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Phylogenetic relationships and species concepts in *Parmelia* s. str. (*Parmeliaceae*) inferred from nuclear ITS rDNA and β-tubulin sequences

María del Carmen MOLINA, Ana CRESPO, Oscar BLANCO, H. Thorsten LUMBSCH and David L. HAWKSWORTH

Abstract: The phylogenetic relationships of 16 species of *Parmelia* s. str. are presented based on sequences of nuITS rDNA from 56 specimens, and β-tubulin gene sequences from 29 collections. *Parmelia serrana* sp. nov. a Mediterranean species morphologically very close to *P. saxatilis* is described. *Parmelia ernstiae* is the sister-group to *P. saxatilis* s. str., and a further undescribed North American species of the *P. saxatilis* complex may require recognition. The isidiate *P. squarrosa* is closely allied to the sorediate *P. sulcata*, which is paraphyletic. Japanese samples of *P. cochleata* form a monophyletic group but too few collections of these were studied to reach firm conclusions regarding their relationships. An epitype is selected for the lectotype of *Lichen saxatilis* to unequivocally fix the application of that epithet.

Key words: Ascomycota, cryptic species, *Lecanorales*, lichens, Linnean typification

Introduction

The family *Parmeliaceae* is one of the largest lichen-forming ascomycete families; in the wide sense it encompasses around 2300 species placed in about 85 genera, 49 of them being described in the last two decades (Hawksworth *et al.* 1995). The segregates have been generally accepted when they were based on relationships between anatomical features of the sexual or conidial structures as well as morphological features of the thallus (Mattsson & Wedin 1998, 1999), but not always where no such correlations exist (Clauzade & Roux 1985; Eriksson & Hawksworth 1986; Purvis *et al.* 1992; Nimis 1993; Santesson 1993), pending clarification by molecular or other studies. Molecular analyses have been carried out to determine phylogenies in parmelioid genera using several regions of DNA: nuclear ITS and 5·8S (Mattsson & Wedin 1998; Crespo & Cubero 1998); nuclear SSU and ITS rDNA (Wedin *et al.* 1999); mitochondrial SSU rDNA (Crespo *et al.* 2001); and ITS and β-tubulin (Crespo *et al.* 2002; Thell *et al.* 2002). Overwhelming molecular evidence is now starting to result in the synonymization of some genera introduced in recent decades, for example *Neofuscelia* and *Paraparmelia* into *Xanthoparmelia* (Elix 2003; Hawksworth & Crespo 2002).

Molecular information is also assisting in species delimitation in the family, and the developing species concept in lichenology has been extensively discussed (e.g. Clerc

M. C. Molina (corresponding author): Departamento de Matemáticas y Física Aplicadas y Ciencias Naturales, Escuela Superior de Ciencias Experimentales y Tecnología, Universidad Rey Juan Carlos, Madrid 28933, Spain.
A. Crespo, O. Blanco & D. L. Hawksworth: Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense, Madrid 28040, Spain.
H. T. Lumbsch: Department of Botany, The Field Museum, 1400 S. Lake Shore Drive, Chicago, Illinois 60605, USA.

1998; Grube & Kroken 2000). Traditionally, differences in morphological, and to some extent anatomical, characters have been used to separate species. In addition, geographic origin or apparent differences in secondary chemistry were accorded great importance, and many allopatric populations were named as separate species where there was little or no other discernible difference. A chemical species concept that assumes a lack of variation at the species level was widely applied in macrolichen genera in the 1960s and 1970s (Culberson 1986), but it is now recognized that chemical characters are best used *a posteriori* rather than *a priori* when characterizing lichen species (Lumbsch 1998*a*, *b*).

Increasingly, phylogenetic species concepts based on molecular (DNA) characters are being employed to delimit species, to determine *a posteriori* which types of phenotypic characters are good predictors of phylogenetic species, and demonstrate how these characters evolve in lichenized fungi (Mallet 1995; Grube & Kroken 2000). These molecular data have led to the recognition of cryptic species (Kroken & Taylor 2001), and conversely also to the union of species traditionally regarded as distinct (Myllys *et al.* 2001; Articus *et al.* 2002; Molina *et al.* 2002).

Parmelia s. str. comprises around 57 species, 40 from Asia, seven from Europe and ten from North America (Hale 1987; Kurokawa 1994*a*, *b*, *c*, *d*, *e*; Crespo *et al.* 2002). Crespo & Cubero (1998) included three species of *Parmelia* s. str., *viz.* *P. saxatilis*, *P. sulcata* and *P. omphalodes*, in a molecular approach to *Parmelia* s. lat. phylogeny. They showed that *P. omphalodes* and *P. saxatilis* were more closely related to each other than to *P. sulcata*. In addition, two species have been studied at population level in the genus, *P. saxatilis* (Crespo *et al.* 2002) and *P. sulcata* (Crespo *et al.* 1999). These two *Parmelia* species are amongst the most widespread macrolichens on Earth. Crespo *et al.* (2002) reported that *P. saxatilis* populations from five continents belonged in two monophyletic groups. One (the Atlantic population) occurred in Arctic and Antarctic regions and also included collections from more Atlantic sites in extrapolar regions. The second monophyletic group (the Mediterranean population) included samples from more continental environments in the Mediterranean region. Subsequently, the new species *P. ernstiae* has been segregated from *P. saxatilis* on minor morphological features and differences in the internal transcribed spacer (ITS) sequences of the nuclear rDNA (Feuerer & Thell 2002).

The present study has two objectives. First, to ascertain if the groups of *P. saxatilis* described as Atlantic and Mediterranean populations by Crespo *et al.* (2002) should be considered as separate species, based on molecular characters and also morphological and ecological features. Second, to test the validity of the recently described *P. ernstiae* by comparison with sequences from a larger number of collections of the complex. The phylogeny of the group has been analyzed using phylogenetic methods, using data from the ITS of the nuclear rDNA, and sequences of the protein-coding β-tubulin genes.

Material and Methods

Lichen material

Fifty-six specimens representing 16 species, including all but one (*P. discordans**) of the European species of *Parmelia* s. str., were used in this study. Details of the material, area of collection, and where samples are deposited, are presented in Table 1. Specimens were air-dried and stored at room temperature.

DNA extraction and PCR amplification

Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) with minor modifications as described by Crespo *et al.* (2001). Sequences from the ITS region and the 5·8S gene of the rDNA were used for almost all taxa. Partial sequences of the protein-coding β-tubulin gene were successfully amplified for 31 of the samples. Amplification of the internally transcribed spacer region of the rDNA gene cluster was undertaken with the primers ITS1F (Gardes & Bruns

*Material was obtained and DNA extracted, but the sequences obtained were found to be due to a lichenicolous fungus evidently growing in the lichen, probably *Athelia arachnoidea*.

1993) and ITS4 (White *et al.* 1990). Amplification of the partial β-tubulin sequence was carried out using Bt3-LM and Bt10-LM primers (Myllys *et al.* 2001).

Each PCR reaction contained the following ingredients: 27·75 µl dH_2O, 5 µl 10 × PCR buffer where the $MgCl_2$ 2 mM was already included (10 mM Tris-HCl, pH 8·0; 50 mM KCl; 1 mM EDTA (ethylene diamine tetra-acetate); 0·1% Triton X-100; 59% glycerol v/v), 1 µl dNTP 10 mM (1·25 mM each of dATP, dCTP, dGTP and dTTP), 2·5 µl of a 10 µM dilution of each of the primers and 1·25 µl DNA Polymerase (1 unit/µl, Biotools). This cocktail was mixed with 10 µl of the DNA template. The PCR amplification ran for 30 cycles: denaturation at 94°C for 60 sec, annealing at 54°C (ITS) and 55°–58°C (β-tubulin) for 60 sec, and extension at 72°C for 90 sec. The reaction was carried out in an automatic thermocycler (Techne Progene).

PCR products were purified through a Biotools Bioclean DNA purification column kit according to the manufacturer's specifications. Sequencing was performed on both strands using the ABI PRISM™ Dye terminator cycle Sequencing Ready Reaction Kit (PE Biosystems), with the amplification primer. The following cycling profile was used: denaturation for 3 min at 94°C, then 25 cycles with 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. Sequencing reactions were electrophoresed on an ABI PRISM 377 DNA sequencer (Applied Biosystems).

Sequence analysis

Nuclear ITS and β-tubulin complementary strands were compared with the assistance of Windows SeqMan (DNAStar) to check for reading errors and when possible, resolving ambiguities. Sequences were then aligned using CLUSTAL W (Higgins *et al.* 1992) and visually adjusted. No position was excluded from the matrix data for posterior analyses. Phylogenetic analyses of the sequence data were performed with maximum parsimony (MP) using PAUP version 4.0b10 (Swofford 2002) and with Bayesian inference using MrBAYES version 2.01 (Huelsenbeck & Ronquist 2001).

Maximum parsimony analyses. A heuristic search of 1000 random taxon addition replicate searches was conducted with TBR branch-swapping and the MulTrees option in effect, equally weighted characters, and gaps treated as missing data. Constant characters were excluded. A limit of 10 000 trees was established as no differences in the topology of the trees were found in using limits from 10 000–50 000 trees.

Individual analyses of the nuITS (56 sequences) and β-tubulin (29 sequences) data sets were performed. In cases where data from both genes were available, the data and the topology of the resulting trees were analysed independently. Since no conflict was evident, it was assumed that the two data sets were congruent and could be combined in a single matrix. Strict consensus trees were generated for the three parsimony analyses, and the bootstrap method (Felsenstein 1985) was used to estimate the robustness of the various clades which appeared in the consensus trees. Bootstrap values were estimated from 1000 replicates, and phylogenetic trees were drawn using TREEVIEW (Page 1996).

Bayesian analyses. The data of the combined matrix were also analysed using a Bayesian approach (Huelsenbeck *et al.* 2000; Larget & Simon 1999). Posterior probabilities were approximated to by sampling trees using a Markov Chain Monte Carlo (MCMC) method. The posterior probabilities of each branch were calculated by counting the frequency of trees that were visited during the course of the MCMC analysis. This analysis was performed assuming the general time reversible model (Rodriguez *et al.* 1990) including the estimation of invariant sites and assuming a discrete gamma distribution with six rate categories (GTR+I+G) for the combined analyses. A run with 1 000 000 generations starting with a random tree and employing eight simultaneous chains was executed, and every 100th tree saved into a file. We used the default settings for the priors on the rate matrix, branch lengths, gamma shape parameter, and the proportion of invariable sites. A Dirichlet distribution was assumed for the base frequency parameters, and an uninformative prior was used for the topology (default settings).

We plotted the log-likelihood scores of sample points against generation time and determined that stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium (Huelsenbeck & Ronquist 2001). The initial 600 trees were discarded as burn-in before stationarity was reached. Using PAUP 4.0b10, majority-rule consensus trees were calculated from 9400 trees sampled after reaching likelihood convergence to calculate the posterior probabilities of the tree nodes. Unlike non-parametric bootstrap values (Felsenstein 1985), these are estimated probabilities of the clades under the assumed model (Rannala & Yang 1996), and hence posterior probabilities equal and above 95 are considered significantly supported. The phylogenetic tree was drawn using TREEVIEW (Page 1996).

The polarity of the characters was assessed by outgroup comparison, using *P. pseudotenuirima*, *P. signifera* and *P. erumpens* in the ITS matrix, and only *P. signifera* in the β-tubulin and combined matrix. These species were found to be members of the sister group to all other taxa included here (data not shown).

Constrained-topology analyses. The combined matrix was used to test five phylogenetic null hypotheses. They were tested using a part of the MCMC tree sample described above. The probability of the null hypothesis being correct was calculated by counting the presence of this topology in the MCMC sample (Lewis 2001). The hypotheses tested were that: (1) *P. saxatilis*, *P. serrana* and *P. ernstiae*; (2) *P. saxatilis* and *P. serrana*; (3) *P. ernstiae* and *P. saxatilis*; (4) *P. ernstiae* and *P. serrana*; and (5) *P. omphalodes* and *P. pinnatifida*, belong in the same clade. These hypotheses were tested according to Lumbsch *et al.* (2004). To ensure that the hypothesis testing was independent from the tree estimate, 9400 trees per null hypothesis were sampled. The frequency of trees in the MCMC sample agreeing with the null

TABLE 1. *Specimens of* Parmelia *used in the study, with location, reference collection detail and GenBank accession numbers*

No.	Species	Locality, Country	Voucher specimens	GenBank Nos	
				ITS	β-tubulin
1	*P. squarrosa*	Hokkaido (Tokyo), Japan	MAF 7281	AY036975	—
2	*P. squarrosa*	Braxton Co. (Burnsville), WV, USA	MAF 7288	AY036976	—
3	*P. squarrosa*	Forge Creek, TN, USA	MAF 7293	AY036977	—
4	*P. squarrosa*	Fayette Co. (Glen Ferris), WV, USA	MAF 7289	AY036978	—
5	*P. squarrosa*	Parson Branch,TN, USA	MAF 7270	AY036979	—
10	*P. sulcata*	Scania (Oerkened), Sweden	Thell-Marth SK-9921	AF410840*	AF410844*
12	*P. sulcata*	La Barranca (Madrid), Spain	MAF 9750	AY295103	AY295111
15	*P. fertilis*	Hokkaido (Tokyo), Japan	MAF 7282	AY036982	AF391143
16	*P.* cf. *cochleata*	Hokkaido (Tokyo), Japan	MAF 7271	AY036983	—
17	*P.* cf. *cochleata*	Hokkaido (Tokyo), Japan	MAF 7275	AY036984	—
18	*P. cochleata*	Hokkaido (Tokyo), Japan	MAF 7280	AY036985	—
19	*P. cochleata*	Hokkaido (Tokyo), Japan	MAF 7279	AY036986	—
20	*P. pinnatifida*	Kola Peninsula, Russia	MAF 7274	AY036987	AF391134
21	*P. pinnatifida*	Kola Peninsula, Russia	MAF 7272	AY036988	AF391133
22	*P. saxatilis*	Kola Peninsula, Russia	MAF 7276	AY036989*	AF391136
23	*P. saxatilis*	Kola Peninsula, Russia	MAF 7273	AY036990*	AF391135
24	*P. saxatilis*	Xinjlang Uygur Region (Altay), China	MAF 7030	AF350029*	AF391139
25	*P. saxatilis*	Torres del Paine (Patagonia), Chile	MAF 6895	AF350026*	AF391138
26	*P. saxatilis*	Leonie Is., Antarctica	MAF 6803	AF350022*	AF391137
27	*P. saxatilis*	New Hampshire, USA	5375 May	AF350033*	—
28	*P. saxatilis*	New Hampshire, USA	5374 May	AF350034*	—
29	*P. saxatilis*	Cadiz, Spain	MAF 7668	AY036982*	—
30	*P. saxatilis*	Umeå, Sweden (epitype)	MAF 6882	AF350027*	—
31	*P. saxatilis**	Milodon, Chile	Feuerer 29548	AF412310*	—
32	*P. saxatilis**	Varsinais-Suomi (Turku), Finland	Thell 9926	AF410835*	—
33	*P. saxatilis**	Glacia Serrano, Chile	Feuerer 29547	AF412309*	—
34	*P. saxatilis*	Ruovesi (Helvetinjørven), Finland	Stenroos 5205	AF410836*	—
35	*P. serrana*	El Escorial (Madrid), Spain	MAF 6885	AF350040*	AF391142
36	*P. serrana*	Batuecas (Cáceres), Spain	MAF 7286	AY036996*	AF391140
37	*P. serrana*	Batuecas (Cáceres), Spain	MAF 7287	AY036997*	AF391141
38	*P. serrana*	El Ventorrillo (Madrid), Spain	MAF 6884	AF350037*	—
39	*P. serrana*	El Ventorrillo (Madrid), Spain	MAF 6893	AF350038*	—
40	*P. serrana*	Pto de Navafría (Madrid) Spain	MAF 9755	AY295104	—
41	*P. serrana*	Tyrol, Austria	Hafellner 52306	AF350031*	—
42	*P. serrana*	Tenerife (Canary Islands), Spain	MAF 6889	AF350044*	—
43	*P. serrana*	El Escorial (Madrid), Spain	MAF 9753	AY295105	AY295114
44	*P. serrana*	La Barranca (Madrid), Spain	MAF 9759	AY215907	AY295115
45	*P. serrana*	La Barranca (Madrid), Spain	MAF 9758	AY295108	AY295116
46	*P. serrana*	La Barranca (Madrid), Spain	MAF 9757	AY295106	AY295106
47	*P. serrana*	Navacerrada (Madrid), Spain (holotype)	MAF 9756	AY295109	—
48	*P. ernstiae*	New Forest (Hampshire), UK	MAF 6886	AF350041*	—
49	*P. ernstiae*	Puerto de Corrales (Burgos), Spain	MAF 9749	AY295110	AY295117
50	*P. ernstiae*	Niedersachsen (Reg.-Bez. Lüneburg), Germany (topotype)	MAF 9805	—	AY295118

*Sequences obtained from GenBank.

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TABLE 1. Continued

No.	Species	Locality, Country	Voucher specimens	GenBank Nos ITS	GenBank Nos β-tubulin
51	*P. ernstiae*	Niedersachsen (Reg.-Bez. Lüneburg), Germany	HBG 4619	AF410833*	AF410841*
52	*P. ernstiae*	Schleswig-Holstein (Grossolt), Germany	HBG 64331	AF410834*	AF410842*
53	*P. omphalodes*	Sierra de las Cabrillas (Madrid), Spain	MAF 6055	AF350046	—
54	*P. omphalodes*	La Plataforma del Calvitero (Salamanca), Spain	MAF 7062	AY036998	AF391131
55	*P. omphalodes*	La Plataforma del Calvitero (Salamanca), Spain	MAF 7044	AY036999	AF391132
56	*P. adaugescens*	Hokkaido (Tokyo), Japan	MAF 7277	AY036991	AF391146
57	*P. adaugescens*	Hokkaido (Tokyo), Japan	MAF 7292	AY036992	AF391145
58	*P. adaugescens*	Hokkaido (Tokyo), Japan	MAF 7291	AY036993	AF391144
59	*P. pseudolaevior*	Hokkaido (Tokyo), Japan	MAF 7290	AY036994	AF391147
60	*P. laevior*	Hokkaido (Tokyo), Japan	MAF 7278	AY036995	AF391148
61	*P. submontana*	Hoya Redonda (Sierra de Cazorla), Spain	MAF 3729	AY037000	—
62	*P. discordans*	Rothiemurchus Forest, Inverness, UK	MAF 9804	—	—
63	*P. erumpens*	Tharwa (ACT), Australia	MAF 7284	AY037001	—
64	*P. pseudotenuirima*	Molongo Gorge (ACT), Australia	MAF 7285	AY037002	—
65	*P. signifera*	Molonglo Gorge (ACT), Australia	MAF 7283	AY037003	AF391149

*Sequences obtained from GenBank.

hypothesis was calculated using the filter command in PAUP 4.0b10, with a certain constraint describing the null hypothesis.

GenBank data

Several *Parmelia* s. str. nuITS and partial β-tubulin sequences from GenBank (http://www.ncbi.nlm.nih.gov) were included in the analyses, and pertinent collections are indicated in Table 1.

Chemistry

Secondary chemical compounds were identified by thin layer chromatography (Elix & Ernst-Russell 1993) in the *Parmelia saxatilis* group (Group I) using solvent systems B, C, and G.

Morphology

The lower surfaces of specimens were examined to determine the type of rhizines (i.e. squarrose vs. simple or furcate) using a Leica Wild M8 dissecting microscope. Lobe shape and width were studied under the same microscope, and widths measured to the nearest 0·1 mm using a CBS Beck Kassel calibrated × 8 magnifier; at least ten measurements were made on the various specimens in the *P. saxatilis–P. serrana* complex. Ascospore dimensions were obtained from squash preparations in 10% KOH measured in a Leica Leitz DM RB microscope; in each case ten mature ascospores free of the asci were measured when found.

Results

A total of 59 new sequences have been produced for this study and 26 have been downloaded from GenBank. Fifty-six of the nuITS rDNA and 29 of the partial β-tubulin gene sequences were used for the analyses. The length of the ITS region was 556 bp. Multiple sequence alignment required the inclusion of several gapped positions. The resultant data matrix has 231 variable sites, 141 of them parsimony-informative.

The length of the β-tubulin sequences was 829 bp. In the data matrix, 100 out of 201 variable sites were parsimony informative. The β-tubulin sequences were readily alignable.

Phylogeny

Parsimony analysis of the ITS data set

Parsimony analysis of the ITS region retained 10 000 equally parsimonious trees. The strict consensus tree is shown in Fig. 1. Since several samples from the same species

FIG. 1. Maximum Parsimony strict consensus tree of the 10 000 equally parsimonious trees obtained from the analysis of nuclear ITS sequence data (length=437, CI=0·689, RI=0·832). Numbers above the branches indicate bootstrap values above 50%. Geographical origins of the specimens: (●) Australasia, (■) Asia, (▲) North America, (❍) South America, (★) Europe, and (□) Antarctica.

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were included to check the consistency of sequences at the infraspecific levels, several polytomies are present in terminal branches.

The ITS tree (Fig. 1) reveals three major groups with bootstrap values over 95%. Group I is formed by *P. saxatilis*, *P. serrana*, *P. ernstiae*, *P. omphalodes*, *P. submontana*, *P. pinnatifida*, and *P. adaugescens*. All these species are monophyletic and well supported. Group II includes all specimens of *P. squarrosa*, *P. sulcata* and *P. fertilis*; the isidiate, sorediate, and apotheciate species were not placed in separate groups. *P. sulcata* is shown as paraphyletic and one of the samples constitutes the sister group to the *P. squarrosa/fertilis* clade.

Group III includes only *P. cochleata*, an apotheciate species in which a certain morphological variability has been detected, with the thallus margins lobulate and more or less concave. *Parmelia pseudolaevior* and *P. laevior* are basal to the three groups (Fig. 1).

Parsimony analysis of the β-tubulin partial gene data set

Representative samples from groups I and II were further analysed using the protein-coding β-tubulin partial gene. 10 000 trees were retained and the strict consensus tree shows two monophyletic clades, corresponding to clades I and II of the ITS analysis (Fig. 2). No representative of *P. cochleata* (clade III in the ITS analysis) could be included in the β-tubulin analysis. Group I includes: (1) *P. adaugescens* at the base; and (2) a clade with 90% bootstrap support including two main branches: one includes *P. saxatilis*, *P. ernstiae*, *P. omphalodes* and *P. pinnatifida; P. ernstiae* is the sister group of *P. saxatilis*; and the second branch includes *P. serrana*. Group II is composed of *P. sulcata* and *P. fertilis*, as in the ITS tree.

Parsimony analysis of the combined data set

The parsimony analysis of the combined data matrix of the ITS region and partial β-tubulin gene is shown in Fig. 3. 756 trees were retained, and a strict consensus tree was computed. The topology of this tree was similar to that derived from single gene trees (Figs 1 & 2). Although the β-tubulin gene sequence of *P. submontana* was not available, the taxon was included in the combined data matrix and the nucleotide positions were filled with gaps.

Bayesian analysis of the combined data set

The phylogenetic tree obtained by Bayesian analysis (Fig. 4) is similar to the combined parsimony tree (Fig. 3), and the phylogenetic relationships were confirmed by this method. This tree strongly supported the sister groups formed in parsimony analysis trees with 100% of posterior probability (Groups I and II) and also the basal position of *P. adaugescens* in Group I.

Constrained topology analysis

Although the three clades previously included in the species concept of *Parmelia saxatilis* (i.e. *P. saxatilis*, *P. ernstiae*, and *P. serrana*) appear in distinct clades in the strict consensus tree derived from parsimony analysis and in the majority-rule consensus tree from B/MCMC sampling, the relationships between the taxa in this part of the tree are not supported by significant posterior probabilities. Hence, a monophyly of a wider *P. saxatilis* concept cannot be excluded and may be present in suboptimal trees. The Bayesian inference of phylogeny offers a straightforward estimate of the posterior probabilities of alternative topologies (Huelsenbeck *et al.* 2002; Lewis 2001). Null hypotheses placing (1) *P. saxatilis*, *P. serrana* and *P. ernstiae*, or (2) *P. saxatilis* and *P. serrana* into one clade were tested; the probability of each of these hypotheses was zero, after considering 9400 trees developed in the analysis. The probability of null hypothesis (3), *P. saxatilis* and *P. ernstiae* being in one clade, was 64%; of (4) *P. serrana* and *P. ernstiae* being together was 0·04%; and (5) that *P. omphalodes* and *P. pinnatifida* belong in one clade, was 6·4% (Table 2).

Chemistry

The results of the chemical analyses are summarized in Table 3. All specimens of

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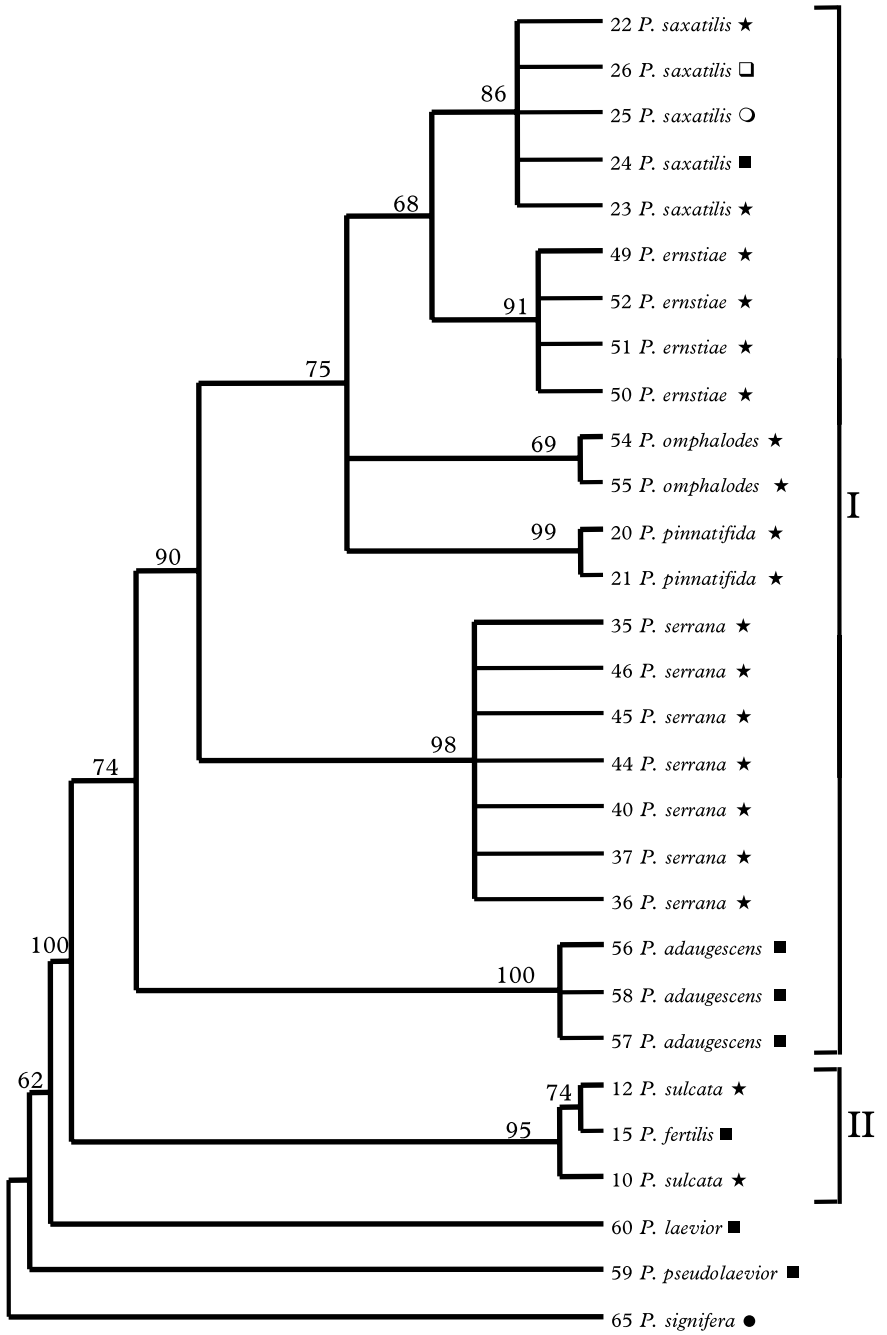

FIG. 2. Maximum Parsimony strict consensus tree of 10 000 equally parsimonious trees obtained from the analysis of protein-coding β-tubulin gene sequence data (length=288, CI=0·809, RI=0·825). Numbers above branches indicate bootstrap values above 50%. Geographical origins as in Fig. 1.

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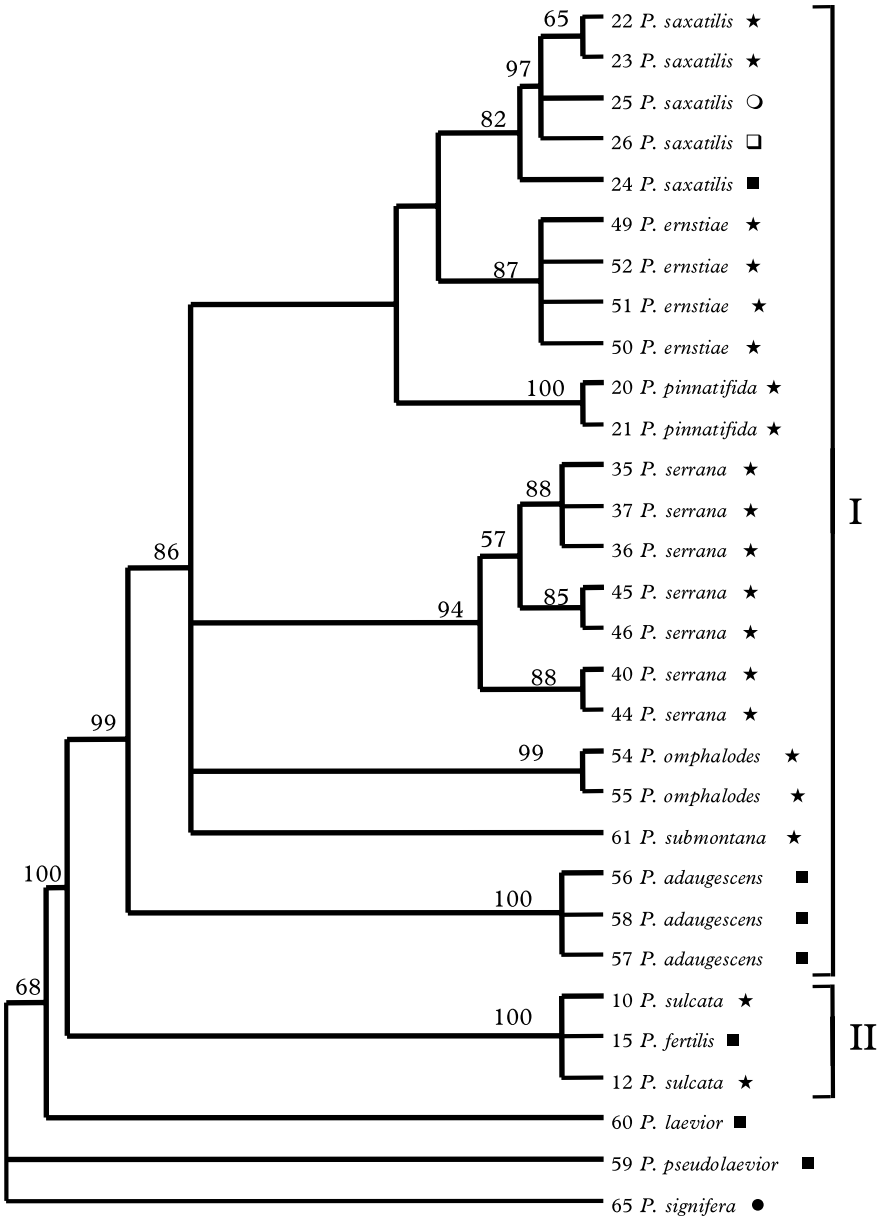

FIG. 3. Maximum Parsimony strict consensus tree of 756 equally parsimonious trees obtained from the analysis of the combined data set (length=590, CI=0·790, RI=0·791). Numbers above branches indicate bootstrap values above 50%. Geographical origins as in Fig. 1.

P. saxatilis s. str. contained atranorin, chloratranorin, salazinic acid, and consalazinic acid; some from the USA also had lobaric acid; protocetraric acid sometimes also occurred. *Parmelia serrana* had a similar chemical composition to *P. saxatilis* s. str., although lobaric acid was more frequent. *P. ernstiae* was also found to have lobaric acid, as did some samples of *P. saxatilis* s. str., *P. omphalodes*, and *P. serrana*. Although

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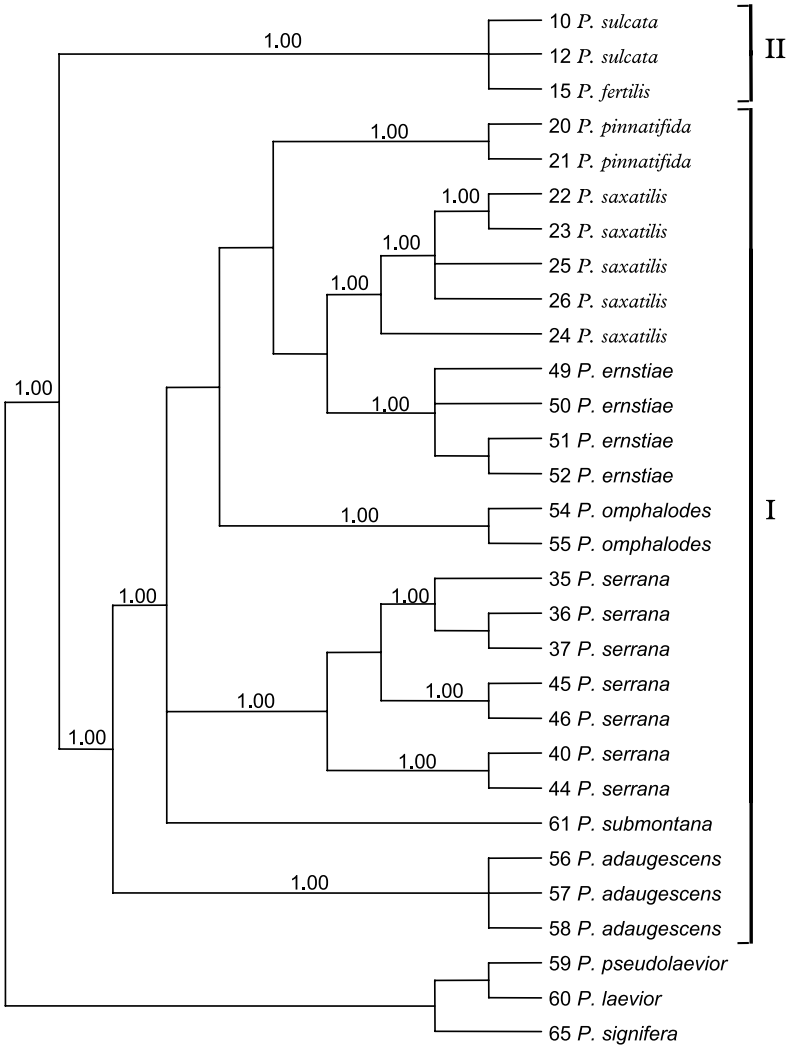

FIG. 4. 50%-majority-rule consensus tree of 8800 trees sampled by a B/MCMC procedure based on the combined data set. Numbers above the branches indicate probability values above 95%.

TABLE 2. *Probabilities of four phylogenetic null hypotheses being correct. Each test is based on a B/MCMC tree sample of 9400 trees*

Null hypothesis	Probability
(1) *P. saxatilis*, *P. serrana* and *P. ernstiae* forming a monophyletic clade	<0·001
(2) *P. serrana* and *P. saxatilis* forming a monophyletic clade	<0·001
(3) *P. saxatilis* and *P. ernstiae* forming a monophyletic clade	64·0
(4) *P. serrana* and *P. ernstiae* forming a monophyletic clade	0·04
(5) *P. omphalodes* and *P. pinnatifida* forming a monophyletic clade	6·40

Probability significant at <0·1%.

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TABLE 3. *Phenolic composition of species of Group I (*Parmelia saxatilis *group)*

Species	Compounds*			
	1	2	3	4
P. adaugescens	+	−	−	+
P. ernstiae	+	−	+	+
P. omphalodes	+	±	+	+
P. pinnatifida	+	−	−	+
P. saxatilis	+	±	±	+
P. serrana	+	±	±	+
P. submontana	+	−	−	+

*1, salazinic acid chemosyndrome; 2, protocetraric acid; 3, lobaric acid; 4, atranorin chemosyndrome.
+, present; −, absent; ±, trace.

norstictic acid was reported in three collections of *P. saxatilis* s. str. from Antarctica, Chile, and China by Crespo *et al.* (2002), using the three solvent systems this could not be unequivocally confirmed.

Parmelia omphalodes was found to contain atranorin, chloratranorin, lobaric acid, salazinic acid, and consalazinic acid, while *P. pinnatifida* had atranorin, chloratranorin, salazinic and consalazinic acid.

Morphology

The results of the morphological studies are incorporated under Taxonomy below.

Taxonomy

As a consequence of our molecular results and the correlation with cryptic morphological differences discussed below, we newly circumscribe *P. saxatilis* s. str., including the selection of an epitype for that species. Further, the *P. saxatilis* Mediterranean population of Crespo *et al.* (2002) is formally described as a separate species.

Parmelia saxatilis (L.) Ach.

Meth. Lich.: 204 (1803).—*Lichen saxatilis* L., *Syst. Pl.* **2:** 1142 (1753); type, Sweden, *sine loc.*, *c.* 1740, *C. Linnaeus* (LINN 1273·62—second from bottom specimen—lectotype designated by Galloway & Elix 1983: 405); Sweden, Västerbotten, Umeå, October 1998, *S. Ott* (MAF 6882–epitypus hic designatus).

The typification of this species was discussed by Jørgensen *et al.* (1994: 354–355) who pointed out that Hale (1987: 38) had wrongly indicated that sheet LINN 1273·61 was the lectotype. However, sheet LINN 1273·62 has been cited as the type at least since Howe (1912: 201) and has the numbers referring to both *Flora Suecica* (Linnaeus 1745) and *Species Plantarum* (Linnaeus 1753) indicating that it was material used in preparing the account for *Flora Suecica*. As there are four specimens on the sheet, one of them had to be chosen as the lectotype, and the second from the bottom was selected by Galloway & Elix (*loc. cit.*); a photograph is provided by Jørgensen *et al.* (1994: 355).

The lectotype specimen is very dark in colour, certainly because of its age, apotheciate, and consists of mainly central parts of an originally much larger thallus. We did not examine the apothecia microscopically nor study its secondary chemistry. The lobes, most distinctly visible near the margins of the specimens, are squared and truncated rather than rounded, do not overlap, and are densely isidiate. The main lobes are 1–2 mm broad, and the ultimate squared lobe ends 0·2–0·4 mm wide.

As no sequence data are available from the lectotype, and as the morphological features are obscure in the aged Linnean collection, we consider it prudent, in order to preclude further debate, to designate a modern

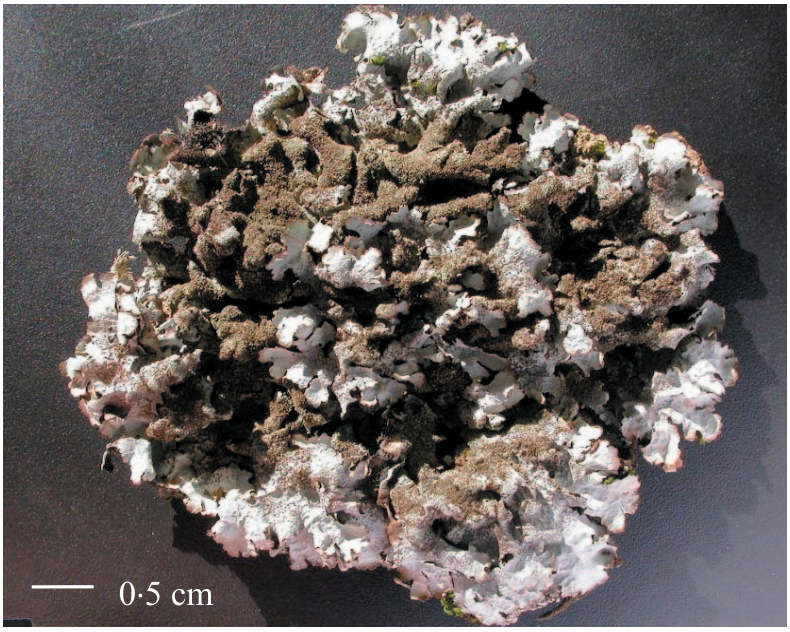

FIG. 5. *Parmelia serrana*, thallus morphology (MAF 9756—holotype).

sequenced collection from northern Sweden as an epitype to fix the application of the name to a member of the Atlantic population of *P. saxatilis*, as understood by Crespo *et al.* (2002). The ITS sequence of the epitype is deposited in GenBank under accession no. AF350027.

Parmelia serrana A. Crespo, M. C. Molina & D. Hawksw. sp. nov.

Similis *Parmeliis saxatilis* sed differt in lobis ultimis rotundatis non truncates, et in sequencis molecularis ITS et β-tubulin.

Typus: Spain, Comunidad de Madrid, Sierra del Guadarrama, Navacerrada, S of Antón Real, close to the junction of roads M 601 and M 607, 40° 43·996′N and 04° 01·438′W, alt. 1300 m, on *Quercus pyrenaica*, 4 February 2003, *A. Crespo & P. K. Divakar* (MAF 9756—holotypus; BM, HBG, TNS, UPS, US—isotypi).

(Fig. 5)

Thallus adnate to loosely adnate, 7–15 cm diam, lobes contiguous to overlapping and imbricate, apically rounded to sublinear, short, 2·5–6 mm wide. Upper surface pale greenish grey to whitish grey, shiny, finely foveolate, becoming reticulately cracked. *Pseudocyphellae* laminal, numerous, mainly linear to irregularly shaped. *Isidia* very dense on older parts of the thallus, cylindrical, simple to branched, to 0·2–0·5 mm tall and 0·08–0·1 mm wide, syncorticate. *Rhizines* on lower surface moderately abundant, simple to furcately branched, not squarrose, 1–1·5 mm long.

Apothecia rather frequent, subpedicellate, disc concave, thalline exciple often sparsely isidiate, 1–4 mm diam. *Ascospores* broadly ellipsoid, 17–18 × 12–13 μm.

Chemistry. Cortex K+ yellow; medulla K+ yellow then red, C −, PD+ red-orange; containing atranorin, chloroatranorin, salazinic acid, consalazinic acid, and protocetraric acid (trace).

Etymology. From the Spanish adjective 'serrano', from the mountains (the sierras).

Ecology. This species is widely distributed on deciduous and coniferous trees, and

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occasionally on mossy rocks. When saxicolous specimens are found, the species is generally also present on nearby trees. The species occurs in submediterranean and Mediterranean areas from around 300–1700 m altitude, and is especially frequent on *Pinus sylvestris* and *Quercus* spp. in the Sierra del Guadarrama.

Distribution. Africa (Canary Islands), and Europe; see Crespo *et al.* (2002) for details of additional collections. *Parmelia serrana* is very common in low to moderately high Mediterranean and submediterranean mountains (sierras). It seems to prefer continental climatic conditions. However, its distribution in Europe is far from fully understood. It also occurs at lower altitudes in more temperate parts of Europe, especially somewhat continental areas, as in Austria. In contrast, *P. saxatilis* s. str. seems to prefer more oceanic areas and to have an essentially bipolar distribution. On rocks, it extends southwards in the oceanic parts of Spain, even to extremely warm sites. In temperate and Mediterranean areas *P. saxatilis* s. str. is common in the alpine and oromediterranean zones, but its distribution at lower elevations outside oceanic areas is poorly known. An ecophysiological comparative study of the two species, and if possible also *P. ernstiae*, would clearly be of interest.*

Observations. Parmelia serrana is morphologically characterized by the broad and round lobes that often overlap each other. In contrast, the lobes of *P. saxatilis* s. str. as typified above are sublinear, narrower, and do not overlap. Further, *P. saxatilis* is often more adnate to the substratum than *P. serrana*, and is also generally darker in colour, especially at the lobe margins. Indeed, darker morphs of *P. saxatilis* s. str. can sometimes resemble paler morphs of *P. omphalodes*, to which it is more closely allied in our trees than to *P. serrana*. However, *P. omphalodes* always lacks isidia. Nevertheless, *P. serrana* and *P. saxatilis* s. str. are morphologically very similar and are likely to be confused, especially when collections are not available for comparison by juxtaposition. However, as the two species appear generally to be allopatric, in practice this seems unlikely to pose field identification problems in ecological and inventory studies.

*In passing, we report that the material of *P. saxatilis* s. str. transported on its original rock from the UK Peak District to the Sierra del Guadarrama mountains in central Spain, and reported on by Crespo *et al.* (2002), did not survive its second year of extreme continental temperatures; the crescent remnants of the thalli had all died and fallen away from the rock by May 2003. However, as this was only a single example with no control, we caution against any conclusions being drawn from this pilot experiment.

Discussion

Our analysis revealed three clades in the predominantly Northern Hemisphere group of *Parmelia* s. str., and further supported the view that these clades are closely related to one another. The precise nature of the relationships of the groups, however, remains unclear and data from additional genes will be necessary to resolve this with confidence.

Within Group I, the generally fertile, large-ascospored and non-isidiate *Parmelia adaugescens* is located at the base. Within this group, seven monophyletic clades were found (Figs 3 & 4). *Parmelia saxatilis* and *P. submontana* were confirmed as distinct, as was *P. pinnatifida* and *P. omphalodes*. *Parmelia submontana*, an uncommon species, with usually contorted lobes, simple or furcate rhizines, orbicular to linear soralia, bearing isidioid soredia, appears as morphologically intermediate between *P. sulcata* and *P. saxatilis*. Although the β-tubulin sequence was not available, the topologies of the trees showed its closest relationship to *P. saxatilis* (Figs 1, 3 & 4). *Parmelia pinnatifida* has been considered as a synonym (Hinds 1998) or subspecies of *P. omphalodes* (Skult 1984), but it differs in having narrower lobes with pseudocyphellae restricted to their margins, and the lack of lobaric acid (Kurokawa 1976; Skult 1984); further, the repeatedly branched and overlapping lobes tend to

form cushion-like pulvinate tufts (Skult 1984). The ITS and β-tubulin trees both suggest that the two species are phylogenetically distinct (Figs 1–4). The null hypothesis, placing *P. omphalodes* and *P. pinnatifida* into a monophyletic clade, was tested using a separate MCMC sampling; the probability of the null hypothesis was low but higher than 0·1%, so this test did not unequivocally confirm the distinction of the two species.

We were unable to obtain DNA from two additional species considered allied to *P. omphalodes*. First, *P. discordans*, which contains protocetraric and lobaric acids, instead of salazinic or norstictic acids (Culberson 1970). *Parmelia discordans* can be recognized in the field by the more uniform darker brown colour and smoother more adpressed and often overlapping narrower lobes [1(–3) mm wide vs. to 4 mm wide in *P. omphalodes*] with more obscure linear pseudocyphellae (Purvis *et al.* 1992; Rose, 1995); it also differs significantly from *P. omphalodes* in protein banding patterns (Skult *et al.* 1990). Second, the arctic circumpolar *P. skultii* (syn. *P. omphalodes* subsp. *glacialis*; Skult 1985) which is distinguished by containing norstictic and salazinic acids (Hale 1987), and in having more rounded and firm-margined lobes (Skult 1985). While we are confident that both *P. discordans* and *P. skultii* merit recognition as species, resolving their relationships will require sequences from fresh collections.

The most striking result of the relationships revealed within Group I is the monophyletic clade, named here as *P. serrana*, for the specimens referred to as the Mediterranean population of *P. saxatilis* by Crespo *et al.* (2002). While the degree of morphological and chemical similarity is remarkable, providing an excellent example of a cryptic species in lichens (Culberson 1986), the taxa can be distinguished on the basis of morphology (see above) and ecology. The null hypothesis test confirms the separation of the new species (Table 2). The probability of *P. serrana*, *P. saxatilis* s. str. and *P. ernstiae* being monophyletic was zero, as was the probability that *P. serrana* and *P. saxatilis* s. str. were genetically closely related.

The name *P. ernstiae*, a species recently separated from *P. saxatilis* (Feuerer & Thell 2002), applies to Clade IV of the Atlantic population of *P. saxatilis* s. lat. of Crespo *et al.* (2002). The species has a strongly pruinose upper surface, made up of calcium oxalate crystals, a mixture of isidia and lobules, as well as being distinguished by molecular data. However, in the combined Bayesian analysis, *P. ernstiae* emerged as the sister-group to *P. saxatilis*; this suggests that the two species are very closely related. The null hypothesis considering that both taxa are monophyletic (Table 2) was also quite high (64%). Additional studies using population genetical approaches will be necessary to further clarify the status of the two morphologically different populations. The two collections of *P. saxatilis* s. lat. from the high mountains of the northern Appalachians in New Hampshire (USA), placed in Clade III of the Atlantic population in the phylogenetic trees of Crespo *et al.* (2002), remained distinct in our new ITS-based tree (Fig. 1). It seems probable that these represent a further undescribed species, but we do not formally describe this as new here pending the outcome of more detailed molecular studies of North American representatives of the genus currently in progress in collaboration with P. May and M. S. Cole.

Group II in the ITS tree includes *P. squarrosa*, *P. fertilis* and *P. sulcata*. *Parmelia squarrosa*, an isidiate species, and *P. fertilis*, a species with sexual reproduction, appear in the same clade, but not with a high supporting value. Hale (1987) considered *P. fertilis* to be related to *P. sulcata* on the basis of several morphological characters, but thought the companion apotheciate morph (*P. fertilis*) was considered to be more closely related to *P. squarrosa*. Our phylogenetic analyses using ITS fragment and partial β-tubulin gene sequences confirm this last assumption, with *P. fertilis* nested within the *P. squarrosa* clade. However, sequences from additional collections of *P. fertilis* are necessary to decide whether *P. squarrosa* and *P. fertilis* are conspecific, in which case they

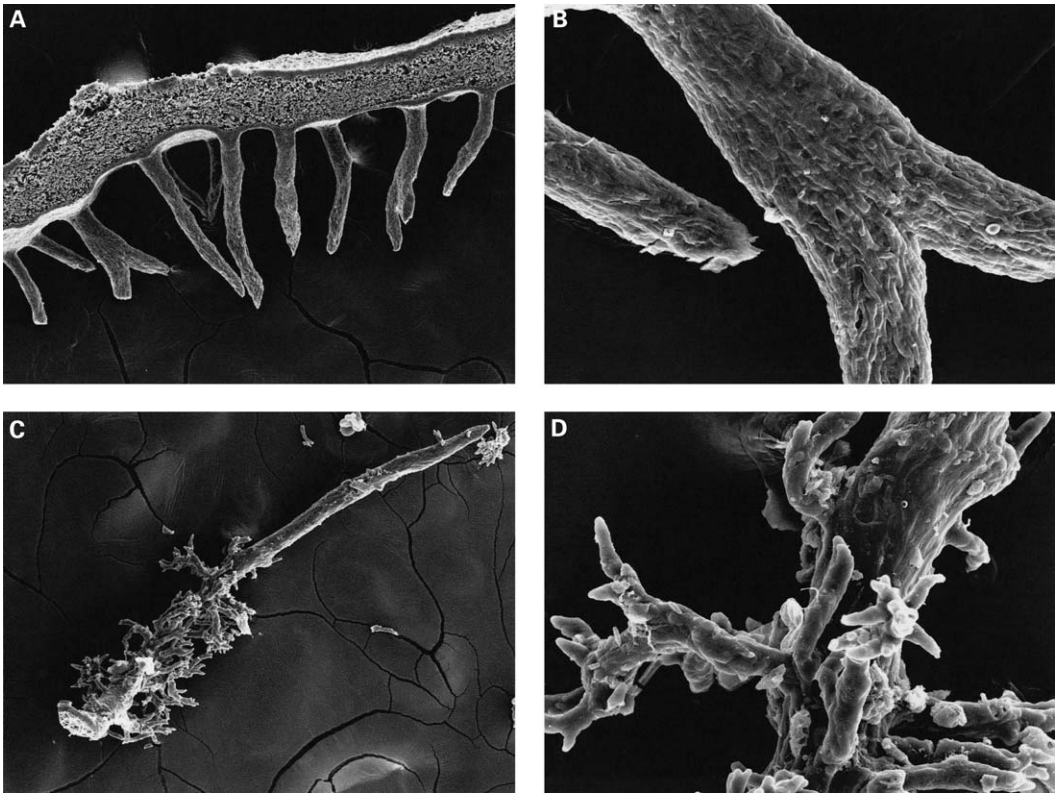

FIG. 6. Scanning electron micrographs showing rhizine morphology. A & B, *Parmelia serrana*, furcate rhizines (MAF 9756—holotype); A, simple and furcate rhizines projecting from the lower surface; B, detail of a single furcate rhizine. C & D, *P. sulcata*, squarrose rhizines (MAF 9750); C, a squarrose rhizine; D, detail showing the perpendicular branching. A & C × 100, B & D × 500.

would have to be united under the older name (i.e. *P. fertilis*). The two taxa are sympatric in eastern North America (Hinds 1998), and the generally asexual morph (*P. squarrosa*) is one of the most common corticolous lichens in North America and Japan.

Parmelia squarrosa has generally been compared with *P. saxatilis* as both are isidiate (e.g. Hinds 1998), and the collections are often confused in herbaria. However, our phylogenetic analyses clearly separate these two species. *Parmelia saxatilis* belongs to Group I in which all members have simple to furcate rhizines, whereas *P. squarrosa* and all other taxa studied of Group II have squarrose rhizines. Some care is nevertheless required in the interpretation of rhizine types. Squarrose rhizines, in which secondary branches arise perpendicularly to the axis (Fig. 6C & D), are sometimes only visible at the base and near the axils of the terminal lobes in 'squarrose' species. In contrast, in what have been termed 'furcate' rhizines in the parmelioid lichens, secondary branches arise obliquely from the axis (Fig. 6A & B). Scanning electron micrographs showing these rhizine types are provided by Hale (1987: fig. 10a–d).

Our results show that the sorediate *P. sulcata* belongs to the same clade as *P. squarrosa*. However, the cosmopolitan *P. sulcata* is polyphyletic in our analysis. Additional studies will be necessary to find out whether the *P. sulcata* complex includes several cryptic species, as demonstrated here in the *P. saxatilis* group. Previous results have already suggested a wide genetic variability in *P. sulcata* (Crespo *et al.* 1997).

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Crespo *et al.* (2002) and Thell *et al.* (2002) reported that *P. sulcata* and *P. saxatilis* were examples of taxa with ITS regions that contain no infraspecific variation between the two Hemispheres. However, the increased number of samples reported on here demonstrates that results based on a very few collections can be misleading, and that it is necessary to conduct more extensive population studies before reaching firm conclusions.

Group III consists only of *P. cochleata*, although the material analysed showed some morphological variability, as already reported by Hale (1987). However, while both *P. adaugescens* and *P. cochleata* have simple to furcate rhizines and similar distributions (Japan and eastern Asia), our phylogenetic analysis did not show any close relationship between them. Further, *P. adaugescens* is distinguished by having larger ascospores (21–27 × 13–15 μm) than *P. cochleata* (12–13 × 6–8 μm).

The results of our study demonstrate once again the power of molecular methods using data from different loci, in this case ITS rDNA and parts of the β-tubulin gene sequence. This approach has enabled us to unravel some complex and hitherto confusing relationships to produce a revised taxonomy of some of the commonest lichens on Earth, which proves to be congruent with morphological and geographical differences.

We are indebted to various collectors for kindly responding to our requests for fresh material of various species, notably Mariette S. Cole, Brian J. Coppins, Jack A. Elix, Ove E. Eriksson, Tassilo Feuerer, Hiroshi Harada, Rosmarie Honegger, Hiroyuki Kashiwadani, Phil May, and Sieglinde Ott. In addition John C. Marsden kindly allowed D.L.H. to study the Linnaean material of *Lichen saxatilis* during a brief visit to his office.

Sequencing was carried out at the Unidad de Genómica (Parque Científico de Madrid, UCM).

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