The Lichenologist **43**(6): 569–583 (2011)
doi:10.1017/S0024282911000582

Using genetic distances in addition to ITS molecular phylogeny to identify potential species in the *Parmotrema reticulatum* complex: a case study

Ruth DEL-PRADO, Pradeep K. DIVAKAR and Ana CRESPO

Abstract: We used a genetic distance approach in conjunction with molecular phylogeny to establish species boundaries and detect cryptic lineages in the *Parmotrema reticulatum – P. pseudoreticulatum* complex. The phylogeny of specimens from a broad geographic distribution was reconstructed from the internal transcribed spacer region. Pairwise genetic distances were calculated and compared to an intraspecific range defined for the parmelioid lichens to circumscribe species-level groups. Our results showed that *P. reticulatum* and *P. pseudoreticulatum* are polyphyletic, being comprised of at least seven well-supported lineages. In contrast, the genetic distance approach revealed ten cryptic lineages within the *P. reticulatum – P. pseudoreticulatum* complex. Neither morphology nor geography was conclusive in attempting to corroborate these genetic lineages. However F_{ST} indices suggest significant genetic differentiation between these lineages. Our results suggest that the morphology-based circumscriptions underestimated species in *Parmotrema* and that, in some cases, genetic distances may be used as an additional tool to determine species boundaries in morphologically cryptic species complexes. The most significant contribution of the present study is the application of a fast and accurate method to identify problematic groups and candidate species using the ITS locus with a genetic distances approach.

Key words: cryptic species, lichens, *Parmeliaceae*, species complexes, species identification

Introduction

Species are fundamental units in biology. Accurate assessment of species-level diversity is essential to systematic research, and also for conservation risk-assessment of threatened species before they become extinct. De Queiroz (2007) argues that the only necessary property of a species is to be a separately evolving metapopulation lineage, although he indicated different criteria to support the existence of a species (e.g. morpho-species and phylogentic species). In lichen-forming fungi, phenotypic criteria have traditionally been used to define species (e.g. different reproductive modes). However, this definition of species may be misleading when diagnostic characters are subject to phenotypic plasticity or mask the presence of distinct species within the same morphological form.

In the last decade, the use of molecular data has revolutionized the delimitation of species in the *Parmeliaceae* and of lichenized fungi in general (Grube & Kroken 2000; Taylor *et al*. 2000; Kroken & Taylor 2001; Molina *et al*. 2004; Divakar *et al*. 2005*a*, 2010*a*; Argüello *et al*. 2007; Crespo & Pérez-Ortega 2009; Elix *et al*. 2009; Wedin *et al*. 2009; Crespo & Lumbsch 2010). These studies have demonstrated that morphology-based species concepts often underestimate diversity in lichen-forming fungi, hiding distinct phylogenetic lineages under a single species name. In some species complexes, re-examination of morphology has revealed previously overlooked morphological and/or chemical characters that support the recognition of these species-level lineages (Molina *et al*. 2004; Divakar *et al*. 2005*a*, 2010*a*; Argüello *et al*. 2007). However, in other

R. Del-Prado, P. K. Divakar and A. Crespo: Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal, Madrid 28040, Spain. Email: rdelprado@farm.ucm.es

complexes, no morphological features have been identified characterizing the phylogenetically distinct lineages (e.g. the case of *Parmelia saxatilis*, *P. sulcata* and *Parmotrema reticulatum*; Molina *et al.* 2004, 2011; Divakar *et al.* 2005*b*). These lineages have been recognized as cryptic species. Cryptic species appear to be common in the *Parmeliaceae*, with up to 80 cryptic lineages counted recently in the phylogenetic revision of parmelioid lichens by Divakar *et al.* (2010*b*).

The *Parmotrema reticulatum – P. pseudoreticulatum* complex includes several species that remain taxonomically controversial. *Parmotrema reticulatum* was described by Taylor (1836; sub *Parmelia reticulata*) from Kerry, Ireland. The species is characterized by a foliose thallus, a minutely reticulate-maculate and sorediate upper surface, a black lower surface, simple to branched rhizines and the presence of atranorin and salazinic acid (Elix 1994*b*; Divakar & Upreti 2005). It grows in a wide range of ecological environments, more frequently as an epiphyte, and normally reproduces asexually from sorediate diaspores. It is widely distributed and is one of the most common taxa in tropical and subtropical regions. *Parmotrema clavuliferum* was segregated from *P. reticulatum* (as *Parmelia clavulifera* Räsänen; Räsänen 1944) based on the capitate soralia on short laciniae and a black mottled white lower surface, especially below the sorediate lobules; it occurs sympatrically with *P. reticulatum*.

Another species, *Parmotrema pseudoreticulatum* also differs from *P. reticulatum* with respect to subtle morphological characters such as leathery thallus, marginal labriform soralia, lower surface with a bare, dark brown marginal rim and sparse marginal cilia (Tavares 1945). It grows mainly on *Quercus* and *Olea* tree trunks in oceanic coastal regions of Portugal, Morocco and Spain (Tavares 1945; Hale & DePriest 1999). The species is considered as a synonym of *P. reticulatum* by several authors (Krog & Swinscow 1981; Clauzade & Roux 1986; Llimona & Hladun 2001) but this synonymy is not universally accepted (Hale & Fletcher 1990; Hale & DePriest 1999).

Parmotrema clavuliferum is accepted as an independent species by some authors (Kurokawa 1991, 2003; Moon *et al.* 2000, 2001), while others have considered it a synonym under *P. reticulatum*, providing evidence of intermediate forms (Hale & Fletcher 1990; Hale & DePriest 1999; Elix 1994*a*; Divakar & Upreti 2005). Divakar *et al.* (2005*b*) studied the *P. reticulatum* complex using a molecular phylogenetic approach. Their results supported the synonymy of *P. clavuliferum* within *P. reticulatum* but resurrected *P. pseudoreticulatum*, extending its distribution to South Africa. This study also detected diverse well-supported clades within *P. reticulatum* s. lat., suggesting the presence of undetected lineages.

The current concept of *P. reticulatum* includes a wide range of morphological variability, including marginal to submarginal soralia on main lobes, capitate soralia on short lacinae, a sparsely to densely reticulate-maculate upper surface, sparsely to densely ciliate margins, and a lower surface with white mottled to black marginal zones.

The use of genetic distances using short standardized gene regions (DNA barcodes) has provided complementary or alternative support for species identification, which is especially useful when distinct morphological characters are scarce or subtle, and also for detecting overlooked taxa as cryptic species (Hebert *et al.* 2003*a*, 2004*a*, 2010; Zemlak *et al.* 2009; Del-Prado *et al.* 2010; Liu *et al.* 2011; Kelly *et al.* 2011). The utility of a single gene sequence for identifying species depends on the extent of separation between intraspecific variation and interspecific divergence (the barcoding gap: Meyer & Paulay 2005). Del-Prado *et al.* (2010) tested the use of nuclear ITS sequences for identification of species using a quantitative method based on the measurement of genetic distances to investigate the gap between intra- and interspecific variation in parmelioid lichens. As a result, a threshold between intra- and interspecific divergence was established, demonstrating that the genetic distances of the ITS sequences are a useful additional tool for establishing species

boundaries in parmelioid lichens. Additionally, the ITS locus has recently been recommended as the barcoding marker for fungal groups (Fungal Barcoding meeting, Amsterdam 2011).

In this study we explore species boundaries and cryptic lineages in the *P. reticulatum* complex to identify potential 'candidate species', applying molecular phylogeny and estimation of genetic distances (using the nuclear ITS region) approaches. To achieve this objective we studied specimens from almost the entire distribution of the species. This comprehensive sampling allows us to investigate the monophyly of taxa and relationships between different specimens. Morphological and chemical features of each clade in the *P. reticulatum* complex are also assessed.

Material and Methods

Taxon sampling

Sequence data of the nuITS gene were analyzed in twelve specimens of *Parmotrema pseudoreticulatum* and sixty-six of *P. reticulatum*, collected from distant geographic regions throughout the species distributions. Samples of other *Parmotrema* species in the molecular phylogenetic analysis included: one individual of *P. austrosinense* and *P. perforatum*; two of *P. cetratum*, *P. crinitum* and *P. hypoleucinum*; and four of *P. perlatum* and *P. tinctorum*. *Flavoparmelia caperata* was selected as the outgroup, (Divakar *et al.* 2005*b*; Crespo *et al.* 2010). Data from 36 individuals were generated for this study and 60 sequences downloaded from GenBank. Detailed collection information and GenBank accession numbers are presented in Table 1.

DNA extraction, PCR and sequencing

Total DNA was extracted from freshly collected materials, using the DNeasy Plant Mini Kit (Qiagen) following the instructions of the manufacturer, with the slight modifications described in Crespo *et al.* (2001). Fungal nuclear ITS rDNA was amplified using the following primers: ITS1F (Gardes & Bruns 1993), ITS4A (Larena *et al.* 1999), ITS1-LM (Myllys *et al.* 1999), and ITS2-KL (Lohtander *et al.* 1998). Amplifications were performed in a 25 µl volume containing 2·5 µl 10 × DNA buffer containing 2 mM $MgCl_2$ (Biotools), 0·5 µl dNTPs (10 mM of each base), 1·25 µl of each primer (10 µM), 0·625 µl DNA polymerase (1 U μl^{-1}), 13·875 µl distilled water and 5 µl of DNA template.

The amplifications for nu ITS rDNA were carried out in an automatic thermocycler (Techne Progene, Jepson Bolton & Co. Ltd., Waltford, Herts, UK) using the following parameters: initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 54 and 56°C for 1 min (ITS1F/ITS4A – ITS1LM/ITS2KL respectively), and 72°C for 1·5 min; and a final extension at 72°C for 10 min. Amplification products were visualized on 1% agarose gels stained with SYBR® Safe DNA (Life Technologies Corporations, USA) gel stain (10 000× concentrated in DMSO) and subsequently purified using the enzyme exoSAP-IT (GE Healthcare, UK) according to the manufacturer's instructions.

Fragments were sequenced using Big Dye Terminator reaction kit (ABI PRISM, Applied Biosystems). Cycle sequencing reactions were performed with the same sets of primers used for PCR amplifications, as described previously (Del-Prado *et al.* 2010). Sequence fragments obtained were assembled with SeqMan 4.03 (DNAStar) and manually edited.

Sequence alignment and phylogenetic analysis

The dataset was aligned using SATé (Liu *et al.* 2009) v. 1.2.1 with the following settings: MAFFT as aligner; Muscle as merger; RAXML as tree estimator; GTRGAMMAI as RAxML model; and size in Max. Subproblem was set at 200, and the rest of the settings were left as default. Ambiguously aligned positions, specifically the first 3 bp (5′ end) and last 35 bp (3′ end), were removed.

The resulting alignment was analyzed using maximum parsimony (MP), maximum likelihood (ML) and a Bayesian Markov Chain Monte Carlo approach (B/MCMC). MP analysis was performed using the program PAUP* 4.0b10 (Swofford 2003). Heuristic searches with 1000 random taxon addition replicates were conducted with the tree-bisection-reconnection (TBR) branch-swapping and MulTrees option in effect, equally weighted characters and gaps treated as missing data. Bootstrapping (Felsenstein 1985) was based on 4000 pseudoreplicates with random sequence additions. To assess homoplasy levels, the consistency index (CI) and retention index (RI) were calculated.

The ML analysis was performed using an online version of the program RAxML 7.0.4 (http://phylobench.vital-it.ch/raxml-bb/; Stamatakis *et al.* 2005, 2008), assuming a general time-reversible model of nucleotide substitution (Rodriguez *et al.* 1990) and a discrete gamma distribution with six rate categories. The bootstrap analysis was run with 100 pseudoreplicates.

The program MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001) was employed to sample trees using an MCMC method. The nucleotide substitution model was selected using the program jModelTest (Posada 2008), and following the AIC criterion. Thus, General Time Reversible substitution model (Rodriguez *et al.* 1990), with estimation of invariant sites and assuming a gamma distribution with six rate categories (GTR+I+G), was used because it had the lowest –lnL value. No molecular clock was assumed. MrBayes was run for 3 million generations with 12 simultaneous chains. Trees were sampled every 200 generations (for a total of 15 000 trees). The first 300 000 generations (i.e., the

TABLE 1. *Specimens used in the study, with location, herbarium and GenBank accession numbers. Specimens from which new sequences were generated are indicated in bold*

Species	Locality	Herbarium acc. no	GenBank acc. no. ITS
Flavoparmelia caperata 1	Spain: Teruel	MAF-Lich 6045	AY581059
F. caperata 2	China: Yunnan	MAF-Lich 10175	AY586561
Parmotrema austrosinense	**Spain: Canary Islands**	**MAF-Lich 16907**	**JN166368**
P. cetratum 1	Uruguay: Maldonado	*H.S. Osorio* 9424	AY586576
P. cetratum 2	Uruguay: Maldonado	*H.S. Osorio* 9425	AY642847
P. crinitum 1	Spain: Canary Islands	MAF-Lich 16170	HM017028
P. crinitum 2	Spain: Canary Islands	MAF-Lich 16174	HM017030
P. hypoleucinum 1	Spain: Cádiz	MAF-Lich 7637	AY586567
P. hypoleucinum 2	Morocco: Rabat	MAF-Lich 16147	HM017036
P. perforatum	USA	*Cole* 7983	AY586568
P. perlatum 1	Spain: Canary Islands	MAF-Lich 16168	HM017050
P. perlatum 2	Spain: Canary Islands	MAF-Lich 16162	HM017052
P. perlatum 3	Portugal: Sintra	MAF-Lich 6965	AY586566
P. perlatum 4	Morocco: Medium Atlas	MAF-Lich 16146	HM017046
P. pseudoreticulatum 1★†	South Africa: Eastern Cape	MAF-Lich 10287	AY642828
P. pseudoreticulatum 2	Morocco: Chaouen	MAF-Lich 16144	HM017055
P. pseudoreticulatum 3★	Morocco: Rabat	MAF-Lich 16149	HM017056
P. pseudoreticulatum 4	Spain: Cádiz	MAF-Lich 7650A	HM017054
P. pseudoreticulatum 5★†	Spain: Canary Islands	MAF-Lich 16185	HM017053
P. pseudoreticulatum 6	Portugal: Estremadura	MAF-Lich 10277	AY642842
P. pseudoreticulatum 7★	Portugal: Estremadura	MAF-Lich 10278	AY642841
P. pseudoreticulatum 8★	South Africa: Eastern Cape	MAF-Lich 10289	AY642830
P. pseudoreticulatum 9	South Africa: Eastern Cape	MAF-Lich 10288	AY642829
P. pseudoreticulatum 10	Portugal: Estremadura	MAF-Lich 10276	AY642839
***P. pseudoreticulatum* 11★†**	**Spain: Balearic Islands**	**MAF-Lich 16912**	**JN166399**
P. pseudoreticulatum 12★	Portugal: Estremadura	MAF-Lich 10292	AY642840
P. reticulatum 1	Portugal: Sintra	MAF-Lich 6067	AY586579
***P. reticulatum* 2★†**	**Spain: Canary Islands**	**MAF-Lich 16901**	**JN166370**
***P. reticulatum* 3★**	**Spain: Canary Islands**	**MAF-Lich 16909**	**JN166371**
***P. reticulatum* 4★**	**Spain: Canary Islands**	**MAF-Lich 16900**	**JN166372**
***P. reticulatum* 5★**	**Fiji Island**	**MAF-Lich 16898**	**JN166373**
***P. reticulatum* 6★**	**Spain: Balearic Islands**	**MAF-Lich 16910**	**JN166375**
***P. reticulatum* 7★**	Morocco: Larache	MAF-Lich 16143	HM016953
***P. reticulatum* 8★**	**Spain: Canary Islands**	**MAF-Lich 16918**	**JN166376**
***P. reticulatum* 9★**	**Spain: Canary Islands**	**MAF-Lich 16904**	**JN166374**
***P. reticulatum* 10★†**	**India: Tamil Nadu**	**MAF-Lich 16917**	**JN166369**
P. reticulatum 11	Spain: Canary Islands	MAF-Lich 16183	HM017064
P. reticulatum 12★	Spain: Canary Islands	MAF-Lich 16175	HM017063
P. reticulatum 13	Spain: Canary Islands	MAF-Lich 16177	HM017062
P. reticulatum 14★†	Spain: Canary Islands	MAF-Lich 10265	AY642844
P. reticulatum 15	Portugal: Evora	MAF-Lich 10275	AY642836
***P. reticulatum* 16★†**	**Peru: Canta**	**MAF-Lich 16893**	**JN166377**
P. reticulatum 17	Morocco: Rabat	MAF-Lich 16152	HM016955
***P. reticulatum* 18★†**	**Spain: Canary Islands**	**MAF-Lich 16923**	**JN166381**
P. reticulatum 19★†	Spain: Galicia	MAF-Lich 10281	HM016954
***P. reticulatum* 20★†**	**Mozambique: Is Inhaca**	**MAF-Lich 16914**	**JN166382**
P. reticulatum 21	Spain: Cádiz	MAF-Lich 7650	AY586578

TABLE 1. *Continued*

Species	Locality	Herbarium acc. no	GenBank acc. no. ITS
***P. reticulatum* 22**★†	**Spain: Canary Islands**	**MAF-Lich 16906**	**JN166378**
P. reticulatum 23★	Portugal: Portalegre	MAF-Lich 10271	AY642838
P. reticulatum 24★	Portugal: Evora	MAF-Lich 10270	AY642837
P. reticulatum 25†	Kenya: Ngong	K 301	AY642845
***P. reticulatum* 26**★†	**India: Tamil Nadu**	**MAF-Lich 16915**	**JN166379**
P. reticulatum 27★†	China: Yunnan	MAF-Lich 10258	AY642818
P. reticulatum 28	China: Yunnan	MAF-Lich 10259	AY642819
P. reticulatum 29★	China: Yunnan	MAF-Lich 10257	AY642817
***P. reticulatum* 30**★†	**India: Tamil Nadu**	**MAF-Lich 16916**	**JN166380**
P. reticulatum 31★†	China: Yunnan	MAF-Lich 10164	AY586577
***P. reticulatum* 32**★†	**Fiji Island**	**MAF-Lich 16897**	**JN166383**
***P. reticulatum* 33**★	**Japan: Tsukuba**	**MAF-Lich 16895**	**JN166384**
***P. reticulatum* 34**★	**Japan: Tsukuba**	**MAF-Lich 16894**	**JN166385**
P. reticulatum 35†	Korea		EU266099
P. reticulatum 36★†	Kenya: Western Province	MAF-Lich 16121	HM016956
P. reticulatum 37★†	China: Yunnan	MAF-Lich 10263	AY642824
P. reticulatum 38★	China: Yunnan	MAF-Lich 10262	AY642823
P. reticulatum 39	China: Yunnan	MAF-Lich 10260	AY642821
P. reticulatum 40	Spain: Cies Islands	MAF-Lich 10285	AY642832
***P. reticulatum* 41**★†	**Spain: Balearic Islands**	**MAF-Lich 16911**	**JN166386**
***P. reticulatum* 42**★	**Spain: Balearic Islands**	**MAF-Lich 16913**	**JN166387**
***P. reticulatum* 43**★	**Spain: Canary Islands**	**MAF-Lich 16899**	**JN166388**
***P. reticulatum* 44**★	**Spain: Canary Islands**	**MAF-Lich 16903**	**JN166389**
***P. reticulatum* 45**★	**Spain: Canary Islands**	**MAF-Lich 16896**	**JN166390**
***P. reticulatum* 46**★	**Spain: Canary Islands**	**MAF-Lich 16921**	**JN166391**
P. reticulatum 47★†	Morocco: Larache	MAF-Lich 16151	HM017058
P. reticulatum 48	Morocco: Larache	MAF-Lich 16150	HM017057
P. reticulatum 49★	Spain: Canary Islands	MAF-Lich 10267	AY642825
P. reticulatum 50★†	Spain: Canary Islands	MAF-Lich 16184	HM017059
P. reticulatum 51★†	Spain: Canary Island	MAF-Lich 10264	HM017060
***P. reticulatum* 52**★†	**Spain: Canary Islands**	**MAF-Lich 16902**	**JN166397**
***P. reticulatum* 53**★	**Spain: Canary Islands**	**MAF-Lich 16920**	**JN166395**
***P. reticulatum* 54**★	**France: Bretagne, Côles d' Amor**	**MAF-Lich 16926**	**JN166393**
***P. reticulatum* 55**★	**Spain: Canary Islands**	**MAF-Lich 16919**	**JN166394**
***P. reticulatum* 56**★	**Spain: Canary Islands**	**MAF-Lich 16925**	**JN166396**
***P. reticulatum* 57**★†	**Spain: Canary Islands**	**MAF-Lich 16924**	**JN166392**
P. reticulatum 58★†	Australia: New South Wales	*Elix* 31555/A (CANB)	AY642843
***P. reticulatum* 59**★†	**Chile: Tierra de Fuego**	**MAF-Lich 16891**	**JN166401**
***P. reticulatum* 60**★	**Chile: Tierra de Fuego**	**MAF-Lich 16892**	**JN166402**
P. reticulatum 61★†	Spain: Canary Islands	MAF-Lich 16182	HM016957
***P. reticulatum* 62**★†	**Spain: Canary Islands**	**MAF-Lich 16922**	**JN166398**
P. reticulatum 63	Spain: Canary Island	MAF-Lich 16180	HM016960
P. reticulatum 64★	Spain: Canary Islands	MAF-Lich 16178	HM016959
P. reticulatum 65	Spain: Canary Islands	MAF-Lich 16181	HM016958
***P. reticulatum* 66**★†	**Spain: Canary Islands**	**MAF-Lich 16905**	**JN166400**
P. tinctorum 1	Japan:Shizuoka	*Y. Ohmura* 5375A	AB177401
P. tinctorum 2	Japan:Shizuoka	*Y. Ohmura* 5399	AB177404

TABLE 1. *Continued*

Species	Locality	Herbarium acc. no	GenBank acc. no. ITS
***P. tinctorum* 3**	**Spain: Balearic Islands**	**MAF-Lich 16908**	**JN166403**
P. tinctorum 4	Yemen: Socotra	*M. Schultz* 14276a (priv. hb), DNA1274 (HBG, LD)	AY251443

* specimens on which chemicals analyses were performed.
† specimens included in the calculation of genetic distances.

first 3000 trees) were deleted as the 'burn-in' of the chains. We plotted the log-likelihood scores of sample points against generation time using the TRACER 1.0 program (http://evolve.zoo.ox.ac.uk/software.html?id=tracer; Rambaut & Drummond 2003) to ensure that stationarity was achieved after the first 300 000 generations (Huelsenbeck & Ronquist 2001). Additionally, we used the program AWTY (Nylander *et al*. 2007) to compare split frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. A majority-rule consensus tree with average branch lengths was calculated for the remaining 12 000 trees using the sumt option in MrBayes. Posterior probabilities were estimated by sampling trees using an MCMC method. Support values were calculated for each branch by counting the occurrences in trees that were visited during the course of the MCMC analysis. Only clades that received $\geq$ 70% bootstrap support in the MP and ML analyses and posterior probabilities $\geq$ 0·95 were considered as strongly supported.

Calculation of genetic distances

Pairwise ML distances (given as the number of nucleotide substitutions per site) among the ITS rDNA sequences of the *Parmotrema reticulatum – P. pseudoreticulatum* complex were calculated. Only one sequence per haplotype was included in the analysis (Table 1). Genetic distances were calculated with TREE-PUZZLE 5.2 (Strimmer & von Haeseler 1997) using the GTR model of nucleotide substitution, assuming a discrete gamma distribution with six rate categories following Del-Prado *et al*. (2010). The program generates an output file which consists of a triangular matrix with all pairwise distances between all the samples included. This matrix was visualized with Microsoft Office program Excel 2000 and genetic distances between different specimens of the *P. reticulatum – P. pseudoreticulatum* complex were manually identified following these criteria: 1) the values of genetic distances among all the specimens morphologically identified as *P. reticulatum* and *P. pseudoreticulatum*; 2) genetic distance values among the specimens nested in the different monophyletic clades defined from the molecular phylogenetic tree topology.

In a complementary approach, we have clustered genetic distances groups, independently of the tree topology obtained in the molecular phylogenetic analysis, based on the threshold of 0·016 substitutions per site (s/s) that separate intra- and interspecific distances in parmelioid lichens (Del-Prado *et al*. 2010). The distance values in the matrix $\leq$ 0·016 s/s have been considered the values between the samples of the single species. We have used the filter provided by Microsoft Excel to separate values $\leq$ 0·016, obtaining for every specimen included in the analysis the group of specimens with which it shares the values that characterize the species range.

Polymorphism analyses

In order to see the degree of genetic isolation between the clusters inferred by the genetic distance threshold, we calculated the numbers of shared and fixed alleles and pairwise fixation indices (F_{ST}) (Hudson *et al*. 1992). We used the program SITES (Hey & Wakeley 1997) to assess genetic differentiation among the groups. Calculations were performed for all pairwise comparisons of inferred distance groups of the two species studied (*P. reticulatum, P. pseudoreticulatum*).

Morphological and chemical studies

Morphology of all specimens of the *P. reticulatum – P. pseudoreticulatum* complex included in the molecular analysis (Table 1) were studied using a Leica Wild M 8 dissecting microscope. Characters investigated were: type of rhizine, relative abundance of marginal cilia, lobe shape, size, upper surface colour, soralia development (marginal, submarginal, or terminal on lacinae), and reticulation on upper surface (presence/absence).The specimens marked with an asterisk in Table 1 were chemically analyzed by thin-layer chromatography (TLC) using solvent system C (Culberson 1972; Elix & Ernst-Russell 1993; Lumbsch 2002).

Results

Phylogenetic analyses

A matrix with 94 *Parmotrema* nu ITS sequences and two *F. caperata* sequences

was compiled, using 36 new sequences and 60 sequences downloaded from GenBank (http://www.ncbi.nlm.nih.gov) (Table 1). The data matrix included 460 unambiguously aligned nucleotide positions (TreeBASE No. S11708).

The MP analysis of the data matrix resulted in 4436 most parsimonious trees (tree length = 264 steps, CI = 0·6174, RI = 0·8969). Twenty-seven positions in the matrix were parsimony uninformative and 107 were informative. For the Bayesian analysis the LnL value was −2328·20 with a standard deviation of ±0·396, and for ML the LnL value was −2093·9609.

Since the topologies of the trees estimated from MP, ML and Bayesian methods did not present any well-supported conflict, only the 50% majority-rule consensus tree of the Bayesian tree sampling is shown, with MP and ML bootstrap values indicated on the Bayesian topology (Fig. 1).

The topology of the tree (Fig. 1) shows that the *Parmotrema* species formed a well-supported monophyletic group, indicated by the three methods. However, the phylogenetic relationships between the species were not well resolved. All the samples morphologically identified as *P. reticulatum* or *P. pseudoreticulatum* nested in a monophyletic clade with strong support in the Bayesian analysis, that included the samples of *P. tinctorum* and *P. austrosinense*. Within this clade, the samples of *P. reticulatum* and of *P. pseudoreticulatum* split into multiple statistically supported clades (Fig. 1). Fifty-eight specimens of *P. reticulatum* formed a monophyletic group (Clade A) with strong support in the Bayesian analysis, two samples from Chile formed a different clade (clade D), five samples from the Canary Islands clustered in an independent group (clade E), and one sample from the Canary Islands (*P. reticulatum* 66) separated from the rest of the clades.

Specimens of *P. pseudoreticulatum* split into two well-supported monophyletic groups (clades B and F). Samples of *P. tinctorum* grouped with *P. austrosinense* in a well-supported monophyletic group (clade C). However, phylogenetic relationships among the clades were not resolved, since they lacked strong statistical support.

Additionally, within clade A, two well-supported monophyletic clades (clades A1 and A2) were recovered. The samples of *P. reticulatum* included in both clades did not correlate with the geographical distribution (see Table 1). Molecular phylogenetic relationships between well-supported lineages within clades A1 and A2 were not resolved as they lacked strong statistical support.

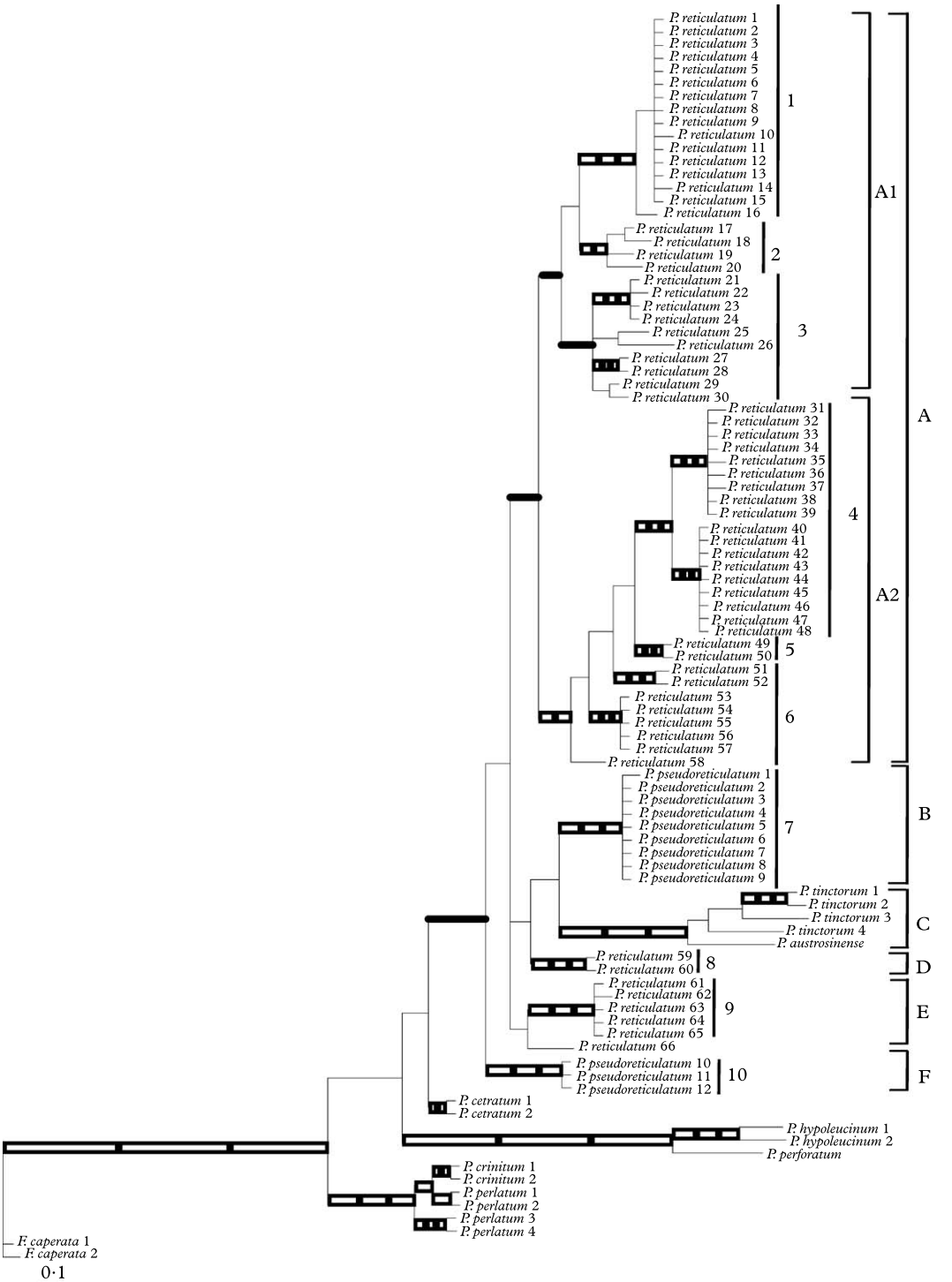
Genetic distances

The results from the genetic distance analysis, comparing the distances values within the groups defined in the *P. reticulatum – P. pseudoreticulatum* complex based on the morphologic, phylogenetic and distances analysis, are compiled in Table 2.

Pairwise genetic distances between all the specimens morphologically identified as *P. reticulatum* (Clades A + D + E + *P. reticulatum* 66 in Fig. 1) ranged from 0·003 substitutions per site (s/s) to 0·054 s/s, while values for the specimens morphologically identified as *P. pseudoreticulatum* (Clades B + F in Fig. 1) ranged from 0·003 s/s to 0·046 s/s. Estimation of the distances between *P. reticulatum* and *P. pseudoreticulatum* samples gave a range of pairwise genetic distances from 0·031 s/s to 0·064 s/s.

Genetic distances were also estimated among the specimens nested in the different supported monophyletic clades based on molecular phylogenetic tree topology (Fig. 1): the range of pairwise genetic distances was 0·003 to 0·047 s/s for clade A1 and 0·003 to 0·030 s/s for clade A2. In clades B and E the distance value between the two different haplotypes of each clade is 0·003 s/s. The specimens included in clades D and F are from different localities but resulted in a single haplotype and thus the distance value is 0·0 s/s.

In a complementary approach, the sequences of *P. reticulatum* and *P. pseudoreticulatum* were grouped on the basis of the threshold of 0·016 s/s that separated the intraspecific and interspecific distances in parmelioid lichens (Del-Prado *et al.* 2010).



https://doi.org/10.1017/S0024282911000582 Published online by Cambridge University Press

Figure 1 shows the groups (numbered 1 to 10) that formed the sequences following this criterion and Table 2 represents the genetic distances within these groups. These clusters, with the exception of the group number 6, correspond to supported monophyletic clades according to the phylogenetic analysis.

Polymorphism analysis

Polymorphisms in the nuITS marker were analyzed to compare the groups delimited in the *P. reticulatum – P. pseudoreticulatum* complex in the genetic distance study, considering a standard threshold of 0·016 s/s (Fig. 1). Table 3 shows the number of fixed differences and the fixation indices (F_{ST}) between the groups. There were fixed differences between the pairwise comparisons of different genetic clusters, although small sample sizes within groups may artificially inflate the number of fixed loci. The lowest F_{ST} value (0·491) was found between groups 2 and 3. The values between group 6 and groups 2, 3, 4 and 5 were approximately 0·6, and all other values were $\geq$ 0·7. Groups 5 and 10 were represented by a small number of sequences and thus F_{ST} values between these groups and the rest are not significant. The shared polymorphisms by different groups, revealed only one nucleotide shared by groups 2 and 3 (position 183) and another by groups 3 and 6 (position 382).

Morphological and chemical studies

There was a wide range of variation in morphological features identified within each individual clade, including specimens with an olive-green to grey upper surface, simple to squarrose rhizines on the lower surface, capitate soralia on lobules to marginal, and submarginal soralia on main lobes. These features were therefore inconclusive when trying to corroborate the monophyletic lineages. Since *P. reticulatum* and *P. pseudoreticulatum* generally reproduce asexually by soralia diaspores, apothecia are rare in these species, so that ascomatal features and ascospore characters were not investigated.

There was no variation in the phenolic constituent in the specimens of the *P. reticulatum – P. pseudoreticulatum* complex; all of them contained atranorin and salazinic acid. Additionally, in *P. reticulatum* 47 (clade A2/4) and *P. reticulatum* 54 (clade A2/6) gyrophoric acid was also found in trace amounts. Thus, phenolic compounds did not corroborate the groups identified using molecular data.

Discussion

Traditionally, morphological and chemical features have often been used to delimit species of lichenized fungi (Hale 1965; Swinscow & Krog 1988; Elix 1994*b*; Divakar *et al.* 2001). In the *Parmotrema reticulatum – P. pseudoreticulatum* complex, characters traditionally considered as diagnostic have been re-evaluated within a molecular phylogenetic framework, and molecular data failed to corroborate traditionally circumscribed taxa. Rather, these data provide evidence of the existence of several cryptic lineages hidden under the names of *P. reticulatum* and *P. pseudoreticulatum*. Lineages inferred from molecular data are not correlated with broad geographical patterns; in fact, specimens from distant geographic regions are clustered in a single clade (clade A1, Fig.1, Table1), which includes specimens from Asia,

FIG. 1. 50% majority-rule consensus tree of the molecular phylogenetic relationships in the *Parmotrema reticulatum – P. pseudoreticulatum* complex, based on 12 000 trees from a B/MCMC tree-sampling procedure, from a dataset of nu ITS sequences. Branches that were strongly supported in all three analyses (i.e., PP $\geq$ 0·95 in the B/MCMC analysis and $\geq$ 70% in the MP and ML bootstraps) are indicated by three open squares or rectangles; those receiving strong support in the B/MCMC analysis and the ML bootstrap are indicated by two open squares. The branches that received strong support only in the ML bootstrap analysis are indicated by an open rectangle, and those that received strong support only in the B/MCMC analysis are indicated in solid bold line. Groups numbered 1 to 10 indicate the phylogenetic clusters obtained when a value of 0·016 s/s is taken as the screening threshold for attempting to determine the species boundaries in the *P. reticulatum – P. pseudoreticulatum* complex.

https://doi.org/10.1017/S0024282911000582 Published online by Cambridge University Press

TABLE 2. *Results from the genetic distance (substitution per site) analysis comparing the pairwise genetic distance values within the defined groups in the P. reticulatum – P. pseudoreticulatum complex. The absence of mean and standard deviation (SD) is due to values from two haplotypes. Zero values correspond to groups with one haplotype*

Morphologically defined groups	Monophyletic clades	Genetic distance clusters	Genetic distance range (mean ± SD)
P. reticulatum			0·003–0·054 (0·031±0·012)
P. pseudoreticulatum			0·003–0·046 (0·030±0·024)
	A1		0·003–0·047 (0·022±0·009)
		1	0·003–0·008 (0·005±0·002)
		2	0·008–0·016 (0·012±0·004)
		3	0·005–0·016 (0·011±0·004)
	A2		0·003–0·030 (0·018±0·009)
		4	0·003–0·016 (0·007±0·004)
		5	0
		6	0·003–0·016 (0·013±0·005)
	B	7	0·003
	D	8	0
	E	9	0·003
	F	10	0

TABLE 3. *Results from nuclear ITS nucleotide polymorphism analyses comparing the groups established in the* P. reticulatum – P. pseudoreticulatum *complex by the use of genetic distances applying a standard threshold of 0·016 s/s to separate intra- and interspecific divergence. Values are number of fixed differences (above diagonal) and fixation index values (F_{ST} – below digonal)*

Group No.	1	2	3	4	5	6	7	8	9	10
1	–	7	5	13	13	7	18	13	14	16
2	0·775	–	3	13	12	6	15	10	13	14
3	0·764	0·491	–	10	9	5	14	10	9	12
4	0·890	0·792	0·758	–	5	3	18	17	13	18
5	0·982	0·854	0·811	0·800	–	2	19	16	12	17
6	0·803	0·657	0·611	0·626	0·661	–	14	10	9	12
7	0·981	0·872	0·848	0·919	0·994	0·870	–	11	15	15
8	0·982	0·830	0·822	0·920	1·000	0·847	0·990	–	12	14
9	0·971	0·852	0·808	0·890	0·984	0·804	0·980	0·984	–	14
10	0·985	0·876	0·845	0·924	1·000	0·859	0·993	1·000	0·986	–

Europe, North and East Africa, and South America. As a consequence, molecular data appears to be essential to characterize and accurately identify species within this complex.

Genetic distance measurements have been used frequently as a tool to investigate species boundaries and to identify cryptic diversity in different groups of animals (e.g. Hebert *et al*. 2003*a*, *b*, 2004*a*, *b*, 2010; Ward *et al*. 2005, 2008; Lefébure *et al*. 2006; Zemlack *et al*. 2009), plants (Kress *et al*. 2005; Fazekas *et al*. 2009; Hollingsworth *et al*. 2009) and fungi (Seifert *et al*. 2007; Smith *et al*. 2007; Nilsson *et al*. 2008; Simon & Weiss 2008; Vialle *et al*. 2009).

Del-Prado *et al*. 2010 measured intra- and interspecific genetic distances in parmelioid lichens, establishing a threshold of 0·015–0·017 s/s to separate ranges of intra- and interspecific divergence. The threshold was established using both phylogenetically and morphologically well delimited species (for detail see Del-Prado *et al*. 2010). Based on these data, the levels of ITS divergence within the specimens morphologically identified as *P. reticulatum* are much higher than those of single-species populations. The same result is obtained when pairwise distances between all the samples identified as *P. pseudoreticulatum* are calculated. Also, the distances within the specimens nested in the monophyletic clades A1 and A2, obtained from the molecular phylogenetic analysis, suggest the presence of different groups of lineages within each clade.

Consequently, based on the threshold established for genetic distances in parmelioid lichens, we have used the *P. reticulatum* – *P. pseudoreticulatum* complex as a case study to formulate an initial hypothesis of species boundaries, as a move towards inferring genetic clusters within this complex. As a result, eight clusters in *P. reticulatum* and two in *P. pseudoreticulatum* were designated as possible candidate cryptic species. Within these clusters, ranges of genetic distances characterized single-species populations. In addition, all of them correspond to well-supported monophyletic groups, except one (group number 6), although the phylogenetic relationships among them remain to be resolved. In the case of group number 6, the phylogenetic relationships between the specimens included in this group also remain to be resolved. Currently we are increasing the taxa sampling and the number of studied loci to evaluate the presence of distances-based groups that could not correspond to monophyletic clades.

Additionally, F_{ST} indices, as a measure of population differentiation (Holsinger & Weir 2009), were calculated to assess the extent to which these candidate populations are genetically isolated. F_{ST} values can range from 0 (complete panmixis) to 1 (complete

https://doi.org/10.1017/S0024282911000582 Published online by Cambridge University Press

isolation between populations). F_{ST} values measured between our targeted clusters were mostly $\geq 0{\cdot}7$. These results, in conjunction with only two shared polymorphisms, suggest that the genetic clusters are significantly isolated and constitute lineages that are evolutionarily independent, or on a trajectory towards becoming independent (Ross *et al*. 2010). Under the unified species concept (De Queiroz 2007), these results provide one layer of evidence supporting that candidate species inferred within the *P. reticulatum – P. pseudoreticulatum* complex represent species-level lineages.

The formal species circumscription, using sequence data exclusively, would be reinforced by strong evidence from multiple independent loci in the subsequent study. In this paper, we have presented an approach to identify candidate species groups using the genetic distances approach with the ITS locus that has recently been proposed as a universal DNA fungal barcode (Fungal Barcoding meeting, Amsterdam 2011). In a subsequent paper, these groups will be evaluated under a multilocus approach to circumscribe species with confidence. Additionally, taxon sampling within this complex is being extended to investigate the reproductive isolation of these clades more completely.

Parmelioid lichens have been shown to include a large number of cryptic lineages hidden under broadly and/or disjunctly distributed species (Crespo & Lumbsch 2010; Divakar *et al*. 2010*b*). This suggests that the morphology-based species concept underestimates diversity in this group of lichenized fungi. Genetic distances/barcoding provides an important approach to more accurately (relative to current morphological interpretation) identify species in parmelioid lichens. DNA barcoding in fungi is in its infancy and recently the ITS locus has been proposed as a universal DNA barcode. To develop DNA barcoding successfully, it is essential to delimit species boundaries accurately. The noteworthy contribution of the present investigation is the application of a rapid and reliable method to identify problematic groups and candidate species within parmelioid lichens using the ITS locus with a genetic distances approach. This study provides a working example of how to use this 'additional tool' for hypothesis testing in parmelioid species complexes that are confounded by a misinterpretation of morphological and chemical characters.

The authors thank two anonymous referees for their valuable suggestions. We acknowledge the Ministerio de Ciencia e Innovación (CGL2010-21646) for providing main funds for this research, Ramón y Cajal grant (RYC02007-01576) to PKD, and the Universidad Complutense de Madrid for all complementary facilities. We also thank Dr Phil Masson for English revision. Sequencing was carried out at the Unidad de Genómica (Parque Científico de Madrid, UCM) and M. Isabel Garcia Saez is especially thanked. We acknowledge the Encyclopedia Of Life (EOL) and all participants at the *Parmeliaceae* meeting in Chicago, May 2010.

References

Argüello, A., Del-Prado, R., Cubas, P. & Crespo, A. (2007) *Parmelina quercina* (*Parmeliaceae, Lecanorales*) includes four phylogenetically supported morphospecies. *Biological Journal of the Linnean Society* **91:** 455–467.

Clauzade, G. & Roux, C. (1986) ['1985'] Likenoj de Okcidenta Europo. Ilustrita Determinlibro. *Bulletin de la Société Botanique du Centre-Ouest, Nouvelle Séries, Numero Special* **7:** 1–893.

Crespo, A. & Lumbsch, H. T. (2010) Cryptic species in lichen-forming fungi. *IMA Fungus* **1:** 167–170.

Crespo, A. & Pérez-Ortega, S. (2009) Cryptic species and species pairs in lichens: a discussion on the relationship between molecular phylogenies and morphological characters. *Anales del Jardín Botánico de Madrid* **66:** 71–81.

Crespo, A., Blanco, O. & Hawksworth, D. L. (2001) The potential of mitochondrial DNA for establishing phylogeny and stabilising generic concepts in the parmelioid lichens. *Taxon* **50:** 807–819.

Crespo, A., Kauff, F., Divakar, P. K., Del-Prado, R., Pérez-Ortega, S., Amo de Paz, G., Ferencova, Z., Blanco, O., Roca-Valiente, B., Núñez-Zapata, J. *et al.* (2010) Phylogenetic generic classification of parmelioid lichens (*Parmeliaceae*, Ascomycota) based on molecular, morphological and chemical evidence. *Taxon* **59:** 1735–1753.

Culberson, C. F. (1972) Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. *Journal of Chromatography* **72:** 113–125.

Del-Prado, R., Cubas, P., Lumbsch, H. T., Divakar, P. K., Blanco, O., Amo de Paz, G., Molina, M. C. & Crespo, A. (2010) Genetic distances within and among species in monophyletic lineages of *Parmeliaceae* (Ascomycota) as a tool for taxon delimitation. *Molecular Phylogenetics and Evolution* **56:** 125–133.

De Queiroz, K. (2007) Species concepts and species delimitation. *Systematic Biology* **56:** 879–886.

Divakar, P. K. & Upreti, D. K. (2005) *Parmelioid Lichens in India* (*A Revisionary Study*). Dehra Dun: Bishen Singh Mahendra Pal Singh.

Divakar, P. K., Upreti, D. K. & Elix, J. A. (2001) New species and new records in the lichen family *Parmeliaceae* (Ascomycotina) from India. *Mycotaxon* **80:** 355–362.

Divakar, P. K., Molina, M. C., Lumbsch, H. T. & Crespo, A. (2005*a*) *Parmelia barrenoae*, a new lichen species related to *Parmelia sulcata* (*Parmeliaceae*) based on molecular and morphological data. *Lichenologist* **37:** 37–46.

Divakar, P. K., Blanco, O., Hawksworth, D. L. & Crespo, A. (2005*b*) Molecular phylogenetic studies on the *Parmotrema reticulatum* (syn. *Rimelia reticulata*) complex, including the confirmation of *P. pseudoreticulatum*. *Lichenologist* **37:** 55–65.

Divakar, P. K., Figueras, G., Hladun, N. & Crespo, A. (2010*a*) Morphological versus phylogenetic species: an example from *Melanelixia glabra* (*Parmeliaceae*, Ascomycota). *Fungal Diversity* **42:** 47–55.

Divakar, P. K., Cubas, P., Blanco, O., Del-Prado, R., Núñez-Zapata, J., Roca-Valiente, B., Lumbsch, H. T & Crespo, A. (2010*b*) An overview on hidden diversity in lichens: *Parmeliaceae*. <*imc9.info/prog_sig3_detail_divakar.htm*>

Elix, J. A. (1994*a*) *Rimelia*. *Flora of Australia* **55:** 186–188.

Elix, J. A. (1994*b*) Lichens, Lecanorales 2, *Parmeliaceae*. *Flora Australia* **55:** 1–360.

Elix, J. A. & Ernst-Russell, K. D. (1993) *A Catalogue of Standardized Thin Layer Chromatographic Data and Biosynthetic Relationships for Lichen Substances*, 2nd edn. Canberra: Australian National University.

Elix, J. A., Corush, J. & Lumbsch, H.T. (2009) Triterpene chemosyndromes and subtle morphological characters characterise lineages in the *Physcia aipolia* group in Australia (Ascomycota). *Systematics and Biodiversity* **7:** 479–487.

Fazekas, A. J., Kesanakurti, P. R., Burgess, K. S., Percy, D. M., Graham, S. W., Barrett, S. C. H., Newmaster, S. G., Hajibabaei, M. & Husband, B. C. (2009) Are plant species inherently harder to discriminate than animal species using DNA barcoding markers? *Molecular Ecology Resources* **9:** 130–139.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

Gardes, M. & Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2:** 113–118.

Grube, M. & Kroken, S. (2000) Molecular approaches and the concept of species and species complexes in lichenized fungi. *Mycological Research* **104:** 1284–1294.

Hale, M. E. (1965) A monograph of *Parmelia* subgenus *Amphigymnia*. *Contributions from the United States National Herbarium* **36:** 193–358.

Hale, B. W. & DePriest, P. T. (1999) Mason E. Hale's list of epithets in the parmelioid genera. *Bryologist* **102:** 462–544.

Hale, M. E. & Fletcher, A. (1990) *Rimelia* Hale & Fletcher, a new lichen genus (Ascomycotina: *Parmeliaceae*). *Bryologist* **93:** 23–29.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & deWaard, J. R. (2003*a*) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London, Series B* **270:** 313–321.

Hebert, P. D. N., Ratnasingham, S. & deWaard, J. R. (2003*b*) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London, Series B* **270:** S96–S99.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H. & Hallwachs, W. (2004*a*) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the Natinal Academy of Sciences of the United States of America* **101**(41)**:** 14812–14817.

Hebert, P. D. N., Stoeckle, M. Y., Zemlak, T. S. & Francis, C. M. (2004*b*) Identification of birds through DNA barcodes. *PLoS Biology* **3:** e422.

Hebert, P. D. N., deWaard, J. R. & Landry, J. F. (2010) DNA barcodes for 1/1000 of the animal kingdom. *Biology Letters* **6:** 359–362.

Hey, J. & Wakeley, J. (1997) A coalescent estimator of the population recombination rate. *Genetics* **145:** 833–846.

Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., Van der Bank, M., Chase, M. W., Cowan, R. S., Erickson, D. L., Fazekas, A. J. *et al.* (2009) A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 12794–12797.

Holsinger, K. E. & Weir, B. S. (2009) Genetics in geographically structured populations: defining, estimating and interpreting F_{ST}. *Nature Review Genetics* **10:** 639–650.

Hudson, R. R., Slatkin, M. & Maddison, W. P. (1992) Estimation of levels of gene flow from DNA sequence data. *Genetics* **132:** 583–589.

Huelsenbeck, J. P. & Ronquist, F. (2001) MrBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17:** 754–755.

Kelly, L. J., Hollingsworth, P. M., Coppins, B. J., Ellis, C .J., Harrold, P., Tosh, J. & Yahr, R. (2011) DNA barcoding of lichenized fungi demonstrates high identification success in a floristic context. *New Phytologist* in press (doi: 10.1111/j.1469-8137.2011.03677.x).

Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A. & Janzen, D. H. (2005) Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America* **102:** 8369–8374.

Krog, H. & Swinscow, T. D. V. (1981) *Parmelia* subgenus *Amphigymnia* (lichens) in East Africa. *Bulletin of the British Museum* (*Natural History*), *Botany Series* **9:** 143–231.

Kroken, S. & Taylor, J. W. (2001) A gene genealogical approach to recognize phylogenetic species boundaries in the lichenized fungus *Letharia*. *Mycologia* **93:** 38–53.

Kurokawa, S. (1991) Japanese species and genera of *Parmeliaceae*. *Journal of Japanese Botany* **66:** 152–159.

Kurokawa, S. (2003) *Checklist of Japanese Lichens*. Tokyo: National Science Museum.

Larena, I., Salazar, O., González, V., Julián, M. C. & Rubio, V. (1999) Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *Journal of Biotechnology* **75:** 187–194.

Lefébure, T., Douady, C. J., Gouy, M. & Gibert, J. (2006) Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. *Molecular Phylogenetics and Evolution* **40:** 435–447.

Liu, K., Raghavan, S., Nelesen, S., Linder, C. R. & Warnow, T. (2009) Rapid and accurate large-scale coestimation of sequence alignments and phylogenetic trees. *Science* **324:** 1561–1564.

Liu, J., Moller, M., Gao, L. M., Zhang, D. Q. & Li, D. Z. (2011) DNA barcoding for the discrimination of Eurasian yews (*Taxus* L., *Taxaceae*) and the discovery of cryptic species. *Molecular Ecology Resources* **11:** 89–100.

Llimona, X. & Hladun, N. L. (2001) Checklist of the lichens and lichenicolous fungi of the Iberian Peninsula and Balearic Islands. *Bocconea* **14:** 1–581.

Lohtander, K., Myllys, L., Sundin, R., Källersjö, M. & Tehler, A. (1998) The species pair concept in the lichen *Dendrographa leucophaea* (*Arthoniales*): analyses based on ITS sequences. *Bryologist* **101:** 404–411.

Lumbsch, H. T. (2002) Analysis of phenolic products in lichens for identification and taxonomy. In *Protocols in Lichenology. Culturing, Biochemistry, Ecophysiology and Use in Biomonitoring* (I. Kranner, R. P. Beckett & A. K. Varma, eds): 281–295. Berlin: Springer.

Meyer, C. P. & Paulay, G. (2005) DNA barcoding: error rates based on comprehensive sampling. *PLoS Biol* **3:** e422.

Molina, M. C., Crespo, A., Blanco, O., Lumbsch, H. T. & Hawksworth, D. L. (2004) Phylogenetic relationships and species concepts in *Parmelia* s. str. (*Parmeliaceae*) inferred from nuclear ITS rDNA and ß-tubulin sequences. *Lichenologist* **36:** 37–54.

Molina, M. C., Del-Prado, R., Divakar, P. K., Sánchez-Mata, D. & Crespo, A. (2011) Another example of cryptic diversity in lichen-forming fungi: the new species *Parmelia mayi* (Ascomycota: *Parmeliaceae*). *Organism Diversity and Evolution*, (in press).

Moon, K. H., Kurokawa, S. & Kashiwadani, H. (2000) A list of Thailand species of *Parmelia* (*sens. lat.*) preserved in the National Science Museum. In *Proceedings of the First and Second Symposia on Collection Building and Natural History Studies in Asia* (K., Matsuura, ed): 97–106. Tokyo: National Science Museum Monographs.

Moon, K. H., Kurokawa, S. & Kashiwadani, H. (2001) The genus *Rimelia* (lichens) from the Hawaiian Islands. *Journal of Japanese Botany* **76:** 321–328.

Myllys, L., Lohtander, K., Källersjö, M. & Tehler, A. (1999) Sequence insertions and ITS data provide congruent information on *Roccella canariensis* and *R. tuberculata* (*Arthoniales*, Euascomycetes) phylogeny. *Molecular Phylogenetics and Evolution* **12:** 295–309.

Nilsson, R. H., Kristiansson, E., Ryberg, M., Hallenberg, N. & Larsson, K. (2008) Intraspecific ITS variability in the Kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics* **4:** 193–201.

Nylander, J. A. A., Wilgenbusch, J. C., Warren, D. L. & Swofford, D. L. (2007) AWTY (Are we there yet?): A system for graphical exploration of MCMC convergence in Bayesian phylogenetics. *Bioinformatics* **24:** 581–583.

Posada, D. (2008) jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25:** 1253–1256.

Rambaut, A. & Drummond, A. J. (2003) Tracer [computer program]. Available from website http://tree.bio.ed.ac.uk/software/tracer/

Räsänen, A. V. (1944) Lichenes Novi I. *Annales Botanici Societatis Zoologicae-Botanicae Fennicae "Vanamo"* **20**(3)**:** 1–34.

Rodríguez, F., Oliver, J. F., Martín, A. & Medina, J. R. (1990) The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* **142:** 485–501.

Ross, K. G., Gotzek, D., Ascunce, M. S. & Shoemaker, D. D. (2010) Species delimitation: a case study in a problematic ant taxon. *Systematic Biology* **59:** 162–184.

Seifert, K. A., Samson, R. A., deWaard, J. R., Houbraken, J., Levesque, C. A., Moncalvo, J.-M., Louis-Seize, G. & Hebert, P. D. N. (2007) Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 3901–3906.

Simon, U. K. & Weiss, M (2008) Intragenomic variation of fungal ribosomal genes is higher than previously thought. *Molecular Biology and Evolution* **25:** 2251–2254.

Smith, M. E., Douhan, G. W. & Rizzo, D. M. (2007) Intra-specific and intra-sporocarp ITS variation of ectomycorrhizal fungi as assessed by rDNA sequencing of sporocarps and pooled ectomycorrhizal roots from a *Quercus* woodland. *Mycorrhiza* **18:** 15–22.

Stamatakis, A., Ludwig, T. & Meier, H. (2005) RAxML-III: A fast program for maximum likelihood-based inference of large phylogenetic trees. *Bioinformatics* **21:** 456–463.

Stamatakis, A., Hoover, P. & Rougemont, J. (2008) A rapid bootstrap algorithm for the RAxML webservers. *Systematic Biology* **57:** 758–771.

Strimmer, K. & von Haeseler, A. (1997) *Puzzle. Maximum Likelihood Analysis for Nucleotide, Amino Acid,*

and two-state Data. Version 4.0. Munich: University of Munich.

Swinscow, T. D. V & Krog, H. (1988) *Macrolichens of East Africa*. London: British Museum (Natural History).

Swofford, D. L. (2003). *PAUP*. Phylogenetic Analysis Using Parsimony* (**and Other Methods*). Sunderland, Massachusetts: Sinauer Associates.

Tavares, C. N. (1945) Contribução para o estudo das Parmeliáceas Portuguesas. *Portugaliae Acta Biologica, serie B*. **1:** 1–210.

Taylor, T. (1836) Musci, hepaticae and lichens. In *Flora Hibernica* (J.T. Mackay, ed.) **2:** 1–156. Dublin: W. Curry.

Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S. & Fisher, M.C. (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31:** 21–32.

Vialle, A., Feau, N., Allaire, M., Didukh, M., Martin, F., Moncalvo, J. M. & Hamelin, R. C. (2009) Evaluation of mitochondrial genes as DNA barcode for Basidiomycota. *Molecular Ecology Resources* **9** (suppl 1)**:** 99–113.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R. & Hebert, P. D. N. (2005) DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society, Biological Sciences* **360:** 1847–1857.

Ward, R. D., Holmes, B. H. & O'Hara, T. D. (2008) DNA barcoding discriminates echinoderm species. *Molecular Ecology Resources* **8:** 1202–1211.

Wedin, M., Westberg, M., Crewe, A. T., Tehler, A. & Purvis, O.W. (2009) Species delimitation and evolution of metal bioaccumulation in the lichenized *Acarospora smaragdula* (ascomycota, fungi) complex. *Cladistics* **25:** 161–172.

Zemlak, T. S., Ward, R. D., Connell, A. D., Holmes, B. H. & Hebert, P. D. N. (2009) DNA barcoding reveals overlooked marine fishes. *Molecular Ecology Resources* **9** (Suppl. 1)**:** 237–242.

Accepted for publication 27 *July* 2011