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Molecular analyses uncover the phylogenetic placement of the lichenized hyphomycetous genus *Cheiromycina*

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ABSTRACT

The genus *Cheiromycina* is one of the few genera of lichenized hyphomycetes for which no sexual reproductive stages have been observed. The genus includes species from boreal to temperate regions of the Northern Hemisphere where it is found growing on bark or wood. Congeners in *Cheiromycina* are characterized by a noncorticate thallus, nearly immersed in the substrate and presenting powdery unpigmented sporodochia, and containing chlorococcoid photobionts. The relationships of members of *Cheiromycina* with other fungi are not known. Here we inferred the phylogenetic placement of *Cheiromycina* using three loci (nuSSU, nuLSU, and mtSSU) representing *C. flabelliformis*, the type species for the genus, *C. petri*, and *C. reimeri*. Our results revealed that the genus *Cheiromycina* is found within the family Malmideaceae (Lecanorales) where members formed a monophyletic clade sister to the genera *Savoronala* and *Malmidea*. This phylogenetic placement and the relationships of *Cheiromycina* with other lichenized hyphomycetous taxa are here discussed.

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INTRODUCTION

Hyphomycetes comprises a polyphyletic assemblage generally recognized by mycologists as one of the major group of anamorphic fungi, including the majority of what are commonly called molds. Fungi generally characterizes as members of Hyphomycetes include parasitic and saprotrophic species growing on diverse natural substrates, such as wood, plant tissues, insects, and other arthropods, and other fungi, including lichens (Seifert et al. 2011). Hyphomycetes represent fungi in asexual stages, and, in many cases, members have subsequently been recognized as anamorphs of several ascomycete and basidiomycete lineages. However, taxa for which no teleomorph has been identified have been hypothesized to have permanently lost the ability of developing sexual structures and are regarded as anamorphic holomorphs (Seifert et al. 2011).

Historically, two successful sorting systems for anamorphic fungi were developed: the sporological system (Saccardo 1886), which focused on conidiomata morphology, and the ontogenetic system (Hughes 1953), which used the conidium ontogeny as a primary diagnostic character. Because Saccardo's sporological system based on conidiomata morphology has been recognized as artificial, and the conidium ontogeny still groups unrelated taxa, Berbee and Taylor (1993) suggested integrating all anamorphic fungi into a unique fungal systematic scheme, which was further expanded by Reynolds and Taylor (1993). More recently, anamorphic fungi are distinguished into three major groups according to the presence and the structures in which conidiospores are produced: (i) blastomycetes, such as the asexually reproducing yeasts; (ii) coelomycetes, fungi producing pycnidia and acervuli; (iii) hyphomycetes, fungi lacking pycnidia or acervular fruting bodies and with conidia developed "out in the air" (Seifert et al. 2011).

In spite of these attempts for a natural classification, the debate for the classification of many anamorphic genera remains unsolved. Recent application of molecular- and culture-based analyses have helped resolving many anamorph-teleomorph connections (e.g., Crous et al. 2001, 2004, 2006; Lizel et al. 2003; Réblová and Seifert 2004, 2011; Huhndorf and Fernández 2005;

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Shenoy et al. 2007; Ertz et al. 2011, 2013, 2014; Pérez-Ortega et al. 2011; Muggia et al. 2015).

Lichenicolous hyphomycetes are recognized within both coelomycetes and hyphomycetes and comprise multiple genera that are known parasites of lichen thalli (Diederich 2011). After Hawksworth's (1979) initial treatment, ongoing research continues to contribute to understanding diversity of lichenicolous hyphomycetes (e.g., Hawksworth and Poelt 1986; Diederich and Scheidegger 1996; Earland-Bennett and Hawksworth 2005; Heuchert and Braun 2006; Zhurbenko et al. 2015). This research has included the description of new species and, to a lesser extent, discovering anamorph-teleomorph relationships (Ertz et al. 2013, 2014; Frisch et al. 2014; Muggia et al. 2015).

The diversity of lichenized and algicolous hyphomycetes has received considerably less attention. The degree to which these anamorphic fungi associate with algae is still argued to be at the edge of lichenization and is further blurred by the saprotrophic and parasitic lifestyles that can be observed in congeneric species.

Vobis and Hawksworth (1981) reported about 41 genera of conidial fungi found to form stable association with algae from temperate and tropical regions. Three genera of lichenized, anamorphic coelomycetes, i.e., *Lichingoldia, Woessia*, and *Hastifera* (Hawksworth and Poelt 1986), were later found representing pycnidial stage of species currently placed in *Bacidina* and *Micarea*, respectively (Ekman 1996; Fryday 2001). Some other anamorphic lichenized species were originally described as lichenicolous fungi on sterile lichens, such as *Reichlingia leopoldii* (Diederich and Scheidegger 1996), which was later recognized as a sporodochiate lichen (e.g., Diederich and Coppins 2009). Conversely, other hyphomycetous species found in loose association with algae are now considered lichen parasites. For example, *Nigropuncta rugulosa* was originally described as a lichenized hyphomycete (Hawksworth and Poelt 1986) but subsequently considered to be a lichenicolous fungus specialized on *Bellemerea cinereorufescens*, on which its infection strongly suppresses the formation of the host apothecia (Hafellner 2012). Alternatively, species believed to be saprotrophic on wood are now recognized to be lichenized, such as *Dictyocatenulata alba* (Diederich et al. 2008). At the time of its original description (Morris and Finley 1967), *D. alba* was poorly understood due to the unclear illustrations and was generally considered to be a bark-inhabiting hyphomycete (Seifert et al. 1987). However, more recently it has been recognized to be a lichen-forming species (Lendemer and Harris 2004).

The nutritional status of some sporodochiate fungi is not easily interpreted, and nutritional modes of various taxa are interpreted differently depending on the author, who may categorized them as either a lichenicolous species or as lichen-forming fungi. *Sclerococcum griseisporodochium* was described as a lichenicolous, parasymbiotic fungus associated with a calcicolous species of *Opegrapha*, but optional lichenization of this taxon was recognized (Etayo 1995). For this reason, *S. griseisporodochium* is sometimes treated as a lichen (e.g., Smith 2009; Ertz et al. 2013), but it is more commonly listed among the lichenicolous fungi in fungal surveys. Similarly, *Milospium graphideorum* is reported as an example of a facultative, lichenized sporodochiate fungus (Diederich and Coppins 2009; Diederich 2013); however, it is usually recorded as a lichenicolous fungus of several epiphytic, crustose lichens associated with trentepohlioid photobionts.

Further, anamorph-teleomorph connections have been described only for few genera to date, and the majority of lichenized hyphomycetes are still known as anamorphic holomorphs. Both *Blarneya hibernica* (Hawksworth et al. 1979) and the type species of the genus *Sporodochiolichen, S. lecanorinus* (Aptroot and Sipman 2011), have been recognized as one anamorph of *Tylophoron* species (Ertz et al. 2013). Sporodochia of the taxon *Cheiromycina ananas* (Aptroot and Schiefelbein 2003) were reinterpreted as representing sessile synnemata corresponding to the widely distributed and polymorphic species *Dictyocatenulata alba* and therefore synonymized with it (Diederich et al. 2008). The generic concept of *Reichlingia* was further enlarged by Frisch et al. (2014) incorporating three additional species with ascocarps and with concordant chemistry (perlatolic acid derivatives).

Lichenized hyphomycetes are still poorly represented in molecular phylogenies. However, molecular data have been generated for a limited number of genera. Based on these data, *Tylophoron* and *Reichlingia* have been placed in the order Arthoniales (Ertz et al. 2011; Ertz and Tehler 2011), and *Dictyocatenulata alba* was recovered within Ostropales but leaving the genus as incerta saedis within this order (An et al. 2012; Lücking et al. 2017). A single specimen of the sporodochiate lichen *Chirleja buckii* was sequenced, and the taxon was suggested to be a member of Icmadophilaceae (Pertusariales; Lendemer and Hodkinson 2012). Further DNA evidence even included *Chirleja buckii* in the genus *Endocena*, and the new combination of *Endocena buckii* was proposed by Fryday et al. (2017). Recently, the description of *Savoronala madagascariensis* from arid regions in southeast Madagascar was coupled with phylogenetic data (Ertz et al. 2013). These latter supported the

introduction of a new family, Malmideaceae, in which both *S. madagascariensis* and *Malmidea* were placed within Lecanorales (Ertz et al. 2013). Presently, additional molecular data are unavailable for other genera of lichenized hyphomycetes.

The genus *Cheiromycina* is unique because of its peculiar, cheiroid (finger/hand forming), multiseptate conidia, which are easily recognizable by microscopy. Cheiroid conidia are also described for other hyphomycetous taxa (Sutton 1985), such as the predominantly heterotrophic genus *Psammina*, including two lichenized species (Earland-Bennett and Hawksworth 2005; Cáceres and Aptroot 2016), and the nonlichenized genera *Cheirospora* (Hughes 1958), *Cheiromyceopsis* (Mercado Sierra and Mena Portales 1988), *Ramoconidiifera* (Sutton et al. 1996), *Digitomyces* (Mercado Sierra et al. 2003), and *Cheirosporium* (Cai et al. 2008). In contrast to *Cheiromycina, Psammina palmata* presents branched, nonseptate conidia (Earland-Bennett and Hawksworth 2005), whereas the recently described taxon *P. tropica* produces septate, only basally branched conidia, and further differentiates by associating with a trentepohlioid photobiont (Cáceres and Aptroot 2016). The other nonlichenized genera are melanized fungi within the class Dothideomycetes (Cai et al. 2008).

Cheiromycina is characterized by a thallus nearly immersed in bark or wood with hemispherical, whitish to pale gray or brownish, powdery sporodochia. The conidia are multicellular, hyaline, rarely pale brownish, smooth, flabelliform to palmate, usually consisting of a basal, subglobose (strongly inflated) conidiogenous cell from which distoseptate dichotomous branches derive. Ecologically, the genus seems to be confined to acidic substrates in humid forests of the boreal zone. The genus was described with *Cheiromycina flabelliformis* B. Sutton as the type species based on material from *Picea* wood from northern Sweden (Sutton and Muhr 1986). It was initially described as a hyphomycete with eustromatic sporodochial conidiomata, resembling the genus *Cheiromyces* due to the presence of distoseptate conidia. A stable association with green algae was confirmed by Hawksworth and Poelt (1986) in the holotype and in further specimens reported from Austria. Later the same authors described an additional species from Austria, *Cheiromycina petri* (Hawksworth and Poelt 1990). According to their description, *C. petri* differs from *C. flabelliformis* by the melanized (noneroding) sporodochia, the branching pattern, and the size of the conidia. Printzen (2007), studying further the type material, emphasized that an indistinctly enlarged conidiogenous cell is the main diagnostic character for *C. petri*. In the previous decade, three other species of *Cheiromycina* have been described: *C. globosa* from Germany (Aptroot and Schiefelbein 2003), *C. ananas* from USA (Aptroot and Schiefelbein 2003), later synonymized under *Dictyocatenulata alba* (Diederich et al. 2008), and *C. reimeri* from Turkey and Russian Far East (Printzen 2007). However, the most rarely recorded taxon, *C. globosa*, does not fit the description of the genus and most likely does not belong to *Cheiromycina* s. str. *Cheiromycina globosa* forms single-celled, globose conidia and differs from other congeners ecologically, being found in nitrophilous lichen communities.

The phylogenetic placement of the genus *Cheiromycina* has not investigated to date, although preliminary sequencing results showed similarity with multiple taxa in Lecanoromycetes. Therefore, in this study, we aimed to infer the phylogenetic placement of *Cheiromycina* within lichen-forming fungi using nuclear and mitochondrial ribosomal genetic markers. Below we discuss the relationship of *Cheiromycina* within Lecanoromycetes and with other lichenized hyphomycetous taxa.

MATERIALS AND METHODS

Sampling and morphological analyses.—Fresh samples of *Cheiromycina* were collected in different localities in six countries including two U.S. states (Czech Republic, Norway, Ukraine, Russia, Poland, and USA [Alaska and Washington]; TABLE 1). Five specimens representing *C. flabelliformis*, seven of *C. petri*, and two of *C. reimeri* were included (TABLE 1). Lichen specimens were sorted under a dissecting microscope, and species identification was performed by analyzing anatomical and squash sections of the sporodochia. Identifications followed Sutton and Muhr (1986), Hawksworth and Poelt (1990), and Printzen (2007). Sections were prepared in water and examined with light microscopy. Digital images of growth habit and anatomical section (FIGS. 1 and 2) were acquired with a ZeissAxioCam MRc5 digital camera (Goettingen, Germany) fitted to the stereo- and light microscopes and were digitally optimized using the image processing software Combine ZM (www.hadleyweb.pwp.blueyonder.co.uk/CZM/).

DNA extraction, amplification, and sequencing.—All samples were checked for contamination, and the sporodochia were carefully dissected under the stereomicroscope and taken for DNA extraction. The material was first frozen in liquid nitrogen and then pulverized with polypropylene pestles. The DNA was extracted according to the protocol of Cubero et al. (1999). Molecular sequence data from *Cheiromycina* samples were generated for the nuclear large and partial nuclear small ribosomal subunits (28S and 18S,

Table 1. Information about *Cheiromycina* samples considered in this study.

Geographic origin	Species (DNA ID)	nuLSU	nuSSU	mtSSU
CZECH REPUBLIC. S BOHEMIA: Šumava Mts, Volary: Mt Plechý, 48°46′38.5″N, 13°51′21.4″E, on mossy bark of *Sorbus aucuparia*, 1320 m a.s.l., 2014, *Z. Palice 18257* (PRA).	*C. flabelliformis* (L2253)	MF431804	MF431795	MF431799
NORWAY. NORD-TRØNDELAG: Snåsa Bergsåsen Nature Reserve, 64°15.264′N, 12°23992′E, on bark, at base of old *Salix caprea* in old mixed coniferous forest, on calcareous ground, 2015, *F. Jonsson LK46*.	*C. flabelliformis* (L2325)	—	MF431798	—
CZECH REPUBLIC. S BOHEMIA: Šumava Mts, Nová Pec, Mt Hraničník, 48°45′05″N, 13°54′45″E, on bark of *Sorbus aucuparia*, 1170 m, 2014, *Z. Palice 17855* (PRA).	*C. petri* (L2222)	MF431805	MF431796	MF431800
USA. ALASKA: Katmai National Park, 200–600 m NE of Mirror Lake, 59.24526°N, 154.75221°W, 440 m a.s.l., corticolous at base of *Salix* near stream in dwarf shrub tundra, 2013, *T. Tønsberg 42848* (BG).	*C. petri* (L2223)	—	MF431797	MF431801
POLAND. NORTH PODLASIE DISTRICT: Bielska Plain, Białowieża National Park, 2015, *M. Kukwa 17681*.	*C. reimeri* (MK17681)	MF431806	—	MF431802
POLAND. NORTH PODLASIE DISTRICT: Bielska Plain, Białowieża National Park, 2015, *M. Kukwa 17422*.	*C. reimeri* (MK17422)	MF431807	—	MF431803
RUSSIA. CAUCASUS, REPUBLIC OF ADYGEA: S of village Guzeripl, Mt Abago, on wood of snag (*Abies nordmanniana*), 1730 m a.s.l., 2016, *Z. Palice 21103* (PRA).	*C. flabelliformis* (L2344)	—	—	—
russia. caucasus, republic of adygea: S of village Guzeripl, Mt Abago, on bark of *Abies nordmanniana*, 1720 m a.s.l., 2016, *Z. Palice 21313* (PRA).	*C. flabelliformis* (L2347)	—	—	—
NORWAY. NORDLAND: Grane, Majavatn, Litlfjellet, 65°09.376 N, 13°22.946 E, 565 m a.s.l., corticolous at base of *Salix lapponum*, 2013, *T. Tønsberg 43128* (BG).	*C. petri* (L2326)	—	—	—
Russia. caucasus, republic of adygea: SE of village Guzeripl, Mt Abago, on wood of *Betula* snag, 1900 m a.s.l., 2016, *Z. Palice 21311* (PRA).	*C. petri* (L2345)	—	—	—
russia. caucasus, republic of adygea: SE of village Guzeripl, Mt Abago, on foot of old *Fagus orientalis*, 1720 m a.s.l., 2016, *Z. Palice 21312* (PRA).	*C. petri* (L2346)	—	—	—
UKRAINE. ZAKARPATSKA OBLAST REGION: Eastern Carpathians, Khust, Velyka Uhol'ka, valley of Velyka Uhol'ka, 48°14′43″N, 23°41′36″E, on bark of *Carpinus*, 420 m a.s.l., 2015, *Z. Palice 19030* (PRA).	*C. petri* (L2327)	—	—	—
USA. WASHINGTON: Cowlitz Co., SW of summit of Mount St. Helens, Goat Marsh, 46.16560°N, 122.28073°W, 900–1000 m a.s.l., corticolous on trunk of *Alnus rubra* at edge of marsh, 2013, *T. Tønsberg 43060* (BG).	*C. petri* (L2224)	—	—	—

Note. Samples that were successfully sequenced and included in the phylogenetic analysis in FIG. 3 are reported together with the NCBI accession numbers. The DNA extraction number is reported in parentheses for each sample.

respectively) and for the mitochondrial small ribosomal subunit (12S). The nuclear 28S locus was amplified using primers LR3R and LR7 (Vilgalys and Hester 1990); the nuclear 18S region was amplified using primers NS1 (White et al. 1990) and nuSSU0852 (Gargas and Taylor 1992); and a fragment of the mitochondrial 12S locus was amplified with primers mtSSU1KL (Zoller et al. 1999) and MSU7 (Zhou and Stanosz 2001). Polymerase chain reaction (PCR) amplifications were carried out using Illustra Ready-To-Go GenomiPhi V3 DNA amplification kit (GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, USA), and the temperature profile followed the "touch-down" PCR conditions reported in Muggia et al. (2015). Complementary strands were sequenced at Microsynth (Vienna, Austria). The sequences were manually assembled and edited in BioEdit (Hall 1999).

Phylogenetic analyses.—The identity of the new generated sequences was checked with sequences available in the GenBank database using the BLAST search algorithm (Altschul et al. 1990); the taxa with the highest sequences similarity, as inferred from the BLAST search, were selected and included in subsequent phylogenetic analyses. In addition, an exploratory analysis was performed, including fungi from a wide phylogenetic spectrum (not shown), comprising the classes Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycets, and Sordariomycetes. As the National Center for Biotechnology Information (NCBI) BLAST results always output as closest matches representatives of Lecanoromycetes, and our first single-locus analyses also placed the sequences within Lecanoromycetes, we reduced the data set of the final phylogenetic analysis to Lecanoromycetes only and selected 10 species of Leotiomycetes as outgroups (TABLE S1). The selection of the outgroups and of the Lecanoromycetes ingroups was based on the recent phylogenetic analyses of Ertz et al. (2013) and Miadlikowska et al. (2014). For a number of specimens, we were unable to generate sequences for all the selected loci (18S, 28S, and mitochondrial 12S), and a complete set of all three sampled loci were not available in GenBank. The sequence alignments were prepared manually in BioEdit and individually for the three loci. Introns and ambiguous single-nucleotide polymorphisms (SNPs) were removed from the alignments. Alignments are deposited in TreeBASE under the reference number TB21301 (http://purl.org/phylo/treebase/phylows/study/TB2:S21301).

Combined data of different loci, whether fully or partially congruent, should be considered when inferring phylogenetic relationships (Dettman et al. 2003), and here we analyzed both single-locus alignments and a combined, three-marker data set. We analyzed the single-locus data sets within a maximum likelihood (ML) framework (Mason-Gamer and Kellogg 1996; Reeb et al. 2004) and the combined data set using both ML and Bayesian approaches. The combined data set was

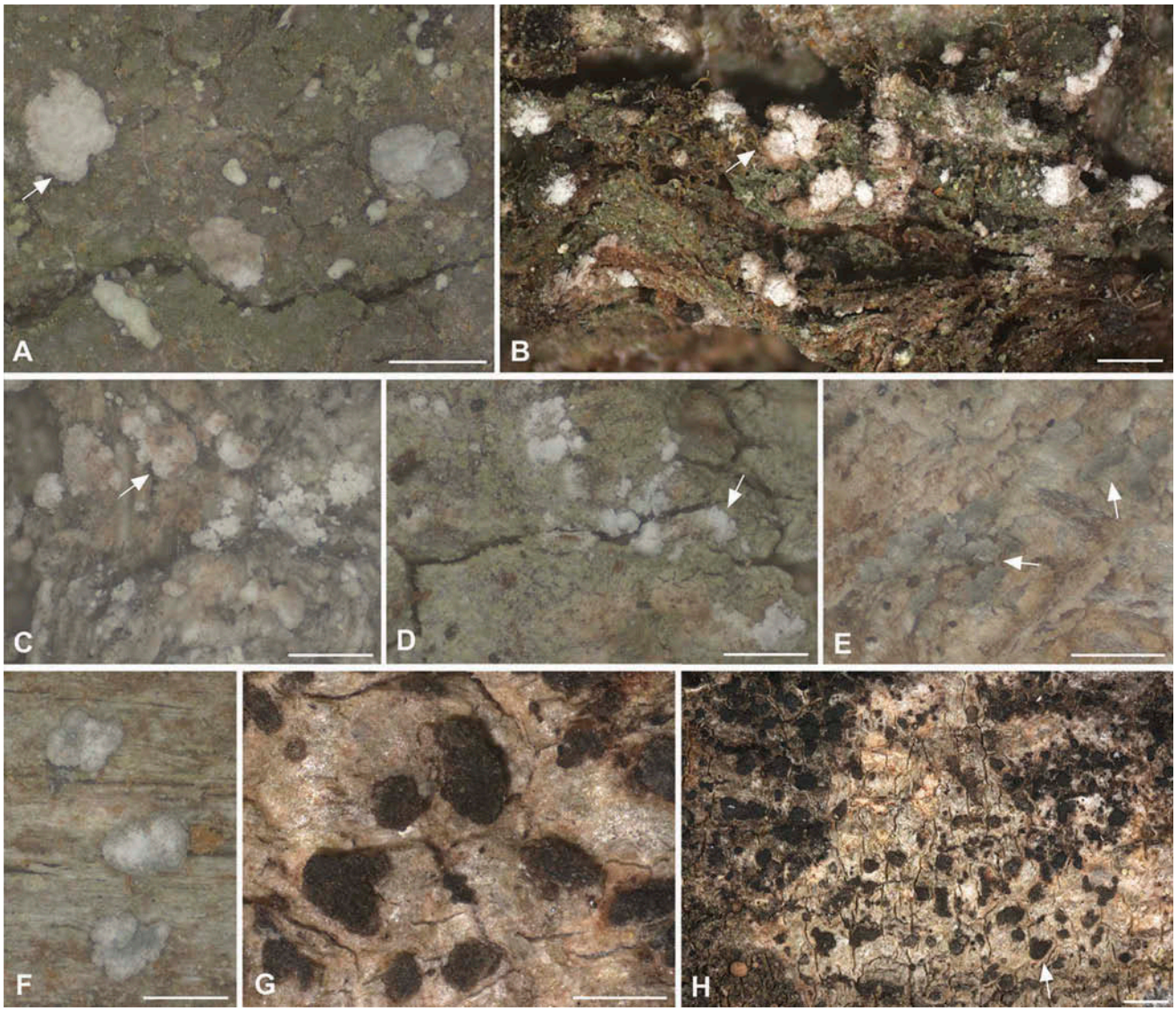

Figure 1. Habit of *Cheiromycina* spp. thalli; samples are reported with their collector and DNA extraction numbers. A, C, D, F. *C. flabelliformis*: A. *Z. Palice 21313*, L2347; C. *Z. Palice 18257*, L2253; D. *F. Jonsson LK46*, L2325; F. *Z. Palice 21313*, L2344. B, E, G, H. *C. petri*: B. *Z. Palice 17855*, L2222; E. *Z. Palice 19030*, L2327; G, H. *T. Tønsberg 42848*, L2223 (G is a detail of H). Arrows point to the sporodochia. Bars: A–F = 0.5 mm; H = 1 mm.

partitioned by individual locus, nuclear 28S and 18S and mitochondrial 16S, in both ML and Bayesian analyses. The program RAxML 7.0.4 (Stamatakis et al. 2005) was used for ML analyses and estimation of bootstrap support. As only a single model of molecular evolution can be used across the partitions, the ML analysis was performed with the GTRMIX model and 1000 bootstrap replicates were run. The model of molecular evolution applied to each gene partition in the Bayesian analysis, GTR+I+G, was estimated in jModeltest 2.1.4 (Darriba et al. 2012) using the Akaike information criterion (Posada and Crandall 1998). The Bayesian Markov chain Monte Carlo (B/MCMC) analyses were run in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2003; Ronquist et al. 2005) with six chains simultaneously, each initiated with a random tree, for 10 million generations; trees were sampled every 100th generation for a total sample of 100 000 trees. Log-likelihood scores against generation time were plotted using Tracer 1.4 (Rambaut and Drummond 2007) to determine when the stationarity of likelihood values had been reached as a guide for where to set the burn-in stage (Ronquist et al. 2005). Burn-in was set at 3 million generations (the first 30 000 sampled trees), and a majority rule consensus tree was calculated from the posterior sample of 70 001 trees. The convergence of the chains was confirmed by the convergent diagnostic of the potential scale reduction factor (PSRF), which approached 1 (Ronquist et al. 2005). The phylogenetic trees were visualized in TreeView (Page 1996).

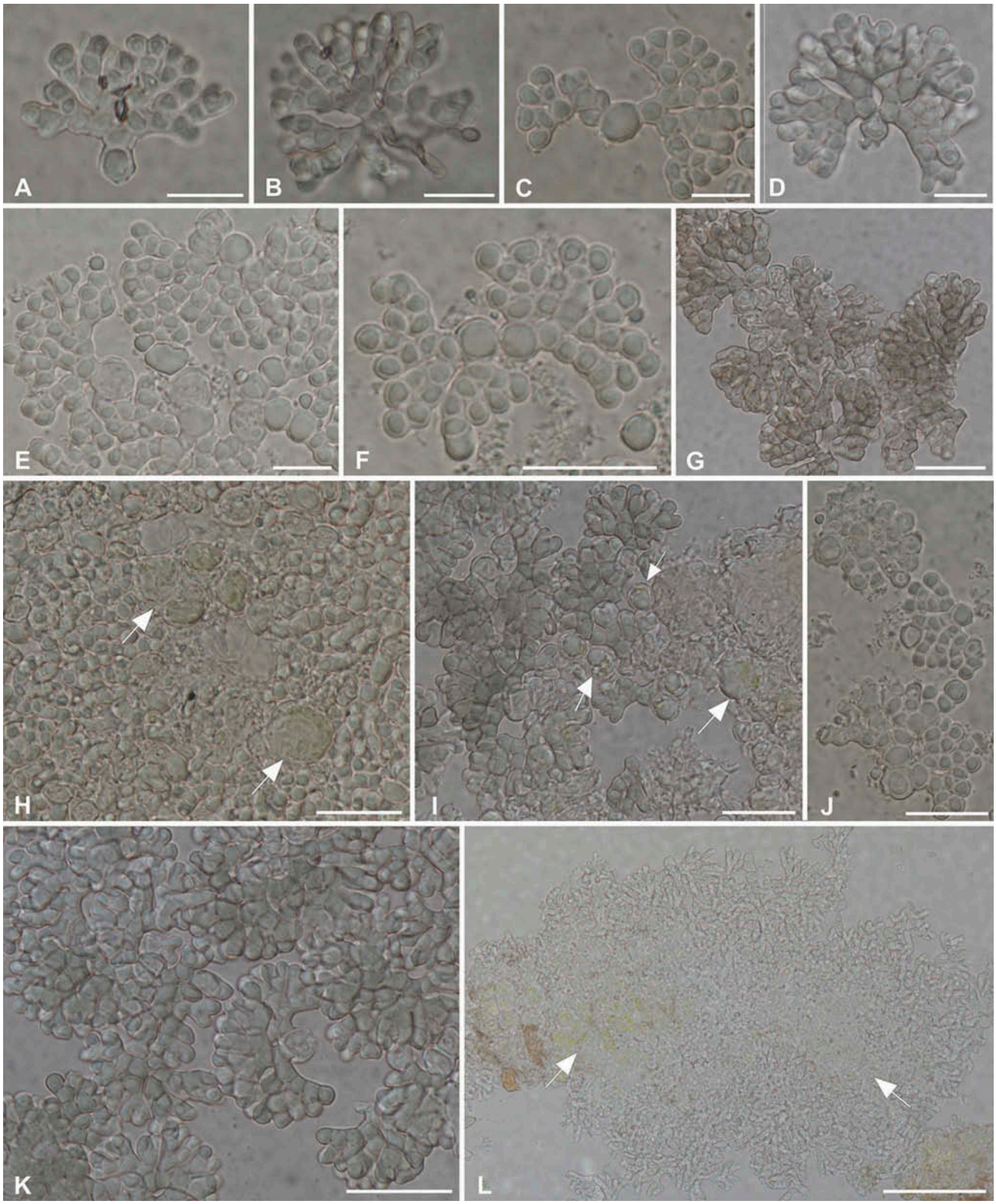

Figure 2. Conidia and sporodochia sections of *Cheiromycina* spp.; samples are reported with their collector and DNA extraction numbers. C, E, F, J, K. Conidia of *C. flabelliformis* in various developmental stages: C, E, F. *Z. Palice 18257*, L2253. A, B, D, G. Pale brown conidia of *C. petri*: A, B. *Z. Palice 17855*, L2222; D. *Z. Palice 19030*, L2327; G. *T. Tønsberg 43060*, L2224. H, I, L. Squash preparation of vertical section of sporodochia: H. *C. flabelliformis, Z. Palice 18257*, L2253; L. *C. flabelliformis, Z. Palice 21313*, L2344; and I. *C. petri, Z. Palice 19030*, L2327. Arrows point to the algal cells scattered among the hyphae in the basal part of the conidioma. Bars: A–E = 10 µm; F–K = 20 µm; L= 50 µm.

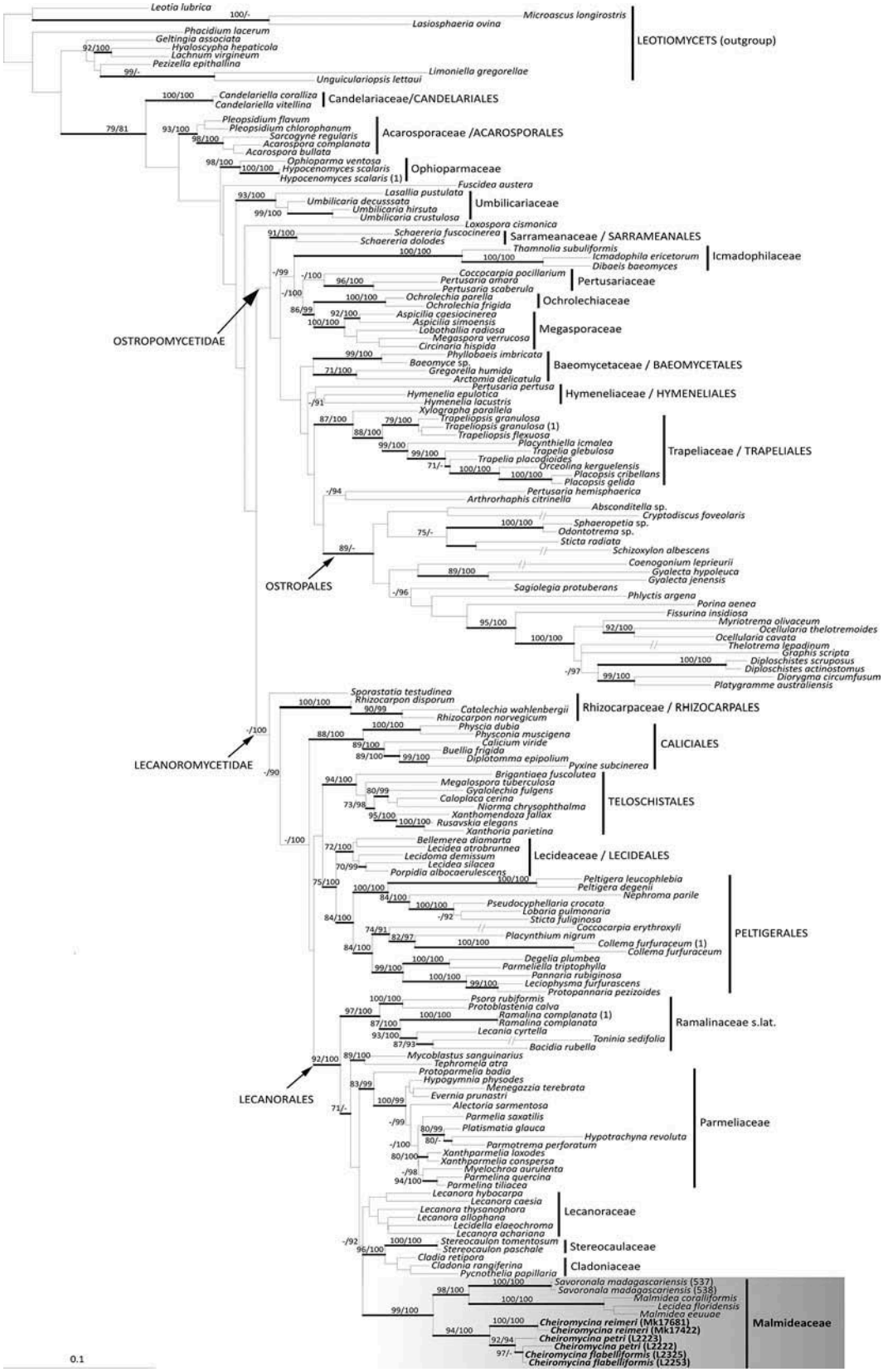

Figure 3. Multigene phylogenetic hypothesis of *Cheiromycina* spp. inferred from the combined data set of nucLSU, nucSSU, and mtSSU markers. Bayesian PP >95% and ML bootstrap support values >70% are reported above branches. Clades are named according to Miadlikowska et al. (2014) and represent a broad selection of taxa within the Lecanoromycetes. The newly sequenced samples (as in TABLE 1) are reported in bold.

RESULTS

We obtained a total of four sequences for *C. flabelliformis* (one nuLSU, two nuSSU, and one mtSSU sequences), five for *C. petri* (one nuLSU, two nuSSU, and two mtSSU sequences), and four for *C. reimeri* (two nuSSU and two mtSSU sequences; TABLE 1). We were unable to generate sequences for the selected loci for nine specimens. Six samples in total were included in the final phylogenetic analysis (TABLE 1). All the retrieved sequences found their closest matches with representatives of Lecanoromycetes.

Our phylogenetic inference (FIG. 3) is topologically congruent with the previous analysis of Miadlikowska et al. (2014). Although this topology was limited in terms of taxon sampling relative to previous studies, the relationships among families and orders within Lecanoromycetes in our phylogenetic inference were largely consistent with previous reconstructions and relationships were generally recovered with high support. All newly sequenced samples, both in the single-locus (not shown) and in the multilocus analyses, form a monophyletic, fully supported clade. This clade was recovered with high support as the sister group of the recently described, monophyletic family Malmideaceae (Ertz et al. 2013), which include "*Lecidea*" *floridensis* (just recently recombined into *Malmidea floridensis* in Cáceres et al. [2017]), *Malmidea coralliformis, M. eeuuae*, and the hyphomycetous species *Savoronala madagascariensis*.

DISCUSSION

This study places the hyphomycetous genus *Cheiromycina* in Lecanorales and within the family Malmideaceae (Kalb et al. 2011). Our phylogeny, inferred from a three-marker data set, recovered *Cheiromycina* as sister lineage to *Savoronala*, another lichenized hyphomycetous genus (Ertz et al. 2013). Apart from sharing the anamorphic state, the two genera occur in different habitats and significantly differ in sporodochia and conidia morphology, as reported in the original description of the two genera by Sutton and Muhr (1986) and Ertz et al. (2013), respectively. In *Savoronala*, sporodochia form at the apex of erected stipes that develop at the center of the thallus and are not branched. The sporodochia are convex, grayish blue in color, and their surface become uneven when covered by the conidial agglomerations (Ertz et al. 2013). In *Cheiromycina*, sporodochia are alternatively eustromatic, usually isolated, white to gray in color, dry, pulverulent, and consist of conidiophores, conidiogenous cells, and conidia intermixed (Sutton and Muhr 1986). Furthermore, thalli of *Savoronala* are well distinguishable, whereas those of *Cheiromycina* are hardly detectable on the substrate surface.

Interestingly, both these hyphomycetous genera are included in a family of lichen-forming fungi for which multiple forms of dispersion are known. In fact, only recently the genera *Crustospathula, Kalbionora*, and *Sprucidea* have been recognized to be part of Malmideaceae (Cáceres et al. 2017; Sodamuk et al. 2017), in addition to the originally included species of *Malmidea, Savonorala*, and two taxa of *Lecidea* s. lat. (Kalb et al. 2011; Ertz et al. 2013). In light of these recent studies, the circumscription of Malmideaceae is in need of a more comprehensive assessment, both in terms of taxon sampling and molecular phylogenetic studies. Future molecular studies in this group of lichen-forming fungi should also consider including the genus *Xyleborus* R.C. Harris & Ladd (Harris and Ladd 2007; Lendemer and Harris 2015) as a potential member of the family Malmideaceae. This unique epixylic genus confined to the Appalachian region of eastern North America is usually fertile, forming dark lecideoid apothecia but simultaneously often produces delimited pale sporodochia with superficial resemblance to the sporodochia of *Cheiromycina*. Harris and Ladd (2007) attributed *Xyleborus* to Stereocaulaceae, which was accepted also in the new classification of Ascomycetes (Lücking et al. 2017). However, this assignment should be understood as tentative due to the lack of molecular data. The potential placement of *Xyleborus* in Malmideaceae would further corroborate the similarity observed with "*Lecidea*" *plebeja* (nowadays known to be a member of Malmideaceae; Ertz et al. 2013; Cáceres et al. 2017) and the presence of sporodochia as known for many members of the family.

Sequence data available for *Dictyocatenulata alba* (An et al. 2012) were purposely not included here for multiple reasons, although *Cheiromycina ananas* was synonymized with *D. alba* (Diederich et al. 2008). First, *D. alba* does not produce cheiroid conidia and, together with *C. globosa*, have a conidiogenesis very distinct from that of *C. flabelliformis* and *C. petri*. *Dictyocatenulata alba* also produces variably tall, long-stiped to sessile synnemata that were misinterpreted to be sporodochia due to the extremely low number of samples bearing sporodochia-like synnemata (which were compared at the time of its description). *D. alba* is a subcosmopolitan lichen reaching also the subtropics and definitely cannot be considered closely related to *Cheiromycina* s. str. Second, our new sequences matched neither with any of the *D. alba* in the GenBank BLAST searches nor with any representatives

of Ostropomycetes, the class in which *D. alba* was placed with *incertae sedis* (An et al. 2012).

Interestingly, An et al. (2012) reported on the relatively fast and successful growth of *D. alba* in culture, but our multiple attempts to isolate *Cheiromycina* spp. failed and the fungi grown in cultures were identified as Sordariomycetes. It is well known that lecanoralean lichen mycobionts grow rather slowly in axenic cultures, whereas Ostropalean fungi, which include also several nonlichenized and optionally lichenized taxa (Wedin et al. 2004, 2006), seem to be easier to culture (Muggia et al. 2011, 2016). This anecdotal observation further supports the likely different phylogenetic placement for the two fungi.

A green, coccoid alga has been observed to be the photobiont in *Cheiromycina* species (Hawksworth and Poelt 1990). However, the identity *Cheiromycina*-associated photobionts has not been studied yet. In this case, all attempts to amplify and sequence and/or culture the algae failed, likely due to the insufficient amount of photobiont cells in our preparations. In fact, algae were found localized only at the base of the sporodochia (FIG. 2H, I, L) and were never observed to be tightly associated with the conidiogenous hyphae or conidia. Co-dispersion of the fungus with the algae might not happen in *Cheiromycina*, although this strategy has been observed for the sister genus *Savoronala*, where the conidia wrap around a single algal cell already at the early stage of the diaspore formation and before the fungal cells become melanized (Ertz et al. 2013).

In general, hyphomycetous lichenicolous and lichenized fungi are less frequently collected than other fungal taxa that develop conspicuous thalli and sexual reproductive structures (apothecia or perithecia). The often subtle morphological characters create unique challenges in effectively circumscribing species boundaries using morphological data alone. Further confounding our understanding of diversity in this enigmatic group is the fact that most specimens of anamorphic fungi usually hold too little material to allow multiple analyses. For example, in the genus *Cheiromycina*, the published descriptions of the three species—*C. ananas, C. globosa*, and *C. petri*—were based on single collections, which definitely have not encompassed the range of intraspecific morphological and anatomical variability that a species comprises (Muggia et al. 2014). External morphological characters are also of little help when trying to distinguish *Cheiromycina* species, as the habit of the sporodochia is rather variable and "not eroding" or "eroding white" sporodochia (Hawksworth and Poelt 1986) are recovered in both species (see also Printzen 2007). More efficient diagnostic, key characters were suggested to be represented by the conidiogenous cells, size, and branching patterns of the conidia (Printzen 2007). *Cheiromycina flabelliformis, C. petri*, and *C. reimeri* are distinguished by their multicellular, palmately branched conidia and have been segregated from each other based on differences in the size of conidiogenous cells and shape and septation of conidial branches. In addition, conidia in *C. flabelliformis* have been reported as displaying a secondary three-dimensional structure. We observed, however, that conidia can be three-dimensional also in specimens otherwise referring to *C. petri* (FIG. 2A, B). The enlarged conidiogenous cells are easily recognized in the majority of the conidia in *C. flabelliformis*, but squash preparation may destroy or break them from the rest of the conidium, thus biasing the species identification. In light of our results, we propose that the genus *Cheiromycina* is in need of taxonomic revision. The current circumscription of the genus may in fact not reflect the phylogenetic position of the other, here not sequenced taxon *C. globosa*, which significantly differs by the lack of cheiroid conidia. However, a taxonomic treatment of the genus is beyond the scope of the present study.

Cheiromycina was described about 30 years ago as "an unusual deuteromycete with the appearance of a sorediate endoxylic lichen" (Sutton and Muhr 1986, p. 831), and no teleomorphic state is known for it so far. In this study, we could detect tiny, pale brown apothecia on the thallus bearing the sporodochia in a single specimen of *C. petri* (L2224, *Tønsberg 43060*) (SUPPLEMENTARY FIG. 1), although the apothecia contained immature asci without ascospores. Unfortunately, PCR amplifications from the apothecia and those from the sporodochia preparations were unsuccessful for this sample; thus, comparison with the other *Cheiromycina* samples was impossible. Recovering *Cheiromycina* and other lichenized hyphomycetous genera within Lecanorales further encourages future investigations of additional anamorphic lichenized fungi for which both no sequence data are available so far and no teleomorphic state is known (Ertz et al. 2011, 2013; Diederich et al. 2013).

In lichen-forming fungi, sexual reproduction is by far the most dominant reproductive mode (Seymour et al. 2005). However, some species-rich genera are exceptionally represented by only asexual species, such as *Lepraria*, which have also evolved strikingly diverse array of secondary metabolites (Ekman and Tønsberg 2002; Elix and Tønsberg 2004; Kukwa 2006; Nelsen and Gargas 2008; Flakus et al. 2011; Lendemer 2013). Whether speciation in *Lepraria* has occurred in the absence of recombination is still a debated question

(Fehrer et al. 2008). Although parasexual processes may play a crucial role in lineages of lichen-forming fungi, it is very difficult to demonstrate and it might be speculated that they act in the successful distribution of the species and their genetic diversity. Due to the limited number of *Cheiromycina* samples available, obtaining detailed, robust insight into intraspecific diversity of these lichenized hyphomycetes will be challenging into the foreseeable future.

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