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Expansion of the Stictidaceae by the addition of the saxicolous lichen-forming genus *Ingvariella*

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Abstract: The monotypic, lichen-forming genus *Ingvariella* originally was segregated from *Diploschistes* and placed within the Thelotremataceae (Ostropales) based on aspects of exciple morphology. However, the I+ hymenium and amyloid ascus wall suggest affinities to families other than the Thelotremataceae. To assess the identity of *Ingvariella* and to investigate its placement within the Ostropales, we inferred phylogenetic relationships of *I. bispora* by comparison of mtSSU rDNA and nuLSU rDNA sequences for 59 species encompassing a broad array of ostropalean fungi by means of Bayesian, maximum likelihood and weighted maximum parsimony methods. Here we report that *Ingvariella* is a member of the Stictidaceae, sister to the mainly saprotrophic genus *Cryptodiscus*. The inclusion of the first saxicolous lichen-forming fungus within this family expands the broad ecological diversity of the Stictidaceae, where saprotrophic fungi, corticicolous lichen-forming fungi and lichenized and non-lichenized conspecific taxa have been described previously. We also present new insights into the relationships among other families within the Ostropales.

Key words: *Diploschistes*, exciple morphology, *Ingvariella*, molecular phylogenetics, Ostropales, Stictidaceae

INTRODUCTION

The genus *Diploschistes* Norman (FIG. 1A) is characterized as having a carbonized pseudoparenchymatous exciple with lateral paraphyses (''periphysoids''), a trebouxioid photobiont and lacking a columella (FIG. 1C, D). In fact, this is the only genus within the Thelotremataceae not having trentepohlioid algae as a photobiont. The species initially described as *Diploschistes bisporus* (Bagl.) Steiner has almost lecideine apothecia (FIG. 1B) that exhibit a pseudoexciple formed by degenerating paraphyses and asci from the outermost part of the hymenium, sometimes with an extremely reduced exciple and no lateral paraphyses (FIG. 1E, F).

Based on these distinctive morphological characters, Guderley et al. (1997) transferred *D. bisporus* to a new monospecific genus, *Ingvariella* Guderley & Lumbsch, within the Thelotremataceae. Traditionally, the generic concepts within this family were based on ascospore septation and pigmentation (Müller 1887, Redinger 1936). Later, Salisbury (1971) used excipular structure to distinguish groups within the Thelotremataceae. This classification then was adopted mainly by Hale (1980, 1981), who modified the generic concept by adding the identity of the photobiont to the excipular characters. As a result, these genera were included: *Diploschistes*, *Myriotrema*, *Ocellularia* and *Thelotrema* (Guderley et al. 1997). However, authors argued later that the characters used to delimit these genera resulted in large and heterogeneous taxa (Frisch 2006). Furthermore, some authors (e.g. Nimis 1998) considered the use of excipular characters as insufficient to segregate *Ingvariella* from *Diploschistes* and proposed to treat *Ingvariella* as a subgenus within *Diploschistes* s. l.

Despite lacking some of the diagnostic characters of the Thelotremataceae (e.g. a true pseudoparenchymatous exciple, non-amyloid ascus wall), Guderley et al. (1997) maintained *Ingvariella* within this family and considered it to be related to *Diploschistes* because both genera are saxicolous, have trebouxioid photobionts and a similar distribution in arid and semiarid areas, whereas the rest of the family is mainly tropical and in association with trentepohlioid algae. On the other hand, they also noted that with the

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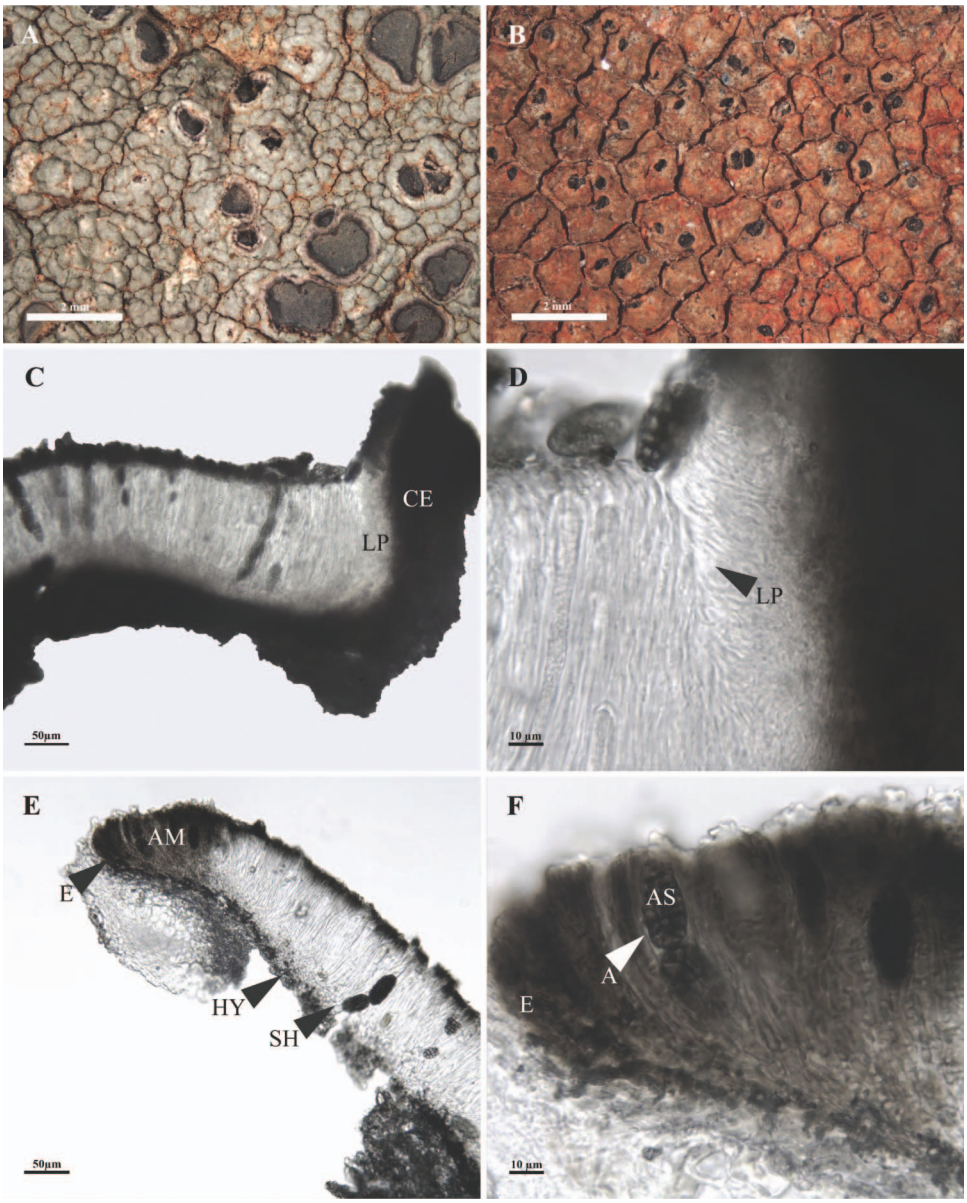

FIG. 1. Thallus morphology and ascomatal features of *Diploschistes scruposus* (A, C, D) and *Ingvariella bispora* (B, E, F). A. Fertile thallus of *Diploschistes scruposus* with well-developed urceolate apothecia. B. Fertile thallus of *Ingvariella bispora* with deeply urceolate apothecia. C. Longitudinal section of an apothecium, showing the well developed carbonized pseudoparenchymatous exciple (CE) with lateral paraphyses (LP). D. Detail of lateral paraphyses (arrow, LP). E. Longitudinal section of an apothecium with the apothecial margin (AM) formed by decaying hymenial elements. A very thin pigmented layer underneath the subhymenium (arrow, SH) can be observed representing the hypothecium/lower ascoma margin (arrow, HY). This layer extends in the outer part of the apothecial margin (arrow, E) and could be considered an extremely reduced exciple. F. Detail of the lateral margin with ascospores (AS), degenerated asci (arrow, A), and reduced exciple (E).

absence of lateral paraphyses and the presence of columella-like structures *Ingvariella* could be related to other genera within Thelotremataceae, such as *Ocellularia* and *Myriotrema*, which also have columella structures.

Ingvariella shares features with other families currently included in the Ostropales. For instance, like the mainly tropical and crustose family Gomphillaceae, *Ingvariella* has urceolate apothecia, muriform ascospores and a chloroccoid photobiont. Henssen (1995) reported that *Sagiolechia atlantica* Henssen (recently transferred to a new family Sagiolechiaceae, Baloch et al. 2010) has an apothecial margin similar to that of *Ingvariella*, in which the marginal parts of the hymenium degenerate in old fruiting bodies, even though a well formed proper exciple is present.

Alternatively, *Ingvariella* also could be ascribed to families such as the Phlyctidaceae and the Stictidaceae, both of which include some members having a chloroccoid photobiont and hymenia, which have unbranched paraphyses, KOH/I+ (blue) ascus walls and lack periphysoids. While the Phlyctidaceae have a true thalline margin and depsidones as secondary compounds, both the Stictidaceae and *Ingvariella* lack this type of margin and depsidones. These shared traits with other families suggest that the traditional placement of *Ingvariella* within the Thelotremataceae is somehow arbitrary and an alternative placement within the Ostropales cannot be completely discarded. The main goal of this study was to revisit the classification of *Ingvariella* by inferring its phylogenetic relationships within the Ostropales.

MATERIALS AND METHODS

Taxon sampling.—To infer the phylogenetic placement of *Ingvariella*, we used the mtSSU and nuLSU loci for a total of 59 species, representing most families within the Ostropales s. l. according to Kauff and Lutzoni (2002) and Lücking et al. (2004) (i.e. Coenogoniaceae, Gomphillaceae, Graphidaceae, Gyalectaceae, Odontotremataceae, Phlyctidaceae, Stictidaceae and Thelotremataceae). *Ingvariella bispora* (Bagl.) Guderley & Lumbsch is represented by three specimens in the mtSSU dataset and by two in the nuLSU. To root the phylogeny two outgroup taxa were selected from the Agyriales based on previous studies that showed its sister relationship to the Ostropales s. l. (Miadlikowska et al. 2006) for a total of 61 species.

In total, 125 sequences were used for this study (see ONLINE DATA SUPPLEMENT 1 for GenBank accession numbers), of which 11 were generated by the first author and the rest were obtained from GenBank and the AFTOL database (AFTOL.org). The concatenated alignment was deposited in TreeBASE (accession number S11040).

DNA extraction, amplification and sequencing.—Genomic DNA was isolated with a phenol-chloroform-isoamyl alcohol extraction protocol based on Lee et al. (1988). Isolated DNA was resuspended in sterile water and stored at −20 C.

We amplified and sequenced these loci: 0.8 kb mtSSU and 1.4 kb nuLSU with primers mrSSU1–mrSSU3R (Zoller et al. 1999) and LR0R (or LIC24R)–LR7 (Vilgalys and Hester 1990, Miadlikowska and Lutzoni 2000), respectively. Symmetric PCR amplifications were prepared as in Gueidan et al. (2007), and amplifications were carried out in a Peltier thermal cycler (Perkin Elmer, GeneAmp PCR System 2400) with programs specified in Zoller et al. (1999) for mtSSU and in Gueidan et al. (2007) for nuLSU. After examination by gel electrophoresis, PCR products were purified with Speedtools PCR Clean-Up Kit (Biotools, Madrid) following manufacturer instructions. Sequencing reactions were prepared in a 10 μL final volume with the same amplification primers and Big Dye Terminator Cycle sequencing kit v3.1 (ABI PRISM, Perkin-Elmer, Applied Biosystems, Foster City, California), following manufacturer instructions. Sequencing products were subjected to electrophoresis with an ABI 3730xl DNA analyzer (Applied Biosystems).

Sequence alignment.—Sequence fragments were subjected to BLAST queries for a first verification of their identities and to rule out fungal contaminants. Subsequently, they were assembled and contigs were edited with Bioedit 7.0 (Hall 1999) and aligned manually with Mesquite 2.6 (Maddison and Maddison 2010, http://mesquiteproject.org). The nuLSU locus was aligned with the help of the secondary structure of *Saccharomyces cerevisiae* as reported by Cannone et al. (2002) following Kjer (1995). Ambiguously aligned regions (sensu Lutzoni et al. 2000) and introns were delimited manually and excluded from the analyses. For the ambiguous regions, their unequivocal coding and the elaboration of symmetric step matrices for each of coded characters were generated with the program INAASE 2.3b (Lutzoni et al. 2000, http://www.lutzonilab.net/downloads).

Phylogenetic analyses.—The mtSSU and nuLSU datasets, with 62 sequences each (including two specimens of *I. bispora*), were analyzed separately with GARLI 0.96 (Zwickl 2006) using maximum likelihood (ML) as the optimization criterion. Models of molecular evolution were estimated for each separate genomic region with the Akaike information criterion (AIC) (Akaike 1973) implemented in Modeltest 3.7 (Posada and Crandall 1998). The selected model for mtSSU was TVM + I + G (Posada 2003) and for nuLSU GTR + I + G (Tavaré 1986). We used GARLI 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.

Topological incongruence between the two datasets was examined with 1000 replicates of ML bootstrapping (ML BS) under the same models described above on each locus separately. A conflict was assumed to be significant if two different relationships (one being monophyletic and the other being non-monophyletic) for the same set of taxa both were supported with bootstrap values ≥ 70% (Mason-Gamer and Kellog 1996). Because no conflicts were detected, we concatenated the two alignments and analyzed this combined dataset phylogenetically.

Phylogenetic relationships and confidence were inferred with maximum likelihood (ML), a Bayesian approach (MB) and weighted maximum parsimony (wMP) based on a combined dataset of 63 OTUs (including an additional specimen of *I. bispora* for which we have only the mtSSU sequence). For the maximum likelihood search, the same settings were used as in the separate analyses with GARLI 0.96, and the same estimated models were specified for each partition for both ML and ML bootstrap (ML BS) analyses.

For the Bayesian analysis, two parallel runs with four independent chains were conducted 10 000 000 generations with MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003), with trees sampled every 100 generations and using GTR + I + G model of nucleotide substitution for both partitions estimated with the AIC in Modeltest 3.7. We plotted the

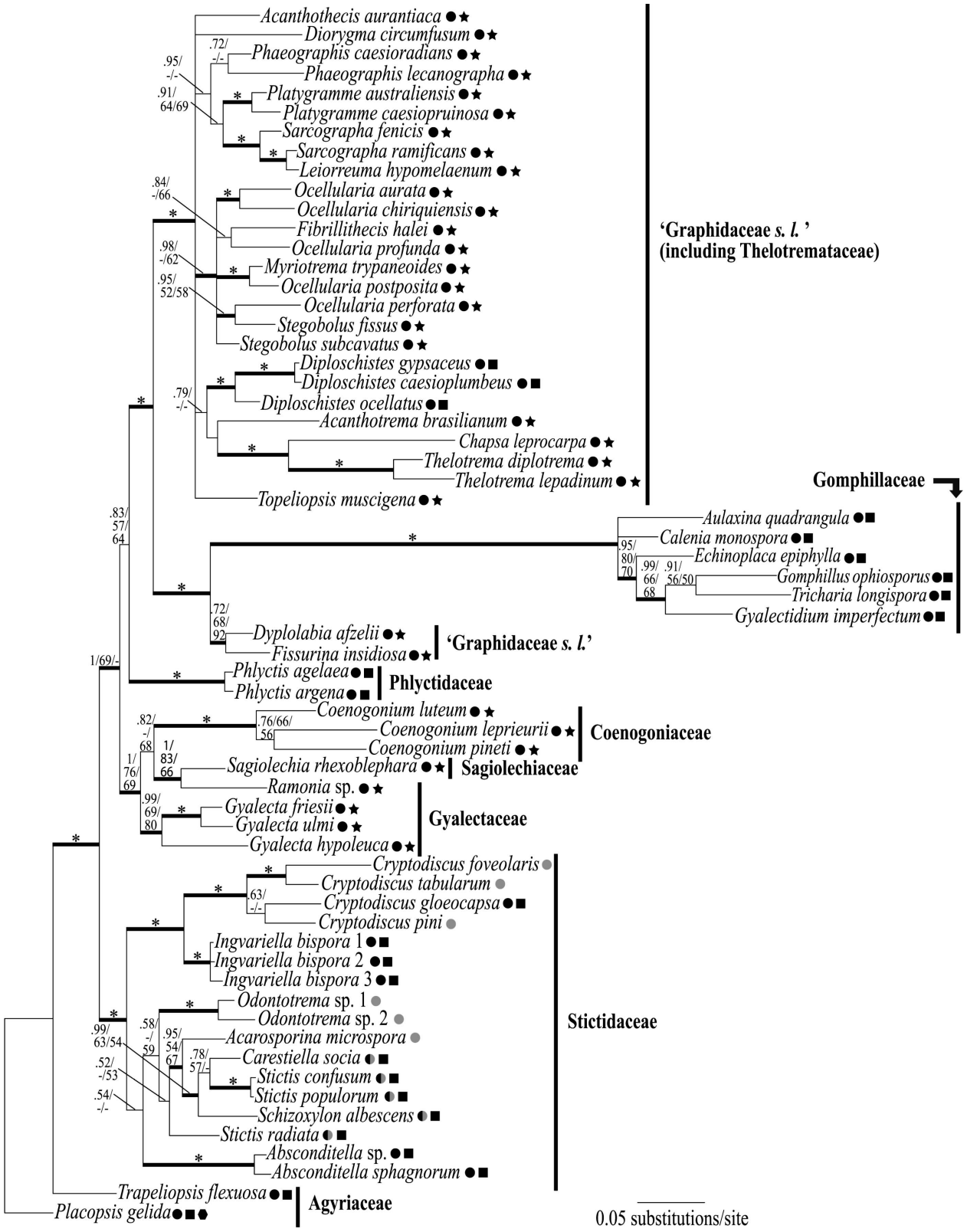

FIG. 2. Bayesian inference of phylogenetic relationships among 59 species, representing 35 genera, from the Ostropales (s. l.) based on concatenated nuLSU and mtSSU. Two genera from the Agyriales were selected as outgroup taxa. Support values above branches are ordered as PP/ML BS/wMP BS. Thicker internodes indicate significant support for at least one statistical method (PP ≥ 0.95, ML BS and wMP BS ≥ 70%). An asterisk above the internode indicates that all three measures of

log-likelihood scores against generation time with Tracer 1.4.1 (Rambaut and Drummond 2007, http://beast.bio.ed.ac.uk/Tracer) and concluded that stationarity was reached when log-likelihood values reached the same stable equilibrium value in both independent runs (Huelsenbeck and Ronquist 2001). A burn-in sample of 10 000 trees was discarded for each run, and the remaining 180 000 (90 000 from each run) were used to calculate posterior probabilities (PP) with the majority rule consensus tree command implemented in PAUP* 4.0b10 (Swofford 2002).

The wMP analysis was performed with PAUP*. Gaps were treated as a fifth character state and symmetric step matrices were created for unambiguous portions of the two loci separately with STMatrix 3.0 (Lutzoni and Zoller, Duke University, http://www.lutzonilab.net/downloads) as outlined in Gaya et al. (2011). Constant sites were removed from wMP searches and wMP bootstrap analyses (wMP BS). Phylogenetic signal from ambiguous regions was recovered without violating positional homology with INAASE 2.3b (Lutzoni et al. 2000). 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 was estimated with 1000 nonparametric bootstrap replicates (Felsenstein 1985) with full heuristic searches, four RAS per bootstrap replicate and the same parameters as for the initial wMP analyses. The number of RAS per bootstrap replicate was calculated taking into account the number of times the shortest tree was hit during the heuristic search with the original dataset.

RESULTS

Alignments and phylogenetic analyses.—The final size of the combined dataset for the 63 specimens (representing 59 species of Ostropales and two outgroup taxa from Agyriales) was 6170 sites (1505 mtSSU sites and 4665 nuLSU sites), leaving 1091 sites (398 mtSSU and 693 nuLSU sites) after exclusion of 67 ambiguous regions and nine introns. Of the 1091 characters included in the ML and MB searches, 623 were constant and 468 were variable. For wMP analyses, the 623 constant sites were excluded and 45 coded INAASE characters replaced 29 and 16 ambiguously aligned regions from the mtSSU and nuLSU datasets, respectively, for a total of 513 variable characters, of which 411 were parsimony informative.

The majority rule consensus tree of 180 000 sampled trees from the Bayesian inference obtained in this study is included (FIG. 2) with branch lengths and support values. With 36 highly supported internodes (PP ≥ 0.95), this was the most resolved and well supported tree, including deep phylogenetic relationships, resulting from the three phylogenetic analyses. The topology of this tree was almost identical to the ML tree and was similar to the wMP majority rule consensus tree. However, the most likely tree (−ln likelihood = 11159.0312) recovered only 28 significantly supported internodes (ML BS ≥ 70%). The wMP analysis with 45 additional (INAASE) characters revealed three equally most parsimonious trees of 4114.59 steps, which were found in one island hit 720 times out of 1000 RAS. The number of internodes with high support (wMP BS ≥ 70%) was 28.

Phylogenetic relationships.—All analyses supported the monophyly of the three specimens of *I. bispora* with high confidence (1 PP, 100% ML BS and wMP BS), sister to the monophyletic *Cryptodiscus* s. str. (1 PP, 100% ML BS and wMP BS). Both genera are nested within the monophyletic family Stictidaceae (sensu Baloch et al. 2009), a result well supported by all measures of phylogenetic confidence (1 PP, 86% ML BS and 77% wMP BS). The Stictidaceae represents the first divergence from the remaining members of the Ostropales s. l. (sensu Miadlikowska et al. 2006), with the Stictidaceae s. str. clade (sensu Baloch et al. 2010) recovered as weakly supported. In our phylogeny, the placement of *Absconditella* and two undescribed taxa of the genus *Odontotrema* is uncertain within the Stictidaceae.

The Gyalectaceae is shown here to form a monophyletic group together with the Coenogoniaceae and Sagiolechiaceae (1 PP, 76% ML BS and 69% wMP BS). The Coenogoniaceae is recovered as monophyletic (1 PP, 100% ML BS and wMP BS), whereas the Gyalectaceae resulted paraphyletic. Our analyses reveal strong evidence for a shared recent common ancestor of *Ramonia* (Gyalectaceae) with *Sagiolechia* (= *Rhexophiale*) *rhexoblephara* (Sagiolechiaceae). Based on our taxon sampling, *Phlyctis* also is supported as a monophyletic group (1 PP, 100% ML BS and wMP BS) but with an uncertain placement within the Ostropales s. l.

We found a strongly supported sister relationship between the monophyletic family Gomphillaceae and two members of the Graphidaceae (1 PP, 90% ML BS and 84% wMP BS), *Fissurina insidiosa* and *Dyplolabia afzelii*. The Gomphillaceae clade showed a remarkably long branch, previously reported and tested for long-branch attraction effect by Lücking et al. (2004). As

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support were significant. Black circles following names of taxa indicate lichen-forming fungi; gray circles, saprotrophic fungi; and circles half black and gray, lichenized and non-lichenized conspecific taxa. Squares indicate lichen-forming taxa associated with trebouxioid or chlorococcal green algae, whereas a star refers to trentepohlioid green algae and a pentagon to cyanobacteria. Graphidaceae is indicated in quotes to highlight that in this phylogeny this family has not resulted monophyletic.

expected, members of the Thelotremataceae appeared nested within the main Graphidaceae clade (1 PP, 92% ML BS, 88% wMP BS), consistent with previously published results indicating that the Thelotremataceae should be treated as a synonym within the Graphidaceae (Kauff and Lutzoni 2002, Grube et al. 2004, Lumbsch et al. 2004, Frisch et al. 2006, Staiger et al. 2006, Mangold et al. 2008, Baloch et al. 2010). Thus, we henceforth will refer to this clade with the family name Graphidaceae. Phylogenetic placements of most taxa within the Graphidaceae s. l. could not be resolved here with high confidence.

DISCUSSION

The establishment of *Ingvariella* as a segregate taxon from *Diploschistes* is in agreement with traditional taxonomy of Guderley et al. (1997), however its placement within the Stictidaceae, as revealed, here is contrary to that of Guderley et al. (1997) who maintained *Ingvariella* within the Graphidaceae. Of interest, *Ingvariella* is the first exclusively lichenized saxicolous species known to date within the Stictidaceae. Although the Stictidaceae traditionally was recognized as a family composed of saprotrophic fungi, some authors included lichenized taxa (Gilenstam 1969, Sherwood 1977) based mainly on the morphology of the apothecial margin, the type of apical apparatus and ascus and ascospore shape. The hypothesis that the Stictidaceae included both lichenized and non-lichenized fungi was confirmed by Winka et al. (1998).

Lutzoni et al. (2001), using *Stictis radiata* to represent the Stictidaceae, concluded that the non-lichenized members of the Ostropales are derived from a lichenized ancestor. Wedin et al. (2004, 2005a, 2006) further demonstrated that specimens of *Conotrema* (lichenized) were nested within *Stictis* (saprotrophic) in different clades and showed that lichenization was present in some taxa of saprotrophic Stictidaceae. Subsequently, Wedin et al. (2004) used the term ''optional lichenization'' to describe this alternation of nutritional modes by the same species. Schoch et al. (2006) revealed the placement of the halotolerant fungus *Glomerobolus gelineus* Kohlm. & Volkm.-Kohlm. as closely related to the Stictidaceae, further expanding the ecological diversity exhibited by the Ostropales. According to Schoch et al. (2006) the ecology and nutritional mode of ostropalean saprobic taxa are best explained as being derived from loss of lichenization (Lutzoni et al. 2001, 2004; Reeb et al. 2004). Ecological and nutritional diversity is not restricted to the Ostropales. Gueidan et al. (2008) showed that rock-inhabiting and human pathogenic fungi (Chaetothyriales) shared a most recent common ancestor with lichen-forming fungi classified within the Verrucariales and Pyrenulales.

In general, relationships among the remaining members of the Stictidaceae revealed in our analyses are similar to phylogenetic results reported by Baloch et al. (2009, 2010) and Wedin et al. (2005a, 2006). Our results add support to the suggestion of Baloch et al. (2009) that *Absconditella* should be retained as a separate genus, apart from *Cryptodiscus*, based on differences in appearance of the ascoma and thickness and features of the ascomatal wall. Our data also support the placement of two undescribed taxa of genus *Odontotrema* within the Stictidaceae, as in Baloch et al. (2009, 2010).

A main feature in the circumscription of the Stictidaceae has been the structure of the ascoma margin: the presence of several wall layers, their pigmentation and the formation of crystals (Wedin et al. 2006). However, several authors have reported a wide range of variation of the apothecial margin in different genera within the family (Gilenstam 1969; Sherwood 1977; Wedin et al. 2005a, 2006) or even among individuals with different nutritional modes (saprotrophs vs. lichens) within the same species (e.g. *Schizoxylon albescens*, *Stictis confusum*, *S. mollis*, *S. populorum*; see Wedin et al. 2006). Regarding *Ingvariella*, none of the samples we examined (see ONLINE DATA SUPPLEMENT 2) or descriptions of this genus (Guderley et al. 1997; Lumbsch 1989, 2004; Mangold et al. 2009) bore any resemblance to the typical ascoma margin of the Stictidaceae, demonstrating that this structure by itself does not provide a reliable set of diagnostic characters for this family. Nor does the exciple of *Ingvariella* share similarities with members of the Graphidaceae, although the exciple structure is also variable in this family. Nevertheless, the hymenium of *Ingvariella* shares several features with some Stictidaceae: KOH/I+ (faint blue) ascus walls and numerous filiform paraphyses. When stained with IKI, the hymenium turns yellow and then brownish red, as reported (e.g. Baloch et al. 2009, Vězda and Vivant 1975) for other Stictidaceae genera (e.g. *Absconditella*, *Cryptodicus*). Conversely, the hymenium in the Graphidaceae differs from *Ingvariella* by the non-amyloid ascus walls and lack of a hymenial iodine reaction, except for a few taxa (e.g. *Glyphis*), where the reaction is blue but never yellow or reddish. In addition, *Ingvariella* has broadly ellipsoid brown muriform spores, while ascospores in the Stictidaceae are typically cylindrically elongated or filiform, colorless, thin-walled and with multiple transversal septa (Sherwood 1977). Although muriform spores occasionally occur (Gilenstam 1969, Sherwood 1977), brown spores are rare within the Stictidaceae (Sherwood 1977).

With regard to the other families in our phylogeny, the familial placement of *Sagiolechia* within the Ostropales has been questioned (Wedin et al. 2005b). Based on morphological and anatomical features, some authors have included the genus within the Graphidaceae (Vězda 1967) and others within the Gomphillaceae (Henssen 1995). A close relationship with the Gyalectaceae was reported by Wedin et al. (2005b). Recently, Baloch et al. (2010) transferred this genus to a new family, Sagiolechiaceae. In our analyses, the sister relationship of *Ramonia* and *Sagiolechia* suggests that the familial assignment of *Ramonia* should be re-evaluated in the context of this new classification.

In our results, the phylogenetic position of the clade *Fissurina insidiosa-Dyplolabia afzelii* agreed with Miadlikowska et al. (2006), where *Fissurina insidiosa* was sister to *Gyalidea hyalinascens* (Gomphillaceae), and to Baloch et al. (2010), where the *Fissurina-Dyplolabia* group was revealed sister to a monophyletic group formed by the Gomphillaceae and Solorinellaceae. These results suggested that clade *Fissurina-Dyplolabia* should be treated separately from the Graphidaceae, otherwise this family would be rendered paraphyletic.

In conclusion, our phylogenetic study based on mtSSU and nuLSU data demonstrated that *Ingvariella* must be transferred to the Stictidaceae. Based on current knowledge, we consider that some hymenium features (e.g. reaction of the hymenium when stained with IKI and amyloid ascus walls), combined with the type of photobiont (chloroccoid when lichenized), could be potential synapomorphies for the Stictidaceae, supporting the placement of *Ingvariella* within this family. However, further studies will be needed to find diagnostic morphological characters that can help in defining natural taxonomic entities within the Stictidaceae and the Ostropales in general.

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