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Conundrums in species concepts: the discovery of a new cryptic species segregated from *Parmelina tiliacea* (Ascomycota: *Parmeliaceae*)

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Abstract: *Parmelina tiliacea* is a common, widely distributed species in south-western Europe, easily identifiable by morphology and much used as an air pollution bioindicator in many regions. A molecular phylogenetic survey of samples from many geographical areas, using Maximum Parsimony and Bayesian inference of nuITS and mtLSU rDNA regions, revealed a group of samples geographically restricted to a small region of the Iberian Peninsula and genetically separated from the other *P. tiliacea* specimens studied. These samples are morphologically indistinguishable from *P. tiliacea*, apart from subtle anatomical characters in the ascomata (hyphae of the exciple and ascospore width), which are frequently absent. Although geographically different, the two taxa occupy similar habitats and are even sympatric in some areas, indicating that they do not exchange genetic material. This previously overlooked, and apparently endemic lineage, is described as *P. cryptotiliacea* sp. nov., and the name *Lichen tiliaceus* is epitypified by a sequenced specimen to fix the application of *Parmelina tiliacea* to the widespread genotype. A second unexpected result was the discovery that the morphologically distinct *P. pastillifera* was nested within *P. tiliacea*. These two cases stress the need to use molecular tools to elucidate species concepts even within widespread morphologically well-characterized macrolichens. Such investigations are necessary to improve our understanding and estimation of biodiversity, and to facilitate the development of sound biodiversity conservation strategies for lichens.

Keywords: genetic distances, mitochondrial LSU, nuclear ITS, *Parmelina cryptotiliacea*, phylogeny

Introduction

Molecular phylogenetic investigations have led to the detection of many cryptic species amongst the parmelioid lichens. Examples include *Parmelia ernstiae* (Feuerer & Thell 2002), *P. serrana* (Molina *et al*. 2004), *P. barrenoae* (Divakar *et al*. 2005), *P. encryptata* (Molina *et al*. 2011), and *Melanelixia californica* (Divakar *et al*. 2010*a*). This approach has been especially useful in *Parmelina*, where the combination of molecular and morphological information provided evidence of polyphyly of the genus: the Australasian species appear in a clade that is unrelated to the Northern Hemisphere species, and are now accommodated in the new genus *Austroparmelina* (Crespo *et al*. 2010). At the species level, as in other organisms, speciation in lichens has not always been accompanied by morphological variation. For instance, phylogenetic analysis and detailed morphological studies found two previously overlooked morphospecies within the widespread *P. quercina*: *P. coleae* and the re-established *P. carporrhizans* (Argüello *et al*. 2007).

At present, *Parmelina* includes seven species distributed in the temperate regions of the Northern Hemisphere (Elix 1993; Argüello *et al*. 2007; Clerc & Truong 2008). *Parmelina coleae* is restricted to North America, *P. gyrophorica* is known only from

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southern China (Wang *et al*. 2000), and five other species occur in western Europe, extending to Asia. Three of those five species always have apothecia: *P. quercina*, *P. carporrhizans*, and *P. atricha* (Poelt & Vězda 1977; Nimis 1993; Clerc & Truong 2008). *Parmelina tiliacea* and *P. pastillifera*, characterized by the presence of isidia, are frequently sterile although individuals with apothecia are not unusual (Schauer 1965; Hale 1976; Divakar & Upreti 2005).

Parmelina tiliacea has a wide distribution, including Europe, northern Africa, the Middle East, Asia and the Indian subcontinent. It is a frequent epiphyte in southwestern Europe, at *c*. 200–1500 m above sea level. However, this species is considered to be threatened in countries such as Denmark, The Netherlands, Poland, and Germany (Liška *et al*. 2006). Phylogenetic information about this species was, however, scarce and based on small samples. This study aimed to establish whether *P. tiliacea* was monophyletic or if cryptic species were hidden within its current circumscription, and to evaluate any implications for species conservation programmes. The phylogenetic relationships were assessed by analysis of two independent loci (nuclear ITS and mitochondrial LSU) with extensive sampling covering most of the distributional range. Samples of the isidiate species, *P. pastillifera*, found to be sister to *P. tiliacea* in previous studies (Argüello *et al*. 2007), were also included in the analysis. Morphological characters were re-evaluated and compared with the relationships found in the molecular analyses.

Material and Methods

Taxon sampling

Specimens of *Parmelina tiliacea* were collected from most of its distributional range, and from different altitudes and ecological areas. Locations included the Iberian Peninsula, the Canary Islands, Morocco, Turkey, France, Germany, Austria, Italy, Slovenia, Tunisia, and India. Details of localities, voucher specimens, and GenBank accession numbers are shown in Table 1. Samples of other *Parmelina* species were included in the phylogenetic analysis: *P. carporrhizans* from Turkey and the Canary Islands, *P. coleae* from the USA, *P. atricha* from France, *P. pastillifera* from the Iberian Peninsula and Turkey, and *P. quercina* from Turkey (Table 1). Two closely related *Parmeliaceae*, *Myelochroa metarevoluta* and *M. galbina*, were used as an outgroup (Blanco *et al*. 2006).

DNA extraction and amplification

Small fragments of vegetative thallus from fresh or frozen herbarium specimens were ground with sterile glass pestles. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Barcelona) according to the manufacturer's instructions, slightly modified as previously described (Crespo *et al*. 2001). Double-stranded DNA amplification of the two regions was performed using the primers: 1) ITS1-LM (Myllys *et al*. 1999) and ITS2-KL (Lohtander *et al*. 1998) for the fungal nuITS rDNA region, and 2) ML3 and ML4 (Printzen 2002) for the fungal mtLSU rDNA region. For ITS amplification we used a reaction mixture of 50 µl, containing 5 µl of ×10 buffer with 2 mM $MgCl_2$, 1 µl of dNTPs (10 mM of each base), 2·5 µl of each primer (10 µM), 1·25 µl of DNA polymerase (1 U μl^{-1}), 27·75 µl of sterile water and 10 µl of dilute DNA template. Amplification of the mtLSU region was performed using PuRe Taq Ready-To-Go PCR Beads (GE Healthcare, UK) in a 25 µl volume containing 13·4 µl of sterile water, 2·5 U of puReTaq DNA Polymerase, 200 µM of each dNTP, BSA, buffer reaction and stabilizers (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1·5 mM $MgCl_2$), 1·5 µl of each primer and 5 µl of dilute DNA template.

The amplifications were run in an automatic thermocycler (Techne Progene) using the following parameters for nuITS rDNA: initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 1·5 min at 72°C, and a final extension of 5 min at 72°C. Parameters for mtLSU rDNA were: initial denaturation for 10 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 47°C and 3 min at 72°C, and a final extension of 10 min at 72°C.

PCR products were cleaned using a Bioclean Columns kit (Biotools, Madrid) according to the manufacturer's instructions. Sequencing was performed using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with the amplification primers. Sequencing reactions were electrophoresed on a 3730 DNA analyzer (Applied Biosystem) at the Unidad de Genómica (Parque Científico de Madrid). Sequence fragments obtained were assembled and manually adjusted in BioEdit 7.0.9.0 (Hall 1999).

Sequence alignments and phylogenetic analysis

Three separate matrices (one for each gene and another with both genes combined) were constructed and aligned using the Clustal W Multiple alignment program (Thompson *et al*. 1994). Ambiguously aligned regions were excluded manually from the matrices. The alignments were analyzed by Maximum Parsimony (MP) and a Bayesian Markov Chain Monte Carlo (B/MCMC) approach (Larget & Simon 1999; Huelsenbeck *et al*. 2000). The trees were rooted using *M. galbina* and *M. metarevoluta* as an outgroup.

TABLE 1. *Specimens included in the phylogenetic study, with details of location, collectors, herbarium code and GenBank accession numbers*

Species	Locality	Substratum	Altitude (m)	Collectors	Herbarium	GenBank.Accession N°	
						nuITS	mtLSU
Parmelina tiliacea 1	Turkey, Bursa, Uludağ	*Quercus cerris*	1330	*A. Crespo, P. K. Divakar, M. Candan & H.T. Lumbsch*	MAF-Lich 16456	JF756977	JF757026
P. tiliacea 2	France, Provence, road between Saint-Tropez and Toulon	–	35	*A. Argüello*	MAF-Lich 16464	JF756982	JF757031
P. tiliacea 3	Tunisia, Jendouba, Al Mawajin	siliceous rock	698	*A. Quintanar*	MAF-Lich 16458	JF756987	JF757036
P. tiliacea 4	Spain, Canary Islands, Gran Canaria, Valleseco	rock	1305	*A. Crespo, P. Cubas, A. Santos & P. K. Divakar*	MAF-Lich 16482	JF756984	JF757033
P. tiliacea 5	Spain, Madrid, El Pardo	*Quercus ilex*	675	*A. Crespo & J. Núñez-Zapata*	MAF-Lich 16452	JF756979	JF757028
P. tiliacea 6	Spain, Ciudad Real, San Quintín mine	*Quercus ilex*	660	*A. Crespo, P. K. Divakar, P. Cubas, J. Núñez-Zapata, R. Oyarzun*	MAF-Lich 16457	JF756986	JF757035
P. tiliacea 7	Morocco, Midle Atlas, Ifrane	*Quercus ilex*	1375	*A. Crespo, P. K. Divakar & R. Del-Prado*	MAF-Lich 16486	JF756976	JF757025
P. tiliacea 8	Spain, Canary Islands, La Palma, road to Roque	rock	794	*A. Crespo, P. Cubas, A. Santos & P. K. Divakar*	MAF-Lich 16470	JF756983	JF757032
P. tiliacea 9	India, Uttarakhand, Badrinath	rock	3200	*P. K. Divakar*	MAF-Lich 16484	JF756978	JF757027
P. tiliacea 10	Italy, Val Trompia, Lodrino	*Quercus* sp.	710	*A. Crespo & P. K. Divakar*	MAF-Lich 16483	JF756985	JF757034
P. tiliacea 11	Germany, Bavaria, Oberfranken	*Tilia platyphyllos*	450	*W & G .v. Brachel*	MAF-Lich 16485	JF756989	JF757038
P. tiliacea 12	Austria, Salzburg, Mittersill	–	812	*J. Núñez-Zapata & F. Candotto*	MAF-Lich 16618	JF756991	JF757040
P. tiliacea 13	Slovenia, Carniola, Bled	*Pinus* sp.	475	*J. Núñez-Zapata*	MAF-Lich 16619	JF756992	JF757041
P. tiliacea 14	Portugal, Braganza,	*Quercus suber*	640	*V. J. Rico*	MAF-Lich 15350	JF756981	JF757030
P. tiliacea 15	Spain, Madrid, El Escorial	*Quercus pyrenaica*	1100	*J. Núñez-Zapata*	MAF-Lich 16467	JF756980	JF757029

TABLE 1. *Continued*

Species	Locality	Substratum	Altitude (m)	Collectors	Herbarium	GenBank Accession N°	
						nuITS	mtLSU
P. tiliacea 16	Spain, Mallorca, Binifaldo	*Quercus ilex*	524	*A. Crespo, P. K. Divakar, G. Amo & J. Nuñez-Zapata*	MAF-Lich 16466	JF756990	JF757039
P. tiliacea 17	Morocco, Middle Atlas, Ifrane, Forest de Yeiva	*Quercus ilex*	1325	*A. Crespo, P. K. Divakar & R. Del-Prado*	MAF-Lich 16468	JF756988	JF757037
P. cryptotiliacea 1	Spain, Madrid, El Pardo	*Quercus ilex*	675	*A. Crespo & J. Núñez-Zapata*	MAF-Lich 16451	JF756970	JF757013
P. cryptotiliacea 2	Spain, Ciudad Real, San Quintín mine	*Quercus ilex*	660	*A. Crespo, P. K. Divakar, P. Cubas, J. Núñez-Zapata, R. Oyarzun*	MAF-Lich 16449	JF756975	JF757018
P. cryptotiliacea 3	Spain, Ciudad Real, Castillo de Calatrava	*Pistacia terebinthus*	666	*A. Crespo, P. K. Divakar & J. Núñez-Zapata*	MAF-Lich 16461	JF756974	JF757017
P. cryptotiliacea 4	Spain, Caceres, Natural Park Monfragüe	*Quercus ilex*	207	*H. T. Lumbsch, A. Green, P. K. Divakar & A. Argüello*	MAF-Lich 16454	JF756972	JF757015
P. cryptotiliacea 5	Spain, Madrid, El Pardo	*Quercus ilex*	675	*A. Crespo & J. Núñez-Zapata*	MAF-Lich 16450	JF756969	JF757012
P. cryptotiliacea 6	Spain, Caceres, Natural Park Monfragüe	*Quercus ilex*	207	*H.T. Lumbsch, A. Green, P. K. Divakar & A. Argüello*	MAF-Lich 16453	JF756973	JF757016
P. cryptotiliacea 7	Spain, Caceres, Natural Park Monfragüe	*Quercus ilex*	207	*H.T. Lumbsch, A. Green, P. K. Divakar & A. Argüello*	MAF-Lich 16455	JF756971	JF757014
P. atricha	France, Midi-Pyrénées, Ariège, Merens-les-Vals	rock	1500	*A. Argüello*	MAF-Lich 15524	JF756965	–
P. carporrhizans 1	Spain, Canary Islands, Gran Canaria, Degollada de Becerra	*Pinus radiata*	1499	*A. Crespo, P. Cubas, A. Santos & P. K. Divakar*	MAF-Lich 16477	JF756995	JF757007
P. carporrhizans 2	Spain, Canary Islands, Tenerife, El Saural	*Fagus orientalis*	894	*A. Crespo, P. Cubas, A. Santos & P. K. Divakar*	MAF-Lich 16474	JF756996	JF757008
P. carporrhizans 3	Turkey, Prov. Canakale, N of Karadag	*Quercus* sp. bark	400	*A. Crespo, P. K. Divakar, M. Candan & H. T. Lumbsch*	MAF-Lich 16476	JF756994	JF757006
P. carporrhizans 4	Turkey, Prov. Bursa, Uludaj roadside	*Fagus orientalis*	1030	*A. Crespo, P. K. Divakar, M. Candan & H. T. Lumbsch*	MAF-Lich 16475	JF756993	JF757005

TABLE 1. *Continued*

Species	Locality	Substratum	Altitude (m)	Collectors	Herbarium	GenBank.Accession N°	
						nuITS	mtLSU
P. coleae 1	USA, California, Mendocino, Sonoma lake	*Quercus* sp.	500	*A. Crespo, P. K. Divakar, R. DelPrado & F. Fernandez-Mendoza*	MAF-Lich 16479	JF756966	JF757009
P. coleae 2	USA, California, Mendocino	*Quercus* sp.	700	*A. Crespo, P. K. Divakar, R. DelPrado & F. Fernandez-Mendoza*	MAF-Lich 16481	JF756967	JF757010
P. coleae 3	USA, California, Mendocino, Sonoma lake	*Quercus* sp.	500	*A. Crespo, P. K. Divakar, R. DelPrado & F. Fernandez-Mendoza*	MAF-Lich 16480	JF756968	JF757011
P. pastillifera 1	Spain, Cantabria, La Lomba	*Fagus sylvatica*	1132	*A. Crespo & J. Núñez-Zapata*	MAF-Lich 16473	JF756998	JF757020
P. pastillifera 2	Spain, Asturias, Saliencia	–	1325	*S. Perez-Ortega*	MAF-Lich 16472	JF756999	JF757021
P. pastillifera 3	Turkey, prov. Canakale, distr. Lapseki, N. of village Dumanli	*Fagus orientalis*	670	*A. Crespo, P. K. Divakar, M. Candan & H. T. Lumbsch*	MAF-Lich 16471	JF756997	JF757019
P. quercina 1	Turkey, Prov. Canakale, Biga, S. of Bakacik	*Quercus* sp. bark	70	*A. Crespo, P. K. Divakar, M. Candan & H. T. Lumbsch*	MAF-Lich 16197	JF757000	JF757022
P. quercina 2	Turkey, Prov. Eskisehir, Tandir village	*Quercus cerris*	1350	*A. Crespo, P. K. Divakar, M. Candan & H. T. Lumbsch*	MAF-Lich 16193	JF757001	JF757023
P. quercina 3	Turkey, Prov. Bursa, Uludaj	*Fagus orientalis*	1030	*A. Crespo, P. K. Divakar, M. Candan & H. T. Lumbsch*	MAF-Lich 16194	JF757002	JF757024
Myelochroa galbina	China, Yunnan, Jianchian County	–	2490	*A. Crespo, O. Blanco & A. Argüello*	MAF-Lich 10414	DQ279531*	
M. metarevoluta	China, Yunnan, Jianchian County	*Quercus* sp.	2490	*A. Crespo, O. Blanco & A. Argüello*	MAF-Lich 10208	AY611102†	

* Divakar *et al.* (2006), † Blanco *et al.* (2004)

Parsimony analyses were carried out using PAUP* 4.0b10 (Swofford 2003) performed at www.bioportal.uio.no, with equally weighted characters and gaps being interpreted as missing data. A heuristic search with 100 random taxon addition replicates was generated with tree-bisection-reconnection (TBR) branch-swapping and the MulTrees option in effect. Nonparametric bootstrap (Felsenstein 1985) was used to assess robustness of clades, running 4000 pseudoreplicates with the same settings as in the heuristic search. Only clades that received bootstrap support above 75% were considered to be strongly supported. To assess homoplasy levels, we calculated the consistency index (CI) and retention index (RI) from each parsimony search. Majority rule consensus trees were drawn using TREEVIEW (Page 1996).

The heterogeneity in phylogenetic signal between the two data partitions was examined by MP and Bayesian approach (Wiens 1998; Buckley *et al*. 2002; Divakar *et al*. 2010*b*). The level of bootstrap support and posterior probabilities were used to detect significance levels of localized incongruence between the two gene partitions and the concatenated analysis. The set of topologies reaching $\geq$ 75% bootstrap under parsimony and the 0·95 posterior probabilities for the Bayesian approach were estimated (Hillis & Bull 1993). If no conflict was evident, it was assumed that the two data sets were congruent and could be combined.

Bayesian analyses were carried out using the program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). Posterior probabilities were approximated 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. The analysis was performed assuming the general time reversible model (Rodriguez *et al*. 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six rate categories (GTR + I + G) for the single-gene and the combined analyses. A run with 2 million generations, starting with a random tree and employing 8 simultaneous chains, was executed. Every 200th tree was saved to a file. We plotted the log-likelihood scores of sample points against generation time using TRACER version 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist 2001). The first 2000 trees were discarded as burn-in before stationarity was reached. Unlike nonparametric boostrap values (Felsenstein 1985), these are estimated probabilities of the clades under the assumed model (Rannala & Yang 1996), and hence posterior probabilities $\geq$ 95% were considered significant support.

Hypothesis testing

As the results of the phylogenetic analysis are incongruent with the current species concept of *Parmelina tiliacea*, we examined whether our data were sufficient to reject the monophyly of *P. tiliacea*. Two methods were employed: 1) the Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa 1999); 2) the expected likelihood weight (ELW) test following Strimmer and Rambaut (2002). We compared the ML tree constrained to have *P. tiliacea* as monophyletic and the unconstrained ML tree. The SH and ELW tests were performed using Tree-PUZZLE 5.2 (Schmidt *et al*. 2004) with the combined dataset from a sample of 200 unique trees (the best trees agreeing with the null hypotheses) and the unconstrained ML tree. These trees were inferred in Tree-PUZZLE employing the GTR + I + G nucleotide substitution model.

Genetic distances

Pairwise maximum likelihood distances, given as the number of nucleotide substitutions per site, between the ITS rDNA sequences were calculated with TREE-PUZZLE 5.2 (Schmidt *et al*. 2004) using the HKY+G (Hasegawa *et al*. 1985) model of nucleotide substitution with between-site variation, and assuming a discrete gamma distribution with six rate categories. Ambiguous characters and repeated haplotypes were removed in the matrix as explained in Del-Prado *et al*. (2010).

Morphological and chemical studies

All specimens included in the molecular analysis were morphologically revised (Table 1). The size and shape of the ascospores were studied in the specimens bearing apothecia. Sections of apothecia were hand-cut with a razor blade under a binocular microscope, and mounted in distilled water. Ascospores and longitudinal sections of apothecia were observed and photographed under a light microscope (Leitz DMRB). Measurements of the length and width were taken in 20 well-developed ascospores per sample at ×1000 magnification. The description of spore shape follows Kirk *et al*. (2008). Statistical tests were performed using STATGRAPHICS Plus 5.1.

Chemical analyses were performed by thin-layer chromatography using standard methods (Culberson 1972; Elix & Ernst-Russell 1993; Orange *et al*. 2001) in all the specimens analyzed.

Results

Phylogenetic analyses

Seventy-six new sequences were generated for this study, including 38 new nuclear ITS rDNA and 39 new mitochondrial LSU rDNA sequences. The aligned matrices had 436 unambiguous nucleotide positions in the nuITS and 668 in the mtLSU. Comparison of the topology of MP and Bayesian trees based on the individual genes showed no supported conflicts (results not shown), thus both regions were combined to increase the resolution of the clades.

The MP analysis of the combined data matrix resulted in the 30 most parsimonious trees (tree length = 259 steps, CI = 0·8147, RI = 0·9159). Thirty-six positions in the matrix were parsimony uninformative and 145 were informative. For the Bayesian analysis the likelihood parameters had the following average values [± one standard deviation (SD)]: likelihood (lnL) = −3095·525 (0·2307), base frequency π(A) = 0·2097 (0·0003), π(C) = 0·2829 (0·0003), π(G) = 0·2644 (0·0003), π(T) = 0·243 (0·0003), rate matrix r(AC) = 0·1256 (0·0007), r(AG) = 0·1365 (0·0007), r(AT) = 0·1667 (0·0009), r(CG) = 0·0764 (0·0005), r(CT) = 0·4096 (0·001), r(GT) = 0·0851 (0·0005), the gamma shape parameter a = 90·139 (2·8764) and the pinvar = 0·5757 (0·0045). Since the topologies of combined dataset analyses using MP and B/MCMC approaches showed no supported conflicts, only the 50% majority rule consensus tree of Bayesian tree sampling is shown (Fig. 1).

The topology of the tree (Fig. 1) showed that the samples of *P. carporrhizans, P. coleae* and *P. quercina* form well-supported monophyletic groups. *Parmelina atricha* is sister to *P. carporrhizans* but other relationships lacked statistical support. However, the samples of *P. tiliacea* separated into two independent groups (clades A and B) with strong statistical support. Clade A included *P. tiliacea* specimens from a wide geographic area and also a nested clade with all the *P. pastillifera* specimens examined. Clade B included only samples from central Spain. As the relationships between the *Parmelina* species are not well supported, the monophyly of *P. tiliacea* as a single species (including clades A and B) was tested and significantly rejected ($P < 0·002$ in the SH and ELW tests). The same results were obtained irrespective of whether *P. pastillifera* was included in clade A.

The pairwise genetic distances between the ITS haplotypes were also calculated to estimate the genetic divergence of clades A and B (Table 2). When all the samples were assigned to a single species, the intraspecific mean was remarkably high (0·030 ± 0·006 s/s), with a maximum distance of 0·093 s/s. On the other hand, when specimens of clades A and B are considered as two separate species, the maximum values of their intraspecific genetic distances fall to 0·0169 s/s for clade A and 0·002 for clade B, and the interspecific distances range from 0·071–0·093. Similar values were obtained irrespective of whether *P. pastillifera* specimens were included in clade A (Table 2).

Morphology and chemistry

Morphological analysis of apothecia of both groups A and B revealed subtle differences in the outer hyphae forming the true exciple. The cells of the exciple in group B had thinner walls than those of group A (Fig. 2B). Measurements of the ascospores of representative specimens of each clade showed that both clades had spores of similar length (clade A, $\bar{x}$ ± SD = 10·53 µm ± 1·17, range = 8–15 µm; clade B, $\bar{x}$ = 10·66 µm ± 0·94; range = 9–13 µm) that did not show a statistically significant difference ($P = 0·4130$). On the contrary, however, the mean width of the ascospores of both clades differed significantly ($P = 0·0000$): those from group A ($\bar{x}$ ± SD = 5·69 ± 0·58, range = 5–7 µm) being wider than those of group B ($\bar{x}$ ± SD = 4·51 ± 0·53, range = 3–5 µm). Spores of clade A (length/breadth ratio of 1·85) can be termed as elongate while those of clade B (length/breadth ratio of 2·36) are cylindrical. Chemical analysis revealed the presence of lecanoric acid and atranorin in all species of *Parmelina* studied.

Taxonomy

Parmelina tiliacea was first described as *Lichen tiliaceus* by Hoffmann in 1784, and was almost certainly collected in southern Germany although no locality was indicated. Based on the results of our phylogenetic analysis, taxonomic changes are made to reflect that the current name *P. tiliacea* encompasses two separate lineages, separated by a large genetic distance, that merit formal recognition. Specimens of clade A are assigned to *P. tiliacea* because it includes samples from

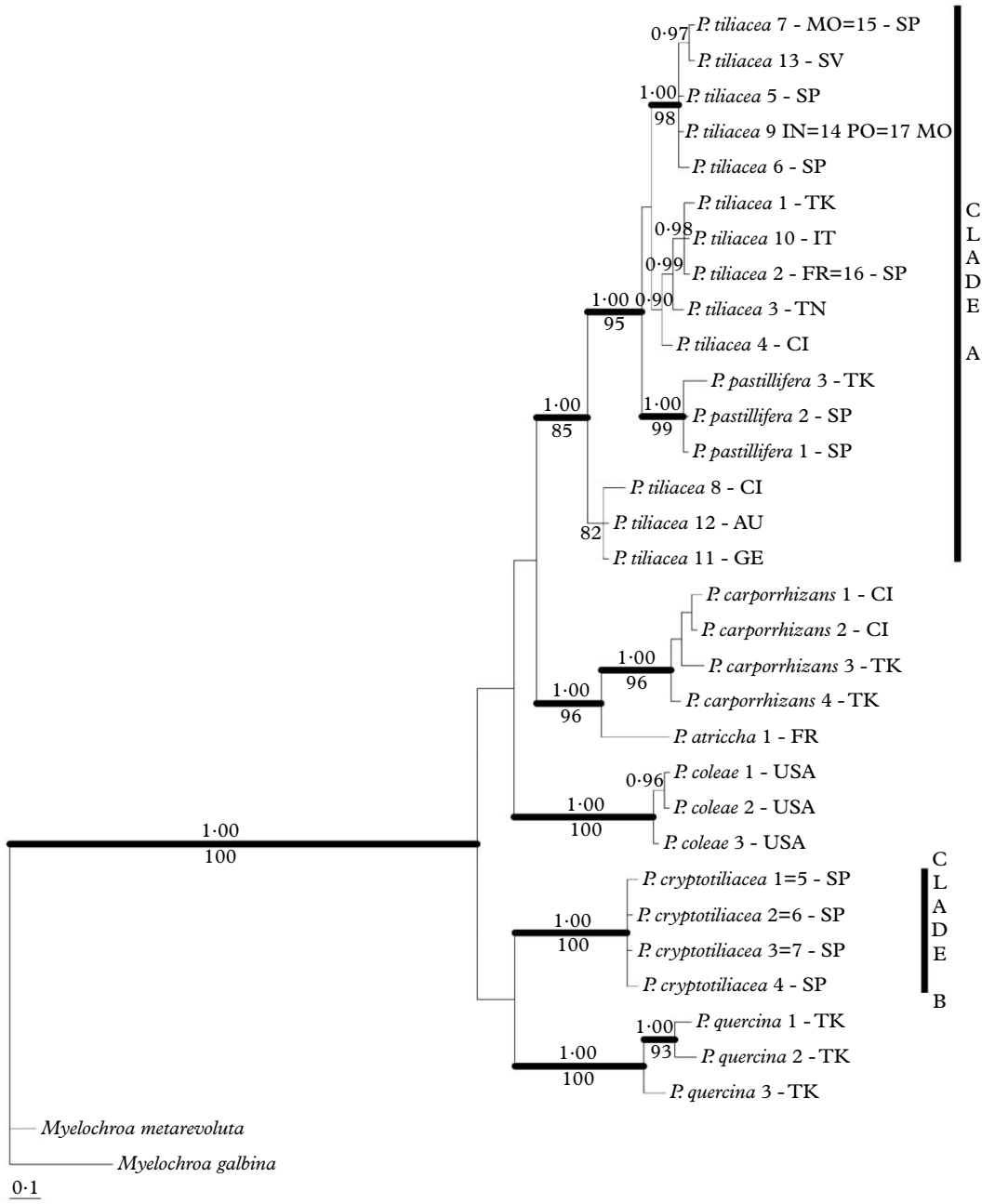

FIG. 1. Majority-rule consensus tree based on 18 000 trees from B/MCMC tree sampling procedure from a combined data set of nuITS rDNA and mtLSU rDNA sequences. Posterior probabilities ≥ 0·95 in the Bayesian analysis are indicated above the branches and MP boostrap values ≥ 0·75 below branches. Branches with significant support in both analyses are in bold. (AU = Austria, CI = Canary Islands, FR = France, GE = Germany, IN = India, IT = Italy, MO = Morocco, PO = Portugal, SP = Spain, SV = Slovenia, TK = Turkey, TN = Tunisia, USA = United States of America).

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TABLE 2. *Pairwise genetic distances between ITS haplotypes*

Intraspecific genetic distances (substitutions/site)	Mean ± standard deviation	Range
Samples of clades A and B (excluding *P. pastillifera*)	0·0305 ± 0·0335 (0·0338 ± 0·0360)	0·0023–0·0929 (0·0023–0·0929)
Samples of clade A (excluding *P. pastillifera*)	0·0100 ± 0·0045 (0·0093 ± 0·0047)	0·0023–0·0169 (0·0023–0·0167)
Samples of Clade B (*P. cryptotiliacea*)	–	0·0023
Interspecific genetic distances (substitutions/site)	**Mean ± standard deviation**	**Range**
Clade A versus Clade B	0·0830 ± 0·0062	0·0712–0·0929
Between all species of *Parmelina*	0·0564 ± 0·0306	0·0023–0·1045

central Europe (Germany, Austria, Italy and Slovenia), and the application of that name is fixed here by designation of a sequenced epitype. Specimens of clade B (which included samples from Spain) are recognized as the new species *Parmelina cryptotiliacea* sp. nov. The new species has a large genetic distance, a more restricted distributional area, and small morphological differences compared with *P. tiliacea*.

Parmelina cryptotiliacea A. Crespo & Núñez-Zapata sp. nov.

MycoBank No.: 561685

Similis *Parmelina tiliacea* sed differte in ascosporis angusta (3–5 μm latis), cellulis in hyphis excipulis cum muris attenuatis, et in sequencis molecularis ITS et mtLSU.

Typus: Spain, Extremadura, Parque Natural de Monfragüe, 39°49′37·9″N 06°03′27·5″W, on *Quercus ilex* subsp. *ballota*, alt. 207 m, June 2005, *H. T. Lumbsch, A. Green, P. K. Divakar & A. Argüello* (MAF-Lich 16454— holotypus).

(Fig. 2)

Thallus adnate on bark, pale mineral grey to mineral grey; lobes irregularly branched, sublinear-elongate, often imbricate, rounded at the apices, 2–7 mm wide, the margins more or less crenate and undulate, not ciliate; upper surface more or less shiny, without maculae, usually pruinose, irregularly cracked, densely isidiate; *medulla* white; lower surface black with brown edge, moderately to densely rhizinate, rhizines black, simple, 1–2 mm long. *Isidia* cylindrical, short 0·5–1·5 mm, rarely branched, usually blackening at the tips.

Apothecia frequent, adnate, to 4 mm diam. *Asci* 8-spored. *Ascospores* cylindrical, length 9–13 μm (10·66 ± 0·94 μm), width 3–5 μm (4·51 μm ± 0·53 μm).

Pycnidia not seen.

Chemistry. Upper cortex K+ yellow; medulla K−, C+ red, KC+ red, P−. Containing atranorin and lecanoric acid, but no fatty acids.

Distribution and ecology. At present the species is known only from four localities in Spain: National Park of Monfragüe (Extremadura), El Pardo (Madrid), Puertollano, and San Quintin mine (both Castilla – La Mancha). It grows on tree trunks and rocks in relatively lowland areas (alt. 250–700 m asl) with a low relative humidity. At low elevations the new species is sympatric with *P. tiliacea*.

Remarks. *Parmelina cryptotiliacea* is a cryptic morph of *P. tiliacea*. When apothecia are present, *P. cryptotiliacea* can be morphologically differentiated from *P. tiliacea* by its narrower ascospores (width 3–5 μm, compared with 5–7 μm in *P. tiliacea*) and the thinner

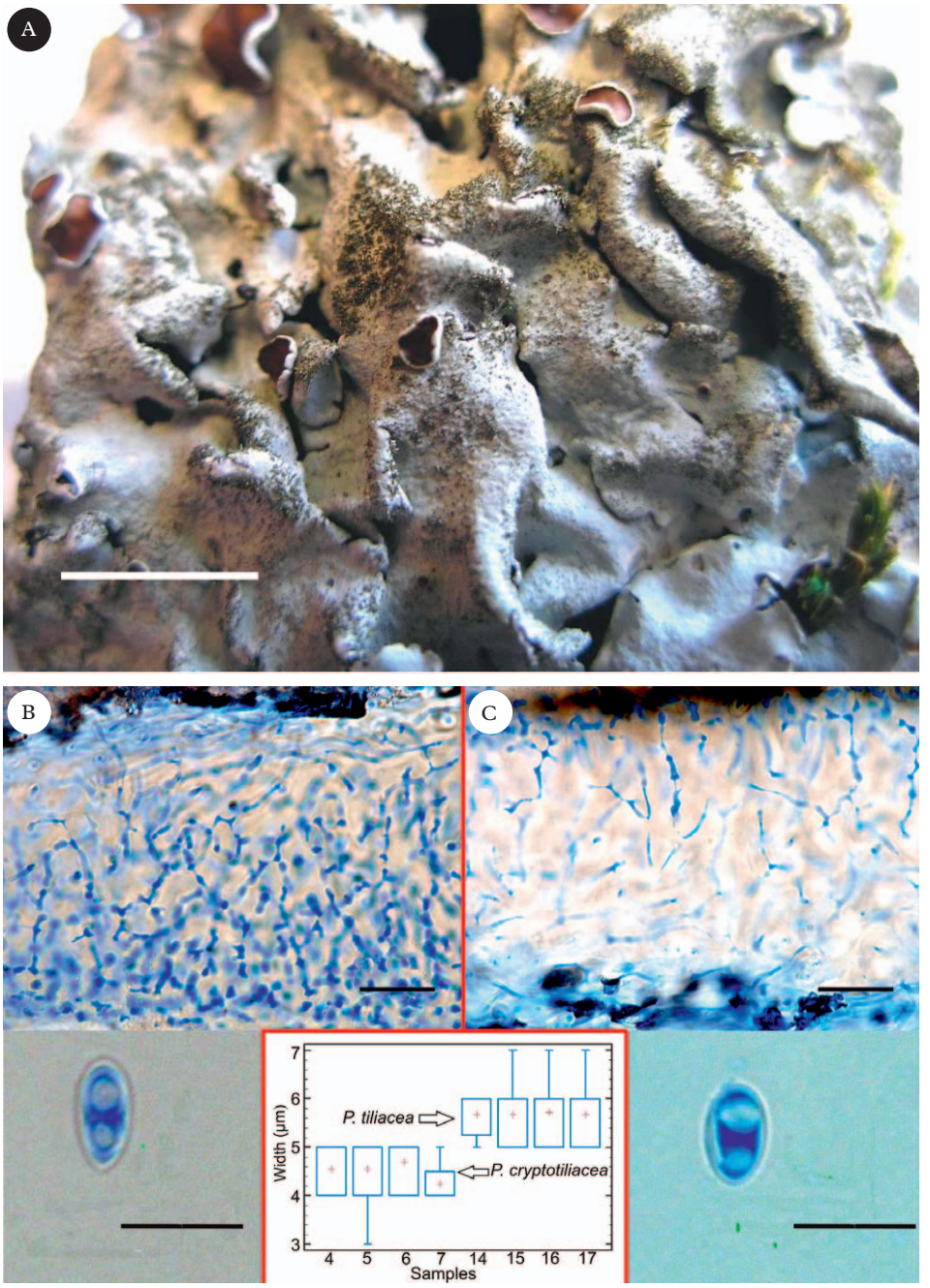

FIG. 2. *Parmelina cryptotiliacea*. A, habit (MAF-Lich 16454, holotype). B & C, comparison of exciple cells and ascospores; B, *P. cryptotiliacea* (MAF-Lich 16453); C, *P. tiliacea* (MAF-Lich 15350); the graph shows the mean (+), the range of the middle 50% of the data and the full range of the width of the ascospores in samples of both species. Mean differences between species are statistically significant ($P = <0{\cdot}0001$). See details of samples in Table 1. Scales: A=50 mm; B & C=20 µm (exciples) and 10 µm (ascospores).

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walls of the exciple cells. However, for samples lacking apothecia, a comparison of the ITS sequences is the only reliable way to distinguish this cryptic species.

Parmelina tiliacea (Hoffm.) Hale

Phytologia **28:** 481 (1974).—*Lichen tiliaceus* Hoffm., *Enum. Lich.*: 96 (1784); type: Europe, *sine loc., op. cit.*: tab. 16 fig. 2 (—lectotype designated by Jørgensen 1972); Germany, Bavaria, Oberfranken, Kteis Forchheim, Ehrenburg, alt. 450 m, on *Tilia platyphyllos*, 27 September 2009, *W. & G. Brachel* (MAF-Lich 16485—**epitypus hic designatus**).

For further synonyms see Hale (1976) and Dobson & Hawksworth (1976).

Remarks. As no material collected and named by Hoffmann prior to 1784 has been located, Jørgensen (1972) designated the original illustration as lectotype for this name. There is Hoffmann material in the Moscow herbarium (MW) which was studied by Peter W. James in 1975 and which belongs to the current concept of the species, but as it is unlocalized and was probably collected after 1804 this cannot be treated as original material appropriate for lectotypification (Dobson & Hawksworth 1976). In order to fix the application of Hoffmann's epithet in the sense it is used in the present paper, we therefore designate a sequenced collection from southern Germany as an epitype for the name.

Discussion

The presence of cryptic species in widely or disjunctly distributed species has often been found in *Parmeliaceae* and seems to be a rather common phenomenon, as noted above. The cryptic species found here within *P. tiliacea* s. lat., was not detected in previous investigations due to the restricted sampling used in earlier phylogenetic studies (Blanco *et al*. 2004, 2006; Thell *et al*. 2004; Argüello *et al*. 2007).

The present investigation, based on more extensive sampling throughout the geographical range of the species, has detected haplotype variability between and within populations of *P. tiliacea* s. lat. The topology of the tree, based on two independent loci nuITS and mtLSU, and the SH and ELW hypothesis tests, indicates that specimens of *P. tiliacea* do not form a monophyletic group but fall in two independent strongly supported clades (clades A and B; Fig.1), named here as *P. tiliacea* s. str. and *P. cryptotiliacea*, respectively. The genetic distances between the ITS haplotypes also supports the separation of the two independent clades at the specific level: assignment of all the samples to a single species gave an extremely high intraspecific mean (0·030 ± 0·006 s/s), with a maximum distance (0·093 s/s) that is four to five times larger than the values found in other *Parmelina* species (e.g. *P. quercina* mean = 0·007 ± 0·004 s/s; maximum distance = 0·013 s/s; Del Prado *et al*. 2010). When clades A and B are considered as separate species (*P. tiliacea* and *P. cryptotiliacea*), their intraspecific distances fall within the species range found in other *Parmeliaceae* ($\leq$ 0·017 s/s; Del Prado *et al*. 2010).

It should be noted that the genetic distances between ITS haplotypes of clade A, fall within the general intraspecific range even if the haplotypes of *P. pastillifera* are included. These data suggest that *P. pastillifera* is genetically very close to *P. tiliacea*, despite the morphological distinctive features (button-like isidia *versus* the cylindrical isidia of *P. tiliacea*; Dobson & Hawksworth 1976) and the different geographical ranges (*P. pastillifera* is much more common in humid western European areas than *P. tiliacea*). A comprehensive population study is being carried out to establish the phylogenetic relationships between *P. tiliacea* and *P. pastillifera*, and no formal taxonomic changes are made pending the outcome of that on-going investigation.

Despite the genetic and small anatomical differences, *P. tiliacea* and *P. cryptotiliacea* do not have clear ecological differences. *Parmelina cryptotiliacea* was collected near the Central System and in the south central area of the Iberian Peninsula between 250 and 700 m above sea level, where climatic conditions are characterized by low summer rainfall. However, *P. tiliacea* is also present in

the same localities where *P. cryptotiliacea* was collected. *Parmelina tiliacea* has a more extensive distribution and altitudinal range (see Table 1), and therefore grows under a wider range of climatic conditions. The known distribution of *P. cryptotilacea* shows that it grows sympatrically with *P. tiliacea* at low elevations and shares the same habitats.

Specimens of *P. tiliacea* and *P. cryptotiliacea* cannot be easily differentiated by morphology alone in the absence of apothecia. Types of perforations of the thallus surface that allow gas exchange, have been regarded as a key character for interspecific characters in parmelioids (e.g. *Parmelina* in Argüello *et al*. 2007, *Melanelia* in Blanco *et al*. 2004, parmotremoid groups in Blanco *et al*. 2005). However, the presence of pruina on the upper surface of the specimens examined prevented us from critically analyzing this feature. Ascospores of parmelioid lichens have not been routinely studied, mainly due to the frequent absence of ascomata, but Argüello *et al*. (2007) showed a correlation between ascospore sizes and phylogenetic hypothesis, especially in the *Parmelina* group. Although the difference in width of ascospores has statistical support in the present study, it is not strong and could be misleading if the degree of maturity of the apothecia and spores is not taken into account. Internal structures of ascoma have also not been considered in any depth in parmelioid lichens (Hale 1976); the other difference between *P. tiliacea* and *P. cryptotiliacea* (the thickness of the cell wall of the exciple) is also subtle and, due to the frequent absence of apothecia in *P. tiliacea*, this structure has been scarcely studied in the past. Chemistry does not provide any diagnostic characters because all species in the genus *Parmelina* share the same compounds (Hale 1976; Diaz-Guerra & Manrique 1984).

Conclusion

As found in other cosmopolitan species of lichenized fungi, molecular phylogeny based on nuITS and mtLSU rDNA regions has revealed that the traditional concept of *Parmelina tiliacea* includes two separate lineages: one corresponding to *P. tiliacea* s. str., a widespread lichen from Europe, and another cryptic, genetically separated lineage that is formally described here as a new species, *P. cryptotiliacea*. *Parmelina cryptotiliacea* can be considered a cryptic species because it can be morphologically separated from *P. tiliacea* only by small anatomical differences in the apothecium and ascospores, and a high proportion of populations lack apothecia. In this and similar cases, molecular data are the most reliable way to distinguish such morphologically close species. Our data indicate that *P. tiliacea* s. str. has a wide distribution, growing in Europe, Middle-East Asia and the Indian subcontinent, while *P. cryptotiliacea* has only been found in Spain, where it is sympatric with *P. tiliacea* at low elevations. The restricted area of *P. cryptotiliacea* suggests that it could be a threatened endemic species, and its conservation status should be evaluated independently of *P. tiliacea* even if molecular tools are necessary for its identification.

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