SYSTEMATICS AND PHYLOGENY

Phylogenetic study of *Diploschistes* (lichen-forming Ascomycota: Ostropales: Graphidaceae), based on morphological, chemical, and molecular data

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Abstract The genus *Diploschistes* includes crustose lichen-forming fungi with a carbonized proper excipulum with lateral paraphyses, and a chemistry dominated by orcinol depsides. However, the taxon *D. ocellatus* lacks these excipular characters and has β-orcinol depsidones, raising doubts about its inclusion within this genus. Using a two-locus dataset (mtSSU, nuLSU), our phylogenetic analyses confirm the classification of *D. ocellatus* within *Diploschistes*. Three different groups have been recognized within this genus, based on ascomatal morphology: Actinostomus (perithecioid), Scruposus (urceolate), and Ocellatus (lecanoroid). These groups have been widely used in monographic studies and keys, but their taxonomic value has not been confirmed yet. Here we inferred phylogenetic relationships within *Diploschistes*, with a special emphasis on the *D. scruposus* complex, using a combined dataset consisting of morphological, chemical, nrITS, and mtSSU data in order to determine if these species groups and phenotypically based species delimitations were monophyletic. Based on our results, a new subgeneric treatment for *Diploschistes* is proposed, and the taxonomic value of fruiting body types is confirmed. The clade corresponding to *D. ocellatus* consists of two well-supported subclades, one of them grouping specimens without ascomata, having only pycnidia. It is also remarkable that the clade containing specimens of *D. diacapsis* subsp. *neutrophilus* appears distantly related to the clade containing all other accessions of *D. diacapsis*. Our analysis revealed that for some taxa, such as *D. scruposus* and *D. interpediens*, molecular variability did not correlate with either morphological or chemical diversity.

Keywords ascoma morphology; *Diploschistes*; fungal classification; INAASE; phenotypically delimited species; phylogenetics

Supplementary Material The Electronic Supplement (Tables S1 and S2, Figs. S1–S6) is available in the Supplementary Data section of the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

Received: 14 June 2012; revision received: 3 Dec. 2012; accepted: 17 Feb. 2013

INTRODUCTION

Currently, the lichen-forming genus *Diploschistes* Norman (Lecanoromycetes: Ostropales: Graphidaceae; Lumbsch & Huhndorf, 2010) includes 43 crustose species (Kirk & al., 2008), which grow on rocks, soil or over mosses and other lichens. *Diploschistes* is widely distributed mostly in arid and semiarid regions of Africa, America, Australia, and Europe (Poelt, 1969; Clauzade & Roux, 1985, 1989; Lumbsch & Elix, 1985, 1989, 2003; Lumbsch, 1989, 1993; Lumbsch & al., 1993; Guderley & Lumbsch 1996; Elix & Lumbsch, 2005; Lumbsch & Mangold, 2007; Mangold & al., 2009). Although this genus is primarily found at subtropical to temperate latitudes, a few species extend into tropical areas (Lumbsch, 1993; Lumbsch & Aptroot, 1993; Pant & Upreti, 1993; Breuss & Brunnbauer, 1997; Umaña & Sipman, 2002).

Traditionally, *Diploschistes* has been characterized by having a carbonized pseudoparenchymatous excipulum with lateral paraphyses, *Trebouxia* Puymaly as its photobiont, and by the absence of a columella (Lumbsch, 1989). Three different ascomatal morphologies are present within the genus (Fig. 1): perithecioid (ascoma enclosed by a wall and opened only by a small ostiole, disc not visible from above, similar to a perithecium with a well-developed carbonized excipulum), urceolate (ascoma with an exposed deeply concave disc and well-developed carbonized excipulum), and lecanoroid (ascoma with an exposed flat to moderately concave disc, and reduced hyaline excipulum). Lettau (1932–1937) first proposed the distinction between the Actinostomus group, with perithecioid ascomata, and the Scruposus group, with urceolate to lecanoroid ascomata. Later, Lumbsch (1985) introduced a third subdivision, the Ocellatus group, to encompass a single species, *D. ocellatus* (Vill.) Norman, characterized by lecanoroid ascomata, an extremely reduced pale excipulum, and lack of lateral paraphyses.

Hale (1980, 1981) had proposed a circumscription of genera within the family Thelotremataceae based on excipular characters. Now this family has been synonymized with the Graphidaceae (Mangold & al., 2008; see also Lumbsch & Huhndorf, 2010) and we follow here this circumscription. Some authors, however, still maintain the two families as

separate (see Hodkinson, 2012). Following Hale's classification, *Diploschistes* was then characterized by having a well-developed carbonized pseudoparenchymatous excipulum with lateral paraphyses. However, *D. ocellatus* did not fit in this generic circumscription. Subsequently, Lumbsch & Tehler (1998) questioned this delimitation since *Diploschistes* shares these excipular traits with other genera of the family (e.g., *Thelotrema* Ach. has lateral paraphyses and *Ocellularia* G. Mey. a carbonized excipulum). For this reason, Lumbsch & Tehler (1998) suggested alternative diagnostic characters for *Diploschistes*: the presence of *Trebouxia* and the absence of the stictic acid complex. Again, *D. ocellatus* would not fit within this circumscription because it contains β-orcinol depsidones belonging to the norstictic acid chemosyndrome (substances included in the stictic acid complex), which it shares with other thelotremoid Graphidaceae (e.g., *Chapsa* A. Massal., *Leptotrema* Mont. & Bosch, *Thelotrema*).

Two phylogenetic studies of *Diploschistes* have been published to date (Lumbsch & Tehler, 1998; Martín & al., 2003). The main focus of these studies was to determine whether *D. ocellatus* belongs, or not, to the genus *Diploschistes* and to investigate the taxonomic value of ascoma morphology in the delimitation of monophyletic entities. In their morphology-based study, Lumbsch & Tehler (1998) showed the Scruposus group, including *D. ocellatus*, as monophyletic, and the Actinostomus group as paraphyletic in relation to the Scruposus group. Conversely, in a nuclear ribosomal internal transcribed spacer (nrITS)-based phylogeny, Martín & al. (2003) recovered the Actinostomus group as monophyletic, whereas the Scruposus group was paraphyletic in relation to the Actinostomus group, with *D. ocellatus* derived from the first split within this genus. Martín & al. (2003) assessed the placement of *D. ocellatus* within *Diploschistes* versus its status as a monotypic genus, by calculating the genetic distance between this species and the rest of the genus. The results

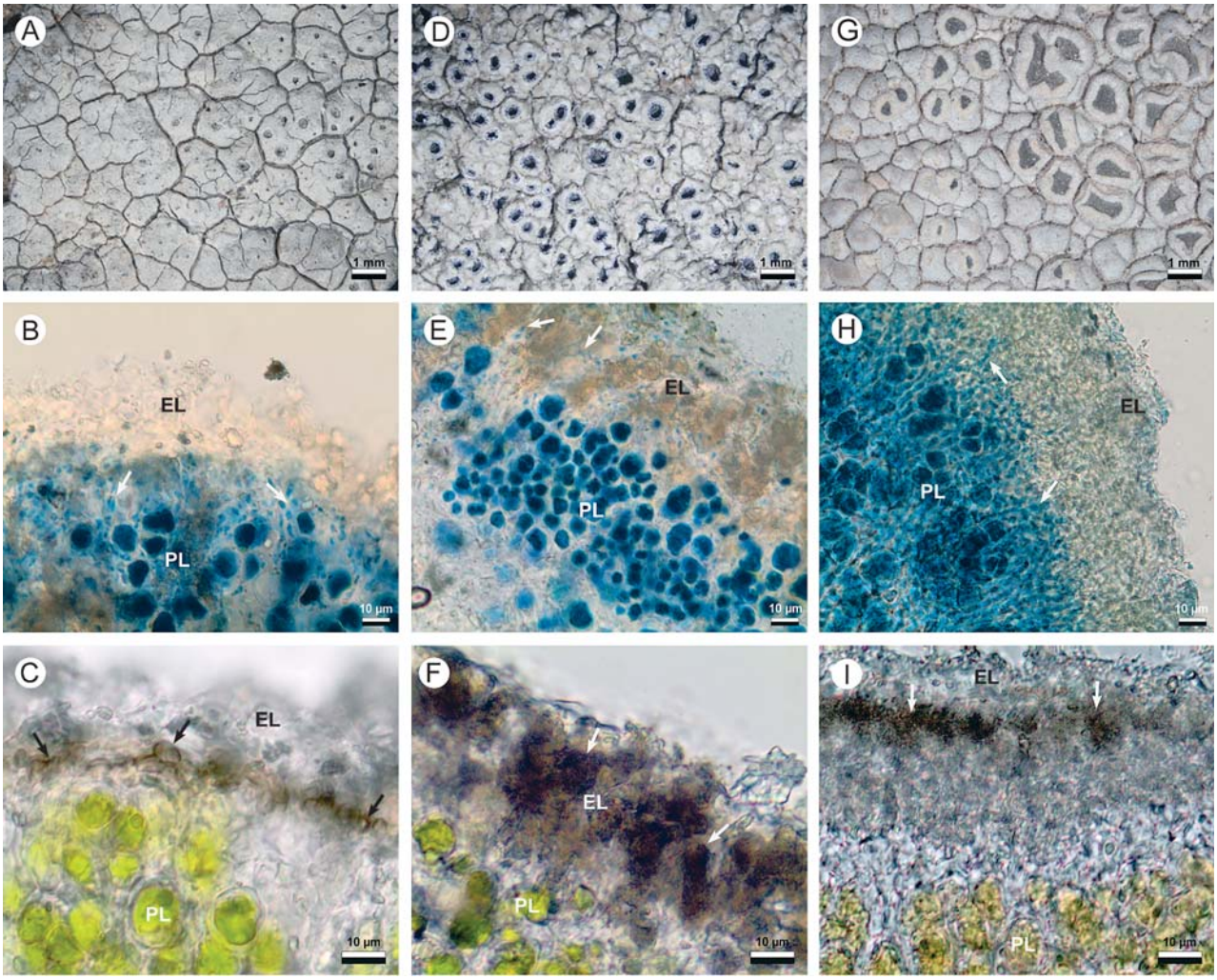

Fig. 1. Morphological and anatomical characters of the thallus of *Diploschistes candidissimus* (*D.* subg. *Limborina*), *D. scruposus* (*D.* subg. *Diploschistes*), and *D. ocellatus* (*D.* subg. *Thorstenia*). **A–C,** *Diploschistes candidissimus* (BCN-Lich no. 19340): **A,** habit; **B,** protocortex formed by loosely organized hyphae (arrows); **C,** dark granules incrusted in hyphal walls (arrows). **D–F,** *Diploschistes scruposus* (BCN-Lich no. 19328): **D,** habit; **E,** epinecral layer containing remnants of hyphae (arrows); **F,** dark pigmentation distributed on the epinecral layer (arrows). **G–I,** *Diploschistes ocellatus* (BCN-Lich no. 19341): **G,** habit; **H,** cortex formed by dense, anticlinally organized hyphae (arrows); **I,** dark pigmentation located on top of the protocortex, not embedded in hyphal walls (arrows). — EL, epinecral layer; PL, photobiont layer.

exceeded the intergeneric distances for euascomycetes, as established in Lumbsch (2002), and Martín & al. (2003) concluded that *D. ocellatus* could be a distant early diverging member of the genus or even a different lineage within the family. However, they did not propose any taxonomical changes for this taxon awaiting more comprehensive phylogenetic analyses. None of these studies drew conclusions with regard to the value of the morphology of the ascoma as a synapomorphic trait to define monophyletic groups within the genus.

Delimitation problems also occur at the species level, as morphological characters used by various researchers to define species resulted in conflicting taxonomies. One example is the number of ascospores per ascus. Poelt (1969) distinguished *D. scruposus* (Schreb.) Norman, with four spores per ascus, from *D. interpediens* (Nyl.) Zahlbr., with octosporous asci. Clauzade & Roux (1985) also considered these two taxa as distinct taxonomic units, but treated *D. interpediens* first as a subspecies of *D. gypsaceus* (Ach.) Zahlbr. and later as a subspecies of *D. diacapsis* (Ach.) Lumbsch (Clauzade & Roux, 1989). Finally, Lumbsch (1989) subsumed *D. interpediens* within *D. scruposus* regardless of the differences in ascospore number. *Diploschistes muscorum* (Scop.) R. Sant., a parasitic species on *Cladonia* P. Browne during the first stages of its development, is another example of controversial taxonomic treatments. Although broadly accepted, Clauzade & Roux (1985) treated this species as a lichenicolous subspecies of *D. scruposus* from which it is barely distinguishable once it becomes independent from *Cladonia*.

Based on the studies cited above, the phylogenetic position of the phenotypically distinct *D. ocellatus* as an early diverging lineage of *Diploschistes* remains to be ascertained. Also, the value of ascomatal morphology to delimit natural groups at the level of generic subdivisions is still untested by molecular phylogenetic methods, and several taxonomical problems, especially within the *D. scruposus* complex, are waiting to be solved. For these reasons, our main goals for this study were to: (1) test the monophyly of *Diploschistes* and determine its placement within the Graphidaceae; (2) investigate the taxonomic value of ascoma morphology within *Diploschistes*; and (3) assess conflicting taxon delimitations within the *D. scruposus* complex.

■ MATERIALS AND METHODS

Taxon sampling. — To test the monophyly of *Diploschistes*, we conducted a set of analyses based on alignments of the mitochondrial ribosomal RNA small subunit gene (mtSSU) and the nuclear ribosomal RNA large subunit gene (nuLSU) for 73 selected specimens from the order Ostropales. Eleven specimens of *Diploschistes* and a broad sampling within the Graphidaceae were included as part of the ingroup. To root the phylogeny, eleven species classified within Ostropales but not belonging to the Graphidaceae were used. In total, 146 sequences were used in the concatenated dataset, of which nine were generated by the first author, and the rest were downloaded from GenBank and the AFTOL database (AFTOL.org).

To further explore relationships within *Diploschistes*, we combined phenotypic and molecular data (nrITS, mtSSU). Fresh material collected in different areas of Spain and Portugal was complemented by selected material from various herbaria (BCN, DUKE, LEB, SANT). For the phylogenetic analyses based on morphological and chemical characters, we included 54 specimens of *Diploschistes*. The sampling was focused on the Scruposus group and, when possible, we included different individuals encompassing the whole morphological and ecological variation ascribed to each taxon. For the Actinostomus group, we included at least two specimens of each species, except for *D. euganeus* (A. Massal.) Steiner for which we only used one specimen. *Thelotrema lepadinum* (Ach.) Ach. and *T. suecicum* (H. Magn.) P. James were used as outgroup species for a total of 56 specimens included in these analyses.

For the molecular phylogenetic analyses, two data matrices (nrITS, mtSSU) were prepared for the same specimens as in the morphological and chemical datasets. All sequences were newly generated for these analyses, except for five sequences of *Diploschistes* and four sequences of *Thelotrema* that were downloaded from GenBank and the AFTOL database. Voucher information and GenBank accession numbers for newly generated sequences, and GenBank ID numbers for downloaded sequences, are listed in Appendix 1.

Morphological and chemical character selection. — A total of 33 morphological, anatomical, and chemical characters were evaluated for being potentially useful. Additionally, one ecological character referring to type of substrate was also examined as it has been traditionally considered of taxonomical value (e.g., Poelt, 1969; Clauzade & Roux, 1985; Lumbsch, 1989). Character scores in the morphological data matrix for the 54 selected specimens of *Diploschistes* are derived from specimen-based studies conducted by SFB. Phenotypic data for *Thelotrema* (outgroup) were drawn from the literature (Culberson & al., 1977; James & Hawksworth, 2009; Mangold & al., 2009) and represent morphological features at the species rather than at the specimen level.

From the 34 initially evaluated characters, 20 were discrete and 14 were continuous. All discrete characters were scored and included directly into the data matrix using Mesquite v.2.6 (Maddison & Maddison, 2009). The continuous characters were converted into discrete characters before they were incorporated into the data matrix (Electr. Suppl.: Fig. S1). The conversion was done following the method described in Lutzoni & Brodo (1995), also applied by McDonald & al. (2003), using the program R v.2.10.1 (R Development Core Team, 2009). First, we performed an analysis of variance, which led to the elimination of two continuous characters (i.e., algal layer thickness and excipulum thickness) for being invariant among taxa. For the remaining twelve continuous characters, for which the null hypothesis (H_0 = no significant differences among means) was rejected, we performed a Tukey's Honest Significant Difference test for pairwise comparisons of means. Test statistic probabilities were used to determine the character state for each taxon. Pairwise comparisons having *P*-values ≤ 0.05 were used to assign a character state to each group; the remaining comparisons, starting with the ones with highest *P*-values, were used to verify the character state assigned to each remaining taxon. Following McDonald & al. (2003), when intermediate P-values

were found ($P \leq 0.8$ and $P > 0.05$), the character was considered polymorphic and two states were given. The final 32 selected characters and their character states are listed in Table S1 and the morphological data matrix in Table S2 (Electr. Suppl.).

Molecular data. — Total DNA was extracted from fresh material and herbarium specimens, and isolated using a phenol-chloroform-isoamyl alcohol extraction protocol based on Lee & al. (1988). Isolated DNA was resuspended in sterile water and stored at −20°C.

Primer combinations for the three loci used in this study were: ITS1F (Gardes & Bruns, 1993) and ITS2, ITS3, and ITS4 (White & al., 1990) for the ≈0.6 kb nrITS; mrSSU1, mrSSU2, mrSSU2R, and mrSSU3R (Zoller & al., 1999) for the ≈0.8 kb of mtSSU; and LIC24R (Miadlikowska & Lutzoni, 2000) and LR7 (Vilgalys & Hester, 1990) for the ≈1.4 kb at the 5′ end of nuLSU. Symmetric PCR amplifications were prepared for a 25 µl final volume as in Gueidan & al. (2007), and were carried out in a Peltier thermal cycler (Perkin Elmer, GeneAmp PCR System 2400). The conditions for thermocycling of nrITS and mtSSU were the following: 94°C for 3 min linked to 35 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min with a final extension of 72°C for 10 min. For the amplification of the nuLSU the following cycling conditions were used: 95°C for 1 min linked to 36 cycles at 95°C for 45 s, 52°C for 40 s, and 72°C for 2 min 30 s with a final extension of 72°C for 10 min. In both PCR programs, after the final extension the samples were kept at 4°C. After examination by gel electrophoresis, amplification products were purified using ExoSAP-IT (USB Corp., Cleveland, Ohio, U.S.A.) and Speedtools PCR Clean-Up Kit (Biotools, Madrid, Spain) following the manufacturers' instructions. Sequencing reactions were prepared in a 10 µl final volume using the same amplification primers as well as LR3 and LR3R (Vilgalys & Hester, 1990) for the nuLSU region, and Big Dye Terminator Cycle sequencing kit v3.1 (ABI PRISM; Perkin-Elmer, Applied Biosystems, Foster City, California, U.S.A.) following the manufacturer's instructions. Sequencing products were subjected to electrophoresis with an ABI 3730xl DNA analyzer (PE Applied Biosystems).

Sequence alignments. — Sequence fragments were subjected to BLAST searches for a first verification of their identities. They were assembled and edited using Bioedit v.7.0 (Hall, 1999), and aligned manually in Mesquite v.2.6. Following Kjer (1995), the nuLSU locus was aligned with the help of the secondary structure of this RNA molecule from *Saccharomyces cerevisiae* Meyen, as reported by Cannone & al. (2002). Introns and ambiguously aligned regions (sensu Lutzoni & al., 2000) were delimited manually and excluded from the analyses. Alignments were submitted to TREEBASE (http://www.treebase.org; ID number 13666).

Phylogenetic analyses to assess the monophyly of *Diploschistes* within the Graphidaceae. — The mtSSU and nuLSU datasets for 73 specimens were analyzed separately using maximum likelihood (ML) as the optimization criterion with GARLI v.0.96 (Zwickl, 2006). Models of molecular evolution were estimated for each locus using the Akaike information criterion (AIC; Akaike, 1973) implemented in jModeltest v.0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). The selected models were TVM+I+G (Posada, 2003) for mtSSU, and GTR+I+G (Tavaré, 1986) for nuLSU. We used GARLI v.0.96 to estimate the values of base frequencies, substitution rates, proportion of invariable sites, and the shape parameter of the gamma distribution. We performed searches setting the program to stop after 10,000 generations if no improvement of the Ln likelihood ≤0.01 was detected, with a maximum of 500,000 generations. Before combining the two loci, topological incongruence between the two datasets was examined using 1000 replicates of ML bootstrapping under the same models described above, on each locus separately (Mason-Gamer & Kellog, 1996). Because there was no conflict detected using a 70% reciprocal threshold, the two alignments were concatenated.

Phylogenetic relationships and confidence were inferred on the combined dataset using maximum likelihood (ML1), a Bayesian approach (MB1), and weighted maximum parsimony (MP1). For ML1, the same settings were used as in the separate analyses using GARLI v.0.96, with the same estimated models specified for each partition, for both ML and ML bootstrap analyses (BS). In MB1, two parallel runs with four independent chains were conducted for 5 million generations using MrBayes v.3.1.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), with trees sampled at intervals of 100 generations. The AIC in MrModeltest v.2.3 (Nylander, 2004) was used to estimate the model of evolution, and GTR+I+G was selected for both partitions. The log-likelihood scores were graphically explored by plotting them against generation time with Tracer v.1.4.1 (Rambaut & Drummond, 2007) and stationarity was assumed when log-likelihood values reached a stable equilibrium (Huelsenbeck & Ronquist, 2001). This was also verified with the AWTY option (Wilgenbusch & al., 2004; Nylander & al., 2008). A burn-in sample of the first 5000 trees was discarded for each run and the remaining 90,000 trees (45,000 from each run) were used to estimate branch lengths and posterior probabilities (PP) with MrBayes v.3.1.1. Finally, MP1 was performed with PAUP* v.4.0b10 (Swofford, 2002). Constant sites were removed from all maximum parsimony analyses, gaps were treated as a fifth character state, and symmetric step matrices were created for unambiguously aligned regions of the two loci separately, using STMatrix v.3.0 (F. Lutzoni & S. Zoller, Dept. of Biology, Duke University), as outlined in Gaya & al. (2003). Phylogenetic signal from ambiguously aligned regions was integrated into the analyses with the programs INAASE v.2.3b (Lutzoni & al., 2000) and ARC v.1.5 (Kauff & al., 2003), as in Gaya & al. (2008). Heuristic searches were performed with 1000 random addition sequences (RAS), TBR (tree bisection-reconnection) branch swapping, MULTREES in effect, and collapsing branches with maximum branch length equal to zero. Branch support (BS) was assessed with 1000 bootstrap replicates (Felsenstein, 1985) with full heuristic searches, 36 RAS per bootstrap replicate and the same parameters as for MP1. The number of RAS per bootstrap replicate was calculated taking into consideration the number of times the shortest tree was found during the heuristic search using the original dataset.

Phylogenetic analyses within *Diploschistes*. — Analyses were performed for 56 taxa, using maximum parsimony (MP) as the optimization criterion, on the following datasets:

(1) morphological-chemical dataset (MP2); (2) nrITS dataset (MP3); (3) mtSSU dataset (MP4); (4) nrITS+mtSSU combined dataset (MP5); (5) morphological-chemical+molecular combined dataset (MP6). For the morphological-chemical dataset analyses, all changes among character states were equally weighted, and to simultaneously accommodate taxa with multiple character states resulting from polymorphy, the Variable option in PAUP* was used. In MP3, MP4, MP5, and MP6 analyses, gaps were treated as a fifth character state, constant sites were removed, unambiguously aligned portions of alignments were subjected to symmetric step matrices (ITS1, 5.8S, and ITS2 were treated separately), and ambiguously aligned regions were recovered using INAASE v.2.3b.

All heuristic searches were performed as in MP1. However, in MP2 and MP4, the high number of equally most parsimonious trees filled the memory before completing the search. For this reason, we executed successive searches progressively incrementing the number of trees saved per RAS. In the first round, we saved only one tree per replicate, in the second we saved 10, in the third we saved 100, and in the fourth and fifth rounds, 1000 and 10,000 trees were saved per replicate, respectively. With this search strategy, we could detect that, even though the number of trees saved per RAS was incremented, the topology of the majority-rule consensus tree remained the same.

Branch support was assessed by bootstrap analyses with full heuristic searches. For MP2, MP4, and MP6, we performed 10,000 bootstrap replicates, using two (in MP2 and MP6) and 10 (in MP4) RAS per bootstrap replicate and saving no more than 10 trees per RAS. For MP3 and MP5, we performed 1000 bootstrap replicates, using five (in MP3) and two (in MP5) RAS per bootstrap replicate and saving all trees per RAS. In all bootstrap analyses, we used the same parameters as in the original maximum parsimony search.

Before combining datasets for MP5 and MP6 searches, we assessed topological congruence among partitions as described above. Since no conflict was detected, datasets were subsequently combined.

Additionally, we analyzed the nrITS and mtSSU combined dataset with maximum likelihood (ML2) and a Bayesian approach (MB2). For ML2, the estimated models with the AIC in jModeltest v.0.1.1 were TIM2ef+G (Posada, 2008) for nrITS, and TIM1+G (Posada, 2008) for mtSSU. The settings for the maximum likelihood analysis and bootstrap searches were the same as for ML1. In MB2, the substitution models selected with the AIC in MrModeltest v.2.3 were SYM+G (Zharkikh, 1994) for nrITS and GTR+G for mtSSU. Bayesian analyses were run as in MB1. Most phylogenetic analyses performed in this study were carried out on the Duke Shared Cluster Resource (DSCR).

■ RESULTS

Alignments and phylogenetic relationships within the Graphidaceae (MB1; ML1; MP1). — The combined dataset for 73 specimens comprised 5526 sites (representing 1276 and 4250 sites for mtSSU and nuLSU, respectively), leaving 1229 sites after exclusion of 4297 sites corresponding to ambiguous regions and introns. In ML1 and MB1, from the included 1229 sites, 709 were constant and 520 were variable. In MP1, the 709 constant sites were excluded and ambiguously aligned regions were incorporated into the analyses as 29 INAASE characters (13 from mtSSU and 16 from nuLSU) and 437 ARC down-weighted characters (161 from mtSSU and 276 from nuLSU), for a total of 986 variable characters, of which 840 were parsimony-informative.

From the three analyses performed on this combined dataset, the majority-rule consensus tree of 90,000 sampled trees from MB1 recovered a topology with 63 resolved internodes, 37 of which were highly supported (PP ≥ 0.95). The most likely tree from ML1 (lnL = −12,848.96) revealed a similar tree, with 62 resolved internodes, 30 of those with BS ≥ 70%. The MP1 search yielded one most parsimonious tree of 6842.55 steps (consistency index [CI] = 0.380, retention index [RI] = 0.560, rescaled consistency index [RC] = 0.213), which was found in one island hit 122 times out of 1000 RAS. MP1 was the most resolved tree with 71 resolved internodes, 34 of those were significantly supported (BS ≥ 70%). The MB1 tree, with the highest number of significantly supported internodes, is shown in Fig. 2 with the statistic support indicated for all three analyses.

In all phylogenetic analyses, *Diploschistes* was recovered as monophyletic with strong support (PP = 1.00, BS = 94% for ML1, BS = 98% for MP1). *Diploschistes ocellatus* diverged first from the rest of the genus, which formed a well-supported clade (PP = 1.00, BS for ML1 and MP1 = 100%) but with relationships within it mostly not significantly resolved. In MB1 and ML1, *Acanthotrema frischii* Lücking appeared as the closest relative to the *Diploschistes* clade, albeit without support. In MP1 (tree not shown), the closest relative of *Diploschistes* was the clade formed by *Wirthiotrema* Rivas Plata & al., *Nadvornikia hawaiiensis* (Tuck.) Tibell, and *Thelotrema bicinctulum* Nyl., but again without support.

In general, deeper internodes within the Graphidaceae mainly lack resolution, although some well-supported clades can be distinguished corresponding to the genera *Chroodiscus* (Müll. Arg.) Müll. Arg., *Diorygma* Eschw., *Glyphis* Ach., *Platygramme* Fée, *Sarcographa* Fée (including *Leiorreuma hypomelaenum* (Müll. Arg.) Staiger), and *Wirthiotrema*. Conversely, *Chapsa*, *Thelotrema*, *Myriotrema* Fée, and *Ocellularia*, were not monophyletic as currently circumscribed. Genera such as *Graphis* Adans. and *Stegobolus* Mont. show an uncertain placement due to lack of resolution. In our phylogeny, the *Dyplolabia afzelii* (Ach.) A. Massal.–*Fissurina insidiosa* C. Knight & Mitt. clade (now subfamily Fissurinoidea sensu Rivas Plata & al., 2012 or family Fissurinaceae sensu Hodkinson, 2012) is sister to other Graphidaceae (PP = 1, BS = 99% for ML1, BS = 97% for MP1).

Morphological and chemical characters within *Diploschistes*. — From the initial 34 selected characters, 32 were finally retained as two continuous characters proved to be not significantly variable among the studied specimens (Electr. Suppl.: Table S1). The character states obtained for all 56 OTUs are summarized in Table S2 (Electr. Suppl.). From these 32 phenotypic characters used in MP2 and MP6 analyses, 30 were parsimony-informative.

Fig. 2. Bayesian phylogenetic inference resulting from a 50% majority-rule consensus of 90,000 sampled trees based on combined mtSSU and nuLSU sequences, depicting phylogenetic relationships among 61 taxa from the Graphidaceae, and 11 species from other families of the Ostropales (i.e., Coenogoniaceae, Gyalectaceae, Phlyctidaceae, Stictidaceae) used as outgroup taxa. Support values above branches are indicated as MB1PP/ML1BS/MP1BS. Thicker internodes show significant support for at least one statistical method (PP ≥ 0.95, MLBS and MPBS ≥ 70%).

Alignments within *Diploschistes*. — The nrITS alignment included 815 sites, from which 510 sites corresponding to 36 ambiguously aligned regions and 227 constant sites were excluded from the MP3 analysis. This high number of ambiguous regions was due to the remarkable variation in length of the sequences of *Diploschistes ocellatus*, especially in ITS1 and ITS2. The signal from 34 ambiguously aligned regions was recovered as 34 INAASE characters that were combined with 78 variable characters for a total of 112 included characters, of which 100 were parsimony-informative.

The final size of the data matrix of mtSSU was 899 sites. A total of eight ambiguously aligned regions were delimited, resulting in the exclusion of 158 sites. A total of 636 constant sites was also excluded from the MP4 analysis. Six ambiguously aligned regions were recovered as six INAASE characters and combined to the 105 remaining characters, for a total of 111 variable characters, 97 of which were parsimony-informative.

The combined nrITS and mtSSU data matrix comprised 1714 sites, from which 668 sites were delimited and excluded from all analyses. From the total of 1046 characters subjected to ML2 and MB2 analyses, 875 were constant and 171 were variable. In MP5 and MP6, constant sites were excluded and 40 INAASE-coded characters were added to the data matrices for a total of 223 variable sites, of which 197 were parsimony-informative.

Phylogenetic relationships within *Diploschistes* based on morphological and chemical data (MP2). — The MP2 search yielded 58,700 equally most parsimonious trees of 293 steps (summarized on a strict consensus tree; Electr. Suppl.: Fig. S2), which were part of 587 islands that were hit 587 times each out of 1000 RAS (CI = 0.901, RI = 0.910, RC = 0.820). In the resulting topology, two major strongly supported clades were recovered: one including all specimens of *Diploschistes ocellatus* (BS = 100%), and the other grouping the remaining members of *Diploschistes* (BS = 87%). Within the larger clade, most relationships were unresolved, and only four internodes were highly supported.

Phylogenetic relationships within *Diploschistes* based on molecular data (MP3, MP4, MP5, ML2, MB2). — Six equally most parsimonious trees of 494.58 steps resulted from the MP3 search (summarized on a strict consensus tree; Electr. Suppl.: Fig. S3), which were part of one island, hit 604 times out of 1000 RAS (CI = 0.874, RI = 0.945, RC = 0.827). The MP4 search resulted in 90,400 equally most parsimonious trees of 227.79 steps (Electr. Suppl.: Fig. S4), which were part of 904 islands that were hit 1000 times out of 1000 RAS (CI = 0.977, RI = 0.987, RC = 0.964). The MP5 search yielded 336 equally most parsimonious trees (Electr. Suppl.: Fig. S5) of 728.37 steps, which were part of one island, hit 995 times out of 1000 RAS (CI = 0.899, RI = 0.952, RC = 0.856).

MP3 (only nrITS; Electr. Suppl.: Fig. S3) and MP5 (combined nrITS and mtSSU; Electr. Suppl.: Fig. S5) revealed similar topologies due to little increase in resolution with the addition of mtSSU (MP4, only mtSSU; Electr. Suppl.: Fig. S4). In MP3 and MP5, specimens of *Diploschistes* appeared in three main clades that correspond to the groups defined by ascoma morphology. In MP3, the Ocellatus and the Actinostomus groups were strongly supported (BS ≥ 70%), whereas in MP5 all three main groups obtained high support (BS ≥ 70%). In MP4, relationships within *Diploschistes* were little resolved. Only *D. ocellatus* and *D. rampoddensis* (Nyl.) Zahlbr. appeared monophyletic with strong support (BS = 98% and 91% respectively), and the Actinostomus and Scruposus groups were grouped together with 100% bootstrap support.

The ML2 search (Electr. Suppl.: Fig. S6) showed a loss of resolution compared to the MP analysis of the same dataset (MP5), with only 21 resolved internodes of which 10 were supported, 12 less than in MP5. The Bayesian topology recovered was almost identical to ML2, with 19 resolved internodes. The differences of resolution among methods are due to the addition of the INAASE characters in the maximum parsimony analyses.

Phylogenetic relationships within *Diploschistes* based on morphological, chemical, and molecular data (MP6). — The final combined data matrix consisted of 1786 sites, which comprised 1714 molecular characters from the concatenated nrITS and mtSSU alignments, 40 INAASE characters, and 32 morphological and chemical characters. From the total of 255 included characters, 227 were parsimony-informative. The MP6 search yielded 152 equally most parsimonious trees of 1052.38 steps (summarized on a strict consensus tree; Fig. 3), which were part of one island that was hit 1000 times out of 1000 RAS (CI = 0.873, RI = 0.928, RC = 0.811).

With the addition of morphological and chemical characters, the MP6 analysis showed a decrease in the number of equally most parsimonious trees compared to MP5 (152 vs. 336 trees, respectively). The recovered topology (Fig. 3) is quite similar to the MP5 tree (Electr. Suppl.: Fig. S5), with slight differences in the relationships within the Scruposus clade, and a general increase in the number of resolved and significantly supported internodes. Within the Actinostomus group, all species represented by more than one specimen were confidently recovered as monophyletic. Regarding the Scruposus group, MP6 recovered four additional monophyletic groups (*D. gypsaceus*, *D. muscorum*, and two additional clades of *D. scruposus*). Two *D. diacapsis* clades, *D. gypsaceus* as well as *D. rampoddensis* were significantly supported. The specimens of *D. interpediens* were placed in two different monophyletic groups, one with strong support (BS = 100%). Finally, the Ocellatus group was revealed once more as significantly monophyletic. With five additional resolved internodes and 26 supported internodes, we consider the phylogeny derived from MP6 as our best estimate of relationships within *Diploschistes*.

■ DISCUSSION

Generic circumscription of *Diploschistes*. — Our phylogenetic analysis of the Graphidaceae, based on mtSSU-nuLSU (Fig. 2), confirms the monophyly of *Diploschistes* including *D. ocellatus*. These results are consistent with the molecular phylogeny presented by Martín & al. (2003). Apart from the morphological and chemical differences between *D. ocellatus* and the remaining members of the genus, remarkable molecular differences are also present, as shown by the long branches on the MB1 and ML2 trees (Fig. 2 and Fig. S6, respectively). Due

to this molecular variation, Martín & al. (2003) considered that *D. ocellatus* could potentially be considered a genus separate from *Diploschistes*. In the present study, with a considerably increased taxon sampling for the Graphidaceae, the same taxonomic conclusions, the erection of a new genus or status quo, could be drawn.

A recent study by Rivas Plata & Lumbsch (2011) revealed the existence of similar morphotypes (referring to thallus, ascoma, and ascospore morphology) in distantly related lineages within the Graphidaceae due to parallel evolution. These results have important consequences for the classification of this group of lichens, since the combination of ascomatal characters (i.e., excipulum structure and pigmentation, occurrence of lateral paraphyses), which have been traditionally used for generic circumscription of thelotremoid Graphidaceae (Hale, 1980, 1981), cannot be considered reliable. Frisch & al. (2006) introduced the use of the character "formation of columella" (which in *Diploschistes* is lacking) to be added to the excipular characters proposed by Hale (1980, 1981) to delimit genera within the thelotremoid Graphidaceae. However, the presence of a columella has been revealed as highly homoplastic in a recent study by Rivas Plata & Lumbsch (2011). Therefore, the only synapomorphy available to circumscribe *Diploschistes* is the type of photobiont. Interestingly, *Diploschistes* is the only genus within the Graphidaceae—after transferring *Ingvariella* (Bagl.) Guderley & Lumbsch to the Stictidaceae (Fernández-Brime & al.,

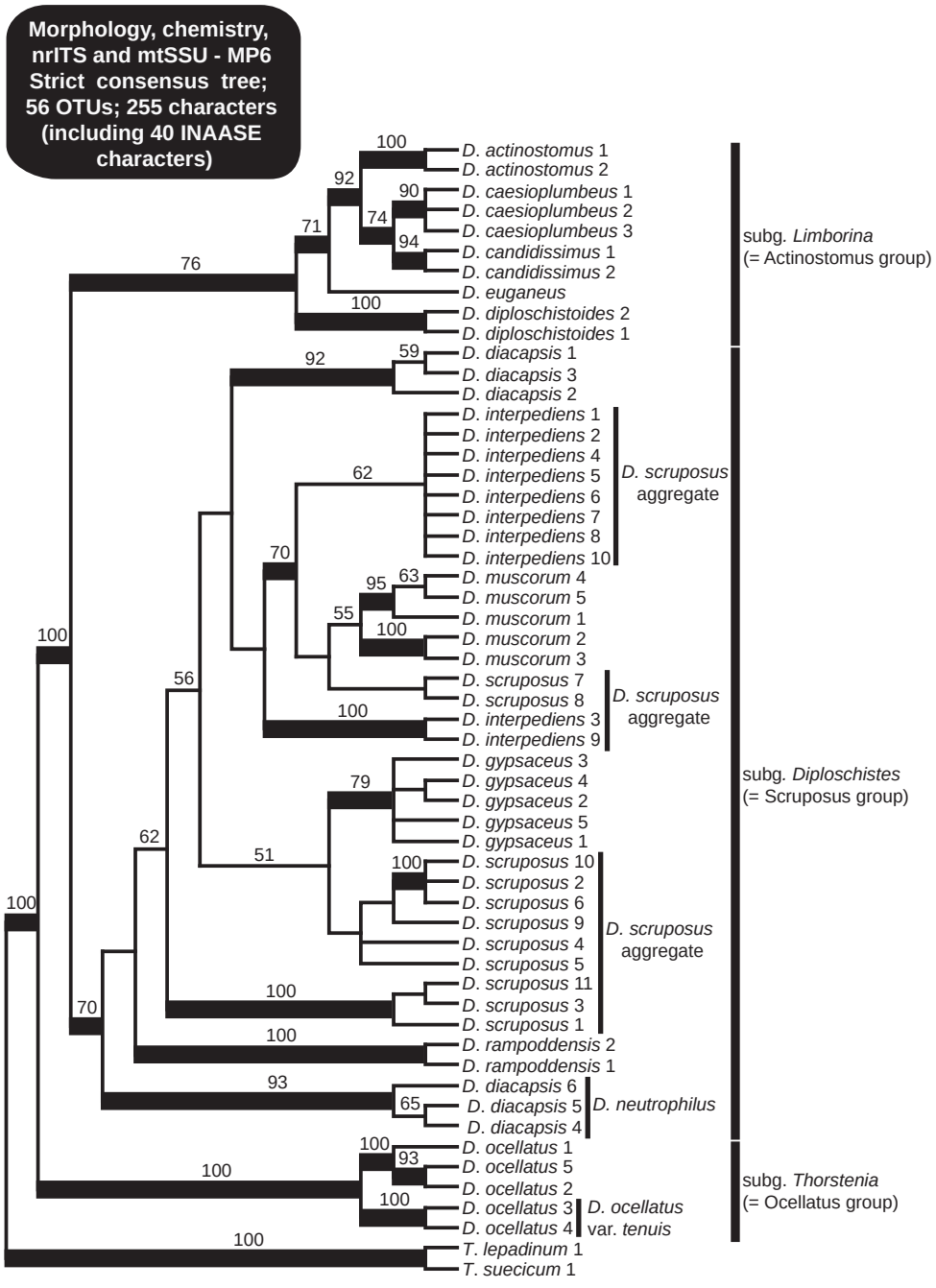

Fig. 3. Strict consensus tree of 152 equally most parsimonious trees based on combined nrITS, mtSSU, and morphological-chemical data of *Diploschistes* taxa. Parsimony bootstrap percentages (BS) greater than 50% are shown above branches. Highly supported branches (BS ≥ 70%) are indicated by bold lines.

2011)—with trebouxioid photobionts and a distribution across both Hemispheres, mainly in arid and semiarid regions. All other taxa of this family have trentepohlioid algae and mainly occur in tropical and in humid subtropical climates. In order to study the evolution of photobiont and habitat switches among these taxa, we need a stable phylogenetic framework for the family. In this study, we could not assess with confidence the relationships of *Diploschistes* with other members of the Graphidaceae. The inclusion of more informative loci, such as protein-coding genes (e.g., Rivas Plata & Lumbsch, 2011), will be necessary to assess deep relationships within the family that we could not resolve here with nuclear and mitochondrial ribosomal RNA-coding loci. For all these reasons we prefer to retain the status quo for the delimitation of the genus *Diploschistes*.

Relative contribution of morphological, chemical, and molecular characters to the *Diploschistes* phylogeny. — In this study (MP2; Electr. Suppl.: Fig. S2)**,** morphological, anatomical, and chemical traits traditionally used in the taxonomy of *Diploschistes* have been shown to be useful to delimit several species (i.e., *D. actinostomus* (Pers. ex Ach.) Zahlbr., *D. candidissimus* (Kremp.) Zahlbr., *D. diploschistoides* (Vain.) G. Salisb., *D. gypsaceus*, *D. muscorum*, *D. ocellatus*). However, they failed in delimiting phenotypically similar species, such as *D. interpediens*, *D. rampoddensis*, and *D. scruposus*, or to group the specimens of *D. diacapsis*.

The topology of the combined molecular datasets (nrITS +mtSSU) was very similar to the nrITS topology. However, the combination of both loci was necessary to confidently recover the Actinostomus, Scruposus, and Ocellatus groups (proposed as subgenera in this study). The addition of morphological and chemical data to the combined molecular dataset recovered for the first time *D. gypsaceus* and *D. muscorum* as monophyletic, and significantly supported *D. candidissimus*, and both clades of *D. diacapsis*. This is concordant with previous studies reporting that the addition of phenotypic characters to molecular datasets can provide additional phylogenetic signal corroborating molecular-based phylogenies (Lutzoni & Vilgalys, 1995; Miadlikowska & Lutzoni, 2000; McDonald & al., 2003; Gaya & al., 2011).

Generic subdivisons of *Diploschistes*. — Since Lettau (1932–1937) and Lumbsch (1989) established the grouping of *Diploschistes* species based on the degree of opening of ascomata, these groups have been widely used in taxonomic treatments of the genus for practical reasons. However, none of these groups has ever been formally recognized, since previous phylogenetic studies (i.e., Lumbsch & Tehler, 1998; Martín & al., 2003) did not recover them as monophyletic entities. Our results, derived from concatenated datasets (Fig. 3), for the first time reveal these three morphological groups as significantly supported monophyletic entities. Consequently, a new subgeneric treatment for this genus is proposed.

Based on molecular data and morphological evidence, we propose here to consider the Actinostomus group as a new subgenus, *Diploschistes* subg. *Limborina* (see Taxonomic conclusions). In this group, all taxa have perithecioid ascomata, and share a mainly continuous to rimose-areolate thallus (Fig. 1A) and the presence of a more or less differentiated protocortex, formed by loosely organized hyphae anticlinally arranged (Fig. 1B). Additionally, in all examined thallus cross-sections, we detected a dark pigment that incrusted in the hyphae walls of the upper part of this protocortex (Fig. 1C).

We place the *scrupous* group in the subgenus *Diploschistes* subg. *Diploschistes*, being *Diploschistes scruposus* (Schreb.) Norman the type of the genus (see Taxonomic conclusions). In this clade all specimens have urceolate ascomata and mainly verrucose thalli (Fig. 1D). There is no distinguishable cortex or protocortex, and only an epinecral layer can be observed with some remnants of hyphae (Fig. 1E), with granules of fuliginous pigmentation (Fig. 1F).

Finally, we propose that *Diploschistes ocellatus* must be recognized as a monotypic subgenus, *Diploschistes* subg. *Thorstenia* (see Taxonomic conclusions). This species is unique in having lecanoroid ascomata, a thallus formed by strongly convex areolae (Fig. 1G), and a well-developed prosoplectenchymatous cortex formed by dense, anticlinally organized hyphae (Fig. 1H). Dark pigmentation granules are present in a thin layer on top of this cortex, but never associated to the hyphae walls (Fig. 1I). These traits can be added to the above-mentioned characters (i.e., lack of a distinguished carbonized excipulum with lateral paraphyses, and presence of β-orcinol depsidones belonging to the norstictic acid chemosyndrome instead of orcinol depsides), which already separated *D. ocellatus* from the rest of the genus.

Species delimitation within *Diploschistes*. — Within subgenus *Thorstenia*, we observed two morphotypes which correspond to two well-supported groups (Fig. 3). One of them contains exclusively fertile specimens, with ascomata and pycnidia (specimens 1, 2, and 5), whereas the other group consists of specimens with only pycnidia (specimens 3 and 4). These groups might represent an example of a lichen "species pair" (Poelt, 1970) where the clade formed by specimens 3 and 4 that reproduce only vegetatively through pycnidiospores would be sister taxon to sexual *D. ocellatus* specimens. Our finding, however, contrasts with other studies also based on molecular data (e.g., Articus & al., 2002; Cubero & al., 2004; Buschbom & Mueller, 2006) in which members of species pairs form a single intermixed monophyletic group without distinction. Independently of the "species pair" consideration, and based on our very limited sampling, we cannot conclude if the specimens that do not form ascomata represent a distinct species. For this reason, we propose to create the variety *D. ocellatus* var. *tenuis* (see Taxonomic conclusions).

Within subgenus *Diploschistes*, the specimens identified as *D. diacapsis* are recovered in two distantly related monophyletic clades, although none of the intervening internodes are well-supported (Fig. 3). During our morphological survey, we found differences in thallus morphology and ecology among the examined specimens, which correlate with the two different clades in our phylogenetic analyses. Specimens 1, 2, and 3 have thick thalli that become convex and very loosely attached to the substratum, and show a preference for gypsiferous soils and for highly calcareous soils from inland continental areas. These specimens fit the morphology and ecology of the type material used to describe *D. diacapsis* (isolectotype: Spain, Lagasca, H-ACH 936a [n.v.]). Specimens 4, 5, and 6 have thinner thalli

that become very flat, as they all grow completely attached to the substratum, on moderately calcareous to decarbonated soils, in coastal areas. These individuals have the same morphology and ecology as the subspecies described by Clauzade & Roux (1989) as *D. diacapsis* subsp. *neutrophilus* (Clauzade & Cl. Roux) Clauzade & Cl. Roux (holotype: Provence, France, *C. Roux*, MARS [hb. Claude Roux no. 99]). Our morphological and molecular results support the recognition of *D. diacapsis* subsp. *neutrophilus* at the species level (see Taxonomic conclusions).

Diploschistes gypsaceus is a species well defined morphologically (heavily whitish-pruinose thallus), chemically (lack of diploschistesic acid), and ecologically (vertical, sheltered surfaces of carbonated rocks), which has been recognized by many authors (e.g., Poelt, 1969; Lumbsch, 1989; Sérusiaux & al., 1999). However, in Clauzade and Roux's classification (1989), *D. gypsaceus* was treated as a subspecies of *D. scruposus*. Based on our results (Fig. 3), the combined phenotypical and molecular data support the recognition of this taxon at the species level.

Diploschistes muscorum is one of the most easily recognizable and collected species of the genus. This species initiates its development as a parasite on *Cladonia* and then becomes independent and able to grow on other substrates (e.g., soil, mosses). The morphology of the thallus changes during the different phases of the life cycle; as a consequence, a large number of infraspecific taxa have been described (Lumbsch, 1989). In our study, we included individuals in different developmental stages to cover this morphological variation. The species was recovered as monophyletic with high support only in the morphological-chemical phylogeny (Electr. Suppl.: Fig. S2). When the phenotypical and molecular data were combined, *D. muscorum* was still monophyletic, but with low support.

Diploschistes scruposus is a cosmopolitan species that occurs in numerous biomes, especially in the Northern Hemisphere, and is one of the most broadly sampled taxa in the genus. This species shows a wide range of morphological variation, which has resulted in the description of numerous varieties and subspecies (see Lumbsch, 1989). Two chemical groups have also been described: one of them contains lecanoric acid as a major compound and orsellinic acid as a minor compound, whereas the second group has lecanoric and diploschistesic acids as major compounds and orsellinic acid as a minor compound. However, no morphological characters have been found related to these two chemotypes (Lumbsch, 1989). In our study, we attempted to cover the wide morphological and ecological variation, including specimens of *D. scruposus* from different climatic areas, as well as from various siliceous substrates (weathered granite, schists, and quartz). The combined phylogeny (Fig. 3) recovered this species as polyphyletic within subgenus *Diploschistes*. Despite the phenotypical variation mentioned above, we have found neither obvious morphological or chemical differences nor a biogeographical pattern that correlates with the placement of the specimens of *D. scruposus* in three different clades. Moreover, two of these clades are not well supported as monophyletic and their placement within the subgenus is uncertain. Therefore, until further studies resolve this species complex with higher confidence, we propose to provisionally treat this taxon as *D. scruposus* agg. following Grube & Kroken (2000).

In the monographic study of *Diploschistes* from the Holarctic region, Lumbsch (1989) included *D. interpediens* within *D. scruposus*, which typically has four spores per ascus instead of eight. With this synonymization, *D. scruposus* became characterized by having four to eight spores, a concept followed by later authors (e.g., Pant & Upreti, 1993; Mangold & al., 2009). In our study, however, we treated them *a priori* as two different taxa. Based on our current phylogeny (Fig. 3), *D. interpediens* appears as polyphyletic, the same way as *D. scruposus*. Due to the lack of resolution and to being distributed over several intermixed clades, we prefer to maintain *D. interpediens* within the *D. scruposus* aggregate following Lumbsch (1989) until more data is gathered. These two taxa with virtually identical ecologies only differ by the number of spores per ascus and a slightly different distribution. Samples named as *D. interpediens* in this study indicate a morphotype with constant 8-spored asci and restricted to the Mediterranean region. It is remarkable that within subgenus *Diploschistes*, these unresolved taxa are embedded in a clade with well-established species (i.e., *D. diacapsis*, *D. gypsaceus*, *D. muscorum*) recovered as monophyletic that correlate well with sets of fixed morphological and ecological traits. This pattern may suggest a case of ongoing speciation, maybe related to ecological diversification, where some taxa have not yet reached the status of species.

Diploschistes rampoddensis shows morphological similarities with the 8-spored specimens of the *D. scruposus* aggregate, as both are saxicolous, have urceolate ascomata, and octosporous asci. However, *D. rampoddensis* has a thinner and friable thallus, lacks diploschistesic acid, and shows a pantropical distribution (Lumbsch, 1993; Pant & Upreti, 1993). In our phylogeny (Fig. 3), *D. rampoddensis* is confidently recovered as monophyletic, separate from closely related taxa.

Apart from the lack of phylogenetic signal to clearly circumscribe several taxa, relationships within subgenus *Diploschistes* remain poorly supported, even when morphological, chemical, and molecular data were combined. Faster evolving molecular markers and denser sampling are needed to conduct population genetic analyses.

Taxa of subgenus *Limborina* were revealed as highly supported monophyletic entities (except for *D. euganeus* represented by a single specimen) with strongly supported interspecies relationships. However, very few samples were included from this group and we cannot exclude more complex relationships among taxa of this subgenus, as has been shown for subgenus *Diploschistes*. Therefore, further studies will need a larger sampling of *Diploschistes* species with perithecioid ascomata.

■ TAXONOMIC CONCLUSIONS

Based on the molecular and morphological evidences presented above, we consider the three lineages within *Diploschistes* should be treated as distinct subgenera. Therefore, two new subgenera are proposed here. The list of species included within each subgenus is given below. The species treated in our molecular study are indicated in bold face. Species for which we did not have molecular data and were not included in our

analyses, were ascribed to each subgenus based on ascoma morphology as stated on their original descriptions.

Diploschistes Norman in Nyt Mag. Naturvidensk. 7: 232. 1853 – Type: *Diploschistes scruposus* (Schreb.) Norman designated by Clements & Shear, Gen. Fung., ed. 2: 320. 1931 (≡ *Lichen scruposus* Schreb., Spic. Fl. Lips.: 133. 1771).

Diploschistes Norman subg. ***Diploschistes***
Mycobank no. MB803206

Diagnosis. – Thallus verrucose-areolate; without cortex or protocortex, with well-developed epinecral layer, generally opaque due to the presence of dark granules; ascomata urceolate, immersed to sessile, sometimes secondarily subdivided, with deeply concave disc visible from above; thalline rime margin thin, occasionally thick, immersed; proper excipulum well developed, radially striated, carbonized in section (dark brown to blackish), with lateral paraphyses.

Included species. – *Diploschistes cinereocaesius* (Sw.) Vain., *D. conceptionis* Vain., ***D. diacapsis*** (Ach.) Lumbsch, *D. isabellinus* Zahlbr., ***D. gypsaceus*** (Ach.) Zahlbr., *D. hypoleucus* Zahlbr., ***D. muscorum*** (Scop.) R. Sant, ***D. neutrophilus*** (Clauzade & Cl. Roux) Fdez.-Brime & Llimona, *D. nepalensis* G. Pant & Upreti, ***D. rampoddensis*** (Nyl.) Zahlbr., ***D. scruposus*** (Schreb.) Norman, *D. thunbergianus* (Ach.) Lumbsch & Vězda.

Diploschistes subg. ***Limborina*** Fdez.-Brime, Gaya & Llimona, **subg. nov.** – Type: *Diploschistes actinostomus* (Pers. ex Ach.) Zahlbr. (≡ *Verrucaria actinostoma* Pers. ex Ach.).
Mycobank no. MB801951

Diagnosis. – Thallus rimose-areolate, sometimes verrucose-areolate; poorly developed protocortex, with dark parietal pigments on hyphae walls, and an upper thin translucent epinecral layer; ascomata perithecioid, immersed, not subdivided, with disc not visible from above, opened only by a small pore; thalline margin thin, immersed; proper excipulum well developed, radially striated, carbonized in section (dark brown to blackish), with lateral paraphyses.

Etymology. – The name *Limborina* was chosen in remembrance of the ancient genus *Limboria* Ach. emend. A. Massal. (1852) used by Massalongo to describe for the first time a separate taxon including *Limboria actinostoma* (Pers. ex Ach.) A. Massal. (≡ *Verrucaria actinostoma* Pers. ex Ach.) and the new species *L. euganea* A. Massal., which shared the same type of ascoma (both species are currently recognized within *Diploschistes*).

Nomenclatural note. – In the process of preparing this manuscript it became apparent that the generic name *Limboria* Ach. (1815) could be an earlier name for *Diploschistes* Norman. This is because *L. constellata* Ach., one of the species included in *Limboria* at the time of its original description, was subsequently combined in *Diploschistes* by Zahlbruckner (1892). To our knowledge neither *Limboria* nor *L. constellata* have been typified. Nonetheless, Zahlbruckner's interpretation of *L. constellata* as a terricolous American species of *Diploschistes* directly conflicts with Acharius' description of a fungus (not clearly specified to be a lichen) growing on wood from India. Indeed the description and illustrations of *L. constellata* published in the protologue do not correlate with any known species of *Diploschistes.* Thus we consider it highly unlikely that the type material of *L. constellata* represents a member of *Diploschistes* and that *Limboria* and *Diploschistes* are synonyms. We refrain from typifying either name here because the types of several other names introduced with *L. constellata* have yet to be evaluated.

Included species. – ***Diploschistes actinostomus*** (Pers. ex Ach.) Zahlbr., *D. aeneus* (Müll. Arg.) Lumbsch, *D. albopruinosus* Pérez-Vargas, Hern. Padrón & Elix, *D. arabiensis* Lumbsch, *D. austroafricanus* Guderley & Lumbsch, *D. awasthii* G. Pant & Upreti, *D. badius* Lumbsch & Elix, ***D. caesioplumbeus*** (Nyl.) Vain., ***D. candidissimus*** (Kremp.) Zahlbr., ***D. diploschistoides*** (Vain.) G. Salisb., *D. elixii* Lumbsch & Mangold, ***D. euganeus*** (A. Massal.) Steiner, *D. gyrophoricus* Lumbsch & Elix, *D. hensseniae* Lumbsch & Elix, *D. megalosporus* Lumbsch & Mayrhofer, *D. microsporus* Lumbsch & Elix, *D. prominens* (Vain.) Lumbsch, *D. sticticus* (Körb.) Müll. Arg., *D. thelenelloides* Lumbsch & Aptroot.

Diploschistes subg. ***Thorstenia*** Fdez.-Brime, Gaya & Llimona, **subg. nov.** – Type: *Diploschistes ocellatus* (Vill.) Norman (≡ *Lichen ocellatus* Vill.).
Mycobank no. MB801955

Diagnosis. – Thallus formed by deeply convex areolae; well-developed prosoplectenchymatous cortex with accumulation of dark granules of pigment on its upper part, not associated to the hyphal walls; ascomata lecanoroid, sessile, with broadly open disc, flat or slightly concave; thalline rime thick and prominent; proper excipulum very reduced, not carbonized in section (pale brown), without lateral paraphyses.

Etymology. – The subgenus is named after H. Thorsten Lumbsch, an expert on the group, who has made major contributions to the knowledge of *Diploschistes*.

Included species. – ***Diploschistes ocellatus*** (Vill.) Norman.

Diploschistes neutrophilus (Clauzade & Cl. Roux) Fdez.-Brime & Llimona, **comb. & stat. nov.** ≡ *Diploschistes gypsaceus* subsp. *neutrophilus* Clauzade & Cl. Roux in Bull. Soc. Bot. Centre-Ouest, nouv. sér., num. spec. 7: 823. 1985 ('*neutrophila*') ≡ *Diploschistes diacapsis* subsp. *neutrophilus* (Clauzade & Cl. Roux) Clauzade & Cl. Roux in Bull. Soc. Linn. Provence 40: 110. 1989 ('*neutrophila*') – 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* (MARS, hb. Claude Roux no. 99).
Mycobank no. MB802353

Diploschistes ocellatus var. ***tenuis*** Fdez.-Brime & Llimona, **var. nov.** – Holotype: Spain, Catalonia, Lleida, La Segarra, Torà, Font de Can Porta, by the road to Solsona, 31TCG6830, 550–600 m, 13 Jun 2008, *Llimona & Fernández-Brime s.n.*, (BCN-Lich no. 19341)
Mycobank no. MB802352

Diagnosis. – Similar to *Diploschistes ocellatus* (Vill.) Norman var. *ocellatus* but having thinner thalli and always lacking ascomata.

Taxa not examined and not assigned to one of the subgenera. — *D. constellatus* (Ach.) Zahlbr., *D. lavicola* H. Magn., *D. mexicanus* B. de Lesd., *D. oceanicus* Zahlbr., *D. ochraceus* Steiner, *D. perrimosus* (Stirt.) Zahlbr., *D. pruiniger* (Steiner & Zahlbr.) C.W. Dodge, *D. sandwiciensis* H. Magn., *D. sanguinescens* Zahlbr., *D. scruposulus* (Nyl.) Steiner, *D. sinensis* H. Magn., *D. steifensandii* (Steiner) Zahlbr., *D. subcupreus* (Nyl.) Zahlbr.

■ ACKNOWLEDGMENTS

The authors thank the curators of the herbaria of BCN, DUKE, LEB, and SANT, in addition to Claude Roux, for providing material used in this study. Thorsten Lumbsch and Claude Roux are kindly thanked for sharing with us their extensive knowledge about *Diploschistes*. We also thank Damien Ertz for generating some of the unpublished sequences in the AFTOL database used for this study; Jolanta Miadlikowska and Frank Kauff for help with the phylogenetic analyses; José Manuel Blanco for the R scripts; Molly McMullen for the English revision and proofreading; Gerry Moore and James Lendemer for their valuable comments regarding the nomenclature; Néstor Hladun for help obtaining digital photos. This study was carried out within the project CGL2007-66734-C03-02 (Spanish government), the project "Filogènia molecular de les Teloschistales i Ostropales Fase II" (Institut d'Estudis Catalans – Secció Ciències Biològiques), and by the "Fons Memorial Salvador Llimona", a Ph.D. scholarship (FPU–Formación del Profesorado Universitario, Ministerio de Educación) to SFB, and the Assembling the Fungal Tree of Life (AFToL) project, supported by NSF Assembling the Tree of Life (ATOL) award DEB-0228668 to FL.

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Appendix 1. List of sequences used in this study. Voucher information and GenBank accession numbers are provided for specimens newly sequenced for this study, whereas GenBank ID numbers are provided for sequences retrieved directly from GenBank.

Species, voucher information (geographic origin, collector, herbarium), nrITS, mtSSU, nuLSU ("–" denotes missing sequence)

Acanthothecis aurantiaca (Müll. Arg.) Staiger & Kalb: –, 90995142, 90995106; ***Acanthotrema frischii*** Lücking [sub ***A. brasilianum*** (Hale) Frisch in GenBank]: –, 110585750, 90995105; ***Acarosporina microspora*** (R.W. Davidson & R.C. Lorenz) Sherwood: –, 46411377, 46411432; ***Ampliotrema auratum*** (Tuck.) Kalb ex Kalb: –, 156739879, 156739928; ***Chapsa astroidea*** (Berk. & Broome) M. Cáceres & Lücking: –, 156739881, 156739929; ***Chapsa leprocarpa*** (Nyl.) Frisch: –, 156739883, 156739930; ***Chapsa phlyctidioides*** (Müll. Arg.) Mangold [sub ***Thelotrema phlyctidioides*** (Müll. Arg.) Hale in GenBank]: –, 156739884, 156739932; ***Chapsa pulchra*** (Müll. Arg.) Mangold [sub ***Ocellularia pulchra*** Müll. Arg. in GenBank]: –, 156739886, 156739934; ***Chroodiscus coccineus*** (Leight.) Müll. Arg.: –, 110585749, 19171976; ***Chroodiscus defectus*** Papong & Lücking: –, 224797138, 224797131; ***Coenogonium leprieurii*** (Mont.) Nyl.: –, 46411453, 19171977; ***Coenogonium luteum*** (Dicks.) Kalb & Lücking: –, 46411454, 12025070; ***Diorygma circumfusum*** (Stirt.) Kalb, Staiger & Elix: –, 90995140, 55139914; ***Diorygma sipmanii*** Kalb, Staiger & Elix: –, 90995138, 55139915; ***Diploschistes actinostomus*** (Pers. ex Ach.) Zahlbr.: (1) U.S.A., *Yahr 4569* (DUKE 0016461), KC166972, KC167025, –; (2) [sub ***D. scruposus*** in GenBank], 336397129, 46411447, –; ***Diploschistes caesioplumbeus*** (Nyl.) Vain.: (1) Spain, *Llimona s.n.* (BCN-Lich 19325), KC166973, KC167026, –; (2) Spain, *Llimona & Fernández-Brime 101* (BCN-Lich 19323), KC166974, KC167027, –; (3) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 17182), KC166975, 330369529, 330369540; ***Diploschistes candidissimus*** (Kremp.) Zahlbr.: (1) U.S.A., *Worthington 23741* (DUKE 0144447), KC166976, KC167028, –; (2) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19340), KC166977, KC167029, –; ***Diploschistes cinereocaesius*** (Sw.) Vain.: –, 119642420, 113958939; ***Diploschistes diacapsis*** (Ach.) Lumbsch: (1) Spain, *Yahr 2431a* (DUKE 0030912), KC166978, KC167030, –; (2) U.S.A., *Nash III 44742* (DUKE 0130126), KC166979, KC167031, –; (3) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19347), KC166980, KC167032, –; ***Diploschistes diploschistoides*** (Vain.) G. Salisb.: (1) [sub ***D. almbornii*** C.W. Dodge] Australia, *Elix 27941* (DUKE 0144445), KC166984, KC167036, 47525220; (2) Australia, *Lumbsch & Guderley 11115n* (DUKE 0018863), KC166985, KC167037, –; ***Diploschistes euganeus*** (A. Massal.) Steiner: Australia, *Lumbsch 5524b* (DUKE 01444451), KC166986, KC167038, –; ***Diploschistes gypsaceus*** (Ach.) Zahlbr.: (1) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 17180), KC166987, 330369530, 330369541; (2) Spain, *Llimona s.n.* (BCN-Lich 19324), KC166988, KC167039, –; (3) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19345), KC166989, KC167040, –; (4) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19346), KC166990, KC167041, KC167075; (5) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19340), KC166991, KC167042, –; ***Diploschistes muscorum*** (Scop.) R. Sant.: (1) U.S.A., *Yahr 4500* (DUKE 0016462), KC167004, KC167055, KC167077; (2) Italy, *Fernández-Brime s.n.* (BCN-Lich 19333), KC167005, KC167056, –; (3) Spain, *Hladun & Muñiz s.n.* (BCN-Lich 14435), KC167006, KC167057, –; (4) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19344), KC167007, KC167058, –; (5) Spain, *Fernández-Brime s.n.* (BCN-Lich 19334), KC167008, KC167059, –; ***D. neutrophilus*** (Clauzade & Cl. Roux) Fdez.-Brime & Llimona: (1) [sub ***D. diacapsis*** 4 in Figures] Spain, *Llimona s.n.* (BCN-Lich 19338), KC166981, KC167033, –; (2) [sub ***D. diacapsis*** 5 in Figures] Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19357), KC166982, KC167034, –; (3) [sub ***D. diacapsis*** 6 in Figures] Spain, *Llimona s.n.* (BCN-Lich 19329), KC166983, KC167035, –; ***Diploschistes ocellatus*** (Vill.) Norman: (1) Spain, *Yahr 2475a* (DUKE 0030907), KC167009, KC167060, –; (2) Australia, *Lumbsch 10734c & Curnow* (DUKE 0144450), KC167010, KC167061, –; (5) Spain, *Terrón s.n.* (LEB 6251), KC167013, KC167063, –; ***Diploschistes ocellatus*** var. ***tenuis*** Fdez.-Brime & Llimona: (1) [sub ***D. ocellatus*** 3 in Figures] Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19341), KC167011, KC167062, –; (2) [sub ***D. ocellatus*** 4 in Figures] Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 17181); KC167012, 330369531, 330369542; ***Diploschistes rampoddensis*** (Nyl.) Zahlbr.: (1) Spain, *Llimona*, *Hladun & Muñiz s.n.* (BCN-Lich. 18009), KC166992, KC167043, –; (2) Spain, *Llimona & Hladun s.n.* (BCN-Lich. 18011), KC166993, KC167044, –; (3) –, 20334361, 8926416; ***Diploschistes scruposus*** (Schreb.) Norman: (1) Spain, *Llimona s.n.* (BCN-Lich 19328), KC167014, KC167064, KC167078; (2) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19351), KC167015, KC167065, –; (3) Spain, *Llimona s.n.* (BCN-Lich 14227), KC167016, KC167066, –; (4) Spain. *Llimona & Hladun s.n.* (BCN-Lich 19326), KC167017, KC167067, –; (5) Spain, *Llimona s.n.* (BCN-Lich 19316), KC167018, KC167068, –; (6) Spain, *Llimona & Fernández-Brime 533* (BCN-Lich 19327), KC167019, KC167069, –; (7) Spain, *Hladun & Muñiz s.n.* (BCN-Lich 14398), KC167020, KC167070, –; (8) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19354), KC167021, KC167071, –; (9) Spain, *Llimona s.n.* (BCN-Lich 19302), KC167022, KC167072, –; (10) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19336), KC167023, KC167073, –; (11) Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19337), KC167024, KC167074, –; ***Diploschistes scruposus*** morphotypus ***interpediens***: (1) [sub ***D. interpediens*** 1 in Figures] Portugal, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19317), KC166994, KC167045, –; (2) [sub ***D. interpediens*** 2 in Figures] Spain, *Llimona & Hladun s.n.* (BCN-Lich 19319), KC166995, KC167046, KC167076; (3) [sub ***D. interpediens*** 3 in Figures] Spain, *Llimona & Paz-Bermúdez s.n.* (BCN-Lich 18007), KC166996, KC167047, –; (4) [sub ***D. interpediens*** 4 in Figures] Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19350), KC166997, KC167048, –; (5) [sub ***D. interpediens*** 5 in Figures] Spain, *Gómez-Bolea & Figueras s.n.* (BCN-Lich 14751), KC166998, KC167049, –; (6) [sub ***D. interpediens*** 6 in Figures] Spain, *Llimona & Fernández-Brime s.n.* (BCN-Lich 19355), KC166999, KC167050, –; (7) [sub ***D. interpediens*** 7 in Figures] Spain, *Fernández-Brime s.n.* (BCN-Lich 19335), KC167000, KC167051, –; (8) [sub ***D. interpediens*** 8 in Figures] Spain, *Paz-Bermúdez s.n.* (SANT 10820)), KC167001, KC167052, –; (9) [sub ***D. interpediens*** 9 in Figures] Spain, *Hladun & Muñiz s.n.* (BCN-Lich 14104), KC167002, KC167053, –; (10) [sub ***D. interpediens*** 10 in Figures] France, *Llimona s.n.* (BCN-Lich 19322), KC167003, KC167054, –; ***Diploschistes thunbergianus*** (Ach.) Lumbsch & Vězda: –, 20334362, 8926417; ***Dyplolabia afzelii*** (Ach.) A. Massal.: –, 90995126, 90995099; ***Fibrillithecis halei*** (Tuck. & Mont.) Mangold [sub ***Myriotrema halei*** (Tuck. & Mont.) Hale) in GenBank]: –, 156739888, 156739936; ***Fissurina insidiosa*** C. Knight & Mitt.: –, 123979266, 123979316; ***Glyphis cicatricosa*** Ach.: –,55831959, 55139920; ***Glyphis scyphulifera*** (Ach.) Staiger: –, 90995133, 55139922; ***Graphis chrysocarpa*** (Raddi) Spreng.:–, 90995164, 19171983; ***Graphis ruiziana*** (Fée) A. Massal.: –, 90995162, 90995122; ***Graphis scripta*** (L.) Ach.: –, 110585705, 62005329; ***Gyalecta hypoleuca*** (Ach.) Zahlbr.: –, 330369539, 19171988; ***Gyalecta jenensis*** (Batsch) Zahlbr.: –, 46411460, 12025074; ***Gyalecta ulmi*** (Sw.) Zahlbr.: –, 46411461, 18481692; ***Leiorreuma hypomelaenum*** (Müll. Arg.) Staiger: –, 90995148, 90995110; ***Leucodecton subcompunctum*** (Nyl.) Frisch: –, 156739890, 156739938; ***Myriotrema minutulum*** (Hale) Hale: –, KC202871, KC202872; ***Myriotrema olivaceum*** Fée: –, 110585734, 156739942; ***Nadvornikia hawaiiensis*** (Tuck.) Tibell: –, 156739896, 47525224; ***Ocellularia cavata*** (Ach.) Müll. Arg.: –, 110585713, 90995112; ***Ocellularia chiriquiensis*** (Hale) Hale: –, 156739897, 156739944; ***Ocellularia massalongoi*** (Mont.) Hale: –, 156739899, 156739946; ***Ocellularia perforata*** (Leight.) Müll. Arg.: –, 156739902, 156739949; ***Ocellularia postposita*** (Nyl.) Frisch: –, 110585707, 55139903; ***Ocellularia profunda*** (Stirt.) Mangold, Elix & Lumbsch [sub ***Thelotrema profundum*** (Stirt.) Shirley) in GenBank]: –, 156739905, 156739951; ***Ocellularia thelotremoides*** (Leight.) Zahlbr.: –, 156739907, 156739953; ***Phaeographis brasiliensis*** (A. Massal.) Kalb & Matthes-Leicht: –, 90995135, 55139917; ***Phaeographis caesioradians*** (Leight.) A.W. Archer: –, 90995145, 55139916; ***Phaeographis lecanographa*** (Nyl.) Staiger: –, 90995160, 90995120; ***Phlyctis agelaea*** (Ach.) Flot.:–, 330369535, 330369536; ***Phlyctis argena*** (Ach.) Flot.: –, 119514137, 119513981; ***Platygramme australiensis*** Staiger: –, 90995147, 55139919; ***Platygramme caesiopruinosa*** (Fée) Fée: –, 90995150, 55139918; ***Ramonia*** sp.: –, 32141094, 37960830; ***Sarcographa fenicis*** (Vain.) Zahlbr.: –, 90995144, 90995108; ***Sarcographa ramificans*** (Kremp.) Staiger: –, 90995158, 90995119; ***Stegobolus fissus*** (Nyl.) Frisch: –, 156739909, 156739955; ***Stegobolus subcavatus*** (Nyl.) Frisch: –, 156739910, 156739956; ***Stictis populorum*** (Gilenstam) Gilenstam [sub ***Conotrema populorum*** Gilenstam in GenBank]:–, 34148564, 48995464; ***Stictis radiata*** (L.) Pers.: –, 46411482, 15216674; ***Thelotrema bicinctulum*** Nyl.: –, 156739913, 156739957; ***Thelotrema diplotrema*** Nyl.: –, 156739914, 156739958; ***Thelotrema gallowayanum*** Mangold, Elix & Lumbsch: –, 156739915, 156739968; ***Thelotrema lepadinum*** (Ach.) Ach.: (1) 336397130, 123979268, –; (2) –, 32141089, 37960825; ***Thelotrema monosporum*** Nyl.: –, 156739916, 156739961; ***Thelotrema nureliyum*** Hale: –, 156739919, 156739964; ***Thelotrema subtile*** Tuck.:–, 156739922, 156739966; ***Thelotrema suecicum*** (H. Magn.) P.James: (1) 45720761, 189039350, –; (2) –, 32141090; 37960826; ***Topeliopsis muscigena*** (Stizenb.) Kalb: –, 156739926, 156739971; ***Wirthiotrema glaucopallens*** (Nyl.) Rivas Plata & Kalb [sub ***Thelotrema glaucopallens*** Nyl. in GenBank]: –, 110585740, 47525213; ***Wirthiotrema trypaneoides*** (Nyl.) Rivas Plata & Lücking [sub ***Myriotrema trypaneoides*** (Nyl.) Hale in GenBank]: –, 156739895, 156739943.