Canoparmelia amazonica, *Myelochroa lindmanii* and *Parmelinella salacinifera* belong to *Parmelinella* (Parmeliaceae)

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Abstract. The lichen family Parmeliaceae is among the best studied groups of lichens. *Canoparmelia amazonica, Myelochroa lindmanii*, and *Parmelinella salacinifera* are species of Parmeliaceae that have yet to be studied in detail with molecular methods. This study used analyses of ITS sequences to examine the phylogenetic position of these three species. *Canoparmelia amazonica* and *M. lindmanii* were recovered within *Parmelinella* rather than the genera to which they are currently assigned. While for the *P. salacinifera* we confirmed its phylogenetic position within the genus. Chemical and morphological descriptions of species are provided, generic placement is discussed, and new combinations are proposed as needed. These results highlight the need for morphological revision of the delimitation of *Parmelinella*, a small genus of Parmeliaceae that has been considered well-defined morphologically and is phylogenetically closely related to *Bulbothrix* s.l.

Keywords. Lichens, taxonomy, ITS, restinga vegetation, Cerrado vegetation, Brazil.

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Parmelinella Elix & Hale, a genus belonging to the Parmeliaceae, was segregated from *Parmelina* s.l. by Elix & Hale (1987) with only three species. The genus was delimited by several characteristics including: a white medulla, emaculate thallus, simple cilia commonly restricted to the lobe axils, salazinic acid, consalazinic acid as main secondary compounds (Benatti 2014; Elix & Hale 1987). *Parmelinella* has a wide geographical distribution, occurring mainly in tropical and subtropical regions (Kirika et al. 2016). Currently, 10 species are known and five of them occur in Brazil: *Parmelinella cinerascens* (Lynge) Benatti & Marcelli, *P. mutata* (Vain.) Benatti, *P. salacinifera* (Hale) Marcelli & Benatti, *P. versiformis* (Kremp.) Marcelli and *P. wallichiana* (Taylor) Elix & Hale (Benatti 2014; Kirika et. al. 2016). *Parmelinella inexplicabilis* Marcelli & C.H.Ribeiro described from Brazil by Marcelli & Ribeiro (2002) is now treated as a synonym of *Remototrachyna costaricensis* (Nyl.) Divakar & A.Crespo (Divakar et al. 2010; Sipman et al. 2009).

The number of species in the genus *Parmelinella* has increased over time, in part, due to morphological reviews of taxa previously placed in other genera, such as *P. cinerascens* and *P. salacinifera*, that were previously placed in *Canoparmelia* Elix & Hale (Benatti 2012, 2014; Elix et al. 1986). *Canoparmelia* species are recognized mainly by the absence of cilia on the margins of the lobes, while *P. cinerascens* and *P. salacinifera* have cilia. Based mainly on this, both were transferred to *Parmelinella* (Benatti 2012, 2014). However, phylogenetic studies have not yet been carried out to confirm the generic placement of these species. *Canoparmelia* is phylogenetically distinct from *Parmelinella*, belonging to the *Parmotrema* clade within the parmelioid lichens (Crespo et al. 2010). *Parmelinella*, while morphologically distinct, is closely related to *Bulbothrix* Hale, and belongs to the *Parmelina* clade, which also includes *Myelochroa* (Asahina) Elix & Hale, *Remototrachyna*

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Divakar & A.Crespo, and *Parmelina* Hale (Crespo et al. 2010; Divakar et al. 2017).

The identification of lichenized fungal species, primarily based on morphology and chemistry characteristics, has changed over time, often associating with molecular tools (Lendemer 2021; Lücking et al. 2020). Recent studies of Parmeliaceae, the most diverse family of lichenized fungi with ca. 2,700 species (Lucking et al. 2017), have led to reviews of numerous genera and species circumscriptions, resulting in many taxonomic changes (Alors et al. 2016; Blanco et al. 2004, 2005; Boluda et al. 2019; Divakar et al. 2010; Kirika et al. 2017; Leavitt et al. 2018). In *Parmelinella*, phylogenetic studies also have expanded the knowledge about diversity within the genus, indicating that the morphological variation among the species may be much broader than previously believed (Eliasaro et al. 2010; Kirika et al. 2016).

During a preliminary study using the DNA barcode of fungi (ITS region) to identify Brazilian Parmeliaceae species, we found that *Canoparmelia amazonica* (Nylander) Elix & Hale and *Myelochroa lindmanii* (Lynge) Elix & Hale appeared to be more closely related to *Parmelinella* species. The aim of this study was to examine the phylogenetic position of *C. amazonica*, *M. lindmanii* and *Parmelinella salacinifera* within of the *Parmelina* clade.

Materials and Methods

Sampling and specimen identification. Samples were collected from trees in two different vegetation domains of Brazil: Cerrado (Brazilian savanna), in the midwest region, and restinga (Atlantic Forest) close to the Brazil-Uruguay border. The Cerrado, distributed between the midwest and northeast regions of Brazil, is considered a global biodiversity hotspot (Mittermeier et al. 2011). It is characterized by heterogeneous vegetation, ranging from grassland formations to dry forests (Bianchi & Haig 2013; Strassburg et al. 2017). On the other hand, the restingas are distributed throughout the entire coastal zone of Brazil, being considered pioneer formations because the vegetation is located on unstable terrain with marine, wind and fluvomarine influences (IBGE 2006; Muylaert et al. 2018). Morphological examination of specimens was carried out using a Nikon SMZ645 stereomicroscope and an Olympus CX22LED optical microscope. Species identification was based on diagnostic characteristics of the thallus, such as medulla coloration, presence/absence and type of vegetative propagules, lower surface coloration, rhizine shape, presence of cilia on the lobe margins, shape and size of the ascospores and conidia. Species descriptions were produced using the Parmeliaceae description protocol adapted from Canêz & Marcelli (2006).

For the initial chemical identification, we also performed spot tests (K, C, KC, P and UV) on the upper cortex and medulla of the specimens, following Orange et al. (2010). Chemical compounds were further studied with thin layer chromatography (TLC) on all specimens, using solvent G in proportions 139:83:8 of toluene/ethyl acetate/formic acid to identify secalonic and protocetraric acid, while for salazinic and consalazinic acid we used the toluene/ethyl acetate/acetic acid in the ratio of 6:4:1 (Culberson 1972; Culberson & Kristinsson 1970; Orange et al. 2010). All studied specimens were deposited in the Herbarium of the Federal University of Mato Grosso do Sul (CGMS).

DNA extraction, PCR amplification and sequencing. For the DNA extraction, we removed a fragment of the thallus (ca. 5mm^2). The samples were placed in acetone for 20 minutes to remove the secondary metabolites that can interfere in DNA extraction and amplification. The fragments were air dried until the acetone evaporated completely. DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega), following the protocol of the manufacturer. The ITS region was amplified using the ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) universal primers. PCR reactions were carried out in 25 μL reactions containing: 1× buffer, 0.2mM dNTPs, 0.2 μM of each primer, 3.0 mM $MgCl_2$, 1U Taq DNA polymerase (Promega) and ca. 20 ng of DNA template. The PCR conditions were: initial denaturation for 2 min at 95°C, followed by 30 cycles of denaturation at 95°C for 30 s, annealing between 54°C to 56°C for 30 s, extension at 72°C for 1.10 min and a final extension at 72°C for 5 min, using a Mastercycler® Gradient thermal cycler. The amplification products were visualized in 1% agarose gel stained with GelRed®. Macrogen Korea performed DNA purification and sequencing.

Dataset selection. Seven new ITS sequences were generated in the study, three of *Canoparmelia*

amazonica, two of *Myelochra lindmanii,* and two of *Parmelinella salacinifera*. These new sequences were compared to 35 GenBank sequences (**Supplementary Table S1**) representing species of the genera *Bulbothrix*, *Myelochroa*, *Parmelina*, *Parmelinella* and *Remototrachyna*, members of the *Parmelina* clade (Crespo et al. 2010). Reference sequences, representing all the main taxa belonging to this clade, were selected according to the genetic proximity identified through the Basic Local Alignment Search Tool (BLAST) (Johnson et al. 2008). The chosen outgroup, *Hypotrachyna osseoalba* (Vain.) Y.S.Park & Hale, was selected according to recent Parmeliaceae phylogenetic studies, showing that *Hypotrachyna* (Vain.) Hale is the closest genus of the *Parmelina* clade (Divakar et al. 2017).

Sequence assembly and alignment. For assembly and quality evaluation of the DNA sequences generated, we used Geneious® 9.1.6 (Kearse et al. 2012). For the sequence alignments, we used MAFFT v7 (Katho & Standley 2013), applying the following parameters: G-INS-i algorithm, 200PAM/K=2 scoring matrix, offset value of 0.0 and the remaining parameters default values (Katho & Standley 2013). To remove ambiguous sites and increase the reliability of the final alignments, we used the Gblocks 0.97b webserver (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) selecting all less stringent options (Talavera & Castresana 2007). The final ITS matrix contained 446 aligned nucleotide positions, included 19 species and is available from TreeBASE, reviewer access URL: http://purl.org/phylo/treebase/phylows/study/TB2:S27491?x-access-code=2670b7a1369dacb53e7f387c08e83b45&format=html.

Phylogenetic analyses. We inferred the phylogenetic position of Parmeliaceae specimens previously identified as *Canoparmelia amazonica*, *Myelochroa lindmanii* and *Parmelinella salacinifera* through the construction of phylogenetic trees using maximum likelihood (ML) and Monte Carlo Bayesian Markov chain (B/MCMC) approaches. Both analyses were run on the Cipres Science Gateway webserver (https://www.phylo.org/). The construction of the ML tree was done using the RAxML v8 program (Stamatakis 2014), with the "GTRGAMMA" model and 1000 bootstrap pseudoreplicates to evaluate the branch support. To determine the best-fitting nucleotide substitution model, we used the program JModelTest 2.1.6 (Darriba et al. 2012) using the Akaike information criterion–AIC (Akaike 1974). The model GTR+I+G was selected. For Bayesian inference, we used MrBayes 3.2.7 (Ronquist et al. 2012). We ran two parallel Markov Chain Monte Carlo (MCMC) chains for 10 million generations, saving every 1.000^{th} tree. The first 25% of the sampled trees were discarded as burn-in. We verified the convergence of the Bayesian analysis chains using Tracer 1.7 (Rambaut et al. 2018), considering as good indicative a sample size (ESS) $\geq$200. Branches with bootstrap values $\geq$70%, and posterior probabilities (pp) $\geq$0.95 were considered supported.

Results and Discussion

Morphological and chemical characters supported the identification of the seven specimens examined as *Canoparmelia amazonica*, *Myelochroa lindmanii* and *Parmelinella salacinifera* (Benatti 2014; Hale 1976a,b). *Canoparmelia amazonica* is characterized by the presence of a white medulla, absence of cilia, laminal isidia, and medullary protocetraric acid. *Myelochroa lindmanii* specimens were identified by the presence of yellow medulla (secalonic acid), marginal cilia and laminal isidia. In case of *Parmelinella salacinifera,* the specimen was recognized mainly by the presence of a white medulla, laminal isidia, cilia on the margins of the lobes, brown lower surface, and presence of salazinic and consalazinic acid in the medulla.

According to the phylogenetic analyses, all genera belonging to the *Parmelina* clade were recovered as monophyletic (support values above 0.95 for posterior probabilities and 70% for bootstrap), except for *Bulbothrix* s.l, which presented two distinct clades (**Fig. 1**). Our results corroborate previous studies that have identified *Bulbothrix* as polyphyletic (Divakar et al. 2017); therefore, we maintained the previous convention in *Bulbothrix*, referring to 'Clade 1′ for species closer to *Remototrachyna*, and 'Clade 2′ for those closer to *Parmelinella*.

The *Parmelinella* clade was supported in the phylogeny (**Fig. 1**), and included sequences of *Canoparmelia amazonica*, *Myelochroa lindmanii*, *Parmelinella salacinifera P. schimperiana* and *P. wallichiana*. Sequences of *M. lindmanii* group *with Parmelia lindmanii* (also from Brazil, GenBank

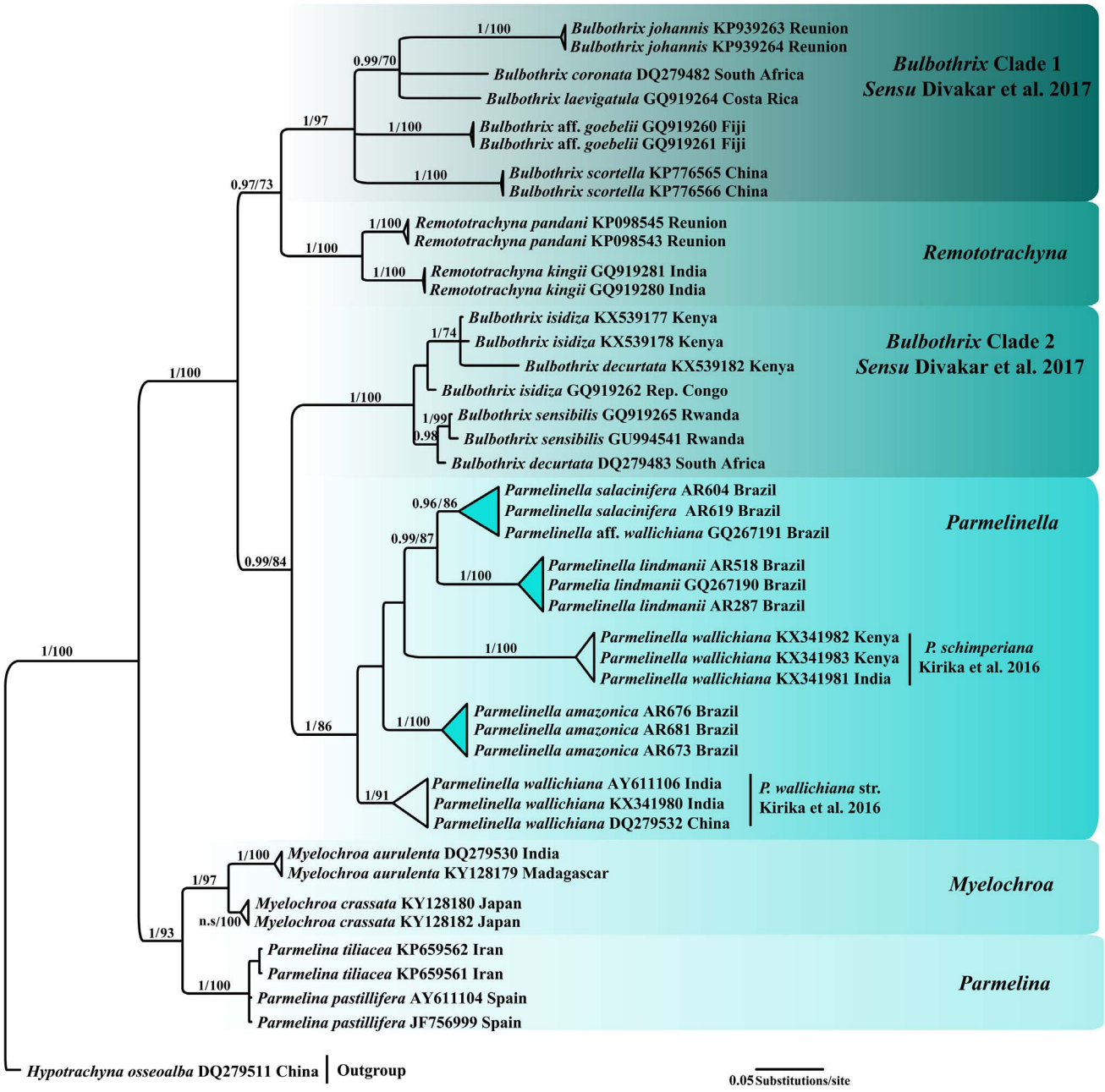

Figure 1. Phylogenetic relationships of *Parmelina* clade based on a maximum-likelihood (ML) and Bayesian analyses from ITS rDNA sequences. The Bayesian tree is shown here. Branches recovered with support values of Posterior probabilities ≥0.95 from the Bayesian analysis and ML bootstrap values ≥70% are indicated in the figure.

accession number GQ267190), indicating that they belong to the same species, however in *Parmelinella*.

The analyses recovered *Canoparmelia amazonica* and *Myelochroa lindmanii* as monophyletic species, with high support values (**Fig. 1**). However, *Parmelinella salacinifera* grouped with a sequence of *Parmelinella* aff. *wallichiana* obtained from a Brazilian specimen (Eliasaro et al. 2010). *Parmelinella salacinifera* and *P. wallichiana* share the same medullary chemistry and have isidia, but differ in lobes sizes, lower surface color and ascospore size (Benatti 2014; Kirika et al. 2016a). Since *Parmelinella wallichiana* s.str. (Kirika et al. 2016) is in another clade, the Brazilian specimen identified as *P.* aff. *wallichiana* should be revised. Due to its genetic similarity (95–96% identity in the ITS region), it can

be a intraspecific variation of *P. salacinifera* or even another species still unknown.

Taxonomy

Parmelinella amazonica (Nyl.) A.S.Rodrigues, A.P.Lorenz & Canêz, *comb. nov*. **Fig. 2**

MycoBank MB839860

ITS Barcoding Sequence Accessions: MW364885, MW364886 and MW364887.

≡ *Parmelia amazonica* Nyl., Flora 68: 611. 1885. ≡ *Pseudoparmelia amazonica* (Nyl.) Hale, Phytologia 29(3): 189. 1974. ≡ *Canoparmelia amazonica* (Nyl.) Elix & Hale, Mycotaxon 27: 278. 1986. Type: BRAZIL. Pará: Santarém, *R. Spruce 111* (h-nyl 35111 [n.v.] lectotype designated by Hale (1959) as holotype; bm [n.v.], g [n.v.], ny [n.v.], w [n.v.], pc [n.v.], isolectotypes) *fide* Hale (1976a).

Description. Thallus greyish green, lobate, corticolous, 7–8 cm broad. Lobes loosely attached, irregularly branched, contiguous to laterally overlapping, 2.5–6.5 mm wide, continuous, smooth and rarely rugose, apical zone rounded, margin crenate; maculae punctiform on the margins of the lobes; cilia absent. Soralia and pustules absent. Isidia present, laminar, concolor to the upper cortex, cylindrical, simple to branched, caducous, 0.1–0.3 × 0.04–0.06 mm. Medulla white. Lower surface black, lustrous, smooth to veined, marginal zone naked, 1–3 mm wide, light brown to brown, slightly lustrous to opaque, papillate to veined. Rhizines black in the central region and brown in the marginal zone, simple, abundant, 0.2–0.4 × 0.02–0.05 mm, evenly distributed. Apothecia present, disc imperforate, substipitate, laminal, margin crenate and involute, amphithecium isidiate; ascospores ellipsoid, 10–15 × 7–9 μm. Pycnidia present, few, submarginal to laminal, conspicuous and immersed, ostiole black, conidia sublageniform to bifusiform, some may appear to be bacilliform, but have a more inflated extremity, similar to sublageniform, 5–8 μm long.

Chemistry. Atranorin and protocetraric acid. We found traces of two other unidentified substances in the specimens studied. One with lower Rf and one higher than protocetraric acid in solvent G. Spot tests: upper cortex: K+ yellow, UV–; medulla K–, C–, KC–, P+ orange, UV–.

Geographic distribution. Africa: Angola and Madagascar (Aptroot 1991; Hale 1976a); North America: Mexico and United States (Lendemer et al. 2016; Sipman & Wolf 1998); Central America: Cuba, Honduras and Puerto Rico (Hale 1976a); South America: Bolivia, Brazil, Colombia, Guyana, Trinidad and Venezuela (Hale 1976a, Flakus et al. 2016; Sipman & Aptroot 1992); and Asia: China, Sri Lanka, Thailand and Taiwan (Ahti et al. 1999; Breuss & Brunnbauer 1997; Hale 1976a; Mongkolsuk et al. 2011).

Notes. *Parmelinella amazonica* is characterized by the absence of cilia on the margins of the lobes, presence of isidia laminar, and protocetraric acid as a medullary chemical compound. *Parmelinella amazonica* was previously treated in *Canoparmelia* due to the absence of cilia, white medulla, and presence of papillae on the lower margin of the lobes (Elix et al. 1986). The similarity between *C. amazonica* and *P. salacinifera,* when both were treated in *Pseudoparmelia*, had already been observed by Hale (1976a) who differentiated them mainly by the medullary chemistry. Later, both taxa were transferred to *Canoparmelia* (Elix et al. 1986) and *Parmelinella* (Benatti 2012).

Parmelinella amazonica differs from other species of *Canoparmelia* that were later transferred to *Parmelinella*, such as *P. cinerascens* and *P. salacinifera*, in that it does not have cilia on the margins of the lobes and in that it produces protocetraric acid in the medulla. The only morphological characters that suggest this species belongs to *Parmelinella* are the white medulla and the absence or traces of triterpenes. Both characteristics are also found in other genera of Parmeliaceae (e.g., *Canoparmelia*, *Parmotrema* A.Massal., *Hypotrachyna* (Vain.) Hale, among others). Therefore, at the moment, this placement is entirely based on molecular data.

Additional specimens examined. BRAZIL. Mato Grosso do Sul: Campo Grande co., Natural Reserve of the Federal University of Mato Grosso do Sul, on bark, 23 May 2019, *A.S. Rodrigues 673, 676, 681* (cgms).

Parmelinella lindmanii (Lynge) A.S.Rodrigues, Canêz & A.P.Lorenz, *comb. nov.* **Fig. 2**

MycoBank MB839859

ITS Barcoding Sequence Accessions: MW364890 and MW364891.

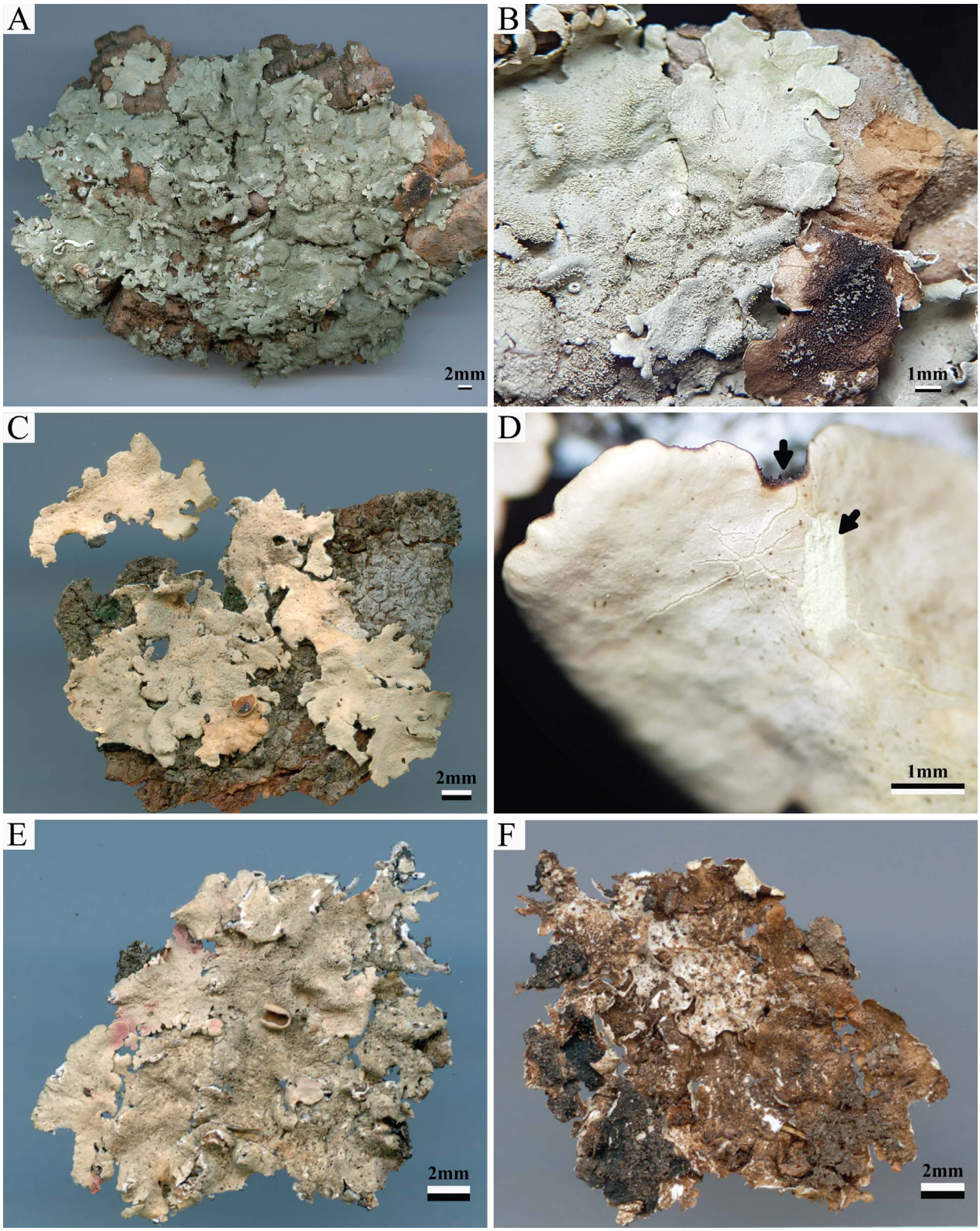

Figure 2. Images of the species studied. **A–B**. Thallus, habitat and eciliated lobes of *Parmelinella amazonica*. **C.** Thallus and habitat of *Parmelinella lindmanii*. **D.** Detail of the morphological characteristics of *Parmelinella lindmanii*. Arrows indicate axillary cilia and yellow medulla. **E–F**. Upper surface, as well as brown lower surface of *Parmelinella salacinifera*.

≡ *Parmelia lindmanii* Lynge, Ark. Bot. 13(13): 74. 1914. ≡ *Parmelina lindmanii* (Lynge) Hale, Phytologia 28(5): 483. 1974. ≡ *Myelochroa lindmanii* (Lynge) Elix & Hale, Mycotaxon 29: 241. 1987. ≡ *Parmotrema lindmanii* (Lynge) Kurok. *in* Kurokwa & Arakawa, Bull. Bot. Gard. Toyama 2: 42. 1997. Type: BRAZIL. Rio Grande do Sul: Porto Alegre, 25 Sept. 1892, *G.O.A. Malme 450* (s [n.v.], holotype) *fide* Lynge (1914).

Description. Thallus greyish green, lobate to sublacinulate, corticolous, 4–6 cm broad. Lobes loosely attached, irregularly branched, contiguous, 2–4.5 mm wide, smooth, continuous to rugose in the older regions, apical zone rounded, margin crenate to slightly crenate; maculae absent; cilia present, black, simple, few and distributed mainly in the axils of the lobes, (0.05) 0.1–0.3 × 0.02–0.05 mm. Soralia and pustules absent. Isidia present, laminar to submarginal, concolor to the upper cortex, cylindrical, simple to slightly branched and very fragile, erect, caducous, 0.1–0.3 × 0.04–0.06 mm. Medulla yellow. Lower surface black, lustrous, smooth to slightly veined, marginal zone naked present, 1–1.5 mm wide, brown, lustrous, papillate to rugose. Rhizines black on the margins with whitish apex becoming totally black in the central region, simple, abundant, 0.2–0.5 × 0.02–0.06 mm, evenly distributed. Apothecia present, disc imperforate, substipitate, laminal, margin smooth, ornamentation absent; ascospores ellipsoid, 10–12 × 6–8 μm. Pycnidia present, submarginal to laminal, conspicuous and immersed, ostiole black, conidia bifusiform 5.5–7 μm long.

Chemistry. Atranorin and secalonic acid. Spot tests: upper cortex: K+ yellow, UV–; medulla K+ weak orange, C–, KC–, P–, UV–.

Geographic distribution. North America: Mexico (Hale 1976b). South America: Argentina, Bolivia, Brazil, Colombia, Paraguay, Uruguay and Venezuela (Flakus et al. 2014; Hale 1976b).

Notes. *Parmelinella lindmanii* is characterized by a yellow medulla, laminal isidia, and secalonic acid as the main medullary chemical compound. Hale (1976b) distinguished two sections in the genus *Parmelina* with distinct morphological and chemical characteristics. Section *Parmelina* included species characterized by and white medulla and absence of triterpenes, while the section *Myelochroa* included species with triterpenes and a pigmented medulla. *Parmelina lindmanii* and *P. immiscens* (Nyl.) Hale, both with yellow medulla and lack of triterpenes, were classified in section *Parmelina*. Elix & Hale (1987) proposed five new genera segregated from *Parmelina* s.l., including *Myelochroa* and *Pamelinella*. The genus *Parmelinella* included species with white medulla, marginal cilia commonly restricted to the lobe axils, salazinic and consalazinic acids, and lack or traces of triterpenes. Species of *Myelochroa* had moderately wide lobes, cilia homogeneously distributed on the margins of the lobes, yellow medulla, presence of medullary secalonic acid, and commonly triterpenes. At that time, *P. immiscens* and *P. lindmanii*, were included in *Myelochroa* (Elix & Hale 1987). Afterwards, Kurokawa & Arakawa (1997) combined *M. lindmanii* into *Parmotrema* because this species had large lobes, with a rounded apex and an evident naked marginal zone in the lower surface. However, *Parmotrema lindmanii* (Lynge) Kurok. was not used by most lichenologists (Eliasaro & Adler 2000; Estrabou et al. 2006; Flakus et al. 2014; Spielmann & Marcelli 2008).

Myelochroa is the sister group to genus *Parmelina*, and together with *Bulbothrix*, *Remototrachyna* and *Parmelinella* belongs to the *Parmelina* clade (Crespo et al. 2010; Divakar et al. 2017). Eliasaro et al. (2010), using ITS sequences, pointed out that *M. lindmanii* probably belonged to *Parmelinella*. They decided not to propose any nomenclatural changes considering that more molecular analyses were needed, instead treating the species under the basionym *Parmelia lindmanii*. Subsequently, a phylogenetic study with *Parmelinella* (Kirika et al. 2016) used the same sequence but referred to it as *Parmelinella lindmanii*. However, neither Eliasaro et al. (2010) nor Kirika et. al. (2016) proposed a formal new combination. Thus, we formally transfer the species to *Parmelinella*.

Based on the morphological characters, *Parmelinella lindmanii* shares with the other species of the genus the presence of marginal cilia commonly restricted to lobes axils and absence of triterpenes. Differing mainly by the yellow medulla and the presence of secalonic acid.

Additional specimens examined. BRAZIL. Rio Grande do Sul: Pelotas co., Dunas Las Acácias, on bark, 28 Jul. 2017, *A.S. Rodrigues 287* (cgms). Rio

Grande co., Barra Falsa, on bark, 15 Jan. 2018, *A.S. Rodrigues 518* (CGMS).

Conclusions

Phylogenetic analyses performed in this study recovered *Canaparmelia amazonica*, *Myelochroa lindmanii,* and *Parmelinella salanifera* as members of the genus *Parmelinella*. Thus, the phenotypic characteristics of *Parmelinella* are broader than those expected, including species with white or yellow medulla, presence or absence of marginal cilia that when present are commonly restricted to the lobe axils, and lower surface varying from black to brown. However, to better delimit *Parmelinella*, studies with greater sampling of species and specimens are needed, including from different geographical regions and using integrative approaches, such as morphological and phylogenetic data.

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Supplementary documents online:

Supplementary Table S1. Sequences of the specimens used in the study, containing the collection site and the accession number of the ITS sequences deposited in GenBank. The new sequences generated in the study are in bold.