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Another example of cryptic diversity in lichen-forming fungi: the new species *Parmelia mayi* (Ascomycota: Parmeliaceae)

M. Carmen Molina · Ruth Del-Prado · Pradeep Kumar Divakar · Daniel Sánchez-Mata · Ana Crespo

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Abstract In the last decade, a number of cryptic species have been discovered in lichenized fungi, especially in species with a cosmopolitan or disjunctive distribution. *Parmelia saxatilis* is one of the most common and widely distributed species. Recent molecular studies have detected two species, *P. ernstiae* and *P. serrana*, within *P. saxatilis* s. lat., suggesting the existence of considerable genetic diversity that may not yet be expressed at the phenotypic level. Due to the complexity in the *P. saxatilis* s. lat. group, we used this as a model to study the species boundary and identify cryptic lineages. We used Phylogenetic (Bayes, ML and MP) and genetic distance approaches to analyze ITS and β-tubulin sequences. Our results confirm the existence of another cryptic lineage within *P. saxatilis* s. lat. This lineage is described herein as a new species, *P. mayi*. It forms an independent, strongly supported, monophyletic lineage, distantly related to the morphologically similar species *P. ernstiae*, *P. saxatilis* and *P. serrana*. Morphologically, it is indistinguishable from *P. saxatilis* but the new species is separated by molecular, bioclimatic, biogeographic and chemical characters. At present, *P. mayi* appears to have a restricted distribution in the northern Appalachian mountain territories of North America. It is found in climatic conditions ranging from hemiboreal and orotemperate to cryorotemperate ultrahyperhumid bioclimates.

M. C. Molina
Departamento de Biología y Geología (Área de Biodiversidad y Conservación), ESCET, Universidad Rey Juan Carlos, Móstoles 28933 Madrid, Spain

R. Del-Prado · P. K. Divakar (✉) · D. Sánchez-Mata · A. Crespo
Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid 28040, Spain
e-mail: pdivakar@farm.ucm.es

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Introduction

The biological species concept was described by Mayr (1970: 12) as: "a group (or population) of individuals who can naturally interbreed with each other, but are reproductively isolated from other similar groups". Contemporary species concepts share the common view that species evolve metapopulation lineages separately (see de Queiroz 1998). Hey (2006), and later de Queiroz (2007), tries to clarify the difference between the 'species concept' and the criteria responsible for 'species delimitation'. According to him, terms like morphospecies, phylogenetic species, etc. should be used as operational criteria for delimiting species but not as a secondary species concept. In lichen-forming fungi, phenotypic criteria have traditionally been used to define species, i.e., individuals that share a set of phenotypic characters. However, this definition of species cannot be used when the characters are plesiomorphic, or when phenotypic plasticity masks the presence of different species with the same morphological appearance. Taylor et al. (2000) contrasted the operational phylogenetic concept with the theoretical species concept, offering a phylogenetic approach to recognizing fungal species based on the concordance of multiple gene genealogies. To delimit fungal species on the basis of this criterion, new operational concepts have been incorporated in an attempt to clarify and recognize genuine fungal diversity. The cryptic species concept was first coined by Hawksworth and Rossman (1997, p 890) and Bickford et al. (2007, p 149) and defined as 'those [that] have been traditionally treated as a

single taxon because they are apparently morphologically indistinguishable'. Vondrák et al. (2009, p 600) introduced the semi-cryptic species concept to refer to those that 'cannot be clearly diagnosed by their morphology, but which are determined by other characters, mainly by their ecology and distribution'.

The number of new species described within Parmeliaceae, and particularly of cryptic species, has increased in recent years, as a result of more extensive research activity and particularly as a consequence of increased use of multiple gene genealogies (e.g., Kroken and Taylor 2001; Molina et al. 2004; Divakar et al. 2005a; b; 2010a; Argüello et al. 2007; Wirtz et al. 2011). It is worth stressing that almost every species in the family studied critically by molecular tools is found to comprise a cryptic lineage, and that, to date, 80 of these have been detected in Parmeliaceae (Divakar et al. 2010b). Parmelioid lichens, which were formerly placed in the genus *Parmelia* Acharius 1803, comprise more than 1,500 species and exhibit significant morphological and chemical diversity (Blanco et al. 2005). Within this large group, *Parmelia* s. str. comprises around 57 species, of which 40 are known from Asia, 7 from Europe and 10 from North America (Hale 1987; Divakar et al. 2001; Molina et al. 2004). This genus is a good model system for recognizing species boundaries and estimating the real level of biodiversity.

P. saxatilis (L.) Acharius 1803, is a well-known lichen species, characterized by simple-to-furcate rhizines and an isidiate upper surface. It is reported from all continents, including Antarctica, and is one of the most common macrolichens on Earth. In North America and Japan, identification of species within the *Parmelia* group has traditionally been difficult. For example, most specimens usually identified as *P. saxatilis* in herbaria, have proved to be *P. squarrosa* Hale 1971, a morphologically related species with densely squarrosely branched rhizines and isidia that are usually most abundant on the lobe margins rather than on the upper surface (Hale 1987). However, Molina et al. (2004) and Divakar et al. (2005b) showed that *P. squarrosa* clustered with *P. sulcata* Taylor 1836, and formed a monophyletic group within *P. sulcata* s. lat. Another species often provoking taxonomic mistakes is *P. kerguelensis* Wilson 1900. In fact, without careful examination, this species could easily be identified as *P. saxatilis*. In North America, *P. kerguelensis* has the same distribution pattern as *P. saxatilis*, but is less common (Hale 1987). Indeed Hinds (1998) and Esslinger (2010; http://lichens.digitalmycology.com/macrolichens/Parmelia.html) did not find this species in eastern North America. Morphologically, *P. saxatilis* closely resembles *P. kerguelensis* but the latter has more closely adpressed, less overlapping lobes and more frequently forked rhizines (Goward et al. 1994; Hinds 1998). The main differences are in the secondary compounds, whereby *P. kerguelensis* contains protocetraric acid (medullary reaction PD+red-orange, K-) instead of salazinic acid (medullary reaction K+red). *P. hygrophila* Goward & Ahti 1983, is also a western North American species related to *P. saxatilis*, but the isidia are ecorticate, granular and more like soredia. Another difference is that *P. hygrophila* is primarily a corticolous lichen in humid, oceanic forests (Hale 1987; Brodo et al. 2001). These taxonomic problems and, in addition, the unexpected morphological variability observed in *P. saxatilis* s. lat from North America (data not shown) justify a detailed study of this group at the molecular level.

The recent use of multiple independent and combined molecular markers has helped greatly to clarify the phylogenetic relationships and the delimitation of species in the Parmeliod group, particularly in *Parmelia* s. lat. (Crespo et al. 2010). This and others works have revealed the variety of evolutionary lineages sheltering beneath the umbrella of a single species name, thereby allowing us to increase our knowledge of the real biodiversity of lichens, justifying the use of terms like 'species complex' or 'cryptic' or 'semi-cryptic' species. For example, *P. saxatilis* s. lat. could be considered to be a species complex from which *P. serrana* A. Crespo, M.C. Molina & D. Hawksw. (Molina et al. 2004) was segregated on the basis of a molecular phylogenetic study using nuITS and partial β-tubulin molecular markers (Crespo et al. 2002; Molina et al. 2004). They are morphologically very similar, although they are unlikely to pose field identification problems since the two species are generally allopatric, *P. saxatilis* being located fundamentally in the Atlantic, Arctic, high mountain regions and Antarctica, whereas *P. serrana* is present mainly in Mediterranean areas.

In this paper, we describe a new species, *Parmelia mayi*, from specimens collected from the Eastern USA and previously identified as *P. saxatilis*. This segregation is carried out on the basis of two operational phylogenetic criteria: monophyly and concordance genealogy (see de Queiroz 2007). The use of an intraspecific threshold of genetic distances provides an objective means of assessing monophyletic clades (Del-Prado et al. 2010), so this approach is used to corroborate the designation of the newly described lineage. Additionally, we evaluate bioclimatic information, biogeography and secondary metabolite data.

Materials and methods

Chemistry and morphology

Secondary chemical compounds were identified in all specimens of the new species in solvent system C using thin layer chromatography (Elix and Ernst-Russell 1993)

and HPLC with reversed-phase columns, gradient elution and benzoic and solorinic acids as standards. The retention index (*I*) calculated from the elution time of the appropriate peak with reference to the standards was used for identification (Feige et al. 1993).

Thallus morphology of all specimens of the *P. saxatilis*–*P. mayi* complex included in the molecular analyses were studied under a Leica Wild M8 dissecting microscope. The characters observed were lobe shape, width, pseudocyphellae, isidia and rhizines, because species in *Parmelia* are differentiated traditionally on the basis of these features. Ascospores were not studied due to the absence of apothecia. Widths were measured to the nearest 0.1 mm using a CBS Beck Kassel calibrated under an 8x magnifier. At least ten measurements of the variables were made in the different specimens of the *P. saxatilis*–*P. mayi* complex.

Molecular methods

Taxon sampling

Thirty-two specimens representing 11 species of *Parmelia* s. lat. were examined in this study. Detailed collection information is presented in Table 1. *P. squarrosa*, *P. sulcata* and *P. barrenoae* Divakar, M. C. Molina & A Crespo (Divakar et al. 2005b), were selected as the out-group, based on Molina et al. (2004) and Divakar et al. (2005b).

DNA extraction and PCR amplification

Samples prepared from freshly collected and frozen herbarium specimens were ground with sterile glass pestles. Total genomic DNA was extracted using the DNAeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions, with the slight modifications described in Crespo et al. (2001). Dilutions of total DNA were used for PCR amplification of the genes coding for the nuclear ITS rDNA and part of the protein-coding β-tubulin gene. Fungal nuITS rDNA was amplified using the primers ITS1F (Gardes and Bruns 1993), ITS4 (White et al. 1990), ITS1-LM (Myllys et al. 1999) and ITS2-KL (Lohtander et al. 1998). The partial β-tubulin sequence was amplified using Bt3-LM and Bt10-LM primers (Myllys et al. 2001). PCR amplifications were performed according Molina et al. (2004). PCR products were cleaned using a Bioclean Columns kit (Biotools; http://www.biotools.eu) according to the manufacturer's instructions. Sequencing was performed using the ABI PRISM BigDye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) at the Unidad de Genómica (Parque Científico de Madrid). Sequences were assembled and manually edited in BioEdit, version 7.0.4 (Hall 1999).

Sequence alignments and phylogenetic analyses

We generated 10 new nuITS and 11 new β-tubulin sequences for this study. These were aligned with previously published nu ITS and β-tubulin sequences (Table 1). Each data set was aligned separately using Clustal W (Thompson et al. 1994) and adjusted manually. We excluded 16 bp in the nuITS rDNA (in ITS1 region) data set that could not be aligned with statistical confidence from the phylogenetic analysis. Ambiguously aligned regions were delimited manually.

The nucleotide substitution models were selected statistically with the help of jModelTest (Posada 2008; program available at http://darwin.uvigo.es). Models were selected by consideration of the Akaike information criterion (AIC; Akaike 1974) and the Bayesian information criterion (BIC; Schwarz 1978). The following models were used: (1) for ITS, the General Time Reversible substitution model (Tavaré 1986) with estimation of invariant sites and the assumption of a gamma distribution with six rate categories (GTR+I+G) had the lowest –lnL value according to the AIC the model used, and the second lowest according to BIC (the lowest value in this case corresponded to the model TIM2+I+G, which was not implemented either in MrBayes or PhyML); (2) for the β-tubulin region, the model selected was also the GTR+I+G, the model with the third according to the AIC and BIC, although the two models with lower−lnL values (TVM+G and TIM3+I+G) were not implemented either in MrBayes or in PhyML. The same models were used for different partitions in the analysis of the combined datasets and also for single loci.

The alignment of the combined data set was analyzed using maximum parsimony (MP), maximum likelihood (ML) and a Bayesian approach (B/MCMC). MP analyses were performed using the PAUP* 4.0b10 program (Swofford 2003). Heuristic searches with 1,000 random taxon addition replicates were conducted with the tree-bisection-reconnection (TBR) branch swapping and MulTrees option, with equally weighted characters, and gaps treated as missing data. Bootstrapping (Felsenstein 1985) was performed on the basis of 2,000 pseudoreplicates with the same settings as for the heuristic search.

We used a MP approach to examine the heterogeneity in phylogenetic signal between the two data partitions (de Queiroz 1993; Buckley et al. 2002; Divakar et al. 2010c). The level of bootstrap was used to detect significance levels of localized incongruence between the two gene partitions. For the two genes and the concatenated analyses, 2,000 bootstrap replicates as described above were performed using PAUP* and the 70% majority-rule bootstrap tree was constructed. We interpreted this bootstrap value as being strong support for a particular node and identified the conflicted nodes by comparing each gene partition with a

Table 1 Specimens used in the study, country, herbarium accession and GenBank accession number. Numbers in the first column (Nº) correspond to the number of the corresponding sequence in the molecular phylogenetic tree. New sequences are indicated in *bold*. ITS Internal transcribed spacer

Nº	Species	Locality	Herbarium acc. no	GenBank accession no.	
				ITS	β-tubulin
1	*P. adaugescens*	Hokkaido (Tokyo), Japan	MAF 7277	AY036991	AF391146
2	*P. adaugescens*	Hokkaido (Tokyo), Japan	MAF 7292	AY036992	AF391145
3	*P. adaugescens*	Hokkaido (Tokyo), Japan	MAF 7291	AY036993	AF391144
4	*P. discordans*	South Aberdeen Mountain (Scotland) UK	MAF 10232	AY583212	AY583213
5	*P. ernstiae*	Puerto de Corrales (Burgos), Spain	MAF 9749	AY295110	AY295117
6	*P. ernstiae*	Niedersachsen (Reg.-Bez-Lüneburg), Germany	HBG 4619	AF410833	AF410841
7	*P. ernstiae*	Schleswig-Holstein (Grossolt), Germany	HBG 64331	AF410834	AF410842
8	*P. hygrophila*	Mendocino, Fick rock road (California), USA	MAF 15770	**JN609436**	-
9	*P. mayi*	Berkshire Co., Mount Washington township, Mount Everett (Massachusetts), USA	MAF 15767	**JN609437**	**JN609425**
10	*P. mayi*	Berkshire Co., Mount Washington township, Mount Everett (Massachusetts), USA	MAF 15766	**JN609438**	**JN609426**
11	*P. mayi*	Berkshire Co., Mount Washington Township, Mount Everett (Massachusetts), USA	MAF 15765	**JN609439**	**JN609427**
12	*P. mayi*	Grafton Co., Franconia, White Mountain National Forest, South Twin Mountain (New Hampshire), USA	MAF 15768	AF350033	-
13	*P. mayi*	Grafton Co., Franconia, Galehead Mountain, White Mountain National Forest, Gale River Trail at Garfield Ridge Trail (New Hampshire), USA	MAF 15769	AF350034	**JN609428**
14	*P. omphalodes*	La Plataforma del Calvitero (Salamanca), Spain	MAF 7062	AY036998	AF391131
15	*P. omphalodes*	La Plataforma del Calvitero (Salamanca), Spain	MAF 7044	AY036999	AF391132
16	*P. pinnatifida*	Kola Peninsula, Russia	MAF 7274	AY036987	AF391134
17	*P. pinnatifida*	Kola Peninsula, Russia	MAF 7272	AY036988	AF991133
18	*P. saxatilis*	South Aberdeenshire, Glen Muick, Tullich & Glengaim, Paish, Dirnett (Scotland), UK	MAF 15763	**JN609440**	**JN609429**
19	*P. saxatilis*	South Aberdeenshire, Glen Muick, Ballater (Scotland), UK	MAF 15764	**JN609441**	**JN609430**
20	*P. saxatilis*	(Montana), USA	MAF 15761	**JN609443**	**JN609432**
21	*P. saxatilis*	(Montana), USA	MAF 15762	**JN609442**	**JN609431**
22	*P. serrana*	Batuecas (Cáceres), Spain	MAF 7287	AY036997	AF391141
23	*P. serrana*	Puerto de Navafría (Madrid), Spain	MAF 9755	AY295104	**JN609433**
24	*P. serrana*	La Barranca (Madrid), Spain	MAF 9759	AY215907	AY295115
25	*P. submontana*	Hoya Redonda (Sierra de Cazorla), Spain	MAF 3729	AY037000	**JN609423**
26	*P. submontana*	Ifrane Medium Atlas, Morocco	MAF 15440	**JN609434**	EU788018
27	*P. submontana*	Ifrane Medium Atlas, Morocco	MAF 15550	**JN609435**	**JN609424**
30	*P. barrenoae*	Marvào (Sao Mamede), Portugal	MAF9900	AY579450	AY579464
31	*P. squarrosa*	Forge Creek (TN), USA	MAF 7293	AY036977	AY580308
32	*P. sulcata*	Ventorrillo (Madrid), Spain	MAF 9901	AY579447	AY579462

threshold between conflicting (≥70% bootstrap) and non-conflicting (≤70% bootstrap) nodes (Hillis and Bull 1993). If no conflict was evident, it was assumed that the two data sets were congruent and could be combined.

Maximum likelihood analyses were performed using PhyML 3.0 on the program's online web server: http://atgc.lirmm.fr/phyml (Guindon and Gascuel 2003) with 2,000 non-parametric bootstrap replicates to assess confidence of the nodes and otherwise, the default settings of the web server were used. The B/MCMC analyses were conducted using the MrBayes 3.1.2 program (Huelsenbeck and Ronquist 2001). Parallel runs were made with 3,000,000 generations starting with a random tree and employing 12 simultaneous chains each. Trees were sampled every 200 generations for a total of 30,000 trees. The first 300,000 generations (i.e., the first 3,000 trees) were discarded as burn-in for the chain. To assess putative lineages across individual gene trees and to identify the presence of the same clades in the single-locus

genealogies, each data set (ITS and β-tubulin) was analyzed separately using the same settings.

We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html/tracer/) to ensure that stationarity was achieved after the first 300,000 generations, checking whether the log-likelihood values of the sample points had reached a stable equilibrium (Huelsenbeck and Ronquist 2001). We also used the AWTY program (Nylander et al. 2007) to compare split frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. For the remaining 54,000 trees (27,000 from each parallel run) a majority-rule consensus tree with average branch lengths was calculated using the sumt option in MrBayes.

In the combined data set, a conservative approach for interpreting support values was considered. Only clades that received ≥70% bootstrap support in MP and ML analyses and posterior probabilities (PPs) ≥0.95 were considered to be strongly supported. Phylogenetic trees were drawn using TREEVIEW (win32) 1.5.2. (Page 1998). Alignments are available at TreeBase (http://www.treebase.org) under study accession number S11404, and matrix and phylogenetic tree accession numbers M8570, M8571, M8572; and Tr43766, Tr43767, and Tr43768.

Calculation of genetic distances

Pairwise maximum likelihood distances (given as the number of nucleotide substitutions per site) between the ITS rDNA sequences in the analysis were calculated with TREE-PUZZLE 5.2 (Strimmer and Von Haeseler 1997) using the HKY+G (Hasegawa et al. 1985) model of nucleotide substitution with among-site variation, and assuming a discrete gamma distribution with six rate categories. We also used a GTR model to compare the pairwise genetic distances obtained by HKY+G. This gave the same results. Only these two models were implemented in TREE-PUZZLE 5.2.

Biogeography and bioclimatic features

In this study, special emphasis was placed on the study of the bioclimatic features of localities of all specimens of *Parmelia mayi* and *P. saxatilis* mentioned in Table 1. Additionally, 30 samples of *P. saxatilis* from different distant geographical regions, 25 of them selected from MAF herbarium material and 5 from Esslinger (2010 checklist), were also examined. For this purpose, we used the bioclimatic proposals of Rivas-Martínez and Rivas Sáenz (2011), and those of the specific studies of North American Boreal and Western Temperate Vegetation (Rivas-Martínez et al. 1999).

Results

Phylogenetic analysis

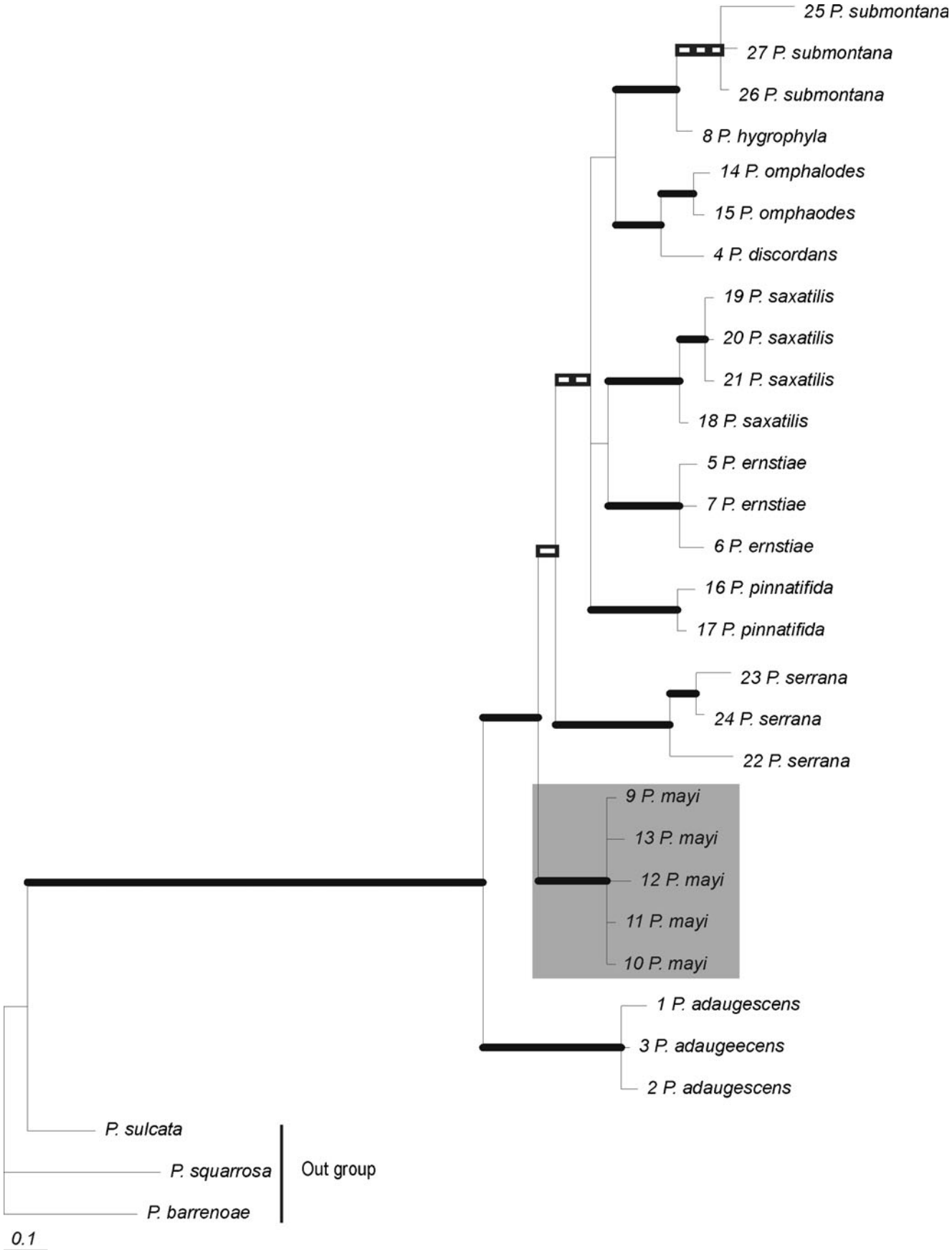
The size of the ITS PCR product obtained ranged between 600 and 800 bp. The differences in size were due to the presence and absence of insertions of ~200 bp identified as group I introns (Gutierrez et al. 2007) and to the different primer pairs used. Using ITS1F and ITS4 primers set enabled us to detect group I introns if they were present in the samples. The product was located at the 3' end of the small ribosomal DNA subunit (SSU). In some cases we used the primer pairs ITS1LM and ITS2KL, in which case this intron could not be sequenced due to the location. We excluded introns and 16 bp of the ITS1 (positions located in the alignment 115–119, 123, 128–139) from the analysis. The data matrix had 495 unambiguously aligned nucleotide positions in the nu ITS and 774 in the β-tubulin partitions. Ninety characters were variable in the nu ITS and 72 in the β-tubulin data set. Testing for topological incongruence showed no supported conflicts (results not shown), so single-gene data sets were combined and analyzed. Since the topologies of the MP, ML and B/MCMC analyses were identical, only the 50% majority-rule consensus tree of Bayesian tree sampling is shown, the nodes in bold being those that received strong support in all three analyses (i.e., PP ≥0.95 in the B/MCMC analysis and ≥70% for the MP and ML bootstraps) (Fig. 1).

In the topology of this tree, it should be noted that all specimens of *P. mayi* collected from East North America and previously identified as *P. saxatilis* s. str. form a supported monophyletic group located at a basal position, as a sister group to the clade including the samples of *P. saxatilis* s. str. (Fig. 1). In the genealogical single locus analyses all specimens of *P. mayi* clustered in a well-supported monophyletic group. In the ITS tree the *P. mayi* clade received support (PP 1.0 and MP bootstrap 98) and the same clade was also found in the β-tubulin tree, supported with PP 0.99 and MP bootstrap 82 (results not shown). Phylogenetic relationships among clades remained unresolved in the ITS and β-tubulin gene trees.

Morphological and chemical analysis

No synapomorphic morphological characters supporting these taxa were detected.

The phenol compositions of several species related morphologically to *Parmelia saxatilis* are presented in Table 2. Some differences in phenol composition are noteworthy: *P. mayi* contains alectorialic acid, aliphatic acids (such as lichesterinic, protolichesterinic, nephrosterinic, isonephrosterinic acids) and alectorialic acid, but lacks chloroatranorin.


25 P. submontana
27 P. submontana
26 P. submontana
8 P. hygrophyla
14 P. omphalodes
15 P. omphaodes
4 P. discordans
19 P. saxatilis
20 P. saxatilis
21 P. saxatilis
18 P. saxatilis
5 P. ernstiae
7 P. ernstiae
6 P. ernstiae
16 P. pinnatifida
17 P. pinnatifida
23 P. serrana
24 P. serrana
22 P. serrana
9 P. mayi
13 P. mayi
12 P. mayi
11 P. mayi
10 P. mayi
1 P. adaugescens
3 P. adaugeecens
2 P. adaugescens
P. sulcata
P. squarrosa
P. barrenoae
Out group
0.1

◀ **Fig. 1** A 50% majority-rule consensus tree of the molecular phylogenetic relationships in the genus *Parmelia* based on 54,000 trees from a B/MCMC tree-sampling procedure from a combined data set of nu ITS and β-tubulin sequences. Branches that received strong support in all three analyses (i.e., PP≥0.95 in B/MCMC analysis and≥ 70% in MP and ML bootstraps) are indicated in *bold*. The branches that received strong support both in B/MCMC analysis and MP bootstrap are indicated by *three empty squares* and the branches that received strong support only either in B/MCMC or ML bootstrap analysis are indicated by *two* and *one empty squares*, respectively. Scale bar=0.1 substitutions per site

Genetic distances

Pairwise maximum likelihood distances between all haplotypes of *Parmelia mayi* ranged from 0.002 to 0.004 nucleotide substitutions per site (s/s), with a mean value of 0.003±0.001 s/s. Pairwise distances between the haplotypes of *P. mayi* and *P. saxatilis* ranged from 0.025 to 0.027 s/s, with a mean value of 0.026±0.002 s/s. Finally, the comparison between the haplotypes of *P. mayi* and other haplotypes within the genus *Parmelia* ranged from 0.017 to 0.035 s/s, with a mean value of 0.0275±0.004 s/s.

Bioclimatology and biogeography

Parmelia mayi seems to be an endemic lichen species currently known to be distributed throughout the orophilous territories (high mountain areas) of the Northern Appalachian Mountains (White Mountains, Taconic Mountains). We recorded populations from the highest elevations, such as the peaks of Mounts Everett and Washington. The precise bioclimatic conditions in these areas are extreme. The general bioclimate, according to the available climatic data, is Temperate (Rivas-Martínez and Rivas Sáenz 2011 website; US-NOAA Eastern Regional Climate Center; http://www.erh.noaa.gov/), ranging from hemiboreal and orotemperate (subalpine) to cryorotemperate thermotypes. A topographic Boreal macrobioclimate can be distinguished clearly on the highest summits and ridge tops.

At higher altitudes, the red spruce-balsam fir forests (*Piceo rubentis-Abietetum balsameae* Marcotte & Grandtner, 1974), which occur in the mid-mountain areas, decline and only balsam fir structures the conifer forests, sometimes with black spruce (*Picea mariana*). This allows us to recognize a southern variant of the association *Abietetum balsameae* Damman, 1964. At the treeline, trees are stunted due to the harsh growing conditions, including extreme wind and ice abrasion. In the 'krummholz', balsam fir and black spruce form prostrate mats or 'flag trees', which indicate the prevailing wind direction. The highest areas have a cryorotemperate bioclimatic character (continental temperate bioclimate) or, in some local areas, a true Boreal macrobioclimate.

The Mount Washington Observatory is located at the summit of Mount Washington, NH, and is the highest mountain of the Presidential range. The weather is very severe here throughout most of the year, with conditions approximating those of much higher latitudes.

We include a bioclimate diagram from the climatic station close to the *P. mayi* collection area, showing temperature (−2.8°C annual average) and rainfall (1,881 mm annual average) graphs (Fig. 2a). We add the bioclimate diagram from a selected *Parmelia saxatilis* locality from the conterminous US: Montana, Red Lodge (Fig. 2b), a comparable locality much further west. All the *P. saxatilis* areas studied have a similar bioclimate, with an annual average temperature of 4.9°C and an annual average rainfall of 506 mm. *P. saxatilis* is a worldwide-distributed species that grows in different macrobioclimates: Temperate (e.g., North and Central Europe, North America), Mediterranean (e.g., South and Southwest Europe) and Tropical (e.g., Southeast Asia). It has even been reported from Antarctica. The climogram of a single locality from North America with a typical Temperate macrobioclimate is presented as a bioclimatic model (see Fig. 2b). *P. saxatilis* did not occur in the bioclimatic conditions where *P. mayi* is currently found.

Table 2 Phenol compositions of *Parmelia saxatilis* s. lat. *Parmelia mayi* analyzed in this study; data for *P. ernstiae, P. saxatilis* and *P. serrana* are taken from Thell et al. (2008). ++=major, +=minor, ±= trace, -=absent

Phenol compounds	*P. mayi*	*P. saxatilis*	*P. serrana*	*P. ernstiae*
Atranorin	+	+	+	+
Chloroatranorin	-	+	+	+
Salazinic acid	++	++	++	++
Consalazinic acid	+	+	+	+
Lobaric acid	+	+	-	+
Alectorialic acid	+	-	-	-
Nephrosterinic acid	+	-	+	+
Lichesterinic acid	+	-	+	+
Protolichesterinic acid	+	-	++	+
Isonephrosterinic acid	+	-	++	+
Protocetraric acid	-	+	±	±

Discussion

Several factors support species status for *P. mayi*, including phylogenetic analyses, ITS pairwise distance values, the unique composition of the secondary compounds and bioclimatic/ecological features, and the presence of the type-I intron.

The combination of nu ITS rDNA and partial β-tubulin genes enabled us to derive the phylogeny of this group. All

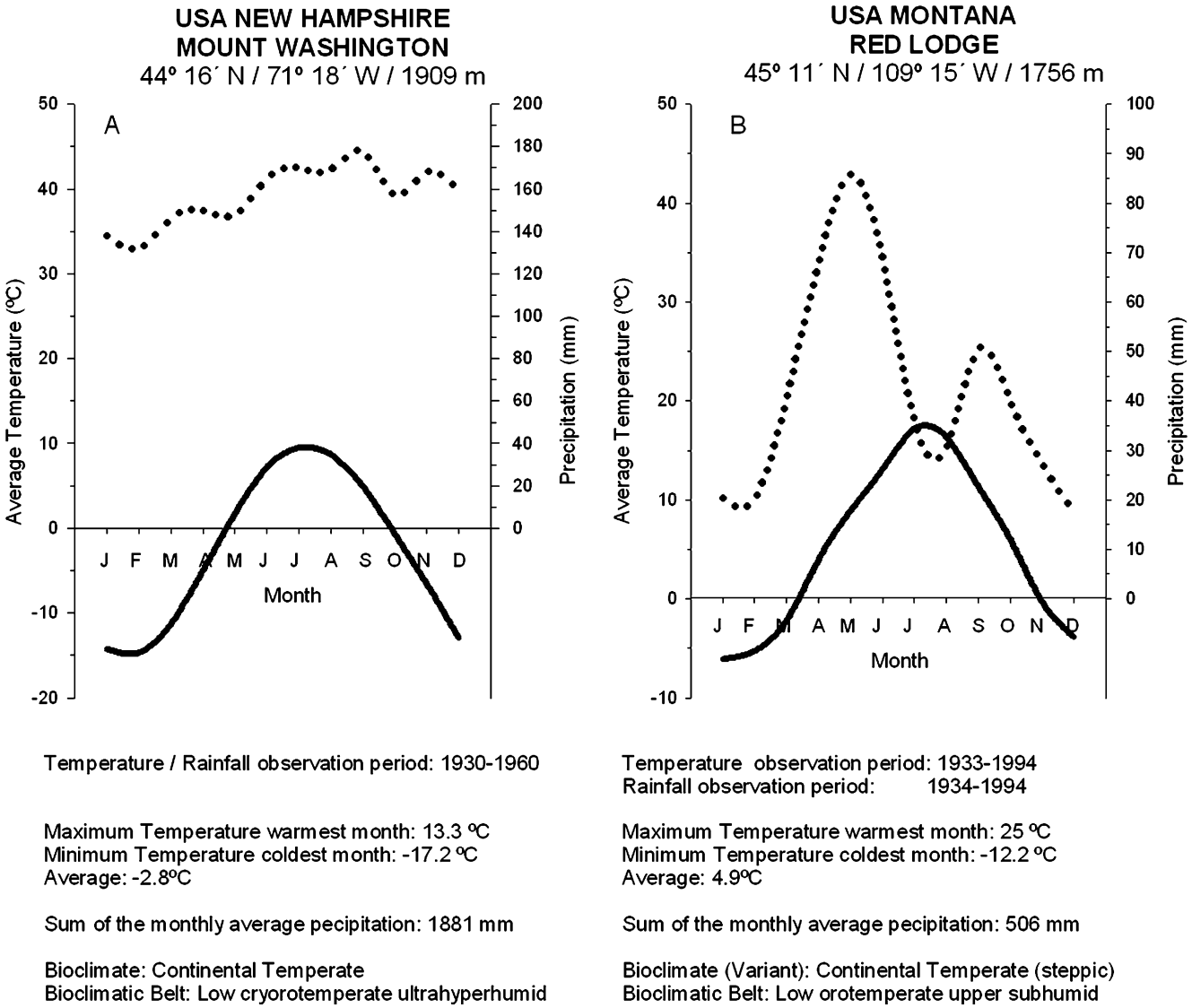


Fig. 2 Climate diagram of the selected localities of *P. mayi* (**a**) and *P. saxatilis* (**b**) from USA. *Solid line*, average temperature; *dotted line*, precipitation. Date source: http://www.globalbioclimatics.org

specimens of *P. mayi* collected from East North America clustered in a strongly supported reciprocal monophyletic group (Fig. 1). However, although the monophyly of this group is clear, the phylogenetic relationships with the other species are not resolved. *P. mayi* could be segregated on the basis of two operational phylogenetic criteria (see de Queiroz 2007): (1) monophyletic criteria, consisting of an ancestor and all of its descendants, are commonly inferred from the possession of shared derived character states (Donoghue 1985); and (2) genealogical criteria, where, over time, there has been an exclusive coalescence of alleles. In other words, all alleles or haplotypes of a given locus are descended from a common ancestral allele not shared with those of other species and are therefore reciprocally monophyletic (Baum and Shaw 1995; Hudson and Coyne 2002). Several authors have highlighted the need to identify the presence of the same clades in different single-locus genealogies, which may be taken as evidence that the clades are reproductively isolated lineages (Dettman et al. 2003; Pringle et al. 2005). Our results show a concordance genealogy (Avise 2000) in which the phylogenetic tree topologies obtained from two independent molecular markers (nuITS and partial β-tubulin gene) are congruent, and the same strongly supported monophyletic clade is found in two independent gene trees. These results provide evidence that the *P. mayi* clade is a reproductively isolated and reciprocal lineage that merits species-level recognition.

With a few exceptions (Kroken and Taylor 2001; Divakar et al. 2010a), in the majority of the cases where potential cryptic species has been identified, only single

locus nuITS rDNA are used (Vondrák et al. 2009). However, multi-locus studies are highly recommended in order to examine critically species boundaries in lichenized fungi (Divakar et al. 2010a).

Another criterion for separating these two species is established by calculating the genetic distances between *P. mayi* and the other morphospecies. Del-Prado et al. (2010) established inter- and intra-specific pairwise distance thresholds in parmelioids using ITS sequences. In the genus *Parmelia*, interspecific distances ranged from 0.019 to 0.116 nucleotide s/s. The results obtained from the present study show a clear threshold gap between *P. mayi* and all other *Parmelia* species. The values obtained are within the interspecific ranges that allow us to consider *P. mayi* as a species different from the others described so far. Moreover, intraspecific distances in the *Parmelia* genus ranged from 0.002 to 0.012 s/s (Del-Prado et al. (2010), with values of 0.002 to 0.004 (s/s) between *P. mayi* haplotypes, indicating relatively low genetic variability within this species. It is worth pointing out that the pairwise distances within *P. mayi* are of the order of 10x lower than between it and the other species.

Additionally, *P. mayi* shows a different pattern of phenolic composition (Table 2) from other *Parmelia* species within the Parmelioid group (Thell et al. 2008). This exclusive feature of the clade is not the reason for the delimitation but should be considered as a synapomorphic character evaluated *a posteriori* (Lumbsch 1998).

Parmelia mayi grows sporadically in orophilous regions throughout the northern Appalachian Mountains. Following the biogeographical approach to North America by Rivas-Martínez et al. (1999), its natural area covers the 'Appalachian sector' ('Appalachian Province', 'North America Atlantic Region'). We did not have enough data to expand its area to the southern Appalachian mountain regions ('South Appalachian sector'). The approach of Sayre et al. (2009) based on previous proposals by Comer et al. (2003) includes the area known as 'Laurentian and Acadean'—a region close to the 'Central Interior and Appalachian' region−. This region has extreme weather conditions (Fig. 2a). In contrast, *P. saxatilis* is a cosmopolitan species in temperate and mediterranean areas (Molina et al. 2004) usually located in regions with annual average temperature and low rainfall (Fig. 2b). According to the Esslinger checklist (2010, http://lichens.digitalmycology.com/macrolichens/Parmelia.html) *P. saxatilis* has been described in five localities close to the *P. mayi* collection site. Three of these are at an altitude below 300 m (two in Massachusetts and one in Connecticut), so these specimens probably correspond to *P. saxatilis* s. str. However, the other two were collected from high mountains, one in White Mountain (NH), and so may correspond to samples of *P. mayi,* considering the proximity and altitude of the collected areas. Given the antiquity of the specimens it is not possible to make a molecular confirmation, but it would be worth collecting fresh material from these locations to confirm the identification.

The new *Parmelia* species can be easily confused with other *Parmelia* that are morphologically related to *P. saxatilis* if it is not critically examined by its morphological and chemical characters. For example, *P. hygrophila* is a species related to, and sometimes confused with, *P. saxatilis* s. str. (Hale 1987; Brodo et al. 2001), although the molecular phylogenetic tree obtained (Fig. 1) revealed a closer relationship with *P. submontana* Hale 1987, rather than *P. saxatilis* s. str., and no close relationship with the new species was found. Moreover, it is distributed in the Pacific Northwest. Another species that is often the subject of taxonomic mistakes is *P. kerguelensis*. Unfortunately, we could not obtain a sequence of *P. kerguelensis* for inclusion in this molecular analysis. However, as this species and *P. mayi* appear generally to be allopatric, field identification problems in ecological and inventory studies seem unlikely in practice. Moreover, *P. kerguelensis*, *P. saxatilis* and *P. mayi* seem to have different secondary metabolic patterns, since *P. kergelensis* has protocetraric acid rather than salazinic acid, which is present in *P. saxatilis* and *P. mayi*. Nevertheless, the analysis of *P. kerguelensis* in future studies of this group is highly recommended.

Finally, the tree topology shows that *P. discordans* Nylander, 1886, is a sister group of *P. omphalodes* (L.) Acharius 1803. Many lichenologists do not agree about the status of *P. discordans*, as it appears to be a 'chemical species' (Hawksworth 1976; Hale 1987). The phylogenetic tree confirms the strong relationship between these taxa, although establishing the reciprocal monophyly of both species would require a larger number of samples.

Although lichenologists have refrained from naming cryptic species for several reasons (Crespo and Pérez-Ortega 2009), we consider it essential to describe taxonomically those specimens that fall into clear reciprocal monophyletic clades and whose pairwise genetic distance values are within the interspecific threshold. Otherwise, if we keep similar species in single taxa this would produce polyphyletic taxa, which is not acceptable. Thus, the reciprocal cryptic lineage obtained in the phylogenetic tree (Fig. 1) is described as a new species (*Parmelia mayi*) here and it is also corroborated with secondary chemistry and genetic distances. Additionally, the new species has a distinct geographic distribution and bioclimatic pattern from those of *P. saxatilis*. The strength of this study is that it has identified divergent cryptic lineages (reciprocally monophyletic lineages with genetic diversity within a defined intraspecific threshold) and produced independent

data about geographic distribution, chemistry composition and bioclimatic/ecological features that add to our understanding of the real genetic biodiversity within this group of lichenized fungi. While this approach may recognize divergent lineages that are reciprocally monophyletic, it would not be effective in cases of recent speciation events affected by incomplete lineage sorting, rare or ongoing gene flow, etc. Traditional (morphological and/or chemical) methods used to delimit species have failed to detect the divergent cryptic lineages and thus underestimate the actual level of biodiversity in these groups of lichenized fungi.

Taxonomic section

Parmelia mayi Divakar, A. Crespo, M.C. Molina sp. nov. (Fig. 3)

Etymology

Parmelia mayi is named in honor of North American lichenologist Dr P. May, the collector of the specimens.

Similis Parmelia saxatilis sed differte in acidum lichesterinicum, protolichesterinicum, nephrosterinicum et isonephrosterinicum continente, et distributione in montis Appalachians septentonalis, et in sequencis molecularis ITS et β-tubulin.

Typus: *USA*: Massachusetts; Berkshire county, Mount Washington township, Mount Everett, 75–150 mN to NNW of summit tower, 42º 06'N 73º 26'W, alt. 790 m, on trunk of *Betula papyrifera*, 13 October 2000, *P. May 5443* (MAF-Lich 15767, holotypus; BM, GZU isotypus).

Fig. 3 *Parmelia mayi* habit (MAF-Lich 15767-holotype) (Scale= 2 mm)

Description

Thallus adnate, up to 8 cm across. Lobes short, subirregular, imbricate; margins crenate to deeply notched, 2–4 mm wide. Upper surface gray, foveolate, pseudocyphellate and isidiate, without soralia. Pseudocyphellae laminal, effigurate, raised with white margin, up to 1 mm long, separate towards center but forming a subreticulate network towards periphery. Isidia laminal, crowded towards thallus center, cylindrical, simple to coralloid branched, brown tipped. Medulla white. Lower surface black with brown margin, rhizinate; rhizines evenly distributed, reaching the thallus margin, simple to furcate, up to 1.5 mm long. Apothecia and pycnidia not seen.

Remarks

The new species resembles *Parmelia saxatilis* morphologically but differs in phylogenetic position, containing aliphatic acids (lichesterinic, protolichesterinic, nephrosterinic, isonephrosterinic acids) and orotemperate to cryorotemperate bioclimatic conditions (Fig. 2).

Ecology and distribution

The species grows on siliceous rocks in tundra vegetation, schists, in *Quercus* open forests, and *Abies balsamea*, *Betula papyrifera* tree trunks in spruce-fir forests. At present *P. mayi* is found in northern Appalachian mountain territories (orophilous regions) of North America at altitudes between 700 and 1,500 m.

Bioclimatology and biogeography

P. mayi is known only from the high mountain areas with an orotemperate–hemiboreal bioclimate throughout Appalachian sector territories (Rivas-Martínez et al. 1999).

Molecular data

In the molecular phylogenetic tree (Fig. 1), all samples of *Parmelia mayi* are clustered in a strongly supported monophyletic group, forming an independent lineage. Thus, the new species is related only distantly to morphospecies with a close resemblance such as *P. ernstiae*, *P. saxatilis* and *P. serrana*. Additionally, remarkable differences were found in pairwise maximum likelihood genetic distances. Genetic distances within the haplotypes of *P. mayi* ranged from 0.002 to 0.004 s/s, while pairwise distances between the haplotypes of *P. mayi* and *P. saxatilis* ranged from 0.025 to 0.027 s/s. Furthermore, *P mayii* has a type-I intron in position 1516,

which is also present in *P. saxatilis* but absent from *P. ernstiae* and *P. serrana*.

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