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Standard Paper

A revision of species of the *Parmelia saxatilis* complex in the Iberian Peninsula with the description of *P. rojoi*, a new potentially relict species

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Abstract

The species of the *Parmelia saxatilis* complex occurring in the Iberian Peninsula were revised. Eight species are accepted, including a new species found in southern Spain, described as *P. rojoi* A. Crespo, V. J. Rico & Divakar. The new species, which forms a sister-group relationship with *P. saxatilis* s. str., is rare in the Iberian Peninsula and is restricted to higher altitudes of northern and central Spain. *Parmelia rojoi* differs from *P. saxatilis* by generally narrower isidia and a more fragile thallus. The segregation of the new species is also supported by ITS (rDNA) and *Mcm7* (MS456) phylogeny and multispecies coalescent-based approaches, including StarBEAST and BP&P. Furthermore, the divergence of *P. rojoi* is dated back to the Pleistocene, *c.* 2.13 Ma. A key to the identification of species from the *P. saxatilis* complex with their diagnostic features is provided. All species of the complex known from Europe are also found in the Iberian Peninsula. We hypothesize that *P. rojoi* is a relict species that survived the Pleistocene glaciations in refugia in Spain and has been unable to extend its distributional range in postglacial periods.

Key words: biogeography, glaciations, lichen, *Parmeliaceae*, phylogeny, refugia, systematics, taxonomy

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Introduction

The genus *Parmelia* s. str. belongs to the parmelioid crown of *Parmeliaceae* and includes *c.* 40 currently accepted species (Divakar *et al.* 2015; Molina *et al.* 2017). The genus is characterized by having a foliose thallus with simple to furcate and squarrose rhizines, a non-pored epicortex, effigurate to elongate pseudocyphellae on the upper surface, isolichenan, and cylindrical or bifusiform conidia (Crespo *et al.* 2010; Thell *et al.* 2012). *Parmelia* s. str. is a widespread genus in the Northern Hemisphere distributed in boreal-temperate Europe, North America and eastern Asia (Hale 1987; Hawksworth *et al.* 2008, 2011; Crespo *et al.* 2010). The Australasian species have been segregated in the genus *Notoparmelia* A. Crespo *et al.* (Ferencova *et al.* 2014) and previously some East Asian species with punctate pseudocyphellae at the lobe edges were accommodated in the genus *Nipponoparmelia* (Kurok.) K. H. Moon *et al.* (Crespo *et al.* 2010). Within *Parmelia* s. str., the *P. saxatilis* group is a monophyletic clade that includes *P. discordans* Nyl., *P. ernstiae* Feuerer & A. Thell, *P. hygrophila* Goward & Ahti, *P. imbricaria* Goward *et al.*, *P. mayi* Divakar *et al.*, *P. omphalodes* (L.) Ach., *P. pinnatifida* Kurok., *P. saxatilis* (L.) Ach., *P. serrana* A. Crespo *et al.*, *P. submontana* Nádv. ex Hale and *P. sulymae* Goward *et al.*, all accepted species characterized by the presence of simple to bifurcate rhizines on the lower surface (Divakar *et al.* 2016; Molina *et al.* 2017). Of these, seven species (*P. discordans*, *P. ernstiae*, *P. omphalodes*, *P. pinnatifida*, *P. saxatilis*, *P. serrana* and *P. submontana*) are known from Europe (Hawksworth *et al.* 2008). The group has its centre of diversity in temperate regions of the Northern Hemisphere but has a cosmopolitan distribution (Hale 1987). While its occurrence in Antarctica has been confirmed with molecular data (Øvstedal & Lewis Smith 2001; Crespo *et al.* 2002), its presence in southern South America (Stenroos 1991; Elvebakk *et al.* 2014) and New Zealand (Galloway & Elix 1983) is not well understood and requires additional studies. Several more or less cryptic species within the complex have been discovered recently in Europe and North America, including *P. ernstiae*, *P. imbricaria*, *P. mayi*, *P. serrana* and *P. sulymae* (Feuerer & Thell 2002; Molina *et al.* 2004, 2011*b*, 2017). While the European species of the *P. saxatilis* complex have been thoroughly studied, the Asian species of the complex are currently less well known.

In the Iberian Peninsula, molecular investigations on *Parmelia* s. str and parmelioid lichens in general have been carried out over the last two decades. The first molecular phylogeny on parmelioids was published in 1998 (Crespo & Cubero 1998), and the first intraspecific molecular studies on *P. saxatilis* and *P. sulcata* Taylor were published in 1997, 1999 and 2002 (Crespo *et al.* 1997, 1999, 2002). Subsequently, Molina *et al.* (2004) described *P. serrana* in the *P. saxatilis* group from Spain and confirmed the monophyly of *P. ernstiae*, reporting it from the Iberian

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Table 1. Specimens of *Parmelia* used in this study, including sample code, collection details, voucher and GenBank Accession numbers. Newly obtained sequences for this study are in bold and missing data are indicated with a dash (—).

				GenBank Accession number	
Sample code	Species	Locality, Country, *Collector*	Voucher specimen	ITS	*Mcm7*
Pdisc4	*P. discordans*	S. Aberdeen Mountain, Scotland, UK, *Hawksworth*	MAF-Lich 10232	AY583212	KR995643
Perst8	*P. ernstiae*	Canakale, Turkey, *Crespo et al.*	MAF-Lich 19627	KT625493	KT625534
Perst9	*P. ernstiae*	Bohuslän, Sweden, *Lysevattnet*	Lysevattnet 20 (UPS)	KT625494	KT625535
Pers10	*P. ernstiae*	Killarney, Ireland, *Crespo & Gavilán*	MAF-Lich 20000	KT625495	KT625536
Per6603	*P. ernstiae*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22791	**MT580478**	**MT583819**
Per6604	*P. ernstiae*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22792	**MT580479**	—
Per6605	*P. ernstiae*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22793	**MT580480**	—
Per6606	*P. ernstiae*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22794	**MT580481**	**MT583820**
Per6607	*P. ernstiae*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22795	**MT580482**	**MT583821**
Per6608	*P. ernstiae*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22796	**MT580483**	**MT583822**
Phyg24	*P. hygrophila*	Mendocino, USA, *Crespo et al.*	MAF-Lich 15770	JN609436	KT625550
Phyg25	*P. hygrophila*	Coast Mountains, BC, Canada, *Björk*	CB 25193	KT625508	KT625551
Phyg26	*P. hygrophila*	Clearwater River drainage, BC, Canada, *Goward*	TG 08-119	KT625509	KT625552
Pimbr19	*P. imbricaria*	Grouse Lake, BC, Canada, *Goward*	TG 08-108	KT625503	KT625545
Pimbr20	*P. imbricaria*	Yukon, Canada, *Spribille*	TS 448	KT625504	KT625546
Pimbr22	*P. imbricaria*	Grouse Lake, BC, Canada, *Goward*	TG 08-124	KT625506	KT625548
Pmayii27	*P. mayi*	Mount Everett, USA, *May*	MAF-Lich 15767	JN609437	KT625553
Pmayii28	*P. mayi*	Mount Everett, USA, *May*	MAF-Lich 15766	JN609438	KT625554
Pmayii29	*P. mayi*	Mount Everett, USA, *May*	MAF-Lich 15765	JN609439	KT625555
Pomp30	*P. omphalodes*	La Plataforma del Calvitero, Spain, *Crespo et al.*	MAF-Lich 7062	AY036998	—
Ppin1	*P. pinnatifida*	Kola Peninsula, Russia, *Schelesong*	MAF-Lich 7274	AY036987	—
Ppin2	*P. pinnatifida*	Kola Peninsula, Russia *Schelesong*	MAF-Lich 7272	AY036988	—
PsaAY11	*P. rojoi* sp. nov.	Cádiz, Spain, *Crespo & Hawksworth*	MAF-Lich 7668	AY114359	—
Psa2347	*P. rojoi* sp. nov.	Cádiz-Málaga, Spain, *Crespo et al.*	MAF-Lich 20062	KT892945	—
Psa2354	*P. rojoi* sp. nov.	Cádiz-Málaga, Spain, *Crespo et al.*	MAF-Lich 20065	KT892944	—
Psa6593	*P. rojoi* sp. nov.	Málaga, Spain, *Crespo et al.*	MAF-Lich 22797	**MT580484**	**MT583823**
Psa6600	*P. rojoi* sp. nov.	Málaga, Spain, *Crespo et al.*	MAF-Lich 22798	**MT580485**	**MT583824**
Psaxt32	*P. saxatilis*	South of Clearwater, BC, Canada, *Goward*	TG 08-49a	KT625511	KT625557
Psaxt33	*P. saxatilis*	Gatling Gorge trail, BC, Canada, *Goward*	TG 08-128	KT625512	KT625558
Psaxt34	*P. saxatilis*	Fage Bluffs, BC, Canada, *Goward*	TG 08-153	KT625513	KT625559
Psaxt35	*P. saxatilis*	Clearwater River drainage, BC, Canada, *Goward*	TG 08-142	KT625514	KT625560
Psaxt36	*P. saxatilis*	Grouse Lake, BC, Canada, *Goward*	TG 08-75b	KT625515	KT625561
Psaxt37	*P. saxatilis*	Table Mountain, BC, Canada, *Goward*	TG 08-71f	KT625516	KT625562
Psaxt38	*P. saxatilis*	Montana, USA, *Pérez-Ortega*	MAF-Lich 15762	JN609442	KT625563
Psaxt39	*P. saxatilis*	S. Aberdeenshire, Scotland, UK, *Hawksworth*	MAF-Lich 15764	JN609441	KT625564
Psaxt41	*P. saxatilis*	Umeå, Sweden, *Ott*	MAF-Lich 6804	AF350027	KT625565
Psaxt42	*P. saxatilis*	Västerbotten, Sweden, *Wedin*	Wedin 7091 (UPS)	AF058037	JX974709
Pser43	*P. serrana*	Navacerrada, Madrid, Spain, *Crespo et al.*	MAF-Lich 9756	AY295109	JX974710
Pse6566	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22799	**MT580487**	—
Pse6569	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22803	**MT580488**	**MT583825**
Pse6570	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22800	**MT580489**	—

(*Continued*)

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Table 1. (*Continued*)

Sample code	Species	Locality, Country, *Collector*	Voucher specimen	GenBank Accession number	
				ITS	*Mcm7*
Pse6571	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22801	**MT580490**	—
Pse6572	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22802	**MT580491**	—
Pse6573	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22804	**MT580492**	—
Pse6575	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22806	**MT580493**	—
Pse6576	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22807	**MT580494**	—
Pse6577	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22808	**MT580495**	—
Pse6578	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22809	**MT580496**	**MT583826**
Pse6583	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22810	**MT580497**	—
Pse6584	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22811	**MT580498**	—
Pse6585	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22812	**MT580499**	—
Pse6586	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22813	**MT580500**	—
Pse6587	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22814	**MT580501**	—
Pse6588	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22815	**MT580502**	—
Pse6589	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 22816	**MT580503**	—
Pse2349	*P. serrana*	Cádiz, Spain, *Crespo et al.*	MAF-Lich 21281	**MT580486**	—
Psub45	*P. submontana*	Ifrane, Medium Atlas, Morocco, *Divakar et al.*	MAF-Lich 15440	JN609434	KT625567
Psub46	*P. submontana*	Jaén, Hoya Redonda, Spain, *Aragón*	MAF-Lich 3729	AY037000	KT625568
Psul52	*P. sulcata*	Killarney, Ireland, *Crespo & Gavilan*	MAF-Lich 15421	EU788027	KT625573
Psul53	*P. sulcata*	Dunkerron, Ireland, *Crespo & Gavilan*	MAF-Lich 15418	EU788028	KT625574
Psylm61	*P. sulymae*	Hemp Creek, BC, Canada, *Goward*	TG 08-14b	KT625526	KT625582
Psylm62	*P. sulymae*	Fage Bluffs, BC, Canada, *Goward*	TG 08-87a	KT625527	KT625583
Psylm63	*P. sulymae*	Gatling Gorge, BC, Canada, *Goward*	TG 08-05a	KT625528	KT625584

Peninsula. Later, Divakar *et al.* (2005) detected an additional species from the Iberian Peninsula, *P. barrenoae* Divakar *et al.*, hidden within the collective name *P. sulcata*. Recently, an additional cryptic species, *P. encryptata* A. Crespo *et al.*, which is in part sympatric with *P. sulcata*, was described from the Iberian Peninsula (Molina *et al.* 2011*a*). Apart from these, *P. mayi*, *P. imbricaria* and *P. sulymae* were segregated from *P. saxatilis* in North America (Molina *et al.* 2011*b*, 2017). A first DNA barcode study on *Parmelia* s. str. species was published in 2016 (Divakar *et al.* 2016).

In Europe, seven species are currently recognized in the group: *Parmelia discordans*, *P. ernstiae*, *P. omphalodes*, *P. pinnatifida*, *P. saxatilis*, *P. serrana,* and *P. submontana* (Kurokawa 1976; Hale 1987; Feuerer & Thell 2002; Molina *et al.* 2004; Hawksworth *et al.* 2008, 2011; Thell *et al.* 2008). The delimitation of *P. discordans* and *P. omphalodes* is currently unclear, with ITS sequence data failing to separate the taxa as reciprocally monophyletic clades (Ossowska *et al.* 2019). However, recent studies using RADseq data suggest that closely related lichen-forming fungal species may require genome-wide data to test their delimitation (Grewe *et al.* 2018).

Our studies of parmelioid lichens in the Iberian Peninsula led to the discovery of an additional, hitherto overlooked lineage occurring in southern Spain that is described below, and which represents the eighth species of the *P. saxatilis* group in Europe. Interestingly, all species of the *P. saxatilis* complex known to occur in Europe have also been found in the Iberian Peninsula. We discuss this fact and the discovery of the new species which we interpret as a potential relict species in light of the impact of the Pleistocene glaciations on the European lichen flora.

Materials and Methods

Phenotypic examination

This study stems from a two decade-long investigation about the diversity of parmelioid lichens in the Iberian Peninsula. Morphological and anatomical characteristics of samples included in Table 1 were studied using a Nikon SMZ-1500 dissecting microscope and a Nikon Eclipse-80i compound microscope (Nikon, Badhoevedrop, the Netherlands). The images were taken in natural light with a Zeiss Touit 2.8/50 mm macro lens (Carl Zeiss AG, Leipzig, Germany) attached to a Fujifilm XT-1 camera (Fujifilm, Tokyo, Japan). Spot tests and thin-layer chromatography (TLC) were carried out following standard procedures (Orange *et al.* 2010), using solvent system C, with concentrated acetone extracts at 50 °C spotted onto silica gel 60 F254 aluminium sheets (Merck, Darmstadt, Germany). The aluminium sheets were dried for 10 min in an acetic acid atmosphere to maximize resolution. For synonyms of included species, see

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elsewhere (Hillmann 1936; Hale 1987). For comparison, we examined the morphology of specimens of other *Parmelia* species, deposited in the herbarium MAF.

DNA isolation, PCR amplification and sequencing

Twenty-six specimens representing three species of the *Parmelia saxatilis* group were selected to obtain new sequences of the internal transcribed spacer (ITS) rDNA and the protein coding single-copy gene *Mcm7* (MS456) as molecular markers. For dataset I, a total of 63 specimens were used to generate ITS and *Mcm7* sequences. Of these, 34 were newly generated for this study (Table 1). For dataset II, the ITS sequences of 155 specimens from different geographical regions, especially of species belonging to the *P. saxatilis* complex and representing all species of the *P. saxatilis* group, were gathered (see Supplementary Material Table S1, available online). *Parmelia sulcata* was selected as the outgroup since the *P. sulcata* group is sister to the *P. saxatilis* group (Molina *et al.* 2017).

Total genomic DNA was extracted from fresh and herbarium specimens using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions, with the slight modifications described previously (Crespo *et al.* 2001). Dilutions 1:10 (v:v) of total DNA were used for PCR amplifications of the genes coding for the nuclear ITS and the single-copy gene *Mcm7* (MS456). Primers, PCR and Sanger sequencing conditions were as described previously (Schmitt *et al.* 2009; Molina *et al.* 2017).

Sequence alignment and phylogenetic analyses

Sequence fragments generated for this study were assembled and edited using the program SeqMan v.7 (Lasergene R, DNASTAR, Madison, WI, USA). Sequence identity was assessed using the mega-BLAST search function in GenBank (Sayers *et al.* 2011). Each dataset was aligned separately using MAFFT v.7 (Katoh & Toh 2008) implementing the G-INS-I alignment algorithm, '1PAM/K = 2' scoring matrix with an offset value of 0.0, and the remaining parameters set to default values. In contrast to the alignment of the *Mcm7*, the ITS alignment contained a number of ambiguous regions which were removed using the least stringent option in Gblocks v.0.91b (Castresana 2000; Talavera & Castresana 2007) on the Gblocks web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). Nucleotide substitution models were selected using the Akaike Information Criterion (AIC) (Akaike 1974) as implemented in jModelTest (Posada 2008). The general time-reversible (GTR) substitution model, with the assumption of a gamma distribution (GTR + G), was used for the ITS. The GTR substitution model with estimation of invariant sites (GTR + I) was selected for the complete *Mcm7* region.

The alignments were analyzed using maximum likelihood (ML) and a Bayesian approach. Maximum likelihood analyses were performed using the RAxML v.8.2.6 program (Stamatakis 2006, 2014) as implemented on the CIPRES Web Portal, with the GTRGAMMA model. In the concatenated data matrix, a total of five partitions was used for the MrBayes analysis: the two loci were treated as separate partitions and we used a three-partition approach for the protein-coding *Mcm7* marker, taking the first, second and third codon positions as separate model partitions. Nodal support was evaluated using the 'rapid bootstrapping' option with 1000 replicates (Stamatakis *et al.* 2008). We used an ML approach to examine the heterogeneity in the phylogenetic signal between the two loci in the concatenated dataset. The bootstrap support value was used to detect incongruence between the two loci alignments. We interpreted high bootstrap values as being strong support for a particular node and identified the conflicting nodes by comparing each gene partition with a threshold between conflicting (> 70% bootstrap) and non-conflicting (< 70% bootstrap) nodes (Hillis & Bull 1993). If no conflict was evident, it was assumed that the two datasets were congruent and could be combined.

The Markov chain Monte Carlo (MCMC) analyses were conducted using MrBayes v.3.2.7 (Ronquist *et al.* 2012). The concatenated two-locus dataset was partitioned as described in the ML analysis, specifying the best-fitting model as described above, allowing unlinked parameter estimation and independent rate variation. Four parallel runs of eight million generations were produced, starting with a random tree and employing eight simultaneous chains each, in which one in every 1000 trees was sampled. The first 25% of trees was discarded as burn-in of the chains. We used AWTY (Nylander *et al.* 2008) 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 in MrBayes. Only clades that received bootstrap support ≥ 70% in the ML analysis and posterior probabilities ≥ 0.95 in the MrBayes analysis were considered to be well supported. Phylogenetic trees were illustrated using FigTree v.1.4.2 (Rambaut 2009).

Species trees and divergence time estimates

In addition, we performed a divergence time analysis to estimate the split of *Parmelia saxatilis* and the new taxon described here. Multilocus species tree methods may provide more accurate hypotheses of evolutionary relationships and more biologically realistic estimates of divergence times relative to concatenated gene tree approaches (Heled & Drummond 2010; McCormack *et al.* 2011). Thus, we used the coalescent-based hierarchical Bayesian model StarBEAST2 implemented in BEAST v.2.4.3 (Bouckaert *et al.* 2014) to estimate a species tree and divergence times for the *Parmelia saxatilis* group. StarBEAST2 estimates the species tree directly from the sequence data and incorporates the coalescent process and the uncertainty associated with gene trees and nucleotide substitution model parameters (Heled & Drummond 2010). As population assignments are required *a priori* for StarBEAST2 analyses, we assigned all individuals with multilocus sequence data to a 'species' group based on the monophyletic cluster recovered in the ITS gene tree and that corresponded to previously recognized species-level lineages based on DNA barcode analyses (Divakar *et al.* 2016). We used the molecular evolution rates for ITS estimated for *Melanelixia* O. Blanco *et al.* (2.43×10^{-9} substitution per site per year) (Leavitt *et al.* 2012*a*) to estimate the time to the most recent common ancestor (MRCA). This rate is similar to other estimates of ITS substitution rates for lichen-forming fungi (e.g. 2.38×10^{-9} substitution per site per year, *Oropogon* Th. Fr., *Parmeliaceae*; Leavitt *et al.* 2012*b*) and a non-lichenized fungus (2.52×10^{-9} substitution per site per year, *Erysiphales*; Takamatsu & Matsuda 2004). The partitioned data matrix was analyzed in BEAST v.2.4.3 (Drummond & Rambaut 2007; Drummond *et al.* 2012; Bouckaert *et al.* 2014), using a relaxed clock model (uncorrelated lognormal), implementing a Yule model prior for the node

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heights and gamma-distributed population sizes for the species tree prior and a piecewise linear population size model with a constant root. Analyses were performed using two independent MCMC runs of 50 million generations, with a sampling tree every 5000th generation. The program Tracer was used to evaluate chain mixing and convergence, considering effective sample size (ESS) values > 200 as a good indicator. The trees of the two runs were combined using LogCombiner v.2.4.3 after excluding the first 25% of sampled trees as burn-in. TreeAnotator v.2.4.3 (Drummond *et al.* 2012; Bouckaert *et al.* 2014) was used to obtain the median node heights and posterior distributions of estimated divergence dates of the sampled trees. Node age and 95% highest posterior density (HPD) were mapped on the maximum clade credibility (MCC) tree.

Species delimitation in the *Parmelia saxatilis* group was also tested using the multispecies coalescent model implemented in the program BP&P v.3.2 (Yang & Rannala 2014). BP&P has been shown to be among the most accurate empirical species delimitation methods (Dowton *et al.* 2014; Leavitt *et al.* 2016). The posterior distribution for species delimitation models is sampled using a reversible-jump Markov chain Monte Carlo (rjMCMC) method. We used the unguided species delimitation algorithm (Yang 2015). This algorithm explores different species delimitations and different species phylogenies, with fixed specimen assignments to populations. The program attempts to merge populations into one species and uses the nearest neighbour interchange (NNI) or subtree pruning and regrafting (SPR) algorithms to change the species tree topology (Yang & Rannala 2014). BP&P gives the posterior probability of each delimited species and the posterior probability for the total number of delimited species. The species tree from StarBEAST2 was used to infer the speciation probabilities by BP&P which incorporates the NNI algorithm that allows changes in the species tree topology, eliminating the need for a fixed user-specified guide tree. The data was analyzed with both algorithms 0 and 1: using theta (θ) prior assigned to gamma distributions of G~ (2, 100), assuming intermediate ancestral population sizes; combined with root age (τ0) assigned G~ (2, 2000), assuming relatively shallow divergences among species. Rates were allowed to vary among loci (locus rate = 1), and the analyses were set for automatic fine-tune adjustments. The prior distribution of θ and τ0 can result in strong support for models containing more species (Leaché & Fujita 2010). Therefore, exploratory analyses were also performed using different combinations of the theta (θ) and tau (τ) priors spanning a range of possible population sizes and divergence times (results not shown). Each rjMCMC analysis was run for 100 000 generations, sampling each generation, and specified a burn-in of the first 8000 generations. Each analysis was run twice to confirm consistency between runs.

Results and Discussion

Molecular analyses

Thirty-four new sequences were generated for this study and aligned with 68 sequences obtained from previous studies published by our Parsys working group (Table 1; Divakar *et al.* 2015; Molina *et al.* 2017). The final, concatenated, two-locus alignment contained 1121 aligned positions, of which 147 were variable. Of these variable characters, 90 occurred in the ITS region and 57 in the *Mcm7*. The single-locus phylogenies showed no conflict (data not shown) and hence a concatenated analysis was performed. The partitioned ML analysis of the concatenated data matrix yielded the optimal tree with a likelihood value of Ln = −2753.88. The mean LnL value of the four parallel runs of the Bayesian analysis was −2807.10 with a standard deviation of ±7.84. The Bayesian phylogeny and ML tree were largely congruent and therefore only the 50% majority-rule consensus tree of the Bayesian tree sampling is shown, with bootstrap (BS) values ≥ 70% and posterior probability (PP) values ≥ 0.95 indicated on the nodes (Fig. 1). For the Bayesian analyses, ESS values were high (> 200) for all parameters indicating adequate sampling of the posterior distribution.

The larger dataset of the ITS marker including 155 samples contained 479 aligned positions, of which 117 were variable. The resulting ML phylogenetic tree is illustrated in Supplementary Material Fig. S1 (available online). All new sequences generated for this study have been deposited in GenBank under accession numbers MT580478–MT580503 and MT583819–MT583826.

The phylogenetic tree (Fig. 1) based on the concatenated dataset showed most currently accepted species in the *Parmelia saxatilis* complex, for which more than one sequence was included, as well-supported monophyletic groups with the exception of *P. hygrophila* which was paraphyletic with a monophyletic *P. submontana* nested within as in a previous study (Molina *et al.* 2017). Most relationships among species in the group lacked support, with the exception of the sister-group relationship of *P. serrana* and *P. sulymae*, and the sister-group relationship of *P. saxatilis* s. str. and a clade consisting of samples from southern Spain, which is described below as a new species. The sister-group relationship of the former agreed with a previous study, although only one sample of *P. serrana* was included there (Molina *et al.* 2017).

The MCC species tree is depicted in Fig. 2. The diversification of the *Parmelia saxatilis* group was estimated to have begun during the late Miocene, *c.* 8.47 Ma (95% HPD = 5.28–11.44 Ma) (Fig. 2). Moreover, our analysis of divergence times by the species tree approach revealed estimates for divergences within the *P. saxatilis* group that were similar to those found in a previous study (Molina *et al.* 2017). The separation of the *P. saxatilis* s. str. lineage and the new taxon was estimated to have occurred during the Pleistocene, *c.* 2.13 Ma (95% HPD = 0.52–3.76 Ma). This separation was also supported by the species tree with a PP value of 0.97 (Fig. 2). Climatic changes during the Pleistocene are hypothesized as being a major contributor to biological diversity (Moyle *et al.* 2009). *Parmelia serrana* is another morphologically cryptic species of this complex, which is sympatric with the new taxon. However, it is phylogenetically distinct and formed a strongly supported sister relationship with a North American endemic species, *P. sulymae* (Fig. 1). Remarkably, *P. serrana* diverged from its closest extant relative, *P. sulymae*, much earlier than *Parmelia saxatilis* s. str. and the new taxon, during the Pliocene, *c.* 4.78 Ma (95% HPD = 1.82–7.73) (Fig. 2). This is correlated with the appearance of the Mediterranean climatic rhythm causing evolution of Mediterranean vegetation (Suc 1984). The Mediterranean climatic conditions consist of a temperate climate characterized by dry summers with rainfall concentrated during the other seasons and lower temperatures during winter. The split of *P. discordans* and *P. omphalodes* occurred during the Pleistocene, *c.* 2.09 Ma (95% HPD = 0.52–4.01) and this is in concordance with our previous study (Molina *et al.* 2017).

Parmelia saxatilis s. str. and the new species were supported as distinct taxa (PP values 0.93) by a multispecies coalescent-based species delimitation approach and the BP&P (Fig. 2). BP&P

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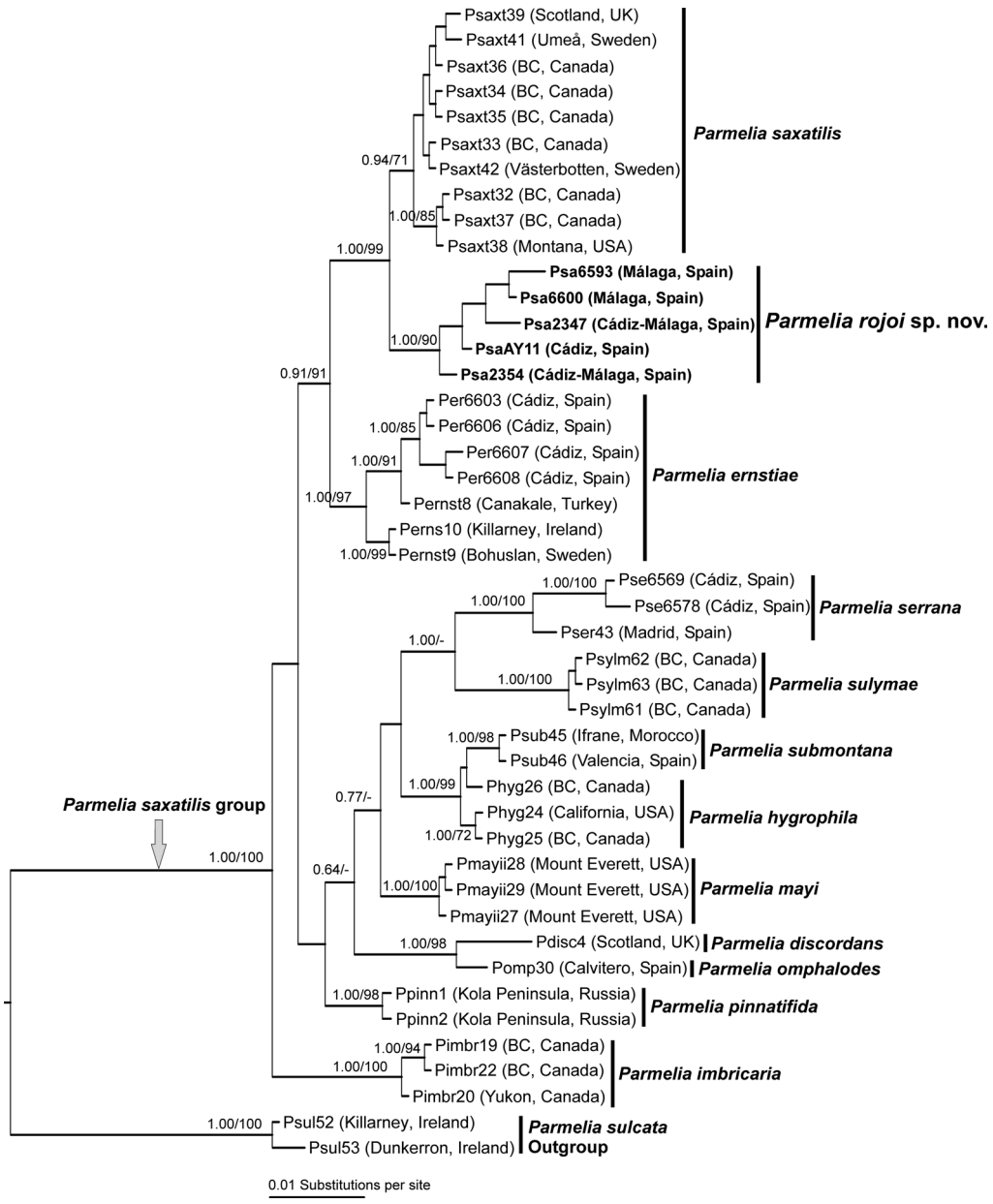

Fig. 1. Phylogenetic relationships of *Parmelia* spp. of the *P. saxatilis* group based on a Bayesian analysis of a concatenated dataset set of ribosomal (ITS) and nuclear protein-coding (*Mcm7*) markers. Bayesian posterior probabilities ≥ 0.95 from the MrBayes analysis and maximum likelihood (ML) bootstrap values ≥ 70% from the RAxML (Stamatakis 2006, 2014) analysis are indicated above branches. The country and province from which individuals were collected is indicated in parentheses. *Parmelia sulcata* was used as outgroup.

provides the posterior probability of each delimited species and the posterior probability for the number of delimited species in a group. Within the *P. saxatilis* group, BP&P supported the presence of 11 species (including the newly discovered taxon) with the highest probability (PP = 0.4964), in contrast to the current 12 species scenario based on phenotypic features. Posterior probabilities of each delimited species are provided in Fig 2. *Parmelia hygrophila* and *P. submontana* were not supported as separate species by BP&P. This is in agreement with the results of our phylogenetic analyses and a previous study (Molina *et al.* 2017). The separation of *P. discordans* and *P. omphalodes* was weakly supported by BP&P analysis (see Fig. 2).

It is worth emphasising that out of 47 samples analyzed of the *Parmelia saxatilis* complex from different localities in the Iberian Peninsula (including two from the Canary Islands), 34 were grouped in the *P. serrana* clade, seven in the *P. ernstiae* clade, five belonged to the newly described species and just one sample grouped in the *P. saxatilis* s. str. clade; this suggests that the presumed widespread *P. saxatilis* s. str. is indeed a rare species in the Iberian Peninsula (Supplementary Material Fig. S1). *Parmelia serrana* seems to be the most widespread taxon in the *P. saxatilis* complex and is more successful in colonizing diverse habitats in the Iberian Peninsula, including the Canary Islands. We hypothesized that the appearance of a Mediterranean climatic rhythm might have played a crucial role in the diversification of *P. serrana* in the Iberian Peninsula. However, this needs to be confirmed by an additional population-scale study of the *P. saxatilis* complex. Within other parts of Europe, different proportions of species in the complex are found, with some strong

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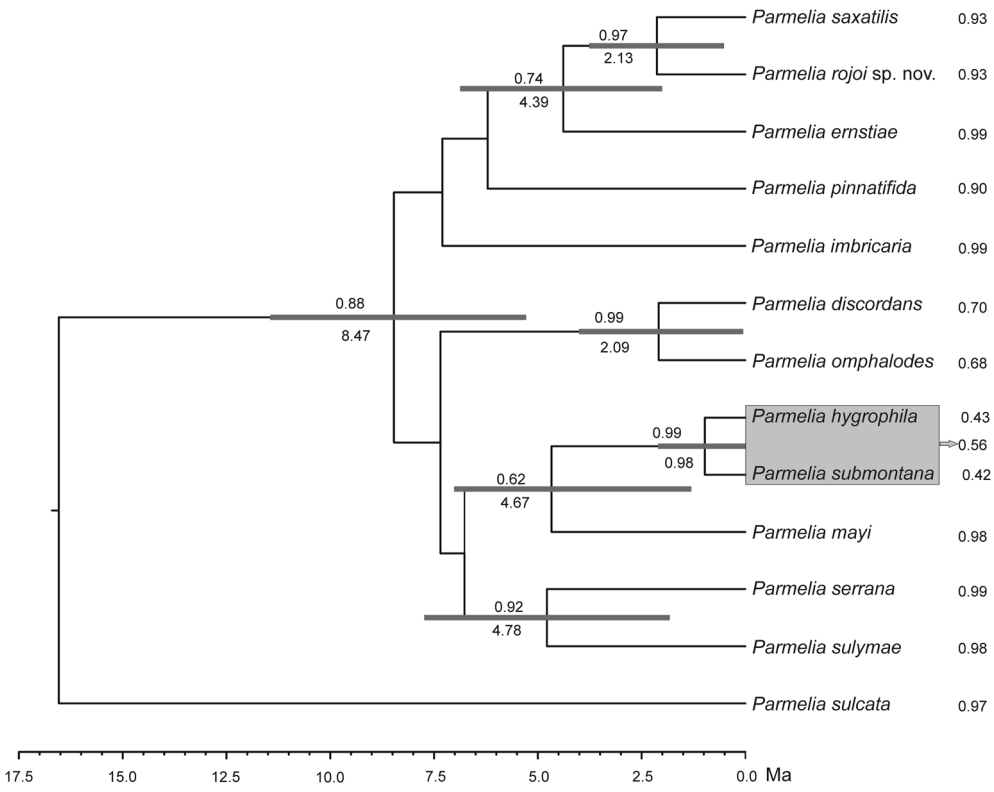

Fig. 2. Time-calibrated species trees for *Parmelia* spp. (*P. saxatilis* group) inferred from two loci (ITS and *Mcm7*) using the program StarBEAST2 (Bouckaert *et al.* 2014). Putative species used in the StarBEAST2 analysis were based on our previous DNA barcoding study of *Parmelia* (Divakar *et al.* 2016). Posterior probabilities (PP) ≥ 0.50 are shown above branches. The 95% highest posterior density intervals (HPD) are shown as dark grey bars, and numbers below the nodes indicate estimated node ages (millions of years ago, Ma). The posterior probabilities of each delimited species calculated by BP&P (Yang & Rannala 2014) are indicated on the right side of each putative species. The grey box indicates species/lineages not supported as separate species by BP&P.

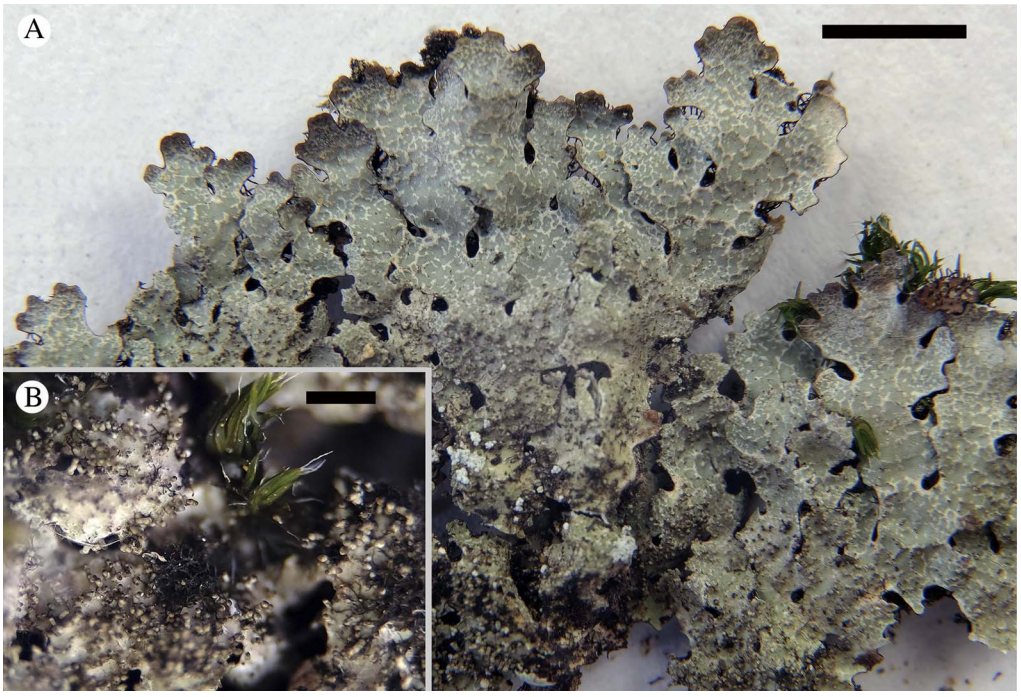

Fig. 3. *Parmelia rojoi* (holotype). A, habitus. B, detail of isidia. Scales: A = 5 mm; B = 1 mm. In colour online.

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signatures of climatic response (Thell *et al.* 2017; Corsie *et al.* 2019; Tsurykau *et al.* 2019). Similarly, samples analyzed from outside Europe such as individuals from Antarctica, Canada, Chile, the Kola Peninsula (Russia) and the USA belonged to *P. saxatilis* s. str. However, samples from Morocco, the Republic of Adygea (Russia), South Korea and Turkey were grouped in the *P. serrana* clade (see Supplementary Material Fig. S1).

The discovery of a new lineage in southern Spain and the fact that all species of the *Parmelia saxatilis* complex occurring in Europe also occur in Spain is remarkable and allows us to hypothesize that the Iberian Peninsula might have served as a refugium for this group of lichens during Pleistocene glaciations. Pleistocene glaciations had major impacts on the temperate flora and fauna (Hewitt 2000, 2004), and were especially severe in Europe where the Alps formed a major barrier for these moving populations (Taberlet *et al.* 1998; Hewitt 2000). In his seminal paper, Poelt (1963) discussed the impact of the glaciations on the lichen flora in Europe and the potential survival of species there with oceanic distributional patterns (such as most species in the *P. saxatilis* complex) in close proximity to glaciers. The Iberian Peninsula was characterized by a cold-adapted fauna and flora during the Pleistocene (Sala *et al.* 2020), so that survival of species of the complex seems plausible. The Iberian Peninsula has been discussed as an important refugial area for plants (de Heredia *et al.* 2007; Postigo-Mijarra *et al.* 2010) and also lichen-forming fungi (Barreno 1991). Recent molecular data are consistent with the Iberian Peninsula having been a refugial area for lichenized fungi (Núñez-Zapata *et al.* 2015; Alors *et al.* 2017; Fackovcova *et al.* 2019). A population-level biological study will be necessary to test our hypothesis of refugial survival of species in the *P. saxatilis* complex in the Iberian Peninsula.

Species of the *Parmelia saxatilis* complex are widely distributed in all continents except Australia and the diversification occurred during the Pleistocene. Our results allow us to hypothesize that long-distance dispersal might have played a crucial role in colonizing transcontinental regions. In lichenized fungi, wide distributional ranges have often been attributed to long-distance dispersal (Geml *et al.* 2010; Amo de Paz *et al.* 2012; Del-Prado *et al.* 2013; Leavitt *et al.* 2013, 2018; Núñez-Zapata *et al.* 2017; Cubas *et al.* 2018; Divakar *et al.* 2019).

Taxonomy

Parmelia discordans Nyl.

Meddn. Soc. Fauna Flora Fenn. **13**, 40 (1886); type: Russia [formerly Finland], Hogland Island [=Gogland, Suursaari], 1868, *Brenner* (H-NYL 34916—lectotype).

Distinguished by an absence of soralia and isidia, a uniformly dark brown thallus, laminal and marginal pseudocyphellae, non-squarrose rhizines, and by containing protocetraric and ±lobaric acids as characteristic substances (Thell *et al.* 2011).

Additional specimen examined. **Spain:** *Asturias*: Caso, Campo de Caso, Parque Natural de Redes, carretera de Campo de Caso a Tarna, km 57, cañón del río Nalón, 43°07′01″N, 05°15′15″W, 870 m, 2012, *V. J. Rico* 4441 (MAF-Lich. 17865).

Parmelia ernstiae Feuerer & A. Thell

Mitt. Inst. Allgem. Bot. Hamburg **30-32**, 52 (2002); type: Germany, Niedersachsen, Lüneburg, Soltau-Fallingbostel, Hof Möhr, Alfred Töpfer Academy of Nature Conservation, on *Fraxinus excelsior*, 80 m, 14 April 2000, *G. Ernst* (HBG 4619—holotype).

Morphologically and chemically close to the *Parmelia saxatilis* agg. (Corsie *et al.* 2019). In our areas it is distinguished from other isidiate species by producing lobaric and salazinic acids as main substances and by occurring only on tree bark, although these features are not consistent in other geographical regions. See Table 1 for specimens studied. The Iberian Peninsula material contained atranorin and chloroatranorin, together with salazinic, consalazinic, lobaric, protolichesterinic and lichesterinic acids.

Parmelia omphalodes (L.) Ach.

Meth. Lich., 204 (1803); type: Dillenius, *Historia Muscorum*, Tab. 24, fig. 80A, (1741) (OXF—lectotype).

The species is characterized by the absence of isidia and soredia, non-squarrose rhizines, marked laminal and marginal pseudocyphellae, laminal pseudocyphellae mostly unconnected with marginal ones and the presence of salazinic and ±lobaric acids as characteristic substances (Thell *et al.* 2011; Ossowska *et al.* 2019). See Table 1 for specimens studied.

Parmelia pinnatifida Kurok.

J. Jap. Bot. **51**, 378 (1976); type: Helvetia, *Schleicher* 257 (H-ACH 1297A—lectotype).

Morphologically close to *Parmelia omphalodes*, but distinguished by a uniform dark brown thallus, narrower and overlapping lobes, mainly marginal pseudocyphellae and salazinic, protolichesterinic and ±lobaric acids as characteristic substances (Thell *et al.* 2011).

Additional specimen examined. **Spain:** *Navarra*: carretera de Elizondo-Bearzun, km 5, 400 m, 22 v 1994, *J. Etayo* (MA-Lichen 5794).

Parmelia rojoi A. Crespo, V. J. Rico & Divakar sp. nov.

MycoBank No.: MB 835851

Differing from *Parmelia saxatilis* by having a more fragile thallus and narrower isidia. It is supported as a distinct lineage from other *Parmelia* species according to ITS sequences and by coalescent-based genetic analyses of multiple loci (Figs 1 & 2).

Type: Spain, Andalucía, Málaga, Cortes de La Frontera, Parque Natural de Los Alcornocales, La Sauceda, 487 m, 36°31′45.4″N, 05°35′10.5″W, 18 October 2018, *A. Crespo, V. J. Rico, P. K. Divakar & C. Ruibal* DNA6593, 6600 (MAF-Lich. 22797—holotype; 22798—isotype). GenBank Accession numbers: MT580485 (ITS), MT583824 (*Mcm7*).

(Fig. 3)

Thallus saxicolous, orbicular to irregular, loosely to moderately attached, up to 5 cm diam. *Lobes* fragile when dry, linear to branched, contiguous to slightly imbricate, up to 4.5 mm wide; upper surface grey, brownish and slightly pruinose mainly on margins, dull, smooth to finely reticulate or foveolate and cracked, tips square to rounded; margins developing sparse secondary

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lobules; lower surface black to brown at margins, with black, simple to furcate, abundant, not squarrose rhizines. *Pseudocyphellae* marginal and laminal, effigurate, numerous across the surface, linear to mainly irregularly shaped but forming a continuous network. *Isidia* laminal, globose to frequently cylindrical and branched, up to 0.15 mm diam., fragile when dry, with brownish tips, rarely spathulate then forming laminal secondary lobules. *Medulla* white.

Apothecia unknown.

Pycnidia sparse and immersed in lobe margins; *conidia* 5.5–7.5 × 1 µm.

Secondary chemistry. Medulla C−, K+ red, KC−, P+ orange. TLC: atranorin (major), chloroatranorin, lichesterinic, protolichesterinic, galbinic, salazinic (major) and consalazinic acids. Lobaric acid absent.

Etymology. The species is named in honour of Juan M. Rojo, Professor of Physics and friend of the authors.

Habitat and distribution. So far known only from the Cádiz and Málaga provinces in southern Spain at lower elevations (*c.* 487 m). It is a saxicolous species growing on sun-exposed sandstones in humid forests of different *Quercus* and *Olea* species. It is sympatric with *Parmelia serrana* and also with the epiphytic *P. ernstiae*.

Notes. This is an almost cryptic species segregated from *Parmelia saxatilis*, from which it is morphologically and chemically only slightly different but genetically distinct (Figs 1 & 2). *Parmelia rojoi* differs from *P. saxatilis* in two minor features: it develops a more fragile thallus and narrower isidia; and contains lichesterinic and protolichesterinic acids, with an absence of lobaric acid. In isidia morphology and chemistry it is also similar to *P. ernstiae* and *P. serrana*; however, *P. ernstiae* from the Iberian Peninsula contains lobaric acid and *P. serrana* has mainly clustered laminal isidia or along margins and ridges. It is worth noting that in the absence of consistent diagnostic features, ITS sequence-based sample identification of this newly described species is highly recommended.

Only five sterile specimens from Málaga and Cádiz (Spain) are known of this taxon (see also Table 1).

Parmelia saxatilis var. *glauca* Maheu & Gillet, described from Agla, close to the coast of Tangier on rocks in Morocco (Maheu & Gillet 1925), could be conspecific with *P. rojoi* but unfortunately we have not been able to study material of this taxon.

Parmelia saxatilis (L.) Ach.

Meth. Lich., 204 (1803); type: Sweden, *sine loc.*, *c.* 1740, *C. Linnaeus* (LINN 1273·62, second specimen from the bottom—-lectotype); Sweden, Västerbotten, Umeå, October 1998, *S. Ott* (MAF-Lich. 6882—epitype).

Morphologically characterized by mainly laminal isidia, which are globose to frequently cylindrical and branched, up to 0.3 mm diam. and a thicker thallus than the closely related *P. rojoi*. The Iberian Peninsula material contained atranorin and chloroatranorin, together with salazinic, consalazinic and sometimes ±lobaric acid. A detailed description of morphological and chemical features can be found in Molina *et al.* (2004, 2011*b*).

Additional specimens examined. **Spain:** *Castilla y León, Ávila*: Candeleda, desde el Refugio Albarea hasta Puerto de Candeleda, sobre roca, 10 vii 2015, *P. K. Divakar & F. Dal Grande* (MAF-Lich. 21288); *ibid.*, Sierra de Gredos, 2300 m, v 1999 (MAF-Lich. 6883).

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

In Molina *et al.*, *Lichenologist* **36**, 48 (2004); type: Spain, Madrid, Sierra del Guadarrama, Navacerrada, S of Antón Real, close to the junction of roads M 601 and M 607, 40°43.996′N, 04°01.438′W, alt. 1300 m, on *Quercus pyrenaica*, 4 February 2003, *A. Crespo & P. K. Divakar* (MAF-Lich. 9756—holotype; BM, HBG, TNS, UPS, US—isotypes).

Morphologically and chemically this species is close to the *Parmelia saxatilis* agg. (Corsie *et al.* 2019). In our analyzed material it is distinguished from other isidiate species by producing salazinic and protolichesterinic acids as main substances, developing mainly clustered laminal isidia or along margins and ridges, and by being mostly saxicolous. See Table 1 for specimens studied. The Iberian Peninsula material contained atranorin and chloroatranorin, together with salazinic, consalazinic, protolichesterinic and lichesterinic acids but lobaric acid was not detected. However, lobaric acid is reported in northern European samples (Thell *et al.* 2017; Corsie *et al.* 2019; Tsurykau *et al.* 2019), and the sample identification requires confirmation in a multilocus phylogeny including a coalescent-based species delimitation approach. A detailed description of morphological and chemical features can be found in Molina *et al.* (2004, 2011*b*).

Parmelia submontana Nádv. ex Hale

Smithson. Contrib. Bot. **66**, 44 (1987); type: Czech Republic, Bohemia, Hlinsko, Planavy, 1931, *Nádvorník* s. n. (PRM—lectotype; US—isolectotype).

Distinguished by long, sparingly branched, down-rolled lobes, numerous orbicular soralia with isidia-like structures, small pseudocyphellae, sparse, mostly simple rhizines, and salazinic acid as the main substance (Hale 1987; Thell *et al.* 2011). See Table 1 for specimens studied.

Key to the species of the *Parmelia saxatilis* group in Europe

1 Thallus without soredia or isidia, saxicolous species 2
Thallus sorediate or isidiate 4

2(1) Medulla K−, with protocetraric and ±lobaric acids as characteristic substances . . . . . **Parmelia discordans**
Medulla K+ red, with salazinic and ±lobaric acids as characteristic substances 3

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3(2) Lobes usually up to 2 mm wide, pseudocyphellae mainly marginal . . . . . **Parmelia pinnatifida**
Lobes usually up to 4 mm wide, pseudocyphellae marginal and laminal . . . . . **Parmelia omphalodes**

4(1) Thallus sorediate to sorediate-isidiate, epiphytic species; lobes elongate, sparingly branched . . . . . **Parmelia submontana**
Thallus isidiate 5

5(4) Mainly epiphytic . . . . . **Parmelia ernstiae**
Mainly saxicolous 6

6(5) Isidia laminal or along margins or ridges, clustered . . . . . **Parmelia serrana**
Isidia mainly laminal, preferably in the central part 7

7(6) Isidia up to 0.3 mm in diameter . . . . . **Parmelia saxatilis**
Isidia up to 0.15 mm in diameter, thallus fragile . . . . . **Parmelia rojoi**

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Supplementary Material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0024282920000341

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