The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

Botanical Journal of the Linnean Society, 2016, **180**, 21–29. With figures

A DNA barcoding approach for identification of hidden diversity in Parmeliaceae (Ascomycota): *Parmelia sensu stricto* as a case study

PRADEEP K. DIVAKAR[1]*, STEVEN D. LEAVITT[2], M. CARMEN MOLINA[3], RUTH DEL-PRADO[1], H. THORSTEN LUMBSCH[2] and ANA CRESPO[1]

[1]*Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain*
[2]*Science and Education, Field Museum, Chicago, IL 60605, USA*
[3]*Departamento de Biología y Geología (Área de Biodiversidad y Conservación), ESCET, Universidad Rey Juan Carlos, Móstoles, 28933 Madrid, Spain*

Received 1 May 2015; revised 17 August 2015; accepted for publication 8 October 2015

Accurate specimen identification is challenging in groups with subtle or scarce taxonomically diagnostic characters, and the use of DNA barcodes can provide an effective means for consistent identification. Here, we investigate the utility of DNA barcode identification of species in a cosmopolitan genus of lichen-forming fungi, *Parmelia* (Parmeliaceae). Two hundred and two internal transcribed spacer (ITS) sequences generated from specimens collected from all continents, including Antarctica, were analysed, and DNA barcodes of 14 species of *Parmelia s.s.* are reported. Almost all species show a barcode gap. Overall, intraspecific divergence values were lower than the threshold previously established for Parmeliaceae. However, the mean and range were elevated by deep barcode divergences in three species, indicating the likely occurrence of overlooked species-level lineages. Here, we provide a DNA barcode reference library with well-identified specimens sampled worldwide and sequences from most of the type material to enable easy and fast accurate sample identification and to assist in uncovering overlooked species in *Parmelia s.s.* Further, our results confirm the efficiency of the ITS region in the identification of species of *Parmelia s.s.* © 2015 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2015, **180**, 21–29.

ADDITIONAL KEYWORDS: cryptic species – DNA barcodes – genetic distances – ITS – lichenized fungi.

INTRODUCTION

Morphology-based species boundaries have traditionally served as the foundation of taxonomy. However, phenotype-based taxonomy may not reflect natural groups, including cases in which morphologically distinct forms formerly recognized as distinct species are shown to represent a polymorphic species, and cases in which multiple morphologically similar species are masked within a single nominal taxon (Bickford *et al.*, 2007; Crespo & Pérez-Ortega, 2009; Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011). The term 'cryptic species' is commonly applied when two or more distinct species are erroneously classified and hidden under a single nominal taxon (Bickford *et al.*, 2007). The occurrence of species-level lineages that defy current phenotype-based taxonomy is now viewed as a common phenomenon in almost all organismal groups, including animals, bacteria, fungi, protists and plants (reviewed in Beheregaray & Caccone, 2007; Bickford *et al.*, 2007; Pfenninger & Schwenk, 2007; Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011; Poulin, 2011; Hawksworth, 2012; Scheffers *et al.*, 2012). The accurate recognition of cryptic diversity has important implications for improving our understanding of ecological, biogeographical, evolutionary and other biological patterns (Kress *et al.*, 2015).

*Corresponding author. E-mail: pdivakar@farm.ucm.es

In lichen-forming fungi, cryptic species-level lineages have already been detected in early molecular studies involving Parmeliaceae, in *Parmelia* Acharius (Crespo *et al*., 1999) and in *Letharia* (Motyka) Krog (Kroken & Taylor, 2001). Since these initial studies, many more cryptic species have been detected in all major lineages of lichen-forming fungi (reviewed by Crespo & Pérez-Ortega, 2009; Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011; see also, for example, Leavitt *et al*., 2012, 2013a, b, 2014; Parnmen *et al*., 2012; Lücking *et al*., 2014). This trend in the discovery of cryptic species over the last decade is largely a result of molecular phylogenetic studies of taxa with putative cosmopolitan and/or widely disjunct distributions. In Parmeliaceae, nearly every nominal species with such a wide distribution pattern that has been critically investigated using molecular sequence data appears to comprise multiple species-level lineages; to date, *c*. 80 of these have been detected in Parmeliaceae (Crespo & Lumbsch, 2010). As a result of more extensive research activity and especially as a consequence of increased use of molecular data, the number of new species described in Parmeliaceae has increased in recent years.

Although Parmeliaceae appears to have relatively high cryptic species-level diversity, high levels of cryptic diversity are also found in a number of other families of lichen-forming Ascomycota, including Graphidaceae and Lobariaceae (Moncada, Reidy & Lücking, 2014; Sohrabi, Lücking & Lumbsch, 2014). Given the overall prevalence of morphologically cryptic species in lichen-forming fungi, the implementation of accurate and efficient methods for the recognition of this diversity is imperative to advancing our understanding of these fungi. Using *Parmelia* as a case study, we implement a DNA barcoding approach for rapid, accurate specimen identification (Hebert & Gregory, 2005) in this taxonomically challenging genus that is known to contain a number of cryptic species.

Parmelia belongs to the parmelioid crown of the family and includes 41 currently accepted species (Crespo & Lumbsch, 2010; Crespo, Divakar & Hawksworth, 2011; Thell *et al*., 2012). Species in this genus are distinguished from those in other genera by having a foliose thallus with simple to furcate and squarrose rhizines on the lower surface, non-pored epicortex, effigurate to elongate pseudocyphellae on the upper surface, isolichenan, cupulate exciple three-layered, thin to thick hyaline layer, thin intermediate layer and cortex-like basal layer, and cylindrical or bifusiform conidia. In this relatively small genus, seven cryptic species have been formally described based on various combinations of subtle morphological differences, distinct ecological and geographical distributions, and evidence from molecular sequence data. In *P. saxatilis* (L.) Ach. *s.l.*, Mediterranean populations were described as a separate species, *P. serrana* A.Crespo, M.C.Molina & D.Hawksw. by Molina *et al*. (2004), *P. ernstiae* Feuerer & A. Thell was described by Feuerer & Thell (2002), eastern North American populations were segregated as a distinct species, *P. mayi* Divakar, A.Crespo & M.C.Molina by Molina *et al*. (2011a) and a fourth species *P. imbricaria* ined. is going to be published in M. C. Molina *et al*. (unpubl. data). In *P. sulcata* Taylor, two species have been described: *P. barrenoae* Divakar, M.C.Molina & A.Crespo (Divakar *et al*., 2005) and *P. encryptata* A.Crespo, Divakar & M.C.Molina (Molina *et al*., 2011b). Another distinct species-level lineage, *P. sulymae* ined., was previously included in the North American morpho-species *P. hygrophila* Goward & Ahti (M. C. Molina *et al*., unpubl. data).

Parmelia s.s. is a widespread genus with centres of distribution in boreal-temperate Europe, North America and in eastern Asia (Hale, 1987; Crespo & Lumbsch, 2010). The Australasian species have recently been accommodated in a new genus: *Notoparmelia* A.Crespo, Ferencova & Divakar (Ferencova *et al*., 2014). Despite the segregation of several cryptic species from *P. saxatilis* and *P. sulcata*, these taxa remain the most widely distributed species in this genus in cold-temperate areas of both hemispheres, the former also being known to occur in Antarctica (Molina *et al*., 2011a, b). Contrasting patterns of broad geographical and ecological distributions in some *Parmelia* spp. and geographically restricted distributions in other congeners further confound accurate sample identification in this group (Crespo & Lumbsch, 2010; Crespo *et al*., 2011; Thell *et al*., 2012).

Despite progress in accurate species circumscriptions in *Parmelia*, morphology-based specimen identification remains challenging because of the difficulties in discerning subtle diagnostic morphological characteristics. Therefore, this genus provides a valuable model system to test the potential of DNA barcoding for the estimation of the level of biodiversity and accurate sample identification. Here, we aim to provide a DNA barcoding tool for rapid, accurate sample identification and detection of cryptic diversity in *Parmelia*. To address this issue, we selected the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, as this locus has been accepted as a universal DNA barcode marker for fungi (Schoch *et al*., 2012), and sampled 202 specimens of *Parmelia s.s.* worldwide for several of the included taxa, representing 13 formally described species and a single undescribed species-level lineage in this genus.

MATERIAL AND METHODS

Taxon sampling

For this study, we sampled 202 specimens representing 14 distinct clades of *Parmelia s.s.* (see Supporting information, Table S1). Thirteen of the 14 clades represent previously described species: *P. barrenoae* (15 specimens); *P. encryptata* (six); *P. ernstiae* (17); *P. fertilis* Müll. Arg. (three); *P. fraudans* (Nyl.) Nyl. (three); *P. imbricaria* ined. (three); *P. mayi* (five); *P. omphalodes* (L.) Ach. (five); *P. saxatilis* (52); *P. serrana* (29); *P. submontana* Hale (six), *P. sulcata* (50); and *P. sulymae* ined. (three). The final clade comprised five specimens in the nominal taxon *P. fertilis* ('*fertilis* B') which were recovered in a distinct, non-sister clade to other representatives of *P. fertilis s.s.* ('*fertilis* A'). Specimen identifications were generally based on diagnostic morphological and chemical characters, although final determinations for a number of morphologically ambiguous specimens were confirmed using phylogenetic reconstructions of ITS sequence data. Samples were gathered worldwide from Antarctica, Asia, Europe, North America, North Africa and South America. *Notoparmelia signifera* (Nyl.) A.Crespo, Ferencova & Divakar and *N. subtestacea* (Hale) A.Crespo, Ferencova & Divakar were used as outgroups based on exploratory analyses.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

For all new material collected for this study, we extracted total genomic DNA from a small portion of the thallus. To reduce the potential for contamination, samples were carefully prepared and visible symptoms of non-target fungal growth were removed. Small pieces (*c.* 2 mm^2) were carefully separated, soaked in acetone for 2 h to remove potential secondary metabolites and dried overnight. Samples were ground with sterile pestles into liquid nitrogen and later into lysis buffer at 65 °C, incubated initially at 65 °C for 2 h, and then kept at room temperature overnight. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions with slight modifications (Crespo, Blanco & Hawksworth, 2001). PCR amplifications of ITS were performed using fungal-specific primers ITS1F (White *et al.*, 1990) and ITS4A (Larena *et al.*, 1999). Genomic DNA (1–10 ng) was used for amplifications of the ITS region. The 25-μL PCRs contained 1 × buffer (containing 10 mM Tris, pH 9.0, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.5 μM each primer, 1.25 units Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 1–10 ng genomic DNA extract. PCR amplifications were carried out in a Techne R TC-3000 thermal cycler, with the following conditions: one initial heating step of 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 54 °C and 1.5 min at 72 °C. A final extension step of 5 min at 72 °C was added, after which the samples were kept at 4 °C. Amplification products were viewed on a 1% agarose gel stained with SYBR Safe DNA (Life Technologies Corporation, Grand Island, NY, USA) and purification was performed by adding 2 μL ExoSAP-IT™ (Exonuclease 1-shrimp alkaline phosphatase) to 10 μL of the PCR products, followed by a heat treatment of 15 min at 37 °C and 15 min at 80 °C. Both complementary strands were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with the same primers as used in the amplification step and with the following settings: initial denaturation at 94 °C for 3 min; 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Sequencing reactions were electrophoresed on a 3730 DNA analyser (Applied Biosystems) at the Unidad de Genómica (Parque Científico de Madrid, Madrid, Spain).

Sequence assembly and multiple sequence alignments

Contigs were assembled and edited using the program SeqMan v.7 (Lasergene R, DNASTAR, Madison, WI, USA). Sequence identity was assessed using the mega-BLAST search function in GenBank (Sayers *et al.*, 2011). Sequences were aligned using the program MAFFT v7, implementing the G-INS-I alignment algorithm, '200PAM/K = 2' scoring matrix and with an offset value of 0.0, with the remaining parameters set to default values. Exploratory analyses revealed that multiple sequence alignments of our ITS sequences were consistent across a broad range of parameter settings. For comparisons of intraspecific genetic distances within each of the 14 species-level clades sampled for this study, intraspecific sequences were aligned using the G-INS-I alignment algorithm, '1PAM/K = 2' scoring matrix and an offset value of 0.2, with the remaining parameters set to default values for all species-specific alignments.

Phylogenetic analysis and genetic distance estimates

We used the program RAxML v8.1.1 (Stamatakis, 2006; Stamatakis, Hoover & Rougemont, 2008) to reconstruct a maximum likelihood (ML) topology from the ITS alignment of all sampled *Parmelia* specimens. The optimal ML topology was inferred using 200 separate ML searches under the 'GTRCAT' model; bootstrap support (BS) for each node was assessed

using 1000 pseudoreplicates. The ML tree was rooted using mid-point rooting. Species were scored as successfully discriminated if sequences formed a species-specific clade with BS ≥ 70% (Kelly *et al*., 2011).

We calculated uncorrected pairwise distances to characterize both intra- and interspecific variation within and among sampled *Parmelia* spp. Intraspecific genetic distances of the sampled taxa were compared with a proposed threshold between intra- and interspecific distances close to 0.015–0.017 substitutions per site in Parmeliaceae (Del-Prado *et al*., 2010). Uncorrected genetic distances based on pairwise comparisons were estimated using the program PAUP* v4.0a.146 (Swofford, 2002). Pairwise distances between different haplotypes were reported as the number of nucleotide substitutions per site.

RESULTS

The ITS data matrix representing *Parmelia s.s.* consisted of 202 sequences and 458 aligned nucleotide position characters (see Tables S1, 1; TreeBase ID: 18060). All new sequences generated for this study have been deposited in GenBank under accession numbers KT892942–KT892945.

Phylogenetic analysis and species discrimination

All species of *Parmelia s.s.* were recovered as monophyletic clades with strong BS (BS ≥ 82%) in the ITS gene tree, with the exception of *P. ernstiae* which was recovered with BS < 50% (Fig. 1; see Supporting information, Fig. S1). Two well-supported clades were recovered in *P. fertilis s.l.* (Fig. 1; Table 1). These two clades were treated as *P.* '*fertilis* A' and *P.* '*fertilis* B' in subsequent comparisons of genetic distances.

Genetic distances

Samples sizes for each species/clade, number of haplotypes, alignment lengths and mean and range of genetic distances are summarized in Table 1. The distribution of intraspecific pairwise distances for each species is shown in Figure 2A. A barcode gap was not detected between intra- and interspecific distances (Fig. 2B). The vast majority of intraspecific pairwise comparisons of genetic distances for all *Parmelia* spp. or clades fell below the estimated 0.015–0.017 substitutions per site intra–interspecific threshold (Fig. 2B), although the overall range of genetic distances exceeded this threshold in *P.* '*fertilis* B', *P. omphalodes* and *P. serrana* (excluding outliers; Table 1; Fig. 2B).

DISCUSSION

Molecular sample identification can provide an effective approach for consistent, accurate specimen identifications in taxonomically challenging groups (Kelly *et al*., 2011). Here, we provide support for the effective implementation of DNA barcode identification of samples from the lichen-forming fungal genus *Parme-*

Table 1. Mean genetic distance values (given as number of nucleotide substitutions per site) and range of intraspecific distances for the sampled species of *Parmelia s.s*. Numbers in parentheses indicate the number of sampled individuals/number of unique haplotypes, and values following the mean genetic distance represent standard deviations

Species	*N*	Alignment length (bp)	Mean	Range
P. barrenoae	15	457 (4/2)	0.0019 ± 0.0018	0.0–0.0070
P. encryptata	6	458 (2/1)	0.0019 ± 0.0018	0.0–0.0045
P. ernstiae	17	458 (11/2)	0.033 ± 0.0044	0.0–0.01825
P. fertilis s.l.	8	458 (21/9)	0.0172 ± 0.0107	0.0–0.0349
P. fertilis A	3	458	0.0 ± 0.0	0.0–0.0
P. fertilis B	5	458 (11/0)	0.0 ± 0.0177	0.0059–0.0094
P. fraudans	3	458	0.0051 ± 0.0013	0.0044–0.0066
P. imbricaria ined.	3	458	0.0015 ± 0.0013	0.0–0.0022
P. mayi	5	458 (0/0)	0.0 ± 0.0	0.0–0.0
P. omphalodes	5	458 (8/3)	0.0085 ± 0.0048	0.0022–0.0181
P. saxatilis	52	458 (22/8)	0.0031 ± 0.0041	0.0–0.0203
P. serrana	29	457 (16/7)	0.0063 ± 0.0050	0.0–0.0229
P. submontana	6	458 (4/0)	0.0029 ± 0.0043	0.0–0.0089
P. sulcata	50	458 (9/8)	0.0034 ± 0.0036	0.0–0.0205
P. sulymae ined.	3	458	0.0 ± 0.0	0.0–0.0
Interspecific	202	458 (135/104)	0.0642 ± 0.0329	0.0044–0.1158

N, number of specimens.

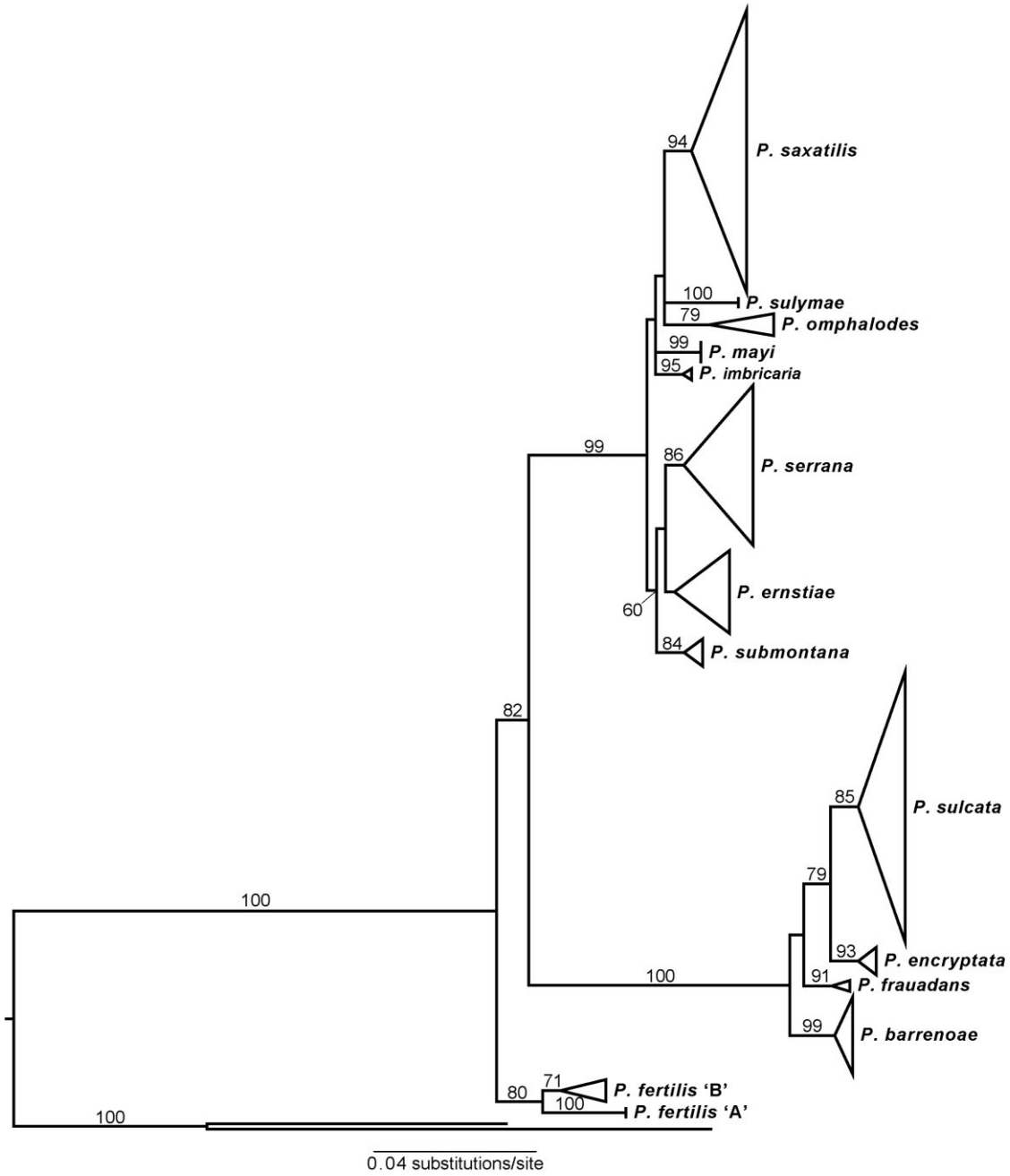

Figure 1. Diagram of the maximum likelihood internal transcribed spacer (ITS) tree obtained from 202 *Parmelia* samples. The complete tree is presented in Figure S1. Values at each node indicate non-parametric bootstrap support. Only support values of ≥ 70% are indicated.

lia representing species collected across broad geographical ranges, including Antarctica. Our results show that the ITS marker can differentiate 13 of the 14 taxa analysed in this study. We detected only one case, *P. ernstiae*, of barcode gap overlap in the 202 samples representing the 14 species analysed in our study. However, the distinction of this taxon as a separate lineage from *P. saxatilis* has been questioned in previous studies (Molina *et al*., 2004; Del-Prado *et al*., 2010), and additional studies are needed to clarify the taxonomic status of *P. ernstiae*. Overall, a molecular barcode identification approach for sample identification in cryptic species in *Parmelia* will aid in increasing our understanding of diversity, biogeography and landscape-level gene flow in this cosmopolitan genus.

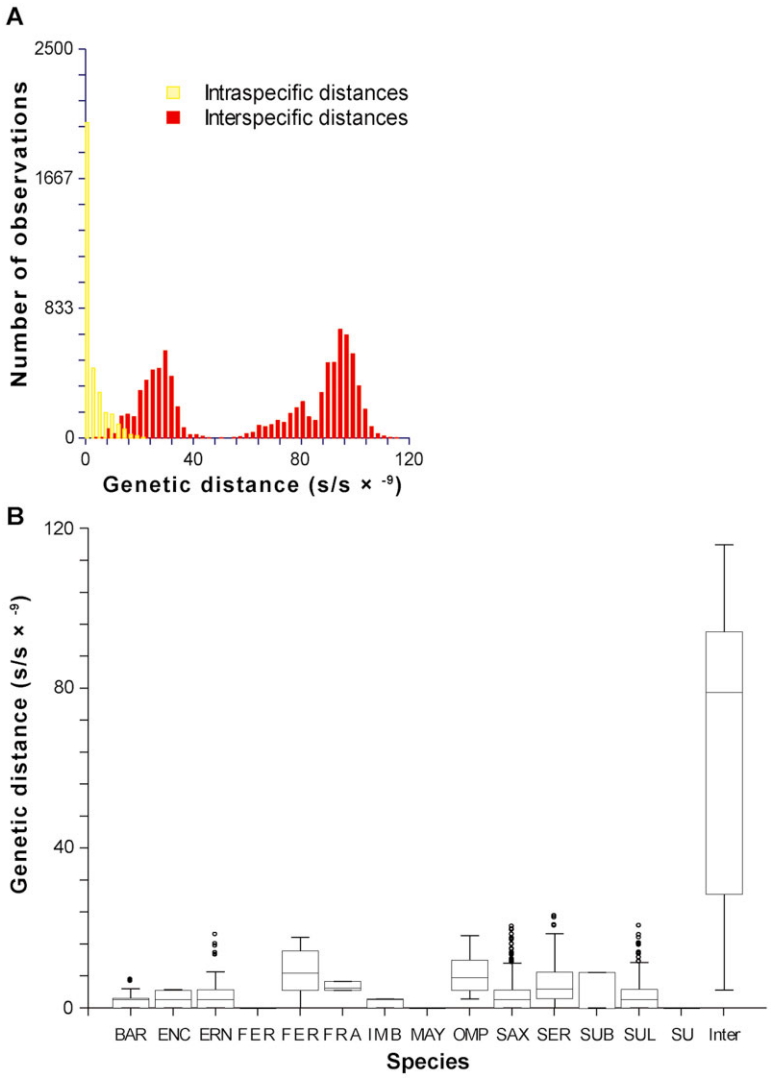

Figure 2. Intra- and interspecific pairwise genetic distances: (A) histogram and (B) box plot comparisons of genetic distances. BAR, *Parmelia barrenoae*; ENC, *P. encryptata*; ERN, *P. ernstiae*; FER, *P. fertilis* A; FER, *P. fertilis* B; FRA, *P. fraudans*; IMB, *P. imbricaria* ined.; MAY, *P. mayi*; OMP, *P. omphalodes*; SAX, *P. saxatilis*; SER, *P. serrana*; SUB, *P. submontana*; SUL, *P. sulcata*; SU, *P. sulymae* ined.; s/s, substitutions per site.

The inclusion of *P. saxatilis* populations from distant geographical regions, such as Antarctica, Asia, Europe and North America, and *P. sulcata* populations from Asia, Europe and North America did not reveal phylogeographical substructure or a significant increase in intraspecific genetic distances using ITS sequence data. This lack of substantial regional variation in ITS barcode sequences may suggest that an effective identification system can be constructed for mycobiota of *Parmelia s.s.* without extensive geographical surveys of each species. The ITS marker has been shown to discriminate a wide range of fungal species successfully (see Schoch *et al.*, 2012). Our results support the potential of ITS to develop effective DNA barcodes of this group of lichenized fungi, in accordance with the previous DNA barcoding studies in lichen-forming fungi (see, for example, Del-Prado *et al.*, 2010; Kelly *et al.*, 2011; Leavitt *et al.*, 2013a, 2014). However, limitations in molecular sample identification using ITS have been reported for a few genera of lichen-forming fungi, including *Cladonia* P.Browne and *Physcia* (Schreb.) Michx. (Myllys, Lohtander & Tehler, 2001; Pino-Bodas *et al.*, 2013).

Although most species studied here revealed relatively low intraspecific divergence compared with values proposed previously for Parmeliaceae (0.015–0.017 substitutions per site; Del-Prado *et al.*, 2010), three taxa exceeded this intraspecific threshold

(Table 1; Fig. 2B). We note that individuals of *P. fertilis* were separated into two groups, named here *P.* '*fertilis* A' and *P.* '*fertilis* B' (Figs 1, S1). Samples in the *P.* '*fertilis* B' clade are morphologically more similar to *P. omphalodes* than to the sister group *P.* '*fertilis* A'. Furthermore, individuals of the *P.* '*fertilis* B' clade are distributed in South-East Asia and north-western North America and the *P.* '*fertilis* A' clade includes samples from Japan, the type locality, whereas *P. omphalodes* occurs exclusively in Europe. Both the DNA divergence and different geographical distributions suggest that the two clades represent distinct species. However, in-depth taxonomic studies, including additional material from these clades, are critical to ascertain their species-level status. Other cases of deep barcode divergence included *P. serrana* and *P. omphalodes* (Table 1), indicating the existence of additional overlooked species. The inclusion of closely related taxa, such as *P. discordans*, is needed to ascertain the species-level status of European populations from the *P. omphalodes* clade, and this may alter the inferences from this study. In contrast, our data suggest that *P. ernstiae* may be conspecific with *P. saxatilis* (Fig. S1). *Parmelia ernstiae* was segregated from *P. saxatilis* based on limited morphological features, including a more pruinose upper surface and isidia often developing into flat laciniae, in addition to differences in ITS sequence (Feuerer & Thell, 2002). Some previous studies have also questioned the distinction of this taxon as a separate lineage (Molina *et al*., 2004; Del-Prado *et al*., 2010) and, ultimately, additional studies will be needed to clarify the taxonomic status of *P. ernstiae*.

Our study supports the validity of most species of *Parmelia s.s.* recognized in previous systematic studies, although we recovered evidence of deeply divergent lineages, indicating the presence of additional previously unrecognized lineages, in three of the 14 sampled species. The incidence of cryptic species encountered in the present study is congruent with the common phenomenon reported in Parmeliaceae and in lichenized fungi in general (reviewed in Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011; see also Leavitt *et al*., 2014).

The DNA barcode reference library from this study, including sequences of well-identified samples and type material of most of the species studied here, could be an asset for easy, rapid and accurate sample identification of species of *Parmelia s.s.* used in biomonitoring and bio-prospecting research. A number of species of *Parmelia s.s.* are frequently used in biomonitoring and climate change studies (Bennett, 2002; Blasco *et al*., 2011; Almeida *et al*., 2012). In addition, secondary metabolites found in *Parmelia* spp. have been shown to have high antimicrobial, antioxidant, cytotoxic and anti-tumour activities (reviewed in Gómez-Serranillos *et al*., 2014). Molecular identification of *Parmelia* spp. used in these types of applications may lead to a more precise interpretation of results in bio-monitoring and bio-prospecting research.

In summary, in this study, we have provided DNA barcodes for 14 clades in *Parmelia s.s.*, comprising 202 specimens collected from all continents, including Antarctica. Our results confirm the effectiveness of the ITS marker for the molecular identification of worldwide mycobiota of *Parmelia s.s.* Our study also provides evidence of additional previously unrecognized species-level diversity in *Parmelia* based on deep barcode divergences, setting the stage for their detailed taxonomic investigation. It is likely that the key findings of this comprehensive investigation will apply to most other taxonomic groups of lichen-forming fungi. We conclude that DNA barcoding can both enable the automated identification of known species and aid in the detection of unrecognized species in this group of lichenized fungi. Further, our study indicates the need for a comprehensive barcode library for massive improvement in our knowledge of the biodiversity of lichen-forming fungi.

ACKNOWLEDGEMENTS

We thank anonymous reviewers for valuable comments and suggestions. This work was supported by the Spanish Ministerio de Ciencia e Innovación (CGL2010-21646/BOS, CGL2013-42498-P), the Universidad Complutense-Banco Santander (GR3/14) and the National Science Foundation ('Hidden diversity in parmelioid lichens', DEB-0949147). Sequencing was performed in the Centro de Genómica y Proteómica del Parque Científico de Madrid, where Maria Isabel García Saez is especially thanked.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Maximum likelihood internal transcribed spacer (ITS) tree of the 202 sampled *Parmelia* specimens, with ≥ 50% bootstrap support indicated at the nodes. Type specimens of each species studied are indicated as 'Typus' or 'Epitypus'.

Table S1. Specimens of *Parmelia* used in this study with location, reference collection details and GenBank accession numbers. Newly obtained sequences are indicated in bold type. Type specimens of each species studied are indicated as 'Typus' or 'Epitypus' and highlighted in grey.