The Lichenologist **47**(1): 43–49 (2015) © British Lichen Society, 2015
doi:10.1017/S0024282914000577

Molecular data support *Pseudoparmelia* as a distinct lineage related to *Relicina* and *Relicinopsis* (Ascomycota, *Lecanorales*)

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Abstract: The phylogenetic position of the genus *Pseudoparmelia* was addressed using molecular data from five loci (mtSSU, nuLSU, ITS, *Mcm7, RPB1*), generated from three species and aligned with sequences from 293 samples representing all major clades of *Parmeliaceae*. *Pseudoparmelia* species form a well-supported monophyletic group that is the sister group of a clade consisting of the genera *Relicina* and *Relicinopsis*. These three genera share a thallus with a pored epicortex, isolichenan as cell wall polysaccharide, and relatively small ascospores. Morphological and chemical characters that distinguish *Pseudoparmelia* from the closely related *Relicina* and *Relicinopsis* are discussed. To further elucidate the relationships of these three genera, we assembled a second dataset including 15 additional samples of *Relicina* and *Relicinopsis* using three loci (mtSSU, nuLSU, ITS). All three genera are monophyletic but monophyly of *Relicina* lacks support and, in the mtSSU single locus tree, the genus is paraphyletic with *Relicinopsis* nested within. Additional studies including more *Relicina* species are necessary to test delimitation of the genera *Relicina* and *Relicinopsis*.

Key words: generic concept, lichens, molecular systematics, *Parmeliaceae*, parmelioid lichens, taxonomy

Accepted for publication 9 *October* 2014

Introduction

Molecular studies have helped to develop a new generic-level classification in the *Parmeliaceae*, in which the delimitation of genera has been vigorously debated (Hale 1984; Hawksworth 1994; Nimis 1998; DePriest 1999; Rambold & Triebel 1999; Crespo *et al.* 2010; Thell *et al.* 2012). The current generic delimitations were recently reviewed (Crespo *et al.* 2011; Thell *et al.* 2012). Presently, the *c.* 2800 recognized species are classified into over 80 genera, the bulk of them belonging to the parmelioid lichens. Despite progress in understanding phylogenetic relationships among parmelioid lichens, the relationships of several groups remain uncertain, including the delimitation of a number of the mostly tropical genera in the *Parmelia* and *Parmelina* clades (Crespo *et al.* 2010). Additionally, a few genera of parmelioid lichens have not yet been studied using molecular markers, including *Bulborrhizina* Kurok., *Parmotremopsis* Elix & Hale, and *Pseudoparmelia* Lynge. Recently, we were able to obtain fresh specimens of the last genus and generated sequences of five loci (mtSSU, nuLSU, ITS, *Mcm7, RPB1*) for three species, including the type species, in order to elucidate the phylogenetic placement of *Pseudoparmelia*.

Pseudoparmelia was initially erected based on the presence of pseudocyphellae on the lower surface (Lynge 1914), a character that

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was subsequently shown to be an artefact caused by tearing of rhizines (Santesson 1942). The genus was not generally accepted until it was resurrected in a redefined circumscription for parmelioid lichens with a pored epicortex and narrow, eciliate lobes (Hale 1974*b*, 1976*b*). However, the genus was subsequently recognized as a heterogeneous assemblage and the majority of species were placed in other genera (Elix *et al.* 1986; Hale 1986), with only a few species remaining in a strict circumscription of *Pseudoparmelia*. Subsequently, a number of additional *Pseudoparmelia* species were described, and currently 16 species are accepted in the genus. In this narrower circumscription, *Pseudoparmelia* is characterized by having small ellipsoid to subspherical ascospores, bifusiform conidia, a yellow-pigmented upper cortex and medulla due to the presence of secalonic acids, a pale lower surface with simple rhizines, isolichenan in the fungal cell walls, *β*-orcinol depsidones in the medulla, and traces of atranorin in the cortex (Elix 1993; Elix & Nash 1997). The centres of distribution of the genus are in the Neotropics and southern Africa.

The tropical genus *Relicina* has sublinear, more or less dichotomously branched lobes with bulbate cilia, a pored epicortex, isolichenan in the cell walls, bifusiform conidia, and usnic acid as cortical substance. The centre of species diversity is in eastern Asia and Australasia, and over 50 species are currently accepted. Originally this genus was thought to be closely related to *Bulbothrix,* since both genera share the presence of bulbate cilia (Hale 1974*a*, 1975, 1976*a*; Elix 1993). However, molecular data show that the two genera are only distantly related, with *Bulbothrix* belonging to the *Parmelina* clade, whereas *Relicina* belongs to the *Parmelia* clade (Crespo *et al.* 2010). Another tropical genus in the *Parmelia* clade, the genus *Relicinopsis*, is similar to *Relicina* and shares key traits with that genus, but differs by lacking bulbate cilia and having fusiform conidia. *Relicinopsis* is a small genus of five species, most diverse in southern Asia and Australasia. Crespo *et al*. (2010) questioned the distinction of *Relicina* and *Relicinopsis*, since *Relicinopsis* was nested within *Relicina* in their 1GENE analysis; however, the two genera have been recovered as separate monophyletic clades in other analyses including more loci but smaller sample sizes.

Our study aims to elucidate whether *Pseudoparmelia* in a strict sense is a distinct lineage and to clarify its phylogenetic relationship within *Parmeliaceae*. We also attempt to elucidate the phylogenetic relationships among tropical genera in the *Parmelia* clade with an extended taxon sampling.

Materials and Methods

Taxon sampling

We prepared two datasets: 1) DNA sequences of nuclear ribosomal internal transcribed spacer (ITS), nuclear ribosomal large subunit (nuLSU), mitochondrial small subunit rDNA (mtSSU) and fragments of the protein-coding markers *RPB1* and *Mcm7* were assembled for five specimens of *Pseudoparmelia* representing three species, *P. cyphellata* (type species), *P. floridensis*, and *P. uleana*. These sequences were added to the 5-Gene dataset obtained (P. Divakar, unpublished data); 2) we assembled a three locus dataset including additional samples representing the genera *Relicina* and *Relicinopsis* in order to better understand the phylogenetic relations of these three target genera in this study. For this second dataset, DNA sequences of ITS, nuLSU and mtSSU were assembled for five samples representing three species including the type species of *Pseudoparmelia*, nine samples representing five species of *Relicina*, and 11 samples representing four species, including the type, of the genus *Relicinopsis*. Three species of *Notoparmelia* were used as outgroup because this genus has previously been shown to be closely related to this clade (Crespo *et al.* 2010). Details of the specimens used in this second dataset, including GenBank accession numbers, are shown in Table 1.

DNA extraction and PCR amplification

Small samples (2 mm^2) prepared from freshly collected and frozen specimens were ground with sterile plastic pestles. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden) according to the manufacturer's instructions but with slight modifications (Crespo *et al.* 2001). Genomic DNA (5–25 ng) was used for PCR amplifications of the ITS, nuLSU and mtSSU rDNA regions, and protein-coding markers *RPB1* and *Mcm7*. Primers, PCR, and cycle sequencing conditions were the same as those described previously (Crespo *et al.* 2010; Leavitt *et al.* 2013). Sequence fragments obtained were assembled with the program SeqMan 4.03 (DNAStar) and manually adjusted.

Sequence editing and alignment

Sequence and species identity was confirmed using the 'megaBLAST' search function in GenBank (Sayers *et al.* 2011). ITS, nuLSU, *RPB1* and *Mcm7* sequences were aligned using the program MAFFT ver. 6 (Katoh & Toh 2008) using the G-INS-I alignment algorithm, '200PAM/K = 2' scoring matrix, and offset value = 0·0, and the remaining parameters set to default values. The mtSSU sequences were aligned with the E-INS-I alignment algorithm, '200PAM/K = 2' scoring matrix, and offset value = 0·0 because long gaps in alignments of this marker are common in *Parmeliaceae* (Crespo *et al.* 2010). The program Gblocks v0.91b (Talavera & Castresana 2007) was used to remove regions of alignment uncertainty, using options for a "less stringent" selection on the Gblocks web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html).

Phylogenetic analyses

The alignments were analyzed using Maximum Likelihood (ML) and a Bayesian approach. ML analyses were performed using the program RAxML v7.2.7, as implemented on the CIPRES Web Portal, with the GTRGAMMA model (Stamatakis 2006; Stamatakis *et al.* 2008) for the single locus and both partitioned combined datasets (1 and 2). Nodal support was assessed using the 'rapid bootstrapping' option with 1000 replicates.

Bayesian analyses were carried out using the program MrBayes 3.2.1 (Huelsenbeck & Ronquist 2001) for the second dataset. Models of DNA sequence evolution for each locus were selected with the program jModeltest v2.1.5 (Posada 2008), using the Akaike Information Criterion (AICc) (Akaike 1974). The concatenated three-locus dataset was partitioned as ITS, nuLSU and mtSSU, specifying the best-fitting model, allowing unlinked parameter estimation and independent rate variation. No molecular clock was assumed. Two parallel runs were made with 10 000 000 generations, starting with a random tree and employing four simultaneous chains each. Every 1000th tree was saved into a file. The first 25% of trees were deleted as the burn-in of the chains.

We used AWTY (Nylander *et al.* 2007) to compare split frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. A majority-rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes.

ML approach was used to examine the heterogeneity in phylogenetic signal among the three data partitions (Lutzoni *et al.* 2004; Divakar *et al.* 2010). For the three loci and the concatenated analyses, the set of topologies reaching ≥70% bootstrap under likelihood was estimated (Hillis & Bull 1993). The combined dataset topology was then compared for conflict with ≥70% bootstrap intervals of the single gene analyses. If no conflict was evident, it was assumed that the two datasets were congruent and could be combined.

Only clades that received bootstrap support ≥70% in ML analysis or posterior probabilities ≥0·95 in MrBayes analysis were considered as well supported. Phylogenetic trees were drawn using FigTree v1.3.1 (Rambaut 2009).

Results and Discussion

We generated 20 new mtSSU, 22 nuLSU, and 17 ITS sequences for this study (Table 1). The matrix of the combined dataset included 3031 unambiguously aligned nucleotide position characters (724 mtSSU, 791 nuLSU, 343 ITS, 512 *Mcm7*, and 661 *RPB1*). In the combined dataset, 2300 positions were constant. ITS PCR products obtained ranged between 600–800 bp. The differences in size were due to the presence or absence of insertions of *c.* 200 bp identified as group I introns (Gutierrez *et al.* 2007) at the 3′ end of the SSU rDNA. Group I introns were excluded from the analyses.

The phylogeny of parmelioid lichens will be discussed in detail elsewhere (P. Divakar, unpublished data) and is not treated here, except for the phylogenetic position of *Pseudoparmelia*. In the analysis of a broad sampling of *Parmeliaceae* (analysis of dataset 1, see Supplementary Materials Figure S1, available on-line), the three sampled *Pseudoparmelia* species formed a well-supported monophyletic group. This clade was recovered with strong support as the sister group to a clade including species of *Relicina* and *Relicinopsis*, each forming well-supported monophyletic groups. The clade consisting of *Pseudoparmelia, Relicina* + *Relicinopsis* was recovered as a sister group to a clade including *Notoparmelia* and *Parmelia,* but this relationship lacked support (Supplementary Materials Figure S1, available online).

In order to better understand the phylogenetic relationships of the three genera *Pseudoparmelia, Relicina* and *Relicinopsis*, we assembled a second dataset including more species represented by three genetic markers (dataset 2, Table 1). In the phylogenetic tree (Fig. 1) resulting from this analysis, the sister group relationship of *Relicina* and *Relicinopsis* was strongly supported, as was the monophyly of *Relicinopsis*. However, the sister group relationship of the two clades found in *Relicina* lacked support in the three-gene phylogeny, and *Relicina* was recovered as paraphyletic, with *Relicinopsis* nested within, in the mtSSU single locus phylogeny (see Supplementary Materials Figure S2, available on-line).

https://doi.org/10.1017/S0024282914000577 Published online by Cambridge University Press

TABLE 1. *Specimens used in the study, with location, reference collection detail and GenBank accession numbers. Newly obtained sequences for this study are in bold.*

Taxon label	Collection details	GenBank Acc. No.		
		ITS	nuLSU	mtSSU
Notoparmelia crambidiocarpa	New Zealand, *Knight* 60590 (OTA)	GU994571	**KM657289**	GU994665
N. cunninghamii	New Zealand, *Knight* 60608 (OTA)	GU994572	**KM657290**	GU994666
N. subtestacea	New Zealand, *Knight* 60609 (OTA)	**GU994573**	**GU994573**	**GU994668**
Pseudoparmelia cyphellata	Mexico, Nayarit *Nash* 46672 (ASU)	**KM657272**	**KM657291**	**KM657311**
P. floridensis	USA, Florida, *Scharnagl* KS3 (F)	**KM657274**	**KM657293**	**KM657313**
P. floridensis	USA, Florida, *Scharnagl* KS11 (F)	**KM657273**	**KM657292**	**KM657312**
P. floridensis	USA, Florida, *Scharnagl* KS30 (F)	**KM657275**	**KM657294**	**KM657314**
P. uleana	USA, Florida, *Seavey* 1386 (LSU)	**KM657276**	**KM657295**	**KM657315**
Relicina abstrusa	Australia, *Elix* 37426 (CANB)	GU994580	GU994580	-
R. abstrusa	Thailand, *Lumbsch* 19756g (F)	**KM657278**	**KM657297**	**KM657317**
R. abstrusa	Thailand, Khao Kew, *Lumbsch* 19754f (F)	**KM657277**	**KM657296**	**KM657316**
R. abstrusa	Thailand, *Buarang et al.* 24368 (RAMK)	**KM657279**	**KM657298**	**KM657318**
R. abstrusa	Thailand, *Buarang et al.* 24369 (RAMK)	**KM657280**	**KM657299**	**KM657319**
R. filsonii	Australia, New South Wales *Elix* 37267 (CANB)	**KM657281**	**-**	**-**
R. subabstrusa	Thailand, *Buarang et al.* 24370 (RAMK)	**KM657282**	**KM657300**	**KM657320**
R. sublanea	Australia, Queensland, *Elix* 36960 (CANB)	**-**	**-**	**KM657321**
R. subnigra	Australia, ACT, *Louwhoff et al.* (MAF-Lich 10184)	AY785274	AY785267	AY785281
R. sydneyensis	Australia, Queensland, *Lumbsch & Mangold* 19179a (F)	GU994581	GU994630	GU994675
Relicinopsis intertexta	Thailand, Khao Khew, *Lumbsch* 19756g (F)	**KM657283**	**KM657301**	**KM657323**
R. intertexta	Thailand, *Buarang et al.* 24372 (RAMK)	**-**	**KM657302**	**KM657324**
R. cf. *intertexta*	Thailand, *Buarang et al.* 24371 (RAMK)	**-**	**-**	**KM657322**
R. malaccensis	Thailand, *Lumbsch* 19752a (F)	**KM657284**	**KM657303**	**KM657325**
R. malaccensis	Thailand, *Buarang et al.* 24373 (RAMK)	**-**	**KM657304**	**KM657326**
R. malaccensis	Thailand, *Buarang et al.* 24374 (RAMK)	**-**	**KM657305**	**KM657327**
R. malaccensis	Thailand, *Buarang et al.* 24375 (RAMK)	**-**	**KM657306**	**KM657328**
R. cf. *malaccensis*	Australia, *Elix* 36972 (hb. Elix)	-	GU994631	GU994677
R. rahengensis	Thailand, *Buarang et al.* 24376 (RAMK)	KM657285	KM657307	-
R. rahengensis	Thailand, *Buarang et al.* 24377 (RAMK)	**KM657286**	**KM657308**	**KM657329**
R. rahengensis	Thailand, *Buarang et al.* 24378 (RAMK)	**KM657287**	**KM657309**	**KM657330**
R. stevensiae	Australia, Northern Territory, *Elix* 37835 (CANB)	**KM657288**	**KM657310**	**-**

The genera *Pseudoparmelia, Relicina,* and *Relicinopsis* have a thallus covered by a pored epicortex, isolichenan as cell wall polysaccharide, and relatively small ascospores in common. In fact, the three genera are morphologically similar and species currently

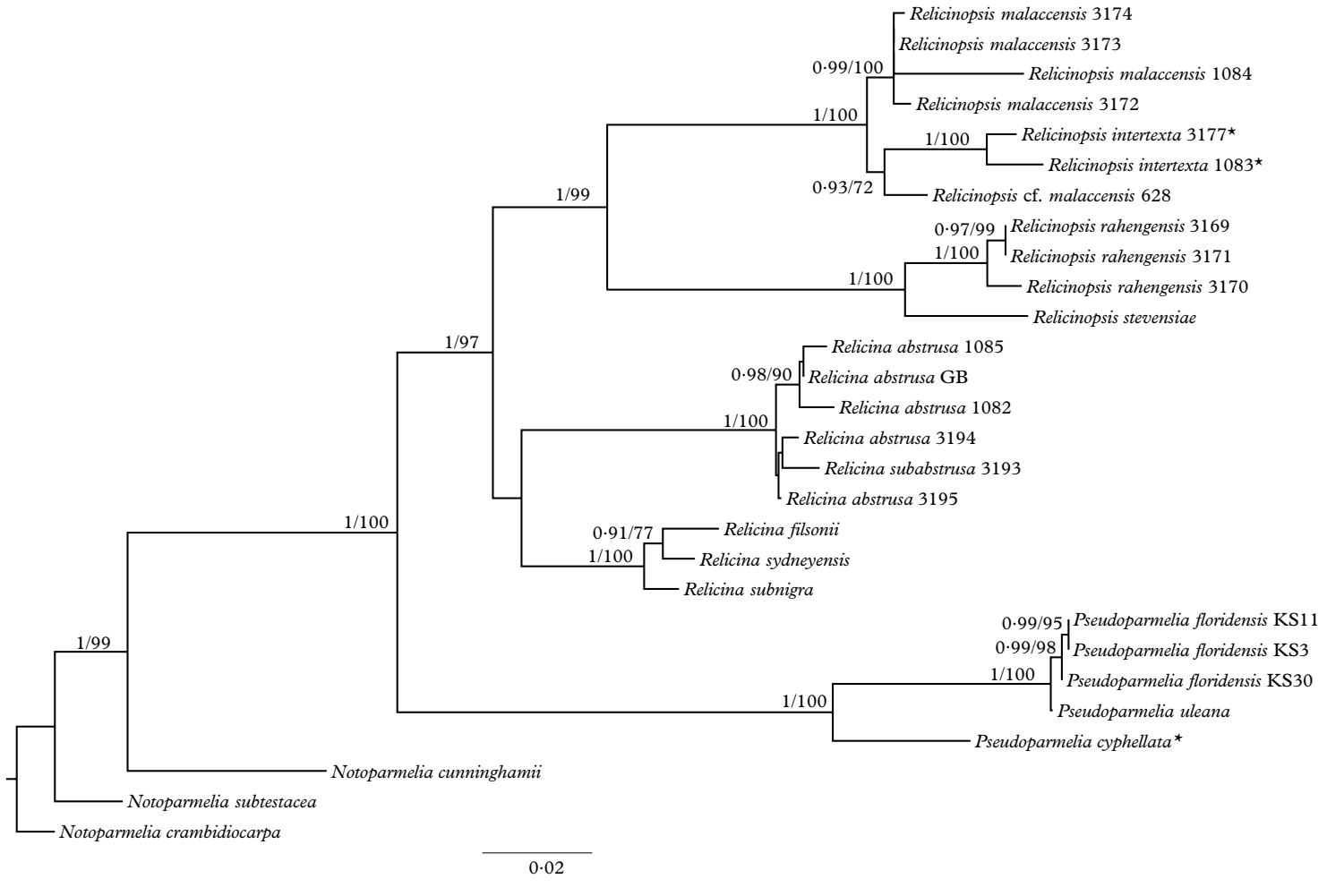

FIG. 1. Phylogenetic relationships of the genera *Pseudoparmelia*, *Relicina*, and *Relicinopsis* samples based on maximum-likelihood and Bayesian analyses using three loci (mtSSU, nuLSU, ITS). Most likely tree obtained with RAxML shown here. ML-bootstrap support ≥70% and posterior probability values ≥ 0·95 are indicated at branches.

TABLE 2. *Diagnostic characters to distinguish* Pseudoparmelia *from the closely related genera* Relicina *and* Relicinopsis.

Character	*Pseudoparmelia*	*Relicina*	*Relicinopsis*
Conidia	bifusiform - filiform	bifusiform	fusiform- cylindrical
Cilia	absent	bulbate	simple or absent
Secalonic acids	present	absent	absent
Usnic acid	absent	present	present

placed in *Relicina* and *Relicinopsis* have been included in the wider circumscription of *Pseudoparmelia* (Hale 1976*b*). Interestingly, a unique group of secondary metabolites, the butlerin derivatives, which are terphenyls, have been found in *Pseudoparmelia* (Elix & Nash 1997) and *Relicina* spp. (Elix *et al.* 1995). Terphenyls are uncommon in lichenized fungi (also occurring in *Parmotrema*), but more common in non-lichenized Basidiomycota. A few *Pseudoparmelia* spp., especially *P. relicinoides* Elix & Nash, resemble the genus *Relicina* in overall growth habit and in having narrow lobes with blackened margins. Characters that distinguish *Pseudoparmelia* from the other genera (Table 2) include the presence of secalonic acids and absence of usnic acid. In addition, *Relicina* differs in having bulbate cilia and *Relicinopsis* in having fusiform-cylindrical conidia. We propose here to accept *Pseudoparmelia* in its strict sense (Elix 1993; Elix & Nash 1997) as a distinct genus within the *Parmelia* clade (Crespo *et al.* 2010). The distinction of the genera *Relicina* and *Relicinopsis* requires further study with a broader sampling of *Relicina* spp., including the type species of the genus, *R. relicinula*. Furthermore, *R. malaccensis* was paraphyletic with *R. intertexta* nested within, suggesting that additional species may be hidden under the current concept of *R. malaccensis*.

Newly obtained DNA sequences were generated in the Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum, and SYSTEMOL Laboratory at the Faculty of Pharmacy, Complutense University of Madrid. We thank Lynika Strozier for making invaluable contributions in the laboratory. This study was supported by the Spanish Ministerio de Ciencia e Innovación (CGL 2010-21646/BOS, CGL2013-42498-P), the Universidad Complutense-Banco Santander (GR 35/10A), Comunidad Autónoma de Madrid (REMEDINAL S-2009/AMB-1783), the Thai Research Fund through the Royal Golden Jubilee Ph.D. program, and the Negaunee Foundation.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0024282914000577

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