Org Divers Evol (2017) 17:351–363
DOI 10.1007/s13127-016-0320-4

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

Using multi-locus sequence data for addressing species boundaries in commonly accepted lichen-forming fungal species

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Received: 9 June 2016 / Accepted: 19 December 2016 / Published online: 3 January 2017

Abstract Accurate species delimitations are of great importance for effectively characterizing biological diversity. Our criteria for delimiting species have changed dramatically over the last decades with the increasing availability of molecular data and improvement of analytical methods to evaluate these data. Whereas reciprocal monophyly is often seen as an indicator for the presence of distinct lineages, recently diverged species often fail to form monophyletic groups. At the same time, cryptic species have repeatedly been detected in numerous organismal groups. In this study, we addressed the species delimitation in the crustose lichen-forming fungal genus *Diploschistes* using multilocus sequence data from specimens representing 16 currently accepted species. Our results indicate the presence of previously undetected, cryptic species-level lineages in the subgenus *Limborina*. In the subgenus *Limborina*, samples from different continents currently classified under the same species were shown to be only distantly related. At the same time, in parts of subgen. *Diploschistes* characterized by short branches, none of the currently accepted species formed monophyletic groups. In spite of the lack of monophyly in phylogenetic reconstructions, a multispecies coalescent method provided support for eight of the nine accepted species in subgen. *Diploschistes* as distinct lineages. We propose to reduce *D. neutrophilus* to synonymy with *D. diacapsis* and point out that additional sampling will be necessary before accepting additional species in subgen. *Limborina.*

Keywords BPP · *Diploschistes* · Graphidaceae · Molecular phylogeny · Species delimitation

Introduction

In a broad sense, species delimitation is the process of identifying how individuals and populations fit into natural, species-level clusters, which are not simply constructs of classification (Carstens et al. 2013). Species are generally considered to represent a fundamental unit in biology and provide valuable context for organizing, evaluating, and communicating important biological concepts and principles (Coyne and Orr 2004; Mayr 1963). Therefore, accurate species circumscriptions are integral to interpreting biological patterns and processes across a wide range of sub-disciplines in biology.

Over the last decade, multi-locus sequence data have increasingly been used for assessing species (Camargo and Sites 2013), and the ongoing development of empirical approaches facilitates more objective species delimitation using molecular data (Leavitt et al. 2015b, 2016). DNA sequence data have revealed previously unrecognized species-level lineages hidden within nominal taxa in all organismal groups studied to date. Furthermore, many traditional taxonomic concepts conflict to various degrees with species-level lineages circumscribed using molecular sequence data. The bulk of species delimitation research highlights the fact that finding

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and applying the appropriate character sets and analytical tools remains one of the greatest challenges with empirical species delimitation (Lumbsch and Leavitt 2011). The phylogenetic species criterion is widely used for species delimitation research using molecular sequence data (Nixon and Wheeler 1990; Sites and Marshall 2004; Fujisawa and Barraclough 2013). Although reciprocal monophyly is intuitively appealing, largely because it provides a utilitarian approach with broad applicability, it may fail to accurately delimit species boundaries, particularly for species that recently diverged (Knowles and Carstens 2007). Hence, we argue that species delimitation using DNA sequence data is best viewed as a statistical inference problem given the stochastic nature of the coalescent and process of sequence evolution (Rannala 2015). Here, we use statistical inference under the multispecies coalescent (MSC) model (Kingman 1982; Degnan and Rosenberg 2009; Yang and Rannala 2010a) to test species boundaries.

Similar to other biological groups, molecular sequence data have been central to improving hypotheses of species boundaries in lichen-forming fungi (Crespo and Lumbsch 2010). Traditionally, differences in morphological, chemical, and ecological features have been the predominant source of diagnostic taxonomic characters for circumscribing lichen-forming fungal species (Printzen 2010). However, lichenized fungi generally display few taxonomically useful characters, and varying levels of intraspecific variation among different species groups may confound accurate taxonomic circumscriptions. Therefore, molecular genetic data play an increasingly prominent role in delimiting fungal species and understanding evolutionary relationships in lichenized fungi.

The lichen-forming genus *Diploschistes* Norman (Lecanoromycetes: Ostropales: Graphidaceae) (Lumbsch and Huhndorf 2010) currently includes about 30 crustose species (Jaklitsch et al. 2016), which grow on rocks, soil, or over mosses and other lichens (Fig. 1). Traditionally, *Diploschistes* has been characterized by having a carbonized proper excipulum with lateral paraphyses, *Trebouxia* Puymaly as its photobiont, and by the absence of a columella (Lumbsch 1989). Morphologically, *Diploschistes* was regarded as consisting of three main groups: the *D. actinostomus* group with perithecioid ascomata, the *D. scrucopus* group with urceolate ascomata, and the *D. ocellatus* group with lecanoroid ascomata. These informal groups were supported as monophyletic clades in phylogenetic studies using phenotypical (Lumbsch and Tehler 1998) or molecular data (Fernández-Brime et al. 2013; Martín et al. 2003).

The monophyly of the genus *Diploschistes* has been discussed in the literature. Morphologically, two species were unique in the genus: *D. bisporus* (Bagl.) J. Steiner and *D. ocellatus* (Vill.) Norman, which both lack lateral paraphyses (Lumbsch 1989). Morphological studies already revealed that the former species is distinct in numerous characters and consequently it was separated at the generic level as *Ingvariella bispora* (Bagl.) Guderley & Lumbsch (Guderley et al. 1997). Recent studies showed that this genus is not only unrelated to other *Diploschistes* but belongs to a different family in Ostropales (Fernandez-Brime et al. 2011). *Diploschistes ocellatus* was also found to be distinct from other *Diploschistes* species with some studies showing the genus being non-monophyletic (Parnmen et al. 2013; Rivas Plata et al. 2013; Kraichak et al. 2014). Consequently, *D. ocellatus* has been segregated as *Xalocoa ocellata* (Kraichak et al. 2014). Currently, *Diploschistes* includes species placed in the *actinostomus* group, which is now recognized as subgenus *Limborina* Fdez.-Brime, Gaya & Llimona, and the *scruposus* group or subgenus *Diploschistes* (Fernández-Brime et al. 2013).

While recent molecular studies yielded in a better understanding of the circumscription and phylogenetic placement of *Diploschistes* and the phylogeny of major clades within the genus, the delimitation of species is still unresolved. Previous studies (Fernández-Brime et al. 2013; Martín et al. 2003) indicated that the circumscription of some species needs reexamination and the species delimitation based on phenotypic characters differs between authors (Clauzade and Roux 1985; Llimona Pages 1974; Lumbsch 1989, 1988; Pant and Upreti 1993). Hence, we assembled a dataset consisting of six loci (two nuclear ribosomal, one mitochondrial ribosomal, and three nuclear protein-coding genes) to address the species delimitations within *Diploschistes* and test morphology-based hypotheses.

Methods and materials

Taxon sampling

Our sampling of *Diploschistes* species included a total of 93 specimens representing 16 currently recognized species (Table 1). Species of the subgenus *Diploschistes* included the following: *D. cinereocaesius*, *D. diacapsis*, *D. gypsaceus*, *D. interpediens*, *D. muscorum*, *D. neutrophilus*, *D. rampoddensis*, *D. scruposus*, and *D. thunbergianus*. Taxa included in this study that belong to subgenus *Limborina* included the following: *D. actinostomus*, *D. caesioplumbeus*, *D. candidissimus*, *D. diploschistoides*, *D. elixii*, *D. euganeus*, and *D. sticticus*. Based on previous studies, the tree was rooted with subgenus *Limborina* (Martín et al. 2003; Fernández-Brime et al. 2013). We attempted to sample specimens across the range of each species' distribution, and overall specimens from Africa, Australia, Central America, Europe, North America, and South America were selected. Additional specimens were selected from GenBank to improve our taxonomic sampling.

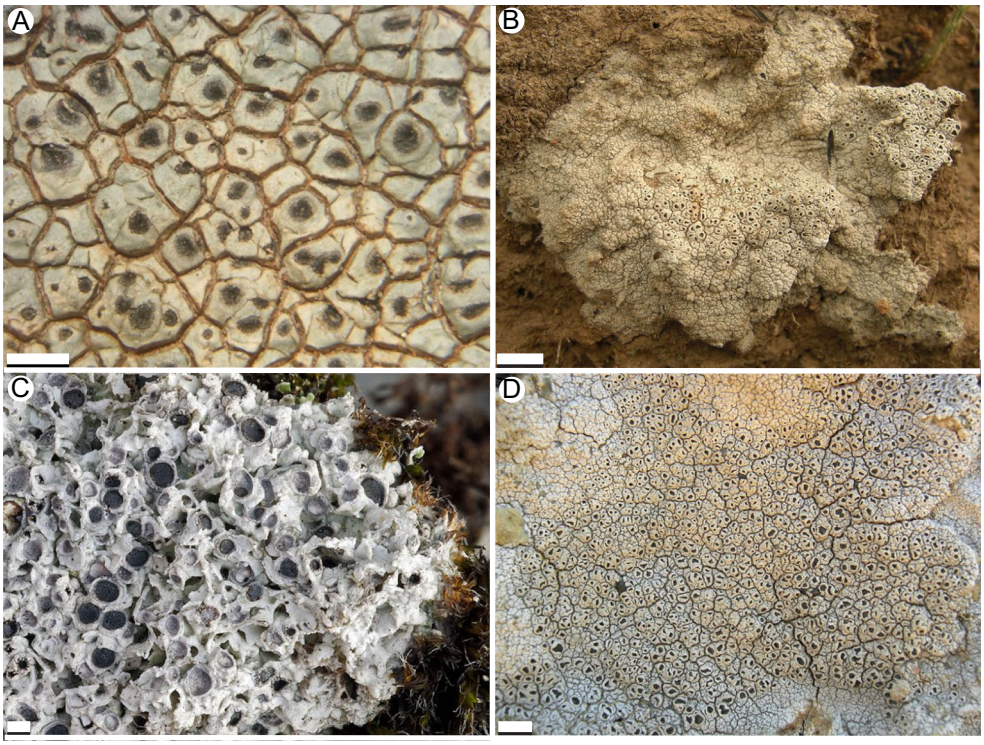

Fig. 1 Habit photographs of *Diploschistes* species. **a** *D. actinostomus.* **b** *D. diacapsis.* **c** *D. muscorum.* **d** *D. scruposus.* Photographs kindly provided by Jason Hollinger and Richard Doker, and the "Ways of enlichenment" ("www.waysofenlichenment.net/#")

Molecular data

Sample preparation, DNA isolation, PCR, and direct sequencing were performed as described previously (Fernández-Brime et al. 2013; Leavitt et al. 2012). Molecular data were generated for six loci: the internal transcribed spacer (ITS), nuclear large subunit (nucLSU), mitochondrial small subunit (mtSSU), minichromosome maintenance complex component 7 (MCM7), the largest subunit of the RNA polymerase II gene (RPB1), and the second largest subunit of RNA polymerase II gene (RPB2). Primers and PCR cycling parameters used for amplifying the six loci are listed in Table 2.

Sequence alignments

New sequences were assembled and edited using the program Sequencher v4.10 (Gene Code Corporation, Ann Arbor, MI) and were subjected to BLAST searches for a first verification of their identities. Sequences of each locus were aligned using the program MAFFT v7 (Katoh et al. 2009) with settings appropriate for the variability of each locus. For ITS sequences, we used the L-ING-i alignment algorithm with the remaining parameters set to default values. For nucLSU, G-ING-i algorithm and "leave gappy regions" were selected. Then, we used E-ING-i algorithm for mtSSU and RPB1, and G-ING-i algorithm for MCM7 and RPB2, with the remaining parameters set to default values. The alignments were adjusted manually to exclude missing data and concatenated. Ambiguous positions of the ITS and mtSSU alignments were removed using the Gblocks web server (Castresana 2000), implementing all the options for a less stringent selection.

Phylogenetic analysis

Exploratory phylogenetic analyses of individual loci revealed a general pattern of poorly resolved topologies. Therefore, the six single-locus alignments were concatenated in Geneious v6.1.2 (Biomatters Ltd., Auckland, NZ) for subsequent phylogenetic analyses. Only specimens that were represented by at least two of six targeted loci were included in the concatenated data matrix (Table 1). A maximum likelihood (ML) analysis was carried out on the multilocus matrix using the locus-specific model partitions (ITS, nucLSU, mtSSU, MCM7, RPB1, and RPB2) in RAxML v8.1.24 (Stamatakis 2006). A search combining 200 separate ML searches was conducted, implementing a GTRGAMMA model, and 1000 pseudoreplicates to evaluate bootstrap support for each node. In addition to the ML analysis, a Bayesian analysis with MrBayes v3.2.3 (Ronquist et al. 2012) was also used for phylogenetic inference from our multilocus dataset. The most appropriate nucleotide substitution model for each of the six loci was selected using the Akaike information criterion in jModelTest v2.1.7 (Darriba et al. 2012). The Bayesian analysis was run for 10,000,000 generations with four independent chains and sampling every 1000th tree. All model parameters were unlinked. Two independent Bayesian runs were conducted to ensure that stationarity was reached and the runs converged at the

Table 1 Information of the species and sequences used in this paper

Species	Isolation; herbarium number	Locality	ITS	nuLSU	mtSSU	RPB1	RPB2	MCM7
Diploschistes actinostomus	Lumbsch3089 (F)	Kenya	KF688486	KF688496	KF688510	KF688530		
Diploschistes actinostomus	SFB004; 0016461 (DUKE)	USA, North Carolina	KC166972		KC167025			
Diploschistes caesioplumbeus	SFB028; 19325 (BCN-LICH)	Spain, Girona	KC166973		KC167026			
Diploschistes caesioplumbeus	SFB030; 19323 (BCN-LICH)	Spain, Girona	KC166974		KC167027		**KX545647**	
Diploschistes candidissimus	SFB008; 0144447 (DUKE)	USA, Delaware	KC166976		KC167028		**KX545634**	
Diploschistes candidissimus	SFB086; 19340 (BCN-LICH)	Spain, Lleida	KC166977		KC167029	**KX545630**	**KX545633**	**KX545555**
Diploschistes cinereocaesius	AFTOL-ID290	Ecuador	KJ542542	**KX545505**	**KX545587**	KJ766850	KJ766951	
Diploschistes cinereocaesius	Luecking 15540 (F)	Costa Rica	KF688481	KF688491	JX421026	KF688518		
Diploschistes cinereocaesius	Lumbsch 19303 (F)	Peru	KF688479	KF688490	KF688502	KF688516		
Diploschistes cinereocaesius	AFTOL-ID328	Costa Rica, San Jose	HQ650715	DQ883799	DQ912306	DQ883742	DQ883755	
Diploschistes diacapsis	AFTOL-ID4860			KJ766550	KJ766384			
Diploschistes diacapsis	SFB001; 0030912 (DUKE)	Spain, Zaragoza	KC166978		KC167030			
Diploschistes diacapsis	SFB052 (BCN)	Spain, Zaragoza	**KX545483**				**KX545643**	**KX545538**
Diploschistes diacapsis	SFB077(BCN)	Spain, La Rioja	**KX545484**		**KX545585**	**KX545612**	**KX545675**	**KX545516**
Diploschistes diacapsis	SFB002; 0130126 (DUKE)	USA, Arizona	KC166979		KC167031			
Diploschistes diacapsis	SFB133; 19347 (BCN-LICH)	Spain, Lleida	KC166980		KC167032			
Diploschistes diacapsis	ALM58	Spain, Almeria	**KX545469**	**KX545512**	**KX545559**	**KX545614**	**KX545654**	**KX545549**
Diploschistes diacapsis	ALM59	Spain, Almeria	**KX545503**	**KX545511**	**KX545561**	**KX545625**	**KX545639**	**KX545552**
Diploschistes diploschistoides	Lumbsch 19073b (F)	Australia, Queensland		AY605076	KF688500	KF688513		
Diploschistes diploschistoides	SFB006; 0144445 (DUKE)	Australia, Western Australia	KC166984		KC167036		**KX545636**	
Diploschistes diploschistoides	SFB010	Australia, Queensland	KC166985		KC167037		**KX545635**	
Diploschistes elixii	Elix 32450 (F)	Australia, Western Australia	KF688482	EU126644	KF688504	KF688520		
Diploschistes euganeus	Lumbsch 20603 (F)	Switzerland, Ticino			KF688507	KF688527		
Diploschistes euganeus	Lumbsch 20605g (F)	Switzerland, Ticino	KF688485	KF688494	KF688508	KF688528		
Diploschistes euganeus	SFB029; 0144451 (DUKE)	Australia, Western Australia	KC166986		KC167038		**KX545632**	
Diploschistes gypsaceus	SFB088; 19324 (BCN-Lich)	Spain, Lleida	KC166988		KC167039			
Diploschistes gypsaceus	SFB020; 17180 (BCN-Lich)	Spain, Lleida	KC166987		**KX545557**	**KX545596**	**KX545644**	**KX545532**
Diploschistes gypsaceus	SFB139; 19340 (BCN-Lich)	Spain, Lleida	KC166991		KC167042			
Diploschistes gypsaceus	SFB127; 19345 (BCN-Lich)	Spain, Lleida	KC166989		KC167040			
Diploschistes gypsaceus	SFB131; 19346 (BCN-Lich)	Spain, Lleida	KC166990	KC167075	KC167041			
Diploschistes interpediens	Lumbsch 20605d (F)	Switzerland, Ticino		KF688495	KF688509	KF688529		
Diploschistes interpediens	SFB057 (BCN)	Spain, La Rioja			**KX545563**	**KX545592**	**KX545650**	
Diploschistes interpediens	SFB064 (BCN)	Spain, La Rioja	**KX545488**				**KX545679**	
Diploschistes interpediens	SFB065; 14751 (BCN-Lich)	Spain, La Rioja	**KX545475**		KC167049	**KX545593**	**KX545659**	**KX545523**
Diploschistes interpediens	SFB021; 19317 (BCN-Lich)	Portugal, Braganca	**KX545473**		KC167045	**KX545591**	**KX545651**	**KX545522**

Table 1 (continued)

Species	Isolation; herbarium number	Locality	ITS	nuLSU	mtSSU	RPB1	RPB2	MCM7
Diploschistes interpediens	SFB026; 18007 (BCN-Lich)	Spain, Santiago de Compostela	**KX545485**		KC167047	**KX545619**	**KX545678**	**KX545553**
Diploschistes interpediens	SFB056 (BCN)	Portugal, Braganca	**KX545487**		**KX545580**	**KX545620**	**KX545676**	
Diploschistes interpediens	SFB025; 19319 (BCN-Lich)	Spain, Girona	**KX545480**	KC167076	KC167046	**KX545621**	**KX545660**	**KX545548**
Diploschistes interpediens	SFB035; 19350 (BCN-Lich)	Spain, Barcelona, Dosrrius	**KX545479**		KC167048	**KX545607**	**KX545662**	**KX545546**
Diploschistes interpediens	SFB036 (BCN)	Spain, Barcelona	**KX545477**		**KX545588**	**KX545609**	**KX545661**	**KX545547**
Diploschistes interpediens	SFB082(BCN)	Spain, Cordoba	**KX545478**			**KX545608**		
Diploschistes interpediens	SFB078 (BCN)	Spain, La Rioja	**KX545476**		**KX545565**	**KX545590**		**KX545525**
Diploschistes muscorum	9038 (BCN)	Spain			**KX545582**		**KX545658**	
Diploschistes muscorum	DMUS140 (BCN)	Spain	**KX545481**		**KX545584**	**KX545631**	**KX545666**	**KX545520**
Diploschistes muscorum	DMUS4500B (BCN)	Spain	**KX545482**		**KX545570**	**KX545617**	**KX545657**	**KX545529**
Diploschistes muscorum	SFB125; 19344 (BCN-Lich)	Spain, Lleida	KC167007		KC167058			
Diploschistes muscorum	SFB140; 19334 (BCN-Lich)	Spain, Barcelona	KC167008		KC167059			
Diploschistes muscorum	SFB045; 19333 (BCN-Lich)	Italy, Sardinia	KC167005		KC167056			
Diploschistes muscorum	SFB079; 14435 (BCN-Lich)	Spain, La Rioja	KC167006		KC167057	**KX545624**	**KX545652**	**KX545528**
Diploschistes muscorum	SFB013 (BCN)	USA, Arizona	**KX545472**		**KX545578**	**KX545618**	**KX545656**	**KX545530**
Diploschistes muscorum	SFB003; 0016462 (DUKE)	USA, Connecticut	KC167004	KC167077	KC167055			
Diploschistes neutrophilus	ALM55 (BCN)	Spain, Almeria	**KX545471**		**KX545579**	**KX545615**	**KX545665**	**KX545550**
Diploschistes neutrophilus	ALM56 (BCN)	Spain, Almeria	**KX545504**		**KX545560**	**KX545628**	**KX545642**	**KX545539**
Diploschistes neutrophilus	ALM57 (BCN)	Spain, Almeria	**KX545470**	**KX545506**		**KX545616**	**KX545653**	**KX545551**
Diploschistes neutrophilus	SFB118; 19329 (BCN-Lich)	Spain, Girona	KC166983		KC167035			
Diploschistes neutrophilus	SFB063; 19357 (BCN-Lich)	Spain, Girona	KC166982		KC167034	**KX545627**	**KX545641**	**KX545541**
Diploschistes neutrophilus	SFB044; 19338 (BCN-Lich)	Spain, Illes Balears	KC166981		KC167033	**KX545626**	**KX545638**	**KX545540**
Diploschistes neutrophilus	SFB146 (BCN)	Spain, Illes Balears			**KX545566**	**KX545629**	**KX545640**	**KX545514**
Diploschistes rampoddensis	SFB101; 18011 (BCN-Lich)	Spain, Girona	KC166993		KC167044			
Diploschistes rampoddensis	SFB068; 18009 (BCN-Lich)	Spain, Girona	KC166992		KC167043	**KX545622**	**KX545637**	**KX545521**
Diploschistes rampoddensis	SFB073; 18008 (BCN-Lich)	Spain, Girona	KJ542543			**KX545623**		**KX545556**
Diploschistes scruposus	9042 (BCN)	Spain					**KX545670**	**KX545519**
Diploschistes scruposus	9044 (BCN)	Spain	**KX545499**		**KX545569**		**KX545673**	
Diploschistes scruposus	9045 (BCN)	Spain			**KX545572**		**KX545685**	
Diploschistes scruposus	Schmitt1376 (F)	Germany, Hesse	KF688478	KF688489	KF688501	KF688515		
Diploschistes scruposus	Schmitt 1375 (F)	Germany, Hesse	KF688477	KF688488		KF688514		
Diploschistes scruposus	9040 (BCN)	Spain	**KX545491**		**KX545571**		**KX545672**	**KX545518**
Diploschistes scruposus	AFTOL-ID4813	USA		KJ766552	KJ766385			
Diploschistes scruposus	SFB014 (BCN)	Canada, Ontario	**KX545492**				**KX545674**	
Diploschistes scruposus	SFB058; 14227 (BCN-Lich)	Spain, La Rioja	KC167016		KC167066	**KX545611**	**KX545684**	**KX545537**

Table 1 (continued)

Species	Isolation; herbarium number	Locality	ITS	nuLSU	mtSSU	RPB1	RPB2	MCM7
Diploschistes scruposus	SFB066; 14398 (BCN-Lich)	Spain, La Rioja	KC167020		KC167070	**KX545594**	**KX545664**	**KX545527**
Diploschistes scruposus	SFB076 (BCN)	Spain, La Rioja	**KX545474**		**KX545562**	**KX545589**	**KX545649**	**KX545524**
Diploschistes scruposus	SFB080 (BCN)	Spain, La Rioja	**KX545486**				**KX545677**	
Diploschistes scruposus	SFB059 (BCN)	Spain, Girona	KJ542545		**KX545586**	**KX545597**	**KX545648**	**KX545533**
Diploschistes scruposus	SFB060; 19326 (BCN-Lich)	Spain, Girona	KC167017		KC167067	**KX545606**	**KX545646**	**KX545535**
Diploschistes scruposus	SFB024; 19328 (BCN-Lich)	Spain, Lleida	KC167014	KC167078	KC167064	**KX545610**	**KX545680**	**KX545536**
Diploschistes scruposus	SFB034 (BCN)	Spain, Barcelona	**KX545501**		**KX545567**	**KX545602**	**KX545668**	**KX545543**
Diploschistes scruposus	SFB037; 19351 (BCN-Lich)	Spain, Barcelona	KC167015		KC167065	**KX545604**	**KX545669**	**KX545544**
Diploschistes scruposus	SFB039 (BCN)	Spain, Barcelona	**KX545502**		**KX545564**	**KX545603**	**KX545667**	**KX545545**
Diploschistes scruposus	SFB069; 19354 (BCN-Lich)	Spain, Barcelona	KC167021		KC167071	**KX545595**	**KX545663**	**KX545526**
Diploschistes scruposus	SFB147 (BCN)	Sweden, Norrbotten	**KX545493**	**KX545508**	**KX545573**			
Diploschistes scruposus	SFB148 (BCN)	Sweden, Norrbotten	**KX545494**		**KX545583**			
Diploschistes scruposus	SFB149 (BCN)	Sweden, Norrbotten	**KX545495**		**KX545574**			
Diploschistes scruposus	SFB150 (BCN)	Sweden, Norrbotten	**KX545500**	**KX545510**	**KX545575**	**KX545598**	**KX545671**	**KX545517**
Diploschistes scruposus	SFB151 (BCN)	Sweden, Norrbotten	**KX545496**	**KX545509**	**KX545576**	**KX545599**	**KX545483**	**KX545554**
Diploschistes scruposus	SFB155 (BCN)	Norway, Finnmark	**KX545498**	**KX545513**	**KX545577**	**KX545600**	**KX545682**	**KX545542**
Diploschistes scruposus	SFB157 (BCN)	Norway, Finnmark	**KX545497**	**KX545507**	**KX545581**	**KX545601**	**KX545681**	**KX545515**
Diploschistes sticticus	Lumbsch 19109b (F)	Australia, Queensland		JX421482	KF688499	KF688512		
Diploschistes sticticus	Lumbsch 3056 (F)	Kenya	KF688487		KF688511	KF688531		
Diploschistes thunbergianus	Eldridge 3800 (F)	Australia, New South Wales	AJ458289	AF274095	AF431955			
Diploschistes thunbergianus	Lumbsch 10728d (F)	Australia, South Australia	AJ458290					
Diploschistes sp.	DMUS159 (BCN)	Spain	**KX545490**		**KX545558**	**KX545613**	**KX545645**	**KX545534**
Diploschistes sp.	DSPY138 (BCN)	Spain	**KX545489**		**KX545568**	**KX545605**	**KX545655**	**KX545531**

Newly generated sequences are in bold

same log-likelihood level (Nylander et al. 2008). After discarding the burn-in, the remaining 7500 trees of each run were pooled to calculate a 50% majority rule consensus tree. Clades that received bootstrap support ≥70% under ML and posterior probabilities ≥0.95 were considered significant. Phylogenetic trees were visualized using FigTree v1.4.2 (Rambaut 2009).

Species delimitation analysis

For a subgroup of species in the *D. scruposus* group, the multispecies coalescent model implemented in the program BPP v3.2 (Yang and Rannala 2010b, 2014; Rannala and Yang 2013) was used to infer support for the separation of the sampled *Diploschistes* species. The *D. scruposus* group has recently been shown to have a recent diversification history (Rivas Plata 2011; Kraichak et al. 2015), and recently diverged species may not be recovered as monophyletic due to incomplete lineage sorting (Knowles and Carstens 2007). Given the recent diversification history for the *D. scruposus* group, lack of resolution and short branches in phylogenetic reconstructions for this group (see Results), and support from phenotypic and ecological evidence (Lumbsch and Tehler 1998), it may be reasonable to assume that traditionally circumscribed species in the *D. scruposus* group represent distinct evolutionary lineages, in spite of their lack of monophyly in phylogenetic reconstructions. Therefore, for the BPP analyses, which accounts for incomplete lineage sorting within a multispecies coalescent framework, specimens within the *D. scruposus* group were assigned to candidate species based on phenotype-based identifications.

BPP incorporates coalescent theory and phylogenetic uncertainty into parameter estimation, and the posterior distribution for species delimitation models is sampled using a reversible-jump Markov Chain Monte Carlo (rjMCMC) chain.

We used the unguided species delimitation analysis "A11" (Yang 2015), which explores different species delimitation models and different species phylogenies, with fixed specimen assignments to populations. Specimens were assigned to nine currently accepted species: *D. cinereocaesius*, *D. diacapsis*, *D. gypsaceus*, *D. interpediens*, *D. muscorum*,

Table 2 Primer information and PCR settings used for this paper

Loci/PCR info	ITS	nucLSU	mtSSU	MCM7	RPB1	RPB2
PCR primers	ITS1f[a]	AL1R[c]	mrSSU1[f]	LecMCM7f[h]	gRPB1a[j]	RPB2-6f[l]
	ITS4a[b]	AL2R[d]	mrSSU2r[f]	LecMCM7r[h]	fRPB1c[k]	RPB2-7cr[l]
		LR5[e]	mrSSU3r[f]	MCM7-709f[i]		
		LR6[e]	MSU1[g]	MCM7-1348r[i]		
			MSU7[g]			
Initial denaturation	95 °C 5 min	95 °C 5 min	94 °C 10 min	94 °C 10 min	94 °C 10 min	94 °C 10 min
Phase 1	10 cycles	10 cycles	34 cycles	34 cycles	34 cycles	34 cycles
	95 °C 30s	95 °C 30s	95 °C 45 s	94 °C 45 s	94 °C 45 s	94 °C 45 s
	66 °C 30s	66 °C 30s	50 °C 45 s	50 °C 50s	50 °C 50s	50 °C 50s
	72 °C 1 min 30s	72 °C 1 min 30s	72 °C 1 min 30s	72 °C 1 min	72 °C 1 min	72 °C 1 min
Phase 2	34 cycles	34 cycles	None	None	None	None
	95 °C 30s	95 °C 30 sec				
	56 °C 30 sec	56 °C 30 sec				
	72 °C 1 min 30 sec	72 °C 1 min 30 sec				
Final extension	72 °C 10 min	72 °C 10 min	72 °C 10 min	72 °C 5 min	72 °C 5 min	72 °C 5 min

[a] Gardes and Bruns (1993)
[b] Larena et al. (1999)
[c] Döring et al. (2000)
[d] Mangold et al. (2008)
[e] Vilgalys and Hester (1990)
[f] Zoller et al. (1999)
[g] Zhou and Stanosz (2001)
[h] Leavitt et al. (2011)
[i] Schmitt et al. (2009)
[j] Stiller and Hall (1997)
[k] Matheny et al. (2002)
[l] Liu et al. (1999)

D. neutrophilus, *D. rampoddensis*, *D. scruposus*, and *D. thunbergianus*. Using analysis A11, the algorithm attempts to merge populations into one species and uses the nearest neighbor interchange (NNI) or subtree pruning and regrafting (SPR) algorithms to change the species tree topology (Yang and Rannala 2014). Analysis "A00" (Yang 2015), a within-model inference, was used to generate the posterior distribution of the parameters theta (θs) and tau (τs) under the multi-species coalescent (MSC) model to infer a reasonable combination of priors given the data (Rannala 2015). Based on the results from the A00 analyses, the gamma prior G for θ was set to ~G(1, 85), and the gamma prior G for tau (τ) was set to ~G(1, 200). Under the unguided species delimitation model, A11, we used two different search algorithms (algorithm 0 or 1), equal probabilities for the labeled histories, to assign probabilities to the models, rates were allowed to vary among loci (locus rate = 1), and the analyses were set for automatic fine-tune adjustments. The rjMCMC analysis was run for 100,000 generations, sampling every 2 generations discarding the first 10% as burn-in. The analysis was run twice to confirm consistency between runs.

Results

Molecular data

For this study, 217 new sequences were generated (Table 1). The multilocus matrix we used in this study was deposited in TreeBase (ID# pending). The concatenated, six-locus matrix consisted of 93 individuals and 5074 aligned nucleotide position characters (Table 3). A summary of alignment information for the multilocus dataset was also provided in Table 3.

Phylogenetic analysis

Phylogenies derived from the ML and B/MCMC analyses were generally concordant. Minor differences in the arrangement of some terminals occurred, but relationships at deeper nodes and in well-supported clades were identical. We chose to present the ML topology, with nodal support values from both ML bootstrap analysis and posterior probabilities from the Bayesian inference (Fig. 2).

The two subgenera of *Diploschistes* were recovered in our phylogenetic trees with strong support (both are BS = 98, PP = 1.0; Fig. 2). In our ML tree, subgen. *Diploschistes* was shown to include several clades. *Diploschistes cinereocaesius* (four specimens) was strongly supported as a monophyletic group forming a strongly supported sister-group (BS = 100, PP = 1.0) with the monophyletic *D. rampoddensis* (three specimens). These two species formed an unsupported sister-group to the monophytletic *D. thunbergianus* (two specimens). Seven specimens of *D. muscorum* formed a strongly supported (BS = 91, PP = 1.0) group, which formed an unsupported relationship with an unsupported clade including five specimens of *D. diacapsis* and seven specimens of *D. neutrophilus*. The bulk of specimens of subgen. *Diploschistes* clustered in an unsupported clade containing five species—*D. diacapsis* (three specimens), *D. gypsaceus* (five specimens), *D. interpediens* (12 specimens), *D. muscorum* (two specimens), and *D. scruposus* (26 specimens), and two samples which could not be identified with certainty, with seven supported internodes. Within subgen. *Limborina*, most relationships were also unresolved, and only two internodes were supported. In this clade, only *D. diploschistoides* (BS = 75, PP = 1.0) was recovered as monophyletic with strong support, whereas all other species were either not monophyletic or their monophyly was not strongly supported. This includes *D. actinostomus* with the samples from North America and Africa being separated, *D. euganeus* with samples from Australia and Europe not clustering together, and *D. sticticus* with samples from Australia and Africa not forming a monophyletic group and *D. caesioplumbeus* forming an unsupported clade.

Branch lengths between clades in subgen. *Limborina* and *D. cinereocaesius*, *D. rampoddensis*, and *D. thunbergianus* differed considerably from those in the other parts of subgen. *Diploschistes*. Branch lengths were generally short, and support for clades was overall low in the latter. Further, species in this part of the phylogenetic tree did not form monophyletic groups. However, their monophyly could not be rejected using alternative topology tests (data not shown). Hence, we employed multispecies coalescent species delimitation using

Table 3 The alignment information for the multilocus dataset

Alignments	ITS	nucLSU	mtSSU	RPB1	RPB2	MCM7	Total
Number of sequences	82	27	84	57	56	43	349
Newly generated sequences	36	9	33	43	54	43	218
Number of sites (including gaps)	629	1404	820	837	768	616	5074
Missing sequences/ the percentages	11/12%	66/71%	9/10%	36/39%	37/40%	50/54%	209/37%
Nucleotide substitution models	GTR + I + G	HKY + G	K80 + G	HKY + I + G	SYM + G	K80 + G	

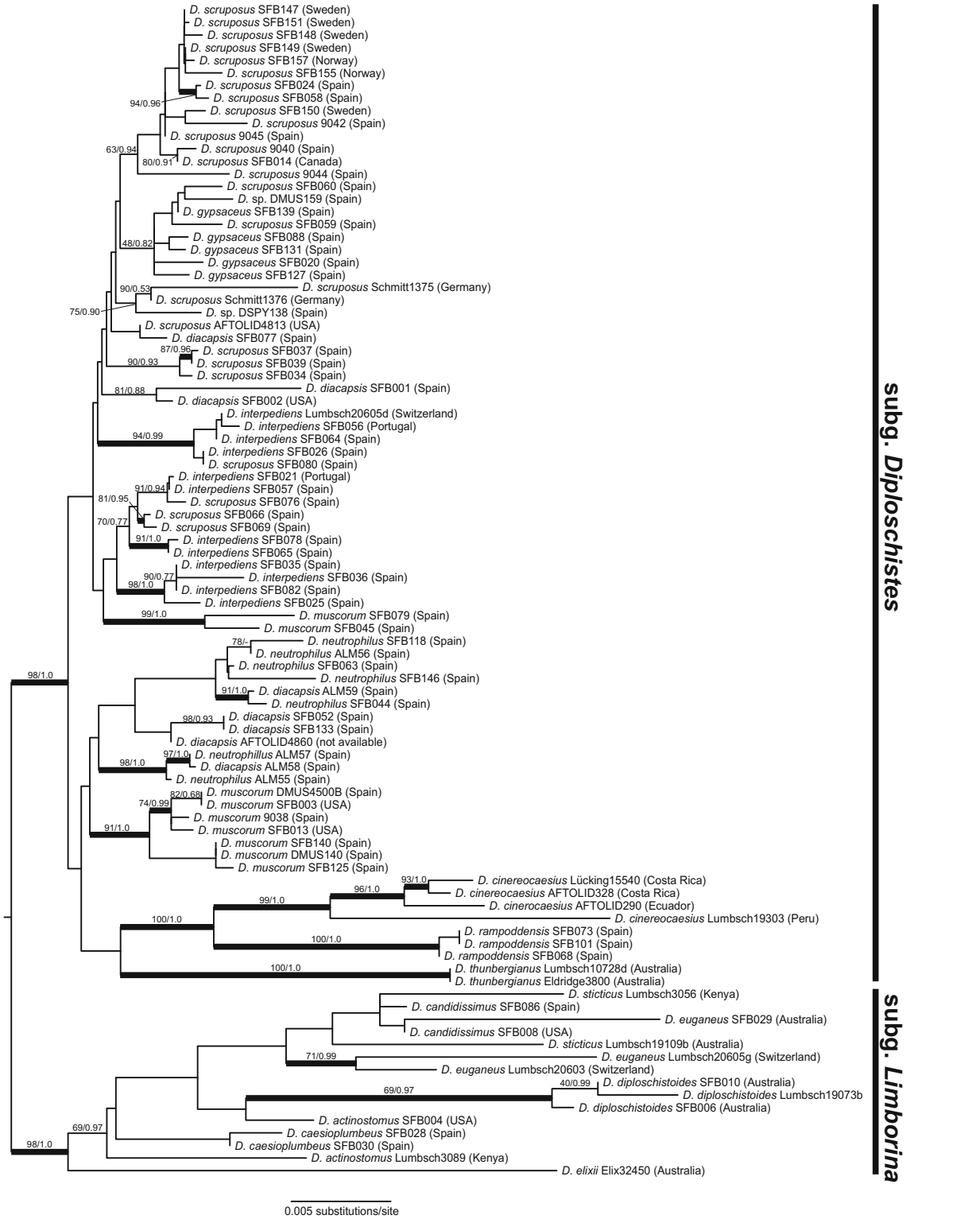

Fig. 2 Maximum likelihood (ML) phylogenetic relationships of *Diploschistes* taxa inferred from a combined 6-locus analysis. Values at each node indicate nonparametric bootstrap support (BS)/posterior probability (PP), branches in bold received maximum likelihood bootstrap support values equal or above 70 and posterior probabilities equal or above 0.95

BPP to evaluate separation of currently accepted species in subgenus *Diploschistes*. An 8-species delimitation scenario had the highest probability, followed by a 7-species scenario (Table 4). All other species delimitation models had probabilities <0.05. Currently accepted species in subgenus *Diploschistes* received the highest supported, with the

Table 4 Species delimitation scenarios and their posterior probabilities inferred under the multispecies coalescent model using the program BPP

Posterior probability	# of inferred species	Taxa supported in each scenario.
0.570	8	(*D. cinereocaesius*; *D. diacapsis* = *D. neutrophilus*; *D. gypsaceus*; *D. interpediens*; *D. muscorum*; *D. rampoddensis*; *D. scruposus*; *D. thunbergianus*)
0.367	7	(*D. cinereocaesius*; *D. diacapsis* = *D. neutrophilus*; *D. gypsaceus* = *D. scruposus*; *D. interpediens*; *D. muscorum*; *D. rampoddensis*; *D. thunbergianus*)
0.041	7	(*D. cinereocaesius*; *D. diacapsis* = *D. neutrophilus*; *D. gypsaceus* = *D. interpediens*; *D. muscorum*; *D. rampoddensis*; *D. scruposus*; *D. thunbergianus*)
0.011	9	(*D. cinereocaesius*; *D. diacapsis*; *D. gypsaceus*; *D. interpediens*; *D. muscorum*; *D. neutrophilus*; *D. rampoddensis*; *D. scruposus*; *D. thunbergianus*)

Cases where multiple taxa were collapsed into a single species are underlined. Only species delimitation models with posterior probabilities >0.01 are reported

exception of *D. diacapsis* and *D. neutrophilus*, which were collapsed into a single species with high probability (Table 5).

Discussion

We used a six-locus dataset including three ribosomal (ITS, nuLSU, mtSSU) and three protein-coding markers (MCM7, RPB1, RPB2) of 93 specimens representing 16 currently accepted species to test the species delimitation in the genus *Diploschistes*. Our results indicate both the presence of previously undetected, cryptic species, and difficulties in separating species using molecular markers. Species in this genus have largely been separated based on ascomatal characters, such as apthecial morphology, exciple thickness, number of ascospores per ascus, ascospore-size, -form and -amyloidity, secondary metabolites, and thallus morphology (Lumbsch 1989; Lumbsch and Elix 1989; Rivas Plata et al. 2010). Whereas species delimitation based on these phenotypical characters have largely been in agreement among authors, variability of number of ascospores, thallus morphology, and ecology of species of the subgenus *Diploschistes* have differed somewhat among authors (Clauzade and Roux 1985; Llimona Pages 1974; Lumbsch 1988, 1989; Pant and Upreti 1993).

Table 5 Delimited species and their posterior probabilities

Probability	Delimited species
1.000	*D. cinereocaesius*
1.000	*D. muscorum*
1.000	*D. rampoddensis*
1.000	*D. thunbergianus*
0.979	*D. diacapsis* = *D. neutrophilus*
0.958	*D. interpediens*
0.624	*D. scruposus*
0.582	*D. gypsaceus*
0.376	*D. gypsaceus* = *D. scruposus*
0.042	*D. gypsaceus* = *D. interpediens*
0.021	*D. diacapsis*
0.021	*D. neutrophilus*
<0.001	*D. diacapsis* = *D. gypsaceus* = *D. neutrophilus*
<0.001	*D. rampoddensis* = *D. thunbergianus*
«0.001	*D. cinereocaesius* = *D. thunbergianus*

Cases where multiple taxa were collapsed into a single species are underlined

Among species of the subgenus *Limborina*, samples from different continents currently classified under the same species were often only distantly related, including *D. actinostomus*, *D. euganeus*, and *D. sticticus*. These results suggest that phenotypically similar specimens occurring on different continents in fact represent distinct lineages. Our taxon sampling is insufficient to address species delimitation in subgen. *Limborina*, but these results demonstrate that additional studies are necessary to better understand species delimitation in this subgenus. However, the presence of distinct lineages within nominal species on different continents has repeatedly been shown in other groups of lichen-forming fungi (Amo de Paz et al. 2012; Arguello et al. 2007; Divakar et al. 2010; Hodkinson and Lendemer 2011; Thell et al. 2009; Otálora et al. 2010; Parnmen et al. 2012; Alors et al. 2016; Leavitt et al. 2015a; Zhao et al. 2015; Cornejo and Scheidegger 2015) and further alternative topology tests rejected monophyly of those species significantly (data not shown). A study addressing the species delimitation in this subgenus will require additional sampling.

In subgen. *Diploschistes*, only three species were monophyletic and strongly supported. In contrast, *D. diacapsis*, *D. interpediens*, *D. muscorum*, *D. neutrophilus*, and *D. scruposus* were not monophyletic, and the relationships among samples of those species were inferred with short branches and mostly poorly supported. Monophyly is not a prerequisite of a species, and thus, a lack thereof is not necessarily evidence that these lineages are conspecific (Leavitt et al. 2016). For example, in North American species of the genus

Xanthoparmelia, recent diversification during Pliocene and Pleistocene was estimated, and independent species-level lineages were not supported by concordant evolutionary histories across multiple, independent loci (Leavitt et al. 2011a, b, 2013).

In spite of our attempt to reconstruct phylogenetic relationships for the *D. scrupsosus* group using multilocus sequence data, species boundaries and relationships within this group remained unresolved. Therefore, we used the program BPP to delimit species boundaries in this group within a statistical framework modeled under the multispecies coalescent. This analysis strongly supported eight of the nine accepted species in subgen. *Diploschistes* as separate species. Previously, it has been shown that the genus *Diploschistes*, and especially subgen. *Diploschistes*, has diversified recently (Rivas Plata 2011; Kraichak et al. 2015). We hypothesize that the difficulties in separating species in some species of subgen. *Diploschistes* are due to recent diversification. The multispecies coalescent analysis, which accounts for incomplete lineage shorting, supports that most of them are in fact distinct species. The only exception is *D. neutrophilus*, which was supported to belong to *D. diacapsis*. Consequently, we propose to reduce *D. neutrophilus* to synonymy with *D. diacapsis* below.

The coalescent-based BPP program accounts for ancestral polymorphisms and incomplete lineage sorting. However, also factors, such as occasional gene flow, hybridization, and recombination, are other evolutionary factors potentially influencing species delimitation inferences. While BPP performs quite robustly under a range of scenarios, speciation probabilities decrease with increasing levels of gene flow (Camargo et al. 2012; Zhang et al. 2011).

BPP also requires a priori assignment of individuals to candidate species, and the impact of incorrectly assigned specimens remains unclear. While traditionally accepted nominal species have been well-studied morphologically (Lumbsch 1989; Lumbsch and Elix 1989; Rivas Plata et al. 2010), our multilocus phylogenetic reconstructions failed to provide strong support either for or against the traditionally, phenotype-based species in the *D. scruposus* group. Therefore, we based our specimen assignments to candidate species for the BPP analysis on phenotype-based identifications. Arguably, as the use of genome-wide molecular data becomes more commonplace in lichen research, species boundaries and evolutionary relationships in lineages with recent diversification histories, including the *D. scruposus* group, will better understood. In the meantime, we propose that the 8-species model inferred for the *D. scruposus* group (Table 4) represents a useful working hypothesis of species boundaries for this group.

Taxonomic conclusions

Diploschistes diacapsis (Ach.) Lumbsch

Lichenologist 20: 20 (1988).—*Urceolaria diacapsis* Ach., *Lich. Univ.*: 339 (1810); lectotype (selected by Lumbsch, *Lichenologist* 20: 20, 1988): Spain, Lagasca (S!).

= *Diploschistes neutrophilus* (Clauzade & Cl. Roux) Fdez.-Brime & Llimona, *Taxon* 62: 275 (2013); holotype: France, Provence, Bouches-du-Rhône Crau, 10 km from Fos-sur-Mer, Clor de Tenque, on neutral clayey sandy soil, 25 Apr 1980, C. Roux (Hb. Claude Roux no. 99!).

In a recent revision of *Diploschistes* (Fernández-Brime et al. 2013), the phylogeny supported two separate clades corresponding to the morphological concepts of *D. diacapsis* and *D. diacapsis* subsp. *neutrophilus*. The authors also noticed morphological and ecological differences: *D. diacapsis* has thicker and convex thalli (up to 2 mm), which were detached from the substrate and is growing on gypsiferous or highly calcareous soils in continental areas, whereas *D. diacapsis* subsp. *neutrophilus* has thin and flat thalli completely attached to the substratum and grows on decarbonized soils in coastal areas. Based on these results, Fernández-Brime et al. (2013) raised *D. dicapsis* ssp., *neutrophilus* to species level. Our present study, however, includes a larger number of samples and loci, and our phylogeny (Fig. 2) shows that specimens with *D. diacapsis* and *D. neutrophilus* morphologies do not form distinct clades. Furthermore, several samples identified as *D. neutrophilus* were collected in the Tabernas desert (Table 1), a typical inland semi-arid *D. diacapsis* locality. In the light of these results based on more data, and finding the thallus thickness and shape a much more inconsistent character than previously believed, we formally synonymize *D. neutrophilus* with *D. diacapsis*.

Acknowledgements This study was financially supported by the National Science Foundation (DEB 0516116 to The Field Museum; PI, H. T. Lumbsch; Co-PI, R. Lücking; and DEB-1025861 to The Field Museum; PI, T. Lumbsch; Co-PI, R. Lücking) and Swedish Research Council grant VR 621-2012-3990 to the Swedish Museum of Natural History (PI M. Wedin). Our colleagues Jason Hollinger and Richard Doker are kindly thanked for permitting us to publish their species images.

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