasonally dry tropical forest, 19° 35′22″N, 105°2′4″W, elev. 144 m, *Miranda* 3823, 3828, 3829, 3886 (MEXU); Ejido Caimán, secondary seasonally dry tropical forest, 19°28′3″N, 105°56′11″W, elev. 54 m, 2010, *Miranda* 2539 (MEXU).—**Bolivia:** *Dept. Santa Cruz*: Prov. Cordillera, PNANMI Kaa-Iya del Gran Chaco, near Peto Blanco, park guard's station, 18°56′26″S, 60°22′39″W, Chiquitano forest, elev. 340 m, 2011, *A. Flakus* 23655 (LPB, KRAM); Prov. Guarayos, RN de Vida Silvestre Ríos Blanco y Negro, Plan de Manejo AISU, 15°09′13″S, 62°47′57″W, lowland Amazon forest, elev. 240 m, 2009, leg. *A. Flakus* 13730 & *P. Rodriguez* (LPB, KRAM). *Dept. Tarija*: Prov. Burnet O'Connor, 28 km from Entre Ríos, near Soledad, 21°41′00″S, 64°07′29″W, Tucumano-Boliviano montano forest, elev. 1500 m, 2012, *M. Kukwa* 11367 (UGDA, LPB).

Discussion

Hawksworth (1983) provided the first thorough description of *Polypyrenula sexlocularis* (as *Polythelis sexlocularis*), but the underlying material was so scarce that he could not verify details such as hamathecium structure. He suggested that the delicate tapering of the interascal filaments was very similar to the true paraphyses of *Pyrenula* and proceeded to accommodate the taxon in *Pyrenulaceae*. Based on Hawksworth's description, Harris (1989) and Aptroot (1991) considered the hamathecium to represent cellular pseudoparaphyses and moved the genus to *Requienellaceae*. Harris (1995) decided to restrict *Requienellaceae* to *Requienella*, which is currently placed in Sordariomycetes in the order *Xylariales* (Jaklitsch *et al.* 2016), and he also moved *Polypyrenula* back to *Pyrenulaceae*.

Our observations based on new collections show that the hamathecium of *P. sexlocularis* is more consistent with

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traberculate pseudoparaphyses (thin interascal filaments that are branched and anastomosing, without visible septation at ×400), which supports the inclusion of the taxon in *Trypetheliaceae*. Although the type material cannot be thoroughly examined for this feature, the description by Müller (1888) stated that the interascal filaments are anastomosing ('*connexæ*').

In our preliminary phylogenetic analysis (Supplementary Material Fig. S1, available online), *P. sexlocularis* was positioned among the core genera of *Trypetheliaceae*; however, the analysis included a limited representation for each of most orders of Dothideomycetes, creating alignment artifacts due to low resolution and widespread taxon sampling. In the final phylogenetic analysis (Fig. 1) that included both mtSSU and nuLSU markers and focused the sampling on *Trypetheliaceae*, we established that *P. sexlocularis* is not part of what was traditionally considered as *Trypetheliaceae* but is positioned among the early diverging lineages of the family, together with other genera only recently included in this family based on molecular data (Nelsen *et al.* 2011, 2014; Lücking *et al.* 2016). The taxon shares with the other basal lineages the ecorticate thallus, exposed black perithecioid ascomata, at least in part euseptate spores that are not astrothelioid, and often weakly to non-lichenized thalli (Nelsen *et al.* 2014; Aptroot & Lücking 2016; Hyde *et al.* 2016; Lücking *et al.* 2016). *Polypyrenula* is the only genus in the family with pronounced eusepta in combination with distosepta.

Currently, the family *Trypetheliaceae* is composed of 17 recognized genera (Hyde *et al.* 2016; Lücking *et al.* 2017), more than 400 species and *c.* 800 predicted species, making it the second largest family of tropical corticolous lichens (Aptroot *et al.* 2016). In particular, the basal lineages in the family are poorly understood and have few DNA sequences available, which might explain the lack of support at the base of the phylogeny. More data are needed for these lineages to test their delimitation and monophyly, and to establish their mutual relationships; the data so far suggest that recently established genera such as *Bogoriella* and *Constrictolumina* remain polyphyletic.

For this study, although not included in our analyses, we decided to also provide the ITS sequences of *P. sexlocularis* for future barcoding purposes, regardless of the infrequent use of the ITS region in this family. Despite ITS being the recommended genetic barcode for fungi (Schoch *et al.* 2012), the number of sequences available is extremely low for *Trypetheliaceae* and other large tropical crustose families, such as *Graphidaceae* and *Pyrenulaceae*. The use of ITS in broad phylogenies may represent a problem because its high variability makes unambiguous alignments difficult, which might be the reason why recent studies in the Dothideomycetes did not include this marker (Nelsen *et al.* 2009, 2011, 2014; Hyde *et al.* 2013; Wijayawardene *et al.* 2014). However, exemplar studies on species complexes in *Trypetheliaceae* such as *Trypethelium eluteriae* (Luangsuphabool *et al.* 2016) show that in this case, the ITS region provides excellent resolution. Expanding the ITS sequence representation of *Trypetheliaceae* will allow the inclusion of these species in biotic, environmental and ecological studies that rely on molecular barcoding. We therefore strongly encourage other researchers to include the ITS marker in their studies of pyrenolichens.

With the new collections now available, *P. sexlocularis* cannot be considered extinct. In fact, six out of the seven samples from Mexico were found in disturbed forests, suggesting that this species might be able to adapt itself to the current conditions of seasonally dry tropical forests: small relicts of pristine areas surrounded by a majority of secondary forests (Quesada *et al.* 2009). Thus, in contrast to the ecosystem as a whole (Janzen 1988; Portillo-Quintero & Sánchez-Azofeifa 2010), this lichen may not be particularly endangered. *Polypyrenula sexlocularis* should be considered an overlooked species. Given that it was found in Mexico, the Caribbean and Bolivia, we expect it to occur throughout the Neotropics in forested ecosystems with a dry season.

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