The Lichenologist **48**(2): 141–146 (2016) doi:10.1017/S0024282915000511

The species pair *Pseudocyphellaria pilosella-piloselloides* (lichenized Ascomycota: *Lobariaceae*) is a single species

María Inés MESSUTI, Alfredo PASSO, Jose Martin SCERVINO and Romina VIDAL-RUSSELL

Abstract: The foliose lichens *Pseudocyphellaria pilosella* and *P. piloselloides* are characterized by a cyanobacterial photobiont, a tomentose upper surface, a yellow medulla and yellow pseudocyphellae. The latter species has long been recognized as the sorediate counterpart of the former. The morphological, anatomical, chemical, and molecular analyses performed for this study support their treatment as a single species.

Key words: haplotypes, lichens, southern South America, synonym, taxonomy

Accepted for publication 14 *October* 2015

Introduction

The genus *Pseudocyphellaria* (*Lobariaceae*, lichenized Ascomycota) is among the best known lichen genera in the Southern Hemisphere (Galloway 1986, 1992). However, during the past decades molecular evidence suggests that the genus, as traditionally understood, is polyphyletic (Miądlikowska & Lutzoni 2000, 2004; Thomas *et al*. 2002; Stenroos *et al*. 2003; Wiklund & Wedin 2003). More recently, Moncada *et al*. (2013, 2014) focused on the phylogeny of *Lobariaceae*, and confirmed the polyphyletic origin of *Pseudocyphellaria*. Furthermore, discrepancies among some species within the genus remain unresolved; many of them are related to the retention of incorrect names or uncertain taxonomic interpretations.

Some of these problems might be solved by studying more material, allowing for a better knowledge of intraspecific variation. One example of this concerns the southern South American endemic species, *Pseudocyphellaria pilosella* Malme and *P. piloselloides* (Räsänen) H. Magn., characterized by a foliose thallus, cyanobacterial photobiont, yellow medulla and pseudocyphellae, and tomentose upper surface. These two species were originally conceived as similar by Räsänen (1932), differing only in their reproductive mode (fertile vs. sorediate). Later, Galloway (1986) cited both as counterparts. However, their diagnostic features and overlapping geographical distribution led us to reconsider whether these are well-defined species. Accordingly, we examined the morphological, anatomical, chemical, and molecular characters in the complex.

M. I. Messuti, A. Passo, J. M. Scervino and R. Vidal-Russell: INIBIOMA (CONICET-Universidad Nacional Comahue), Quintral 1250, Bariloche, Argentina. Email: maria.messuti@crub.uncoma.edu.ar

Materials and Methods

Specimens studied

Specimens of *Pseudocyphellaria pilosella* and *P. piloselloides* were collected by the authors in 2014 during field studies in *Nothofagus* forest in north-western Patagonia, Argentina. All the lichen material was deposited at BCRU.

In order to increase the sampling area, additional specimens from Llao-Llao (Río Negro Province, Argentina), Parque Nacional Puyehue and Parque Nacional Queulat (X and XI Regions, Chile) provided by S. Calvelo (hb. SC) and T. Wheeler together with P. Nelson (hb. Wheeler) respectively, were also included; within these were collections which exhibit intermediate forms, with both soredia and apothecia.

Digital images of the type specimens provided by the S and H herbaria were compared with the material collected from Argentina and Chile.

Morphological and anatomical observations were performed following standardized methods used in lichenology. Secondary chemistry of the material was studied with high performance thin-layer chromatography (HPTLC) using methods described by Arup *et al.* (1993). Photomicrographs were taken with a digital camera USB 2.0.

Molecular studies

Total genomic DNA of *P. pilosella* and *P. piloselloides* was extracted from a total of 12 collections using the commercially available Ultraclean Microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, USA) according to the manufacturer's protocols, and resuspended in 50 µl of ultrapure water. The internal transcribed spacer regions (ITS, including 5.8S) of nuclear ribosomal DNA were sequenced and used in phylogenetic analysis. The ITS regions were amplified with the following pair of fungal-specific primers: ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). The PCR reactions were performed in a 25 µl PCR mixture containing 5× GoGreen Buffer (5 µl), 10 mM of each PCR nucleotide mix (1·6 µl), 10 µM of upstream (1 µl) and 10 µM downstream primer (1 µl), 5 u/µl GoTaq DNA polymerase (Promega, USA) (0·125 µl), 2 ng of template DNA and nuclease-free water to final volume (25 µl). PCR amplification was carried out in a Veriti 96-well thermal cycler (Applied Biosystems Inc., USA) using the following profile: one cycle of 2 min at 94 °C; 35 cycles of 1 min at 94 °C, 55 s at 53 °C, 3 min at 72 °C; one cycle of a final extension for 10 min at 72 °C. DNA fragments were detected by staining with SYBR® Safe DNA gel stain (Invitrogen, Life Technologies, USA). Amplicons generated by PCR were sequenced by the Macrogen Inc. (Korea) sequencing service.

Sequences of three *Pseudocyphellaria* species were used as outgroups: two of them, *P. coriifolia* (Müll. Arg.) Malme and *P. crocata* (L.) Vain., are closely related to *P. pilosella*, and the third one, *P. faveolata* (Delise) Malme, is more distantly related, according to Moncada *et al.* (2014) and the MycoBank website (http://www.mycobank.org/BioloMICSSequences.aspx?expandparm=f&file=all). Sequences of these species were downloaded from GenBank. The matrix consisted of four individuals of *P. coriifolia*, three of *P. crocata*, one of *P. faveolata*, eight of *P. pilosella*, and five of *P. piloselloides* (Table 1). Sequences were aligned and trimmed manually in Aliview (Larsson 2014). Genetic distances were calculated as uncorrected *P*-values in R (R Development Core Team 2011) with the seqinR package (Charif & Lobry 2007). Intermediate forms were not amplified due to low quality DNA.

The phylogenetic reconstruction was carried out with maximum likelihood criterion using RAxML (Stamatakis 2014) with a Gamma model of rate heterogeneity. Equal sequences were collapsed and only one representative was left for the analysis to avoid redundancy. Branch support was attained through non-parametric bootstrap with 1000 replicates.

TABLE 1. *List of the species, specimens, collection data and accession numbers for the marker ITS of* Pseudocyphellaria *used in the estimation of genetic distances and phylogenetic analyses. Newly obtained sequences for this study are in bold. Numbers in parentheses indicate specimens used in the calculation of genetic distances.*

Species	Haplotype	Country	Collection/Herbaria	GenBank Acc.No. (ITS)
P. coriifolia (1)		Argentina	*Stenroos* 5476 (TUR)	EU558706
P. coriifolia		Argentina	*Stenroos* 5563 (TUR)	EU558707
P. coriifolia (2)		Argentina	*Stenroos* 5264 (TUR)	EU558708
P. coriifolia		Argentina	*Stenroos* 5477 (TUR)	EU558709
P. crocata		Chile	*Galloway* PC12	AJ888205
P. crocata		Argentina	*Stenroos* 5474 (TUR)	EU558701
P. crocata (1)		Reunion I.	*LG: DNALIST* 688	JQ735976
P. faveolata		New Zealand	*Thomas* 994	AF350311
P. pilosella (1)	I	Argentina	*Stenroos* 5283 (TUR)	EU558740
P. pilosella	II	Argentina	*BCRU* 05398	**KT722796**
P. pilosella (2)	I	Argentina	*BCRU* 05407	**KT722797**
P. pilosella	III	Argentina	*BCRU* 05397	**KT722798**
P. pilosella	II	Argentina	*BCRU* 05400	**KT861464**
P. pilosella	II	Argentina	*BCRU* 05396	**KT861465**
P. pilosella	II	Argentina	*BCRU* 05395	**KT861466**
P. pilosella	II	Chile	*Wheeler & Nelson* 4394 (hb. Wheeler)	**KT861467**
P. piloselloides	II	Argentina	*BCRU* 05403	**KT722799**
P. piloselloides	II	Argentina	*BCRU* 05399	**KT861460**
P. piloselloides	II	Argentina	*BCRU* 05404a	**KT861461**
P. piloselloides	II	Argentina	*BCRU* 05401	**KT861462**
P. piloselloides	II	Argentina	*BCRU* 05404b	**KT861463**

Results and Discussion

Molecular analyses

The total length of the ITS alignment consisted of 420 characters. In the maximum likelihood tree, *Pseudocyphellaria coriifolia* and *P. crocata* are sister taxa (with 96% bootstrap support) and both are monophyletic (with 91% and 98% bootstrap support respectively). All individuals of *P. pilosella* and *P. piloselloides* form a clade with 99% bootstrap support (Fig. 1). The 13 sequences of "*P. pilosella-piloselloides*" analyzed were grouped in three haplotypes (Table 2 and Fig. 1). Haplotype I was found in two individuals, both identified as *P. pilosella*; Haplotype II, represented in Fig. 1 by only two sequences, was found in 10 individuals, five identified as *P. pilosella* and five as *P. piloselloides*; Haplotype III includes only one individual, identified as *P. pilosella* (Table 1). The pairwise genetic distance between the three haplotypes ranges from 0·002–0·007 (Table 2), where the number of site differences between each pair of sequences in the alignment ranges from 1–4. When the sequences of these individuals of *P. pilosella* and *P. piloselloides* were compared with sequences from individuals of *P. coriifolia* and *P. crocata* the site differences between each pair of sequences in the alignment increased to a minimum of eight sites. The pairwise genetic distance between species used in this study ranges from 0·029–0·038. The average genetic distance within *P. coriifolia* was higher than that between *P. pilosella* and *P. piloselloides* (Table 2). Consequently, the phylogenetic results, together with low genetic distances of ITS sequences found between *P. pilosella* and *P. piloselloides*, confirm that they should be regarded as a single species.

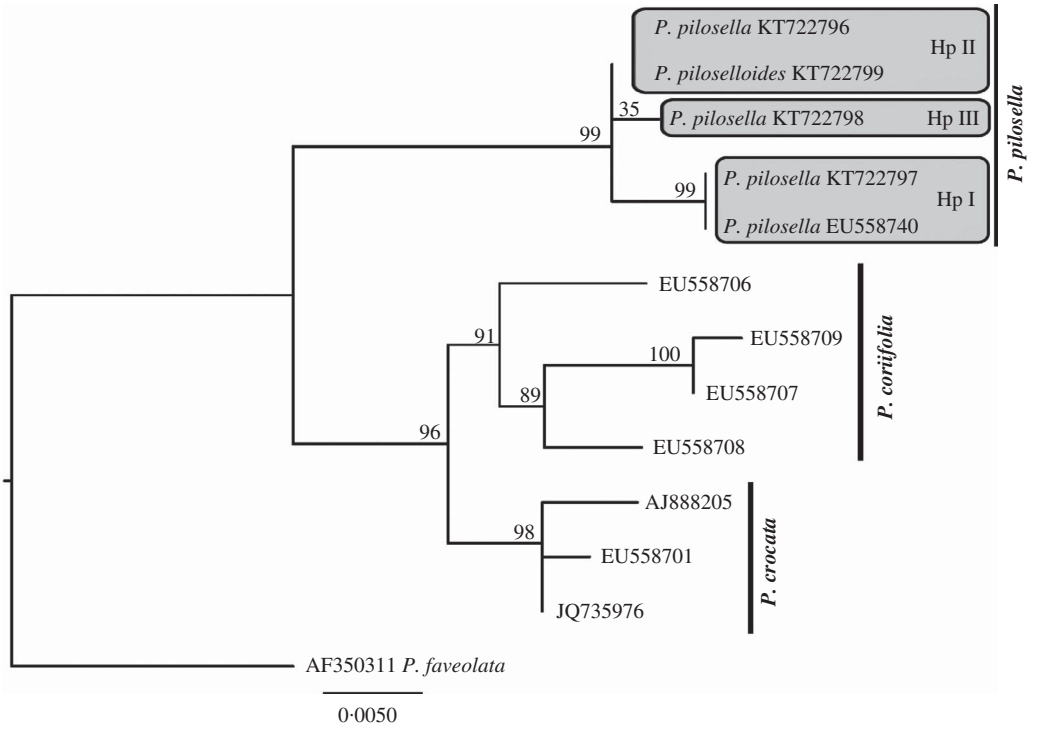

FIG. 1. Phylogenetic relationships of *Pseudocyphellaria pilosella* and *P. piloselloides*. Maximum likelihood tree based on ITS data with bootstrap branch support above branches. Haplotypes I, II, III (Hp I, Hp II, Hp III) are indicated in grey boxes.

TABLE 2. *Genetic distances of ITS sequences calculated as uncorrected* P-*values for haplotypes of* Pseudocyphellaria pilosella *and* P. piloselloides *and a comparison with outgroup species. See Table 1 for numbers in parentheses.*

Species	Haplotype I *P. pilosella* (1)	Haplotype I *P. pilosella* (2)	Haplotype II	Haplotype III	*P. coriifolia* (1)	*P. coriifolia* (2)	*P. crocata* (1)
Haplotype I *P. pilosella* (1)	-						
Haplotype I *P. pilosella* (2)	0·000	-					
Haplotype II	0·005	0·005	-				
Haplotype III	0·007	0·007	0·002	-			
P. coriifolia (1)	0·034	0·034	0·029	0·031	-		
P. coriifolia (2)	0·038	0·038	0·034	0·036	0·017	-	
P. crocata (1)	0·034	0·034	0·029	0·031	0·019	0·014	-

Taxonomy

Pseudocyphellaria pilosella Malme

Bih. K. svenska VetenskAkad. Handl., ser. 3, **25** (6)**:** 30 (1899); type: Argentina, Puerto Blest, *ad lacum* Nahuel Huapi, 1897, *P. Dusén* 180 (S!— holotype).

Pseudocyphellaria piloselloides (Räsänen) H. Magn., *Acta Horti gothoburg* **14:** 7 (1940).—*Cyanisticta piloselloides* Räsänen, *Ann. Bot. Soc. Zool.-Bot. Fenn. "Vanamo"* **2** (1)**:** 39 (1932); type: Chile, Fuegia, Fjordo Finlandia, *Berberis ilicifolia*, 28 February 1929, *H. Roivainen* (H!—lectotype).

(Fig. 2 A–D)

For a detailed description of the morphology and anatomy see Galloway (1986: 144 and 147). A short emended description with significant diagnostic features of *P. pilosella* is proposed below.

Thallus foliose, submonophyllous to lobulate, margins entire, irregular or notched. *Upper surface* white-pubescent tomentose and shallowly faveolate. *Medulla* yellow. *Lower surface* white-tomentose. *Pseudocyphellae* yellow, scattered, round to irregular, conspicuous. *Soralia* absent or present, mainly marginal, rarely laminal, glomerulate to coralloid. *Photobiont* cyanobacterial (*Nostoc*).

Apothecia absent or present, subpedicellate, rounded; *disc* dark red-brown, epruinose. *Pycnidia* present only in non-sorediate specimens.

Secondary chemistry. Calycin (+), pulvinic acid (+), norstictic acid (±), gyrophoric acid (+) and two unidentified triterpenoids (+).

Distribution and habitat. Pseudocyphellaria pilosella s. lat. occurs in the southern region of Argentina (Neuquén, Río Negro and Tierra del Fuego Provinces) and in Chile (VIII, IX, X, XI, XII Regions). This corticolous species is an epiphytic component of the Valdivian rainforest and Magellanic forest, occurring on trunks and twigs of trees and shrubs. Our field observations in Parque Nacional Nahuel Huapi, which exhibits a sharp precipitation gradient along a short distance, suggest that fertile individuals are found only in more humid, shaded habitats, while sorediate individuals are associated with drier, open areas.

Taxonomical notes. Pseudocyphellaria pilosella and *P. piloselloides*, as traditionally interpreted, were an easily recognizable species pair. Both share a combination of characters, namely a yellow medulla, a tomentose upper surface, and a cyanobacterial photobiont, which distinguishes them from most species of *Pseudocyphellaria* s. lat. in southern South America. The two species were separated from each other by the presence or absence of apothecia and the presence or absence of soredia, and by a more laciniate thallus in *P. pilosella* in contrast to a submonophyllous

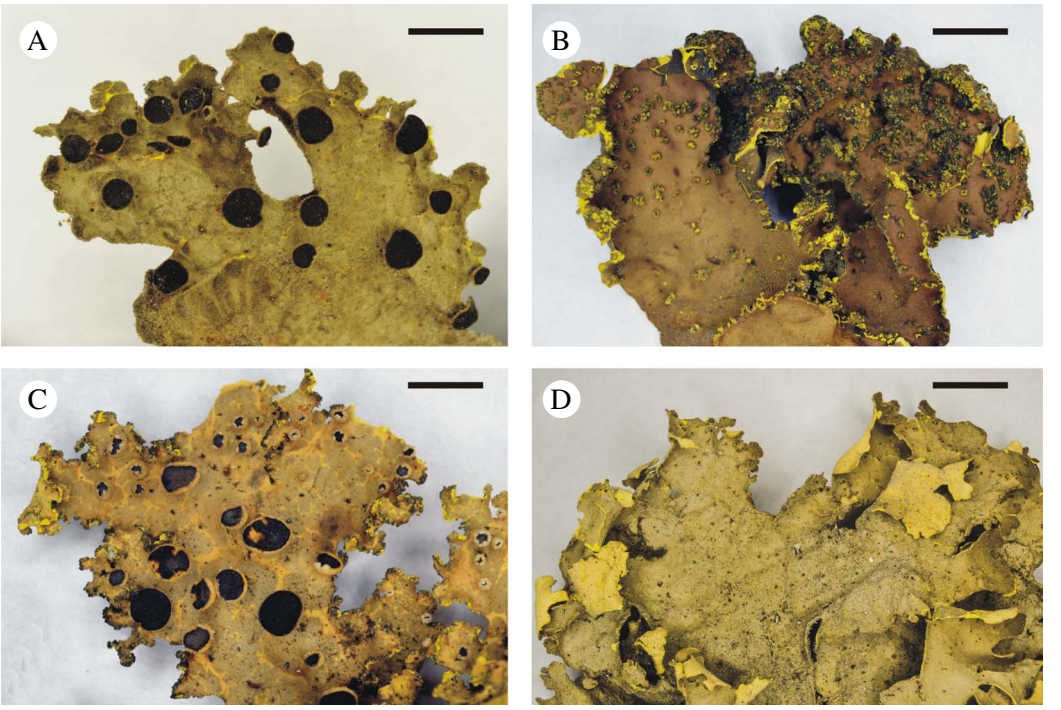

FIG. 2. Thallus variability in *Pseudocyphellaria pilosella*. A, Argentina, Provincia Neuquén, Puerto Blest, *M. I. Messuti et al.* (BCRU 05407) (apotheciate thallus); B, Chile, Parque Nacional Puyehue, along trail to Volcán Puyehue, *T. Wheeler & P. Nelson* 5820 (hb. Wheeler) (sorediate thallus); C, Chile, Parque Nacional Queulat, *T. Wheeler & P. Nelson* 7082 (hb. Wheeler) (apotheciate and sorediate thallus); D, Argentina, Provincia Río Negro, Puerto Blest, *M. I. Messuti et al.* (BCRU 05400) (sterile and non-sorediate thallus). Scales: A–D = 5 mm.

one in *P. piloselloides*. Accordingly, the two species were considered as fertile-sorediate counterparts (Galloway 1986). Regarding the lobe shape, when the type material of both species (*P. Dusén* 180, S; *H. Roivainen* s/n, H) was observed it became evident that the thallus of *P. pilosella* is not laciniate. Furthermore, revision of the several specimens collected from Argentina and Chile reveals that the shape of the lobes of both species is extremely variable, from submonophyllous to laciniate. Moreover, we found individuals (*S. Calvelo* 1360, *T. Wheeler & P. Nelson* 5801, 7082) with intermediate forms bearing apothecia and soredia in the same thallus. Therefore, the cited differences between the species, mentioned by Galloway (1986, 1992), were not reliable.

In addition, it has been suggested that species pairs with a sympatric distribution and intermediate forms should not be treated as separate species (Mattson & Lumbsch 1989). Differences in reproductive mode (sorediate vs. fertile forms) can be associated with environmental conditions and, thus, part of the phenotypic plasticity of the taxon; therefore difference in reproductive mode alone appears not to be a sufficient reason to recognize different species (Crespo & Pérez-Ortega 2009). All these assertions are supported by molecular evidence, as shown in Table 2 and Fig. 1, and consequently *P. piloselloides* is reduced here to synonymy of *P. pilosella*.

Additional specimens examined. **Chile:** *X Región*: Parque Nacional Puyehue, along trail to Volcán Puyehue, 40·620°S, 72·145°W, on bark of *Nothofagus dombeyi*, 2008, *T. Wheeler & P. Nelson* 5801, 5820 (hb. Wheeler); *ibid.*, 40·744°S, 72·296°W, on bark of *Ribes* sp., 2008, *T. Wheeler & P. Nelson* 5055

(hb. Wheeler); Parque Nacional Alerce Andino, camp at the western head of Laguna Sargazo, 41°30·631'S, 72°35·022'W, on *Berberis* spp., 2006, *T. Wheeler & P. Nelson* 1369 (hb. Wheeler). *XI Región*: Parque Nacional Queulat, 44·469°S, 72·548°W, on *Weinmannia trichosperma*, 2008, *T. Wheeler & P. Nelson* 6729 (hb. Wheeler); 44·471°S, 72·560°W, on bark of *Nothofagus dombeyi*, 2008, *T. Wheeler & P. Nelson* 7082 (hb. Wheeler); Reserva Nacional Río Simpson, trail from Sector Correntoso heading into R. N. Río Simpson, 45·4865°S, 72·251°W, on *Fuchsia magellanica*, 2008, *T. Wheeler & P. Nelson* 6161 (hb. Wheeler). — **Argentina:** *Provincia Neuquén*: Puerto Blest, 41°01'16·5"S, 71° 49'26·4"W, on bark of *Saxegothaea conspicua*, 2014, *M. I. Messuti et al.* (BCRU 05394). *Provincia Río Negro*: Puerto Blest, trail to Puerto Alegre, 2004, *S. Calvelo* #23 (hb. SC); *ibid.*, coast of Río Frías, on bark of *Saxegothaea conspicua*, *S. Calvelo* s. n. (hb. SC); S.C. de Bariloche, Llao-Llao, Villa Tacul, on bark of *Lomatia hirsuta*, 1993, *S. Calvelo* 1360 (hb. SC); *ibid.*, Puente Romano, 41° 02'40'3"S, 71°49'26·4"W, on bark of *Lomatia hirsuta*, 2014, *A. Passo* (BCRU 05402); *ibid.*, on bark of *Lomatia hirsuta*, 2014, *M. I. Messuti et al.* (BCRU 05405, 05406, 5408).

This study was financially supported by UNComahue (Secretaría de Investigación, No B0177), CONICET (PIP 11220120100564) and ANPCyT (PICT 1036E2). We wish to thank Leena Myllys, curator of H, for providing images of type specimens, a bibliography and comments. The critical reviews of one anonymous reviewer and Arve Elvebakk have improved the paper considerably. We are grateful to Susana Calvelo, Peter Nelson and Tim Wheeler who kindly sent us additional *P. pilosella* and *P. piloselloides* specimens for examination. Thanks to María Fernanda Artola del Pozo for her assistance with the herbarium material.

References

Arup, U., Ekman, S., Lindblom, L. & Mattsson, J.-E. (1993) High performance thin layer chromatography (HPTLC), an improved technique for screening lichen substances. *Lichenologist* **25:** 61–71.

Charif, D. & Lobry, J. R. (2007) SeqinR 1.0-2: a contributed package to the R project for statistical computing devoted to biological sequences retrieval and analysis. In *Structural Approaches to Sequence Evolution: Molecules, Networks, Populations* (U. Bastolla, M. Porto, H. E. Roman & M. Vendruscolo, eds): 207–232. Heidelberg: Springer Berlin.

Crespo, A. & Pérez-Ortega, S. (2009) Cryptic species and species pairs in lichens: a discussion on the relationship between molecular phylogenies and morphological characters. *Anales del Jardín Botánico de Madrid* **66:** 71–81.

Galloway, D. J. (1986) Non-glabrous species of *Pseudocyphellaria* from southern South America. *Lichenologist* **18:** 105–168.

Galloway, D. J. (1992) Studies in *Pseudocyphellaria* (lichens) III. The South American species. *Bibliotheca Lichenologica* **46:** 1–275.

Gardes, M. & Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2:** 113–118.

Larsson, A. (2014) AliView: a fast and lightweight alignment viewer and editor for large data sets. *Bioinformatics* **30:** 3276–3278.

Mattson, J.-E. & Lumbsch, H. T. (1989) The use of the species pair concept in lichen taxonomy. *Taxon* **38:** 238–241.

Miądlikowska, J. & Lutzoni, F. (2000) Phylogenetic revision of the genus *Peltigera* (lichen-forming Ascomycota) based on morphological, chemical and large subunit nuclear ribosomal DNA data. *International Journal of Plant Science* **161:** 925–968.

Miądlikowska, J. & Lutzoni, F. (2004) Phylogenetic classification of Peltigeralean fungi (*Peltigerales*, Ascomycota). *American Journal of Botany* **91:** 449–464.

Moncada, B., Lücking, R. & Betancourt-Macuase, L. (2013) Phylogeny of the *Lobariaceae* (lichenized Ascomycota: *Peltigerales*), with a reappraisal of the genus *Lobariella*. *Lichenologist* **45:** 203–263.

Moncada, B., Reidy, B. & Lücking, R. (2014) A phylogenetic revision of Hawaiian *Pseudocyphellaria sensu lato* (lichenized Ascomycota: *Lobariaceae*) reveals eight new species and a high degree of inferred endemism. *Bryologist* **117:** 119–160.

R Development Core Team (2011) *R: a Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available online at http://www.R-project.org/.

Räsänen, V. (1932) Zur kenntnis der Flechtenflora Feuerlands, sowie der Prov. de Magellanes, Prov. de Chiloë und Prov. de Ñuble in Chile auf Grund des von H. Roivainen gesammelten Materiales. *Annales Botanici Societatis Zoologicae-Botanicae Fennicae "Vanamo"* **2:** 1–65.

Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30:** 1312–1313.

Stenroos, S., Stocker-Wörgötter, E., Yoshimura, I., Myllys, L., Thell, A. & Hyvönen, J. (2003) Culture experiments and DNA sequence data confirm the identity of *Lobaria* photomorphs. *Canadian Journal of Botany* **81:** 232–247.

Thomas, M. A., Ryan, D. J., Farnden, K. J. F. & Galloway, D. J. (2002) Observations on phylogenetic relationships within *Lobariaceae* Chevall. (*Lecanorales*, Ascomycota) in New Zealand, based on ITS-5.8s molecular sequence data. *Bibliotheca Lichenologica* **82:** 123–138.

White, T. J., Bruns, T., Lee, S. & Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. San Diego: Academic Press.

Wiklund, E. & Wedin, M. (2003) The phylogenetic relationships of the cyanobacterial lichens in the *Lecanorales* suborder *Peltigerineae*. *Cladistics* **19:** 419–431.