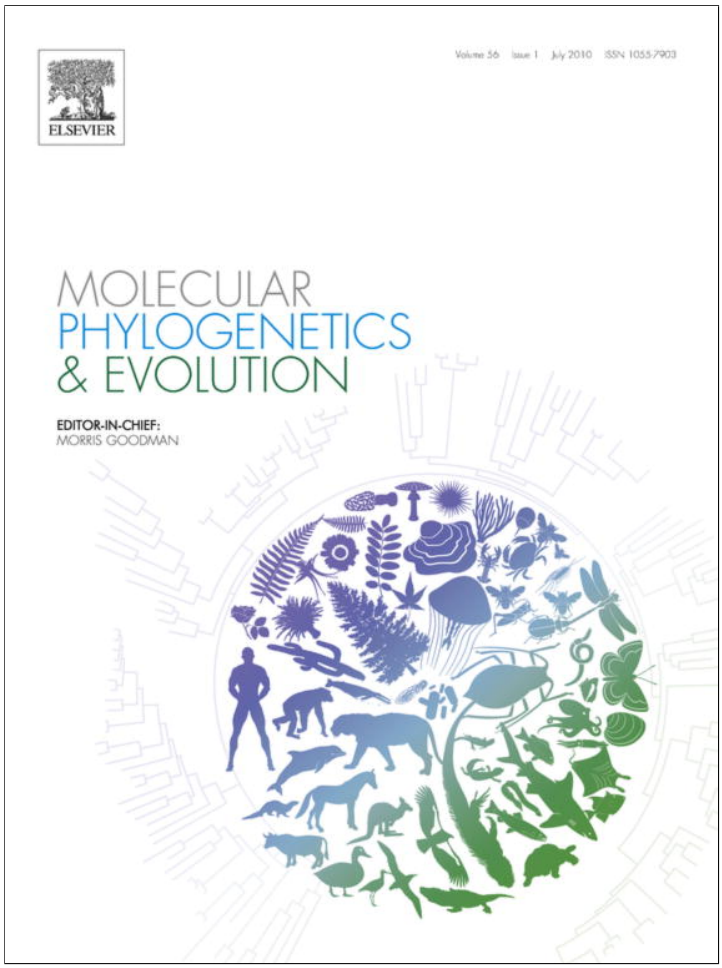

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Genetic distances within and among species in monophyletic lineages of Parmeliaceae (Ascomycota) as a tool for taxon delimitation

Ruth Del-Prado [a], Paloma Cubas [a,*], H. Thorsten Lumbsch [b], Pradeep K. Divakar [a], Oscar Blanco [c], Guillermo Amo de Paz [a], M. Carmen Molina [d], Ana Crespo [a]

[a] Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense, E-28040 Madrid, Spain
[b] Department of Botany, The Field Museum, 1400 S. Lake Shore Drive, Chicago, IL 60605-2496, USA
[c] Unidad de Bioanálisis, Centro de Investigación y Control de Calidad, Instituto Nacional del Consumo, Av. Cantabria s/n, 28042 Madrid, Spain
[d] Departamento de Biología y Geología, Universidad Rey Juan Carlos, 28933 Móstoles, Madrid, Spain

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ABSTRACT

The species delimitation in fungi is currently in flux. A growing body of evidence shows that the morphology-based species circumscription underestimates the number of existing species. The large and ever growing number of DNA sequence data of fungi makes it possible to use these to identify potential cases of hidden species, which then need to be studied with extensive taxon samplings. We used Parmeliaceae, one of the largest families of lichenized fungi as a model. Intra- and interspecific distances derived from maximum-likelihood phylogenetic trees inferred from 491 nuclear ITS rDNA sequences were examined for five major clades of parmelioid lichens. The intra- and interspecific distances were well separated in most cases allowing the calculation of a threshold, with exceptions of highly deviating distances in a few cases. These situations are shown to be taxa in which the current delimitation needs revision. Thus the analysis of the distance distributions is shown to be a powerful tool for identifying species complexes.

1. Introduction

The delimitation of species in fungi is currently in a state of flux. A growing body of evidence suggests that the current morphology-based species recognition method in fungi underestimates the true number of species. Numerous studies have found distinct phylogenetic lineages hidden under a single species name. Re-examination of morphology with the background of a molecular phylogenetic estimate revealed morphological and/or chemical characters, supporting the distinction of these clades at species level (Argüello et al., 2007; Baloch and Grube, 2009; Divakar et al., 2005a,b; Geml et al., 2006; Giraud et al., 2008; Grube and Kroken, 2000; Kauserud et al., 2006; Kroken and Taylor, 2001; Molina et al., 2004; Pringle et al., 2005; Wirtz et al., 2008). There are also cases of cryptic species in which no morphological characters could be identified to distinguish distinct lineages. In several cases distinct lineages are correlated with distinct biogeographical patterns (Argüello et al., 2007; Crespo et al., 2010; Molina et al., 2004; Wirtz et al., 2008).

Parmeliaceae (Ascomycota, Lecanorales) is one of the largest families of lichen-forming fungi, comprising more than 2000 species placed in about 90 genera (Crespo et al., 2007). One large group within this family is constituted by the parmelioid core with approximately 1500 species (Hale and DePriest, 1999) that were formerly placed in a broadly defined *Parmelia s.l.* genus (DePriest, 1999) and later split into many different genera. Circumscription of genera in lichen-forming fungi has been traditionally based on ascomatal characters as most relevant features. Generative characters were generally believed to be uniform within parmelioid lichens and hence, vegetative characters and secondary chemistry was employed to delineate genera (Elix, 1993; Hale, 1974, 1990; Krog, 1982). The use of vegetative and chemical characters in circumscribing genera in the absence of ascomatal differences has been criticized and has resulted in a lack of consensus of the generic circumscription within parmelioids. Consequently, acceptance of new genera has not been uniform (Clauzade and Roux, 1985; Eriksson and Hawksworth, 1998; Llimona and Hladun, 2001; Nimis, 1993). Molecular studies have indicated the existence of seven well-supported clades within the monophyletic parmelioid core group (Blanco et al., 2006). Several genera within these groups have been re-evaluated combining molecular and morphological data. These studies have resulted in the merging of some of the existing genera (Blanco et al., 2004a, 2005) and the segregation of new genera to recognize distinct clades taxonomically (Blanco et al., 2004b).

In addition to the generic limits, species boundaries have been intensively discussed in Parmeliaceae. A combination of morphological and molecular data has been used for revising species circumscriptions in several parmelioid genera (e.g. *Melanelixia,*

* Corresponding author. Fax: +34 913941774.
E-mail address: cubas@farm.ucm.es (P. Cubas).

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Parmelia, Parmelina, Parmotrema and *Punctelia*). This process has shed light onto some critical issues regarding cryptic and misunderstood taxa (Argüello et al., 2007; Crespo and Pérez-Ortega, 2009; Crespo et al., 2002; Divakar et al., 2005a; Fuerer and Thell, 2002; Molina et al., 2004).

The increase of DNA sequence data in public data bases is accelerated by intensified interest in using phylogenetic approaches to address biological questions and large scale sequencing initiatives, such as barcoding (Hebert et al., 2003, 2004; Kress et al., 2005; Moritz and Cicero, 2004; Seifert, 2009; Seifert et al., 2007). The use of molecular tools allows delimitation of monophyletic groups; however, the taxonomical rank attributed to these groups should not be based only on the topology of the tree but also on the correlation between morphological, anatomical, chemical and molecular features. The use of molecular data for species circumscription, using a genealogical concordance phylogenetic species recognition (GCPSR) (Matute et al., 2006; Pringle et al., 2005; Taylor et al., 2000) or cohesion species recognition (CSR) (Templeton, 2001; Wirtz et al., 2008) requires intense sampling of populations that cannot be done randomly for all taxa. Alternatively, genetic distance measurement can be used as a tool to investigate species limits and to identify 'genetic gaps' between monophyletic groups. In this approach species delineation relies on the use of threshold sets to differentiate between intraspecific variation and interspecific divergence. Once these thresholds are established, genetic distances help to elucidate species limits and/or to rank taxonomically monophyletic groups. Besides, in thoroughly sampled clades, genetic distances thresholds help to identify and assign specimens to taxonomic groups.

A large number of data is now available for ranges of intra- and interspecific distances in animals. Some key papers on this issue are those of Castresana (2001) (mammals), Hebert et al. (2004) (birds), Meyer and Paulay (2005) (marine gastropods), and Lefébure et al. (2006) (crustaceans) among others. The available literature for plants has also greatly increased during the last decade (Fazekas et al., 2009). Some general studies address species-level distinctions for economically important fungi (Seifert et al., 2007) and relationships between genetic distances and genus delimitation in fungal families (Lumbsch, 2002).

In this study we want to develop a quantitative method based on measurements of genetic distances that can be used for (1) identification of species complexes (i.e. species where morphologically discrete groups are not obvious but present polymorphisms in morphology, chemistry, reproductive modes or habitat preferences; Grube and Kroken, 2000), and (2) delimitation of species within the Parmeliaceae. The study is based on nuclear ITS sequences, since this marker has been widely used and has sufficient genetic variability at the species level (Gaya et al., 2008; Seifert, 2009; Summerbell et al., 2007). We use a thoroughly sampled clade (parmelioid genera) as a model group to assess the extent of and overlapping between intra- and interspecific genetic variation, in order to find potential relationships between the range of genetic distances and taxonomical ranks at lower levels (genera and species). The parmelioid core of Parmeliaceae is an ideal model because it encompasses numerous genera and species, it has been subject to many molecular and classical taxonomical studies, and several of its lower level taxonomic groups have recently been revised (Blanco et al., 2005; Divakar et al., 2005b; Molina et al., 2004; Thell et al., 2008).

2. Materials and methods

2.1. Taxon sampling

Representative taxa of five main monophyletic clades (Fig. 1 and Supplementary material) of the parmelioid core of Parmeliaceae (Parmelia, Parmelina, Parmotrema, Melanelixia and Xanthoparmelia clades; Lumbsch et al., 2008) were included in this study. We compiled a matrix of 124 species and 491 sequences. GenBank accession numbers are given in Table 1, and details of the number of species, specimens, haplotypes and matrix lengths are given in Tables 2 and 3.

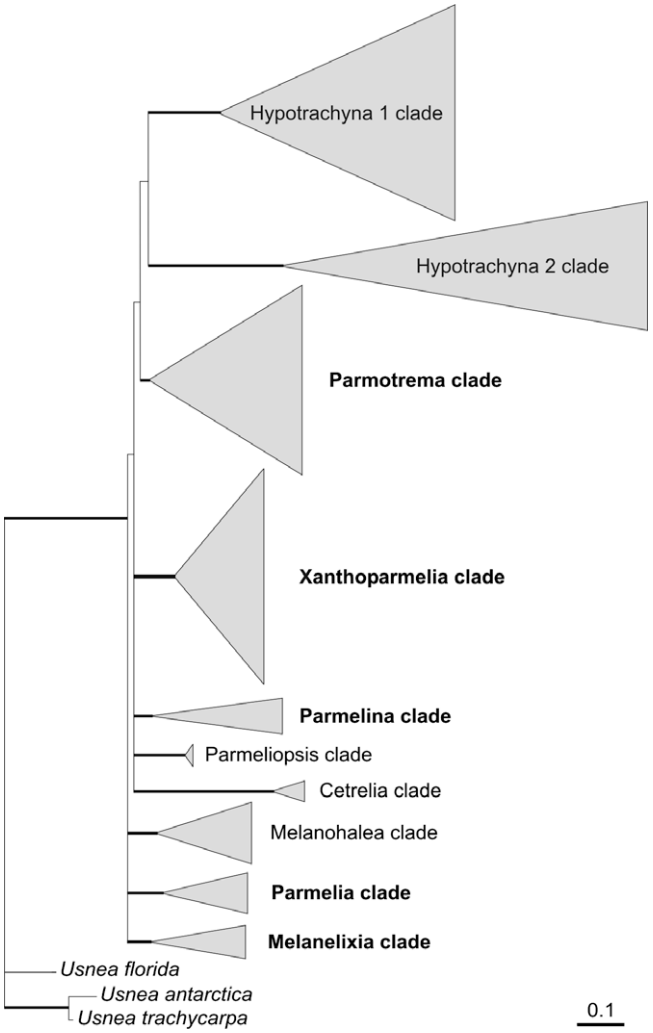

Fig. 1. Phylogenetic position of major clades of parmelioid lichens inferred from a combined analysis of nuclear ITS, nuclear LSU and mitochondrial SSU rDNA, sequences. Fifty percentage of majority-rule consensus tree of 56,000 trees sampled using a Bayesian MC/MCMC analysis. Branches with posterior probabilities above 0.94 and bootstrap support under parsimony equal or above 75% are indicated in bold (data from Lumbsch et al., 2008).

2.2. DNA extraction, PCR and sequencing

Total DNA was extracted from freshly collected material specimens, using the DNeasy Plant Mini Kit (Qiagen) following the instructions of the manufacturer, with slight modifications described in Crespo et al. (2001). Fungal nuclear ITS rDNA was amplified using the primers: ITS1F (Gardes and Bruns, 1993), ITS4 (White et al., 1990), ITS1-LM (Myllys et al., 1999), and ITS2-KL (Lohtander et al., 1998). Amplifications were performed in 50 μl volume containing 5 μl 10 × DNA buffer containing 2 mM $MgCl_2$ (Biotools), 1 μl dNTPs (10 mM of each base), 2.5 μl of each primer (10 μM), 1.25 μl DNA polymerase (1 U μl^{-1}), 27.75 μl distilled water and 10 μl of DNA template.

Table 1
GenBank accession numbers of the ITS sequences used in this study.

Species	GenBank accession numbers nuITS
Parmotrema clade	
Flavoparmelia aff. rutidota	HM010925, HM010926
F. baltimorensis	AY586559, AY586560
F. caperata	AY581059, AY586561, HM014172–HM014209
F. euplecta	HM010927, HM010928
F. ferax	HM010929
F. haysomii	DQ299904, HM010930, HM010931
F. haywardiana	HM010932–HM010934
F. Marchantii	DQ299905, HM010935
F. papillosa	HM010936
F. rutidota	DQ299906, HM010937–HM010939
F. secalonica	HM010940
F. soredians	AY586562, HM010941–HM010945, HM014210–HM014231
Flavoparmelia sp. 1	HM014232
Flavoparmelia sp. 2	HM014233
F. virensica	HM010946, HM010947
Parmotrema aff. abessinicum	HM017025
P. aff cetratum	AY642848–AY642850
P. aff. gardneri	HM017034
P. aff. perlatum	AF451749, HM017026, HM017027
P. cetratum	AY251449, AY586576, AY642847
P. crinitum	AY251442, AY586565, HM017028–HM017033
P. fistulatum	AY251415, AY581057
P. haitiense	AY581055
P. hypoleucinum	AY586567, HM017035–HM017037
P. margaritatum	HM017038
P. perlatum	AY586566, HM017039–HM017052
P. perforatum	AY586568
P. pilosum	AY581056
P. pseudoreticulatum	AY642828–AY642830, AY642841, AY642842, HM017053–HM017056
P. reticulatum	AY586577–AY586579, AY642817–AY642827, AY642831–AY642838, AY642843–AY642846, HM016953–HM016956, HM017057–HM017064
P. robustum	AY586569
Parmotrema sp. 1	HM016957–HM016960
Parmotrema sp. 2	AY642839, AY642840
Parmotrema sp. 3	HM016961
Parmotrema sp. 4	HM016962
P. subcaperatum	AY586557
P. subtinctorium	AY586558
P. tinctorum	AB177401–AB177404, AY251443, AY586570, DQ394372
P. xanthinum	HM016963
Punctelia aff. borreri 1	AY773111
P. aff. borreri 2	AY773115
P. borreri	AF451769, AY581088, AY613399–AY613401, AY613404, AY613405, AY613409, AY773110, AY773112–AY773114, AY773122, AY773124, DQ394373, HM016964–HM016966
P. perreticulata	AY613391, AY773123, HM016967
P. pseudocoralloidea	AY586572
P. reddenda	AY613410
P. rudecta	AY586573, AY586574, AY613402, AY613403
P. subrudecta	AY581089, AY613392–AY613398, AY773116–AY773118, HM016968–HM016980
P. subflava	AY586575
P. ulophylla	AY613406, AY613407
Parmelia clade	
Parmelia aff cochleata	AY036983
P. aff. saxatilis	AF350034, HM016981–HM016983
P. adaugescens	AY036991, AY036993
P. barrenoae	AY295103, AY579444, AY579446, AY579448, AY579450, AY579451, HM016984–HM016988
P. fertilis	AY036982
P. omphalodes	AF350046, AY036998, AY036999, AY251440, EF611295
P. pinnatifida	AY036988, EF611300
P. saxatilis	AF058037, AF141370, AF350020–AF350028, AF350030, AF350035, AF410835–AF410837, AF412309, AF412310, AF451770–AF451772, AY036989, AY036990, AY114359, HM016989–HM017000
P. serrana	AF350031, AF350036–AF350039, AF350044, AF350045, AY036996, AY036997, AY295104–AY295109, EU034668, HM030805, HM017001–HM017005
P. sulcata	AF410838–AF410840, AF451773, AF451774, AY036980, AY579447, AY579452, AY579453, AY580313, AY581083, EU788026–EU788028, HM017006–HM017020
P. squarrosa	AY036975–AY036979
P. submontana	AY037000, AY579458
Parmelina clade	
Parmelina carporrhizans	AY611105, DQ273849–DQ273854,
P. coleae	DQ273855, DQ273857, DQ273858
P. pastillifera	AY611104, DQ273860
P. quercina	DQ273842–DQ273848, HM017021–HM017024
P. tiliacea	AY581084, DQ273861
Melanelixia clade	
Melanelixia glabra	EU761204, EU761206–EU761209, EU761213–EU761215, AY581064
Xanthoparmelia clade	
Xanthoparmelia aff. delisei	AY581067
X. aff. glabrans	AY581072
X. atticoides	AY581066
X. amplexula	DQ167456
X. azaniensis	EF042900
X. bibax	GU992341
X. brachinaensis	AY581062
X. conspersa	AF451748, AY581096, DQ394369
X. convolutella	DQ167452
X. crespoae	AY581098
X. dayiana	DQ167457
X. delisei	AY581068
X. digitiformis	AY581099
X. exornata	EF042908
X. fissurina	GU992327
X. flindersiana	DQ167458
X. fumigata	DQ167459
X. glabrans	AY581069
X. hottentota	AY251452, AY340875, GU992326
X. hueana	AY581090
X. hypoleiella	DQ167455
X. ianthina	GU992331
X. incrustata	DQ167448
X. isidiigera	DQ167451
X. lineola	EF591823, EF591824
X. lithophila	AY581077
X. lithophiloides	AY251437, AY581078
X. loxodes	AY581070, AY581076
X. mougeotii	AY037006, AY581100
X. murina	AY251438, AY581079
X. neopropaguloides	GU992334
X. neorimalis	EF591821
X. neotinctina	DQ167460
X. norcapnodes	AY581080
X. ovealmbornii	EF042901
X. peltata	DQ980021
X. perfissa	GU992336
X. perspersa	GU902328–GU902330, GU902332, GU902333, GU902335, GU902337–GU902339, GU903338–GU903339
X. pertinax	DQ167462
X. pokornyi	AY581075
X. protomatrae	EU034671
X. pulla	AY581071
X. pulloides	AY037004
X. reptans	AY581102
X. ryssolea	GU902340
X. scotophylla	AY581081
X. subcrustacea	DQ167449
X. subincerta	AY581073

(*continued on next page*)

Table 1 (*continued*)

Species	GenBank accession numbers nuITS
X. sublaevis	AY581106
X. subspodochroa	AY581082
X. substrigosa	DQ167450
X. tasmanica	DQ167463, DQ167464
X. tegeta	AY581107
X. tinctina	AY581108, AY581110
X. transvaalensis	AY581095
X. versicolor	DQ167454
X. verrucigera	AY581111

The amplifications for nuITS rDNA were carried out in an automatic thermocycler (Techne Progene, Jepson Bolton & Co. Ltd., Waltford, Herts) using the following parameters: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 54–58 °C for 1 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. Amplification products were visualised on 1% agarose gels stained with ethidium bromide and subsequently purified using the Bioclean Columns kit (Biotools Madrid) according to the manufacturer's instructions. Fragments were sequenced using Big Dye Terminator reaction kit (ABI PRISM, Applied Biosystems). Sequencing and PCR amplifications were performed using the same sets of primers. Cycle sequencing was executed with the following settings: initial denaturation for 3 min at 94 °C followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min. Sequencing reactions were electrophoresed on a 3730 DNA analyser (Applied Biosystems). Sequence fragments obtained were assembled with SeqMan 4.03 (DNAStar Madison) and manually edited.

2.3. Sequence alignments, phylogenetics analyses, and calculation of genetic distances

ITS sequences were aligned separately for each clade (Parmelia, Parmotrema, Xanthoparmelia, Melanelixia and Parmelina clades). The alignments were made with Clustal W (Thompson et al., 1994) and ambiguously aligned regions were excluded using Gblocks (Castresana, 2000). If sequences had different lengths, only the part shared by all the sequences was used. The accuracy of the

Table 2
Parameters of the analysis of interspecific distances between the haplotypes of each genus. Mean, standard deviation (SD) and range are number of substitutions per site (s/s). Matrix length is indicated after name clade in base pairs (bp).

Genus	No. species	No. samples ()[a]	No. haplotypes	Mean ± SD	Range
Parmotrema clade (472 bp)					
Flavoparmelia	15	93 (85)	33	0.076 ± 0.029	0.016–0.160
Parmotrema	25	110 (52)	55	0.068 ± 0.024	0.018–0.150
Punctelia	10	56 (17)	27	0.076 ± 0.012	0.038–0.119
Parmelia clade (441 bp)					
Parmelia	12	120 (44)	51	0.060 ± 0.024	0.019–0.116
Parmelina clade (482 bp)					
Parmelina	5	25 (4)	12	0.082 ± 0.018	0.015–0.103
Melanelixia clade (441 bp)					
Melanelixia	1	9 (–)	8	–	–
Xanthoparmelia clade (474 bp)					
Xanthoparmelia[b]	56	78 (19)	72	0.103 ± 0.040	0.002–0.257
Total	124	491 (221)	258	–	–

[a] Number of new sequences obtained for this work.
[b] Taxa with anomalous ranges.

Table 3
Parameters of the analysis of intraspecific distances between haplotypes. Mean, standard deviation (SD) and range are number of substitutions per site (s/s). CD: Clonal diversity (Ellstrand and Roose, 1987).

Species	No. samples	No. haplotypes	CD	Mean ± SD	Range
Parmotrema clade					
Flavoparmelia caperata	40	7	0.17	0.005 ± 0.002	0.002–0.009
F. soredians	28	4	0.14	0.003 ± 0.001	0.002–0.004
Parmotrema pseudoreticulatum	9	2	0.22	0.002	0.002
P. reticulatum[a]	38	15	0.42	0.028 ± 0.011	0.002–0.047
Punctelia borreri	18	12	0.67	0.006 ± 0.003	0.002–0.011
P. subrudecta	24	4	0.17	0.006 ± 0.002	0.002–0.009
Parmelia clade					
Parmelia barrenoae	11	3	0.27	0.003 ± 0.001	0.002–0.005
P. saxatilis	36	12	0.33	0.007 ± 0.002	0.002–0.012
P. serrana	22	9	0.41	0.009 ± 0.004	0.002–0.016
P. sulcata	29	5	0.17	0.005 ± 0.003	0.002–0.009
Parmelina clade					
Parmelina carporrhizans	7	2	0.29	0.002	0.002
P. quercina	11	6	0.46	0.007 ± 0.004	0.002–0.013
Melanelixia clade					
Melanelixia glabra	9	8	0.89	0.008 ± 0.004	0.002–0.017
Xanthoparmelia clade					
Xanthoparmelia perspersa[a]	11	8	0.73	0.042 ± 0.015	0.002–0.066

[a] Taxa with anomalous ranges.

identifications and the monophyly of the clades were checked using a phylogenetic estimate with MrBAYES 3.1.1 (Huelsenbeck and Ronquist, 2001). The analyses were run as in Del-Prado et al. (2006). Pairwise maximum likelihood distances among sequences of each clade were calculated with TREE-PUZZLE 5.2 (Strimmer and Von Haeseler, 1997) using the HKY+G (Hasegawa et al., 1985) model of nucleotide substitution with among-site variation, and assuming a discrete gamma distribution with six rate categories.

Pairwise distances between different haplotypes are given as number of nucleotide substitutions per site (s/s), that is, number of different sites between two sequences divided by sequence length. The distances can be viewed as a rough measure for the overall sequence divergence. The genetic distances were separated into interspecific and intraspecific parameters. Intraspecific distances were calculated as the mean value of the pairwise distances between the haplotypes of each species (conspecific haplotypes). Distances were estimated in species with more than 10 different specimens, covering the distribution area as much as possible except in some species with restricted distribution where less than 10 sequences were used (e.g. *Parmelina carporrhizans* and *Melanelixia glabra*). Interspecific distances were calculated as the mean value of all pairwise distances between the haplotypes found in each genus (congeneric haplotypes), excluding distances between conspecific haplotypes. Graphs were plotted with SigmaPlot 8.0. ANOVA analyses were run with StatGraphics 5.1 to detect significative differences between mean values of interspecific and intraspecific distances.

3. Results

Two hundred and twenty-one new sequences were used in this study. Initially, four clades of Parmeliaceae (Parmelia, Parmotrema, Melanelixia and Parmelina clades) were selected. The matrix of each clade was aligned and analysed separately to verify identifications of the specimens. For each clade the pairwise distances between the different haplotypes were estimated and the distribution of distances plotted. Tables 2 and 3 show the length of matrices, number of haplotypes found at the specific and generic level, and mean values of distances between congeneric haplotypes (interspecific distances) and conspecific haplotypes (intraspecific distances).

Table 4
Analysis of variance of the interspecific and intraspecific pairwise distances between haplotypes of ITS sequences in parmelioids.

Source	Sum of squares	df	Mean square	F-ratio	*P*-value
Interspecific distances					
Among genera	0.107816	4	0.026954	47.02	0.0000
Within genera	1.79073	3124	0.000573215		
Total	1.89854	3128			
Intraspecific distances					
Among species within genera	0.000399545	11	0.0000363223	3.97	0.0000
Within species	0.00213271	233	0.00000915326		
Total	0.00253226	244			

3.1. Pairwise distances between congeneric haplotypes: interspecific distances

Mean values, standard deviation and range of interspecific distances within *Parmelia*, *Flavoparmelia, Parmotrema, Punctelia* and *Parmelina* are shown in Table 2 and Fig. 2. Mean values are of the same order of magnitude, ranging from 0.060 substitutions per site (s/s) in *Parmelia* to 0.082 s/s in *Parmelina*. The ANOVA analysis (Table 4) indicates a statistically significant difference between the means of the pairwise distances of the haplotypes of each genus at the 95% confidence level. These differences are due to the genera *Parmelia* and *Parmotrema* (lowest means), while *Flavoparmelia*, *Punctelia* and *Parmelina* form a homogeneous group in a multiple range test. The histograms of the interspecific distances distribution for each genus (Figs. 3 and 4), show only minor differences in the range of distances. The minimum values of interspecific haplotype distances are the following: 0.015 s/s (*Parmelina*), 0.016 s/s (*Flavoparmelia*), 0.018 s/s (*Parmotrema*), 0.019 s/s (*Parmelia*), and 0.038 s/s (*Punctelia*).

Xanthoparmelia is a large monophyletic genus with approximately 750 species, only partially studied by molecular analysis

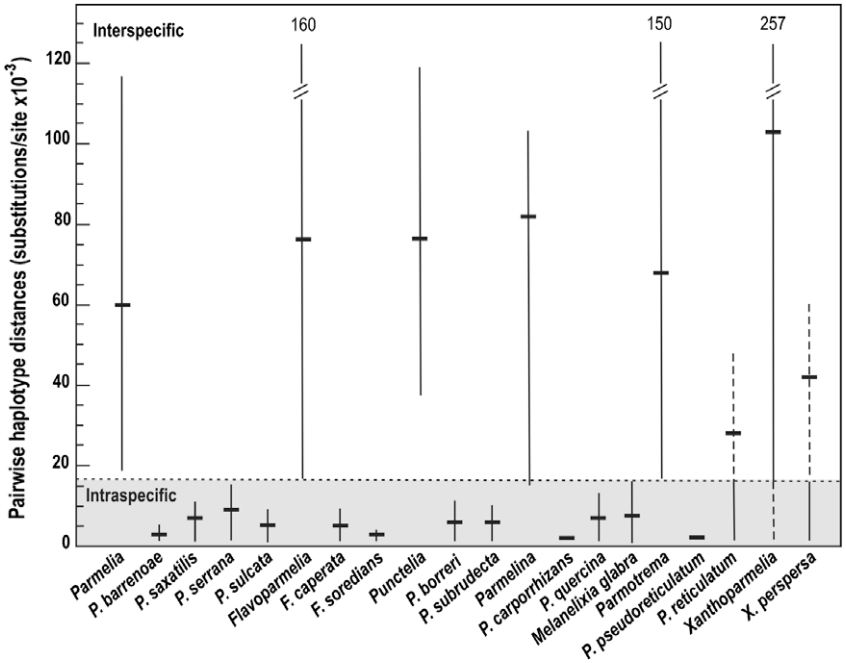

Fig. 2. Mean values and range of interspecific and intraspecific haplotype distances.

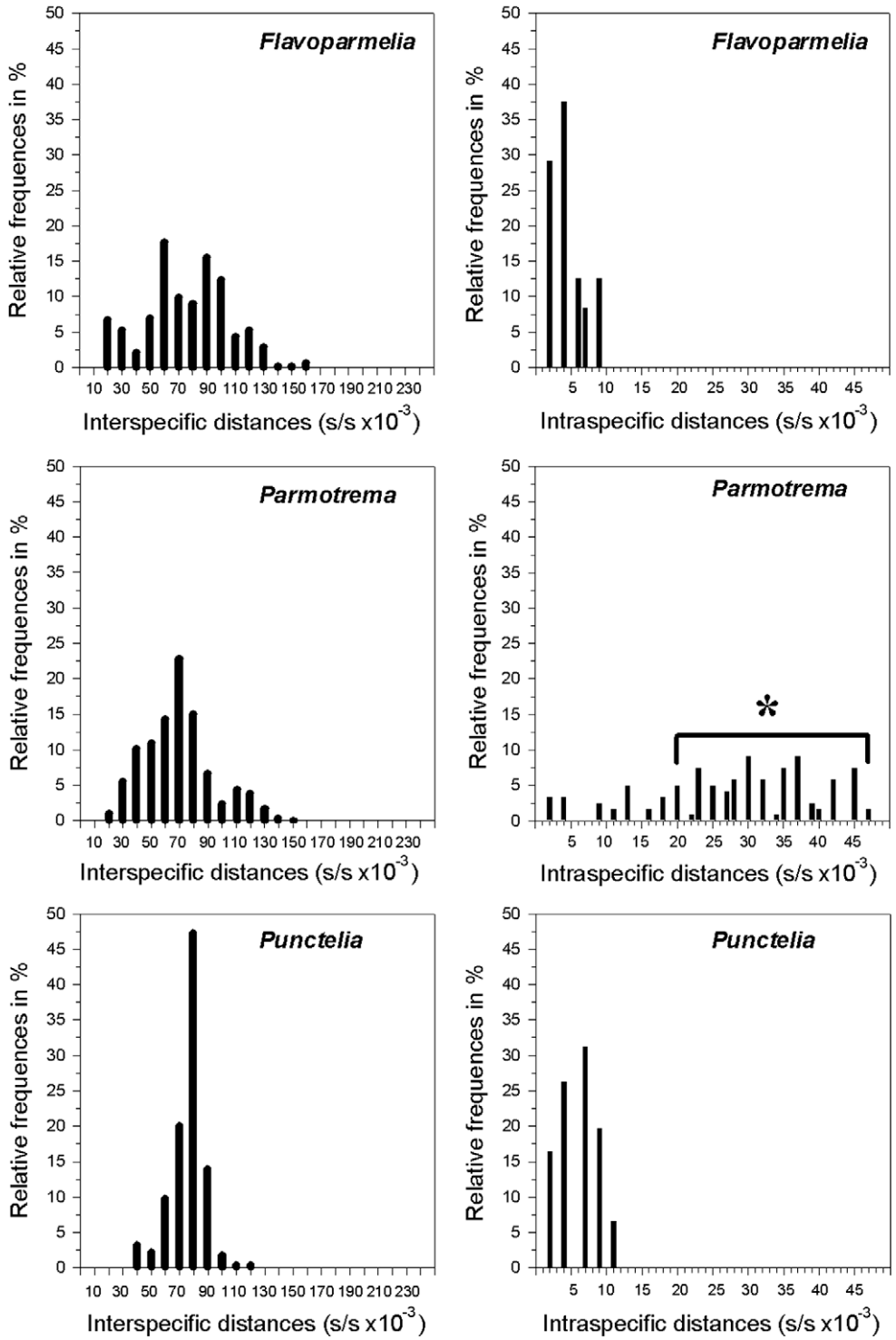

Fig. 3. Distribution of interspecific (left) and intraspecific (right) pairwise haplotype distances in the Parmotrema clade. Distances are in number of substitutions per site (s/s). *: Anomalous distances.

(Crespo et al., 2007; Thell et al., 2006). Seventy-two haplotypes of *Xanthoparmelia* were included in the analysis to test the results found for the above genera. Compared to the other genera *Xanthoparmelia* has a slightly higher (0.103 s/s) mean value of interspecific distances. However, the range of interspecific distances is different (Table 2, Fig. 2). *Xanthoparmelia* shows a minimum range value (0.002 s/s) that is 10 times lower than those of the other genera, whereas the maximum value (0.257 s/s) is almost twice the others (Table 2, Fig. 2). Low values in *Xanthoparmelia* (<0.015 s/s) are given by the distances between species that are either delimited by chemical characteristics and subtle morphological traits (e.g. *X. murina* and *X. norcapnodes*; *X. lithophiloides*, *X. lithophila* and *X. subspodochroa*; *X. dayiana* and *X. flindersiana*), or morphologically well differentiated (e.g. *X. pertinax*, *X neotinctina*). The higher values (>0.16 s/s) are due to distances between haplotypes of species previously included in two separate genera: *Almbornia* and *Omphalodiella*. These taxa share the general morphological and geographical characteristics of the other *Xanthoparmelia* species (Elix, 1993).

Besides, they are phylogenetically nested within *Xanthoparmelia* (Thell et al., 2006). However, the large genetic distances cause the long branches that leads to these species within *Xanthoparmelia* (Crespo et al., 2007).

3.2. Pairwise distances between conspecific haplotypes: intraspecific distances

We selected 13 species that were studied recently using phylogenetic analyses of molecular data (Argüello et al., 2007; Crespo et al., 2004; Divakar et al., 2005a,b, 2010; Molina et al., 2004) for the estimation of intraspecific distances (Table 3). Clonal diversity, mean values, standard deviation and range of distances between haplotypes of each species (intraspecific distances) are shown in Fig. 2 and Table 3. Clonal diversity (i.e. the proportion of distinguishable haplotypes divided by the sample size) is a good overall descriptor of the genetic diversity (Ellstrand and Roose, 1987). This value ranges from 0.14 in *Flavoparmelia soredians* (only four haplotypes were found in 28 specimens) to 0.89 in *Melanelixia glabra* (eight different haplotypes found in nine samples). Mean intraspecific values are $\leqslant$0.009 s/s. The ANOVA analysis indicates that there is a statistically significant difference between the means of the distances of the haplotypes of each species (95% confidence level) (Table 4). A multiple range test does not detect homogeneous

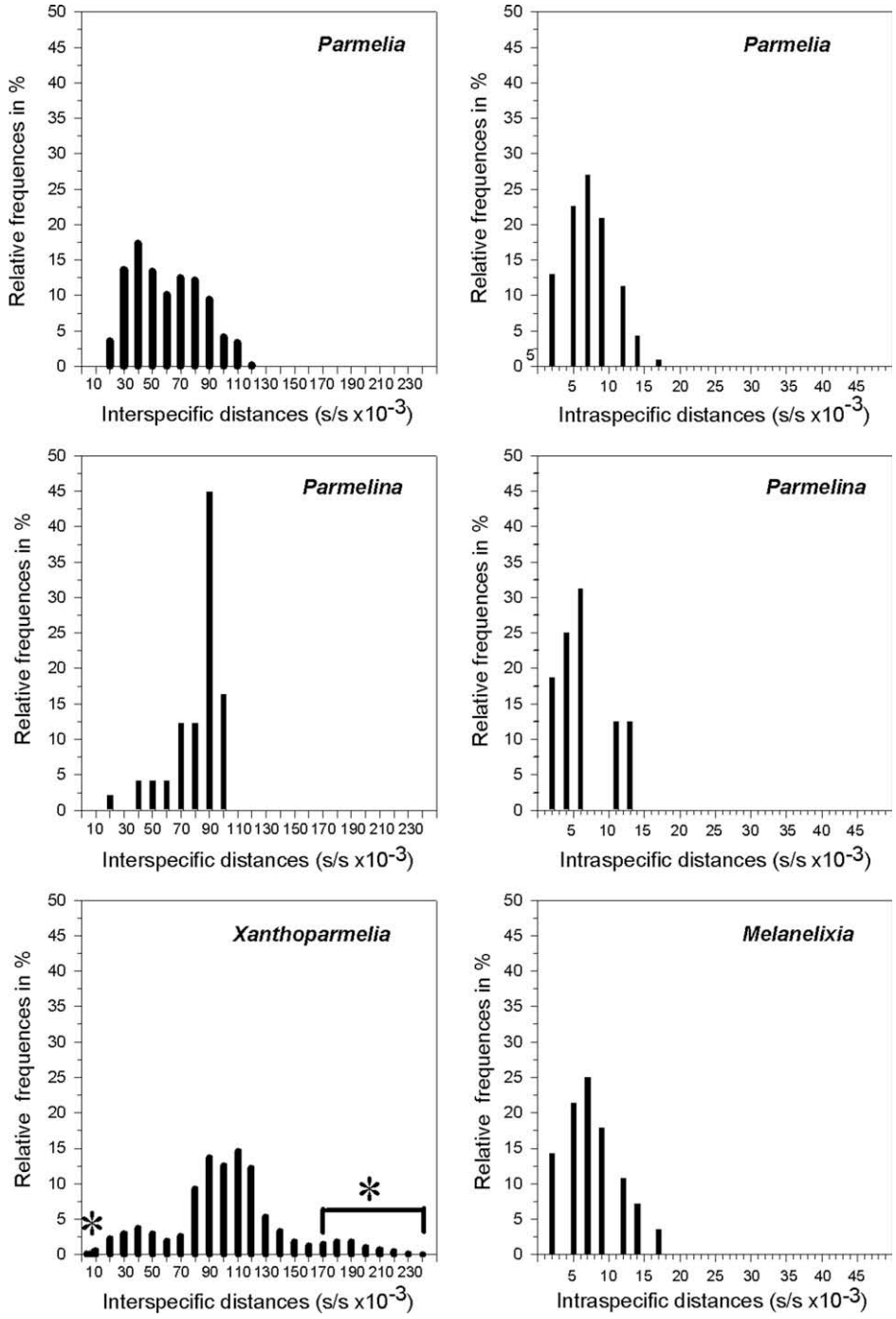

Fig. 4. Distribution of interspecific (left) and intraspecific (right) pairwise haplotype distances in the Parmelia, Parmelina, Xanthoparmelia and Melanelixia clades. Distances are in number of substitutions per site (s/s). *: Anomalous distances.

groups. The histograms of the intraspecific pairwise distances distribution for each genus (Figs. 3 and 4) show only minor differences in the range of distances between the different genera (Table 3). The maximum range values of intraspecific haplotype distances are ⩽0.017 s/s. No correlation between sexual reproductive mode (apothecia present vs absent) and intraspecific distances was found.

3.3. Threshold between intra- and interspecific distances

Distribution and ranges of intra- and interspecific pairwise distances (Tables 2 and 3 and Figs. 2–4) are separated in the studied taxa by a threshold close to 0.015–0.017 s/s. This threshold was tested in two species where we have preliminary evidence for the presence of cryptic species: *Parmotrema reticulatum* (Divakar et al., 2005b) and *Xanthoparmelia perspersa* (Crespo et al., unpublished results). Thirty-eight samples of *P. reticulatum* (16 haplotypes) and eleven samples of *X. perspersa* (8 haplotypes) were included in the analysis. Multiple range tests clearly indicated that the mean values of *X. perspersa* and *P. reticulatum* are significantly different from the other taxa (Table 3). Pairwise distances between haplotypes of *P. reticulatum* yield a remarkably high mean (0.028 ± 0.011), with a maximum (0.047 s/s) that is three times higher than the values found in other genera. This intraspecific maximum and the values of interspecific distances overlap (Fig. 2). The molecular data suggest the existence of cryptic lineages hidden under the name *P. reticulatum* (Divakar et al., 2005b). *Xanthoparmelia perspersa* also showed a remarkable high mean value of intraspecific distances (0.042 s/s), with a maximum (0.066 s/s) that is four times those of other genera.

To check the validity of the threshold concept to distinguish between inter- and intraspecific distance, we tested closely related species in the genus *Parmelia*, which have recently been studied. For this test we merged *P. serrana* and *P. saxatilis* (Molina et al., 2004); and *P. barrenoae* and *P. sulcata* (Divakar et al., 2005a), respectively. These pairs have been regarded as two variable species before a molecular analysis revealed that they actually represent distinct lineages. When *P. serrana* is merged into *P. saxatilis*, and *P. barrenoae* into *P. sulcata*, the range of *Parmelia* intraspecific distances increases with a maximum of 0.045 s/s that overlap the interspecific range consistent with our threshold concept.

4. Discussion

Using Parmeliaceae, the most speciose family of lichen-forming macrolichens as a model, we show that the comparison of inter- and intraspecific genetic distances is a powerful tool to identify species complexes that require thorough molecular studies to address the species delimitation in these taxa. While the intra- and interspecific distances showed generally no overlap, those taxa in which overlap was demonstrated are considered species complexes based on previous evidence. Further testing the threshold concept using two previously studied *Parmelia* species corroborated these results: splitting of taxa decreases intraspecific variation and interspecific divergence, whereas lumping of taxa increases both parameters (Meyer and Paulay, 2005). The results of our study indicate that in parmelioid lichens pairwise distances between haplotypes of the same species (intraspecific) are ⩽0.017 s/s. The higher distances in *Parmotrema reticulatum* suggest that this lineage has already undergone molecular divergence, although not yet accompanied by morphological or ecological differences. Similarly intraspecific distances and interspecific values overlap in *Xanthoparmelia*, suggesting that several distinct lineages are hidden under the name *X. perspersa*, a species defined on chemical and morphological grounds (Hale, 1989). With the sole exception of *Xanthoparmelia*, pairwise interspecific distances are ⩾0.015 s/s. The results indicate that in parmelioid genera a threshold between intraspecific and interspecific distances is between 0.015 and 0.017 s/s for ITS sequences.

Although molecular tools allow delimitation of monophyletic groups, the attribution of a taxonomic rank for a given lineage it is not straightforward. Different authors have suggested that genetic distances could help in decision making regarding taxonomic ranks (Avise and Aquadro, 1982; Castresana, 2001; Johns and Avise, 1998; Nimis, 1998; Lumbsch, 2002). More recently Stuessy (2009) stressed the importance of specific quantitative methods that could help to make decisions in the recognition of groups in formal classification. Our results indicate that the study of genetic distances is a useful additional tool for determination of species boundaries in parmelioid lichens. Furthermore, it may also help to detect cryptic lineages (i.e. with little morphological divergence but genetically differentiated). The range of intraspecific distances found in this work can also be useful for rapid species identification, especially in cases where the diagnostic morphological characters are subtle and require a great expertise on the group. As a consequence, results of this study are likely to aid non-taxonomists make accurate identifications, facilitating biomonitoring studies and development of conservation strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.04.014.

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