



Multilocus phylogenetic analysis reveals that Cyttariales is a synonym of Helotiales

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Abstract

Cyttaria is a morphologically and biologically distinct genus comprising wood-inhabiting species that are biotrophic associates of trees in the genera *Nothofagus* sensu stricto and *Lophozonia* in southern South America, Australia, and New Zealand. The uniqueness of the fruit bodies and habitat of *Cyttaria* has led taxonomists to justify its placement in its own order, Cyttariales, in Leotiomyces. A multilocus phylogenetic reconstruction of the class Leotiomyces incorporating new sequence data from *Cyttaria nigra* shows Cyttariaceae to have an isolated position near the base of Helotiales, with weak support for a relationship with *Polydesmia pruinosa* (incertae sedis) and Chlorociboriaceae. Cyttariales is here proposed as a synonym of Helotiales.

Keywords Leotiomyces · Molecular systematics · Multilocus phylogeny · Plant-pathogenic fungi · Systematics

Introduction

Cyttaria is a morphologically and biologically distinctive genus of wood-inhabiting biotrophic fungi restricted to the host genera *Nothofagus* sensu stricto and *Lophozonia*. Species of *Cyttaria* produce numerous apothecial locules embedded in highly gelatinous stromata that themselves form in groups on galls on living branches and twigs of their hosts. Asci are inoperculate with an amyloid apical ring, and some species form pycnidial anamorph immersed in young stromata (Gamundí 1971; Mengoni 1986; Peterson and Pfister 2010; Jaklitsch et al. 2016; Quandt and Haelewaters 2021). *Cyttaria* species are known from *Nothofagus* and *Lophozonia* from southern South America and from *Lophozonia* from Australia and New Zealand. Its specialization to host genera, often considered one of the keys to

understanding Southern Hemisphere biogeography (Darlington 1965; Steenis 1971), has meant that *Cyttaria* has been subject to several fungal biogeographic studies (e.g., Korf 1983; Crisci et al. 1988; Setoguchi 2005; Peterson et al. 2010). As is the case with Nothofagaceae, explanations for the present geographic distribution of *Cyttaria* have changed over time. Since the widespread acceptance of continental drift, the classic Gondwanan geographic distribution of Nothofagaceae and *Cyttaria* initially was thought to reflect geologically ancient patterns of vicariance related to the breakup of Gondwana. Later molecular phylogenetic and molecular clock studies, and an appreciation of the role that extinction has played in the geological history of Nothofagaceae, suggest less simple explanations for present day distributions, requiring a combination of vicariance and geologically more recent transoceanic dispersals (Knapp et al. 2005). The analyses of Peterson et al. (2010) show a strong cophylogenetic relationship between *Cyttaria* spp. and their *Nothofagus* and *Lophozonia* hosts and support a geologically relatively recent, transoceanic dispersal of *Cyttaria* from Australia to New Zealand.

The unusual morphology of *Cyttaria* has resulted in an uncertain taxonomy above the level of genus. The family Cyttariaceae was proposed invalidly by L veill  (1846) and subsequently validated by Spegazzini (1888). A few other genera have been incorrectly placed in the family at times, but it is now generally accepted as monotypic (Korf 1983).

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The relationship of Cyttariaceae to other ascomycetes has long been unsettled, for example, with Korf (1983) and Santesson (1945) using different interpretations of the ascus morphology to conclude a position in Pezizales and Helotiales, respectively. Our morphological observations support Gamundí (1971) and Mengoni (1986) that the asci, with a thickened ascus apex and amyloid pore, are typical of Leotiomycetes. Figure 1 shows a thickened ascus apex with a very broad pore, the margins of the pore amyloid, flaring towards the outside of the ascus wall, and the amyloid zone appearing to have a complex series of fine bands. This agrees with the descriptions of Mengoni (1986), who interpreted this as the *Bulgaria*-type ascus.

Gamundí (1971) proposed the monotypic order Cyttariales that she hypothesized was morphologically intermediate between Helotiales and Sphaeriales. The first molecular study incorporating *Cyttaria* data showed it to be Leotiomycetes (Landvik and Eriksson 1994) and that has been confirmed in later analyses (Wang et al. 2006; Peterson and Pfister 2010; Pärtel et al. 2017). Wang et al. (2006) showed

a strongly supported sister relationship with *Chlorociboria*, but this was not so clear in the analyses of Peterson and Pfister (2010) or Pärtel et al. (2017). Ekanayaka et al. (2019) accepted a broader concept of Cyttariales, to include Cyttariaceae, Cordieritidaceae, and Deltopyxidaceae, but the phylogeny on which they based this conclusion has no support above the level of family. Note that Ekanayaka et al. (2019) included *Deltopyxis* and *Phaeopyxis* in their new family Deltopyxidaceae. However, *Phaeopyxis* was shown by Suija et al. (2015) and Pino-Bodas et al. (2017) to be Ostropomycetidae (Lecanoromycetes), and based on the available sequence data for *Deltopyxis* on NCBI GenBank (ITS and LSU only), Baral et al. (2020) excluded *Deltopyxis* from Leotiomycetes, placing it within Lecanoromycetes.

To help clarify the relationships of Cyttariales, we present a phylogeny incorporating new sequence data from multiple loci for *Cyttaria nigra*. Previously, the genus was represented only by ribosomal DNA (rDNA) data in addition to 16 mtSSU and 3 *TEF1* sequences (Peterson and Pfister 2010).



Fig. 1 *Cyttaria* ascomata and asci. **A–E** *Cyttaria nigra*. **F** *Cyttaria* ‘*gunnii*’. **A** Fresh specimen from which DNA was extracted, ascomata immature showing prominent papillae characteristic of *C. nigra* (PDD 117571). **B** Dried specimen, ascoma starting to mature, apothecia still covered by membranous tissue (PDD 68277, New Zealand: Westland, 24 October 1997, ex. *Lophozonia menziesii*, leg. H. Setoguchi). **C** Dried specimen, mature ascoma with individual apothecia fully exposed (PDD 68277). **D** Asci from ascoma in **B**. **E** Ascus from ascoma in **C**, with detail of ascus apex. **F** Detail of ascus apex from *C. ‘gunnii’* sensu New Zealand (Peterson and Pfister 2010) (PDD 76512, New Zealand: Buller, 22 October 2002, ex. *Lophozonia menziesii*, leg. P.R. Johnston & R.E. Beever). **D–F** Dried specimen rehydrated in 3% KOH and mounted in Melzer’s reagent. Scale bars **A** = 50 mm, **B–C** = 1 mm, **D–F** = 10 μ m

Materials and methods

DNA extraction, PCR amplification, sequencing

An ascoma of *Cyttaria nigra* (PDD 117571, New Zealand: Fiordland, 2 December 2019, ex. *Lophozonia menziesii*, leg. P.R. Johnston) was rehydrated in water, and the outer layer was completely removed. The internal part of the ascoma (sterile tissue plus maturing apothecia) was then vacuum dried and powdered using a TissueLyser (Qiagen, Stanford, CA, USA) at 30 Hz for 60 s. DNA was extracted from 100 mg of the powder using a CTAB/phenol/chloroform method (Schwessinger and McDonald 2017). Partial sequences of the small subunit (SSU), large subunit (LSU), mitochondrial small subunit (mtSSU), *MCM7*, translation elongation factor 1 alpha (*TEF1*), RNA polymerase subunit I (*RPB1*), RNA polymerase subunit II (*RPB2*), and β -tubulin (*bTUB*) were amplified using the following primer pairs: NS1/NS4 for SSU (White et al. 1990), LR0R/LR5 for LSU (Vilgalys and Hester 1990; Hopple 1994), mtSSU1/mtSSU3R for mtSSU (Zoller et al. 1999), Mcm7-709for/Mcm7-1348rev for *MCM7* (Schmitt et al. 2009), EF1-526F/EF1-983F/EF1-1567R/EF1-2218R for *TEF1* (Rehner and Buckley 2005), RPB1-Af/RPB1-Cr for *RPB1* (Stiller and Hall 1997; Matheny et al. 2002), fRPB2-5F/fRPB2-7cR for *RPB2* (Liu et al. 1999), and TUB2Fd/TUB4Rd for *bTUB* (Aveskamp et al. 2009).

Cycling conditions followed the protocols in the above references for the respective primers. In most cases, however, PCR needed optimization with 0.6 μ M concentration of forward and reverse primers, 2.5 mM MgCl₂ concentration, annealing temperature set at 45 °C, and 40 cycles. When PCR resulted in multiple bands on gel, a gel extraction of the band with the desired size was performed before sequencing. When the concentration of the PCR products was very low, 25 additional cycles of PCR were performed using the PCR product as template. Sanger sequencing was done in both directions on a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA). Generated sequences were assembled and edited in Sequencher v. 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). All sequences are deposited in GenBank under the following accession numbers: MW364564 (SSU), MW364561 (LSU), MW350086 (*MCM7*), MW363493 (*RPB1*), MW350084 (*RPB2*), MW350088 (*TEF1*), MW364562 (mtSSU), and MW350089 (*bTUB*).

Sequence alignment and phylogenetic analysis

Data from Johnston et al. (2019) were used as a basis for our phylogenetic analyses. Their 15-locus data matrix is available in the Manaaki Whenua–Landcare Research Datastore (<https://doi.org/10.7931/T5YV-BE95>). The SSU, LSU, mtSSU, *MCM7*, *TEF1*, *RPB1*, *RPB2*, and *bTUB* regions were extracted from the matrix separately, and sequences of *Cyttaria nigra* were added to the respective dataset. Alignments were done for each locus using Muscle v. 3.7 (Edgar 2004) on the Cipres Science Gateway (Miller et al. 2010). The aligned sequences of all loci were concatenated in MEGA7 (Kumar et al. 2016) to form a supermatrix of 19,290 bp (Supplemental Materials S1 = matrix, S2 = partition file). Phylogenetic relationships were inferred by analyzing the combined 15-locus dataset with maximum likelihood (ML). We used the command line version of IQ-TREE (Nguyen et al. 2015) under partitioned models (Chernomor et al. 2016). Appropriate models of nucleotide substitution were selected according to the corrected Akaike information criterion (AICc) through the built-in ModelFinder (Kalyaanamoorthy et al. 2017). Selected models are presented in Table 1. Ultrafast bootstrapping was done with 1000 replicates (Hoang et al. 2018). The final tree with ML bootstrap support values (BS) was visualized in FigTree v. 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) and edited in Adobe Illustrator version 25.1 (San Jose, CA, USA).

Results

Our 15-locus dataset included 380 isolates and 19,290 characters, of which 7660 were constant and 9908 were parsimony-informative (Table 1). The tree topology resulting from the ML analysis is shown in Fig. 2. Compared to Johnston et al. (2019), this ML tree supports family Lauriomycetaceae (Lauriomycetales) at the base of class Leotiomycetes and includes newly erected families Chlorospleniaceae, Discinellaceae (Ekanayaka et al. 2019), Leptodontiaceae (Hernández-Restrepo et al. 2017), Lichinodiaceae (Prieto et al. 2019), Micraspidaceae (Quijada et al. 2020), Neolauriomycetaceae (Crous et al. 2018), Tribliadiaceae (Karakehian et al. 2019), and Tricliadiaceae (Johnston and Baschien 2020).

Cyttaria nigra PDD 117571 is retrieved as sister to *C. darwinii* and *C. hariotii* with maximum support. This Cyttariaceae clade is placed sister to *Polydesmia pruinoso*, deep within Helotiales sensu Johnston et al. (2019). Likewise, also the Erysiphaceae clade (*Blumeria graminis*, *Erysiphe necator*, *Golovinomyces cichoracearum*) is placed within Helotiales, sister to Arachnopezizaceae and the *Psychrophila* clade. Leotiales, with increased taxon sampling compared

Table 1 Gene regions included in the 15-locus phylogenetic analysis, with for every partition the number of sequences, total number of sites, informative sites, invariable sites, and model of nucleotide substitution as selected by ModelFinder

Partition	Locus	Sequences	Sites	Informative	Invariable	Model
1	5.8S	321	158	42	61	TNe + R3
2	<i>aTUB</i>	82	2056	1021	872	GTR + F + I + G4
3	<i>bTUB</i>	136	2139	1040	907	GTR + F + R5
4	LSU	334	898	397	398	SYM + R6
5	<i>MCM7</i>	117	732	386	302	GTR + F + R5
6	mtSSU	135	869	326	453	GTR + F + R5
7	<i>RPA1</i>	81	1079	647	362	GTR + F + R5
8	<i>RPA2</i>	83	1744	979	660	TIM3 + F + R5
9	<i>RPB1</i>	212	1450	857	482	SYM + R6
10	<i>RPB2</i>	284	2026	1215	639	GTR + F + R10
11	<i>RPC2</i>	82	1569	947	498	GTR + F + R7
12	<i>SF3B1</i>	88	1104	491	571	GTR + F + R7
13	SSU	253	1153	339	579	GTR + F + R5
14	<i>TEF</i>	193	1135	500	516	SYM + R5
15	<i>TFB4</i>	79	1178	721	360	TVM + F + R6
Concatenated	15 loci	380	19,290	9908	7660	N/A

to Prieto et al. (2019), includes Leotiaceae, Lichinodiaceae, Mniaeciaceae, Tympanidaceae (sensu stricto), and *Flagellospora curvula* CCM F-18699.

Taxonomy

Based on our phylogenetic analysis, we propose the following order-level synonymies. Both the Erysiphales and Lichinodiales synonymies were implicitly included in previous work (Johnston et al. 2019; Quijada et al. 2020), but are here presented as formal taxonomic changes towards a stable classification for Leotiomycetes.

Helotiales Nannf., *Nova Acta Reg. Soc. Scient. Upsala*, Ser. 4 8: 68 (1932).

= Cyttariales Luttr. ex Gamundí, *Darwiniana* 16: 502 (1971).

= Erysiphales Warm., *Haandbog i den systematiske Botanik*, ed. 2: 63 (1884).

Leotiales Korf & Lizoň, *Czech Mycol.* 52(4): 256 (2001).

= Lichinodiales M. Prieto, M. Schultz, Olariaga & Wedin, *Fungal Divers.* 94: 36 (2019).

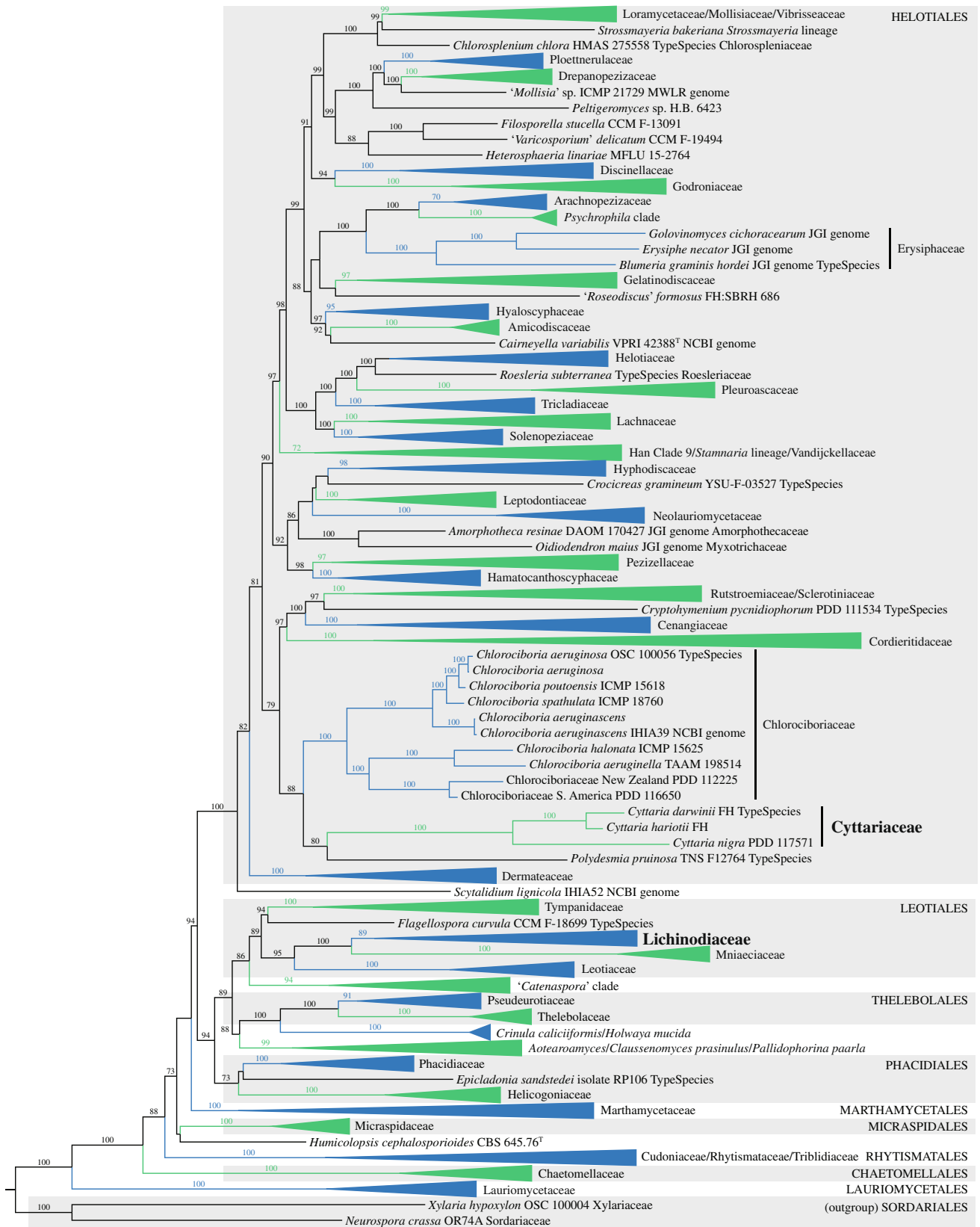
Discussion

The *Dictionary of Fungi* (Kirk et al. 2008) mentioned five orders within Leotiomycetes: Cyttariales, Erysiphales, Helotiales, Leotiales, and Rhytismatales, as well as Thelebolales with uncertainty. In their overview of class Leotiomycetes, Jaklitsch et al. (2016) listed ten orders: Cyttariales, Erysiphales, Helotiales, Lahmiales, Leotiales, Medeolariales, Phacidiales, Rhytismatales, Thelebolales, and Triblidiales.

After the publication of this chapter, several new leotiomycetous orders were described: Chaetomellales (Crous et al. 2017), Laureomycetales (Hernández-Restrepo et al. 2017), Lichinodiales (Prieto et al. 2019), Marthamycetales (Johnston et al. 2019), and Micraspidales (Quijada et al. 2020). Conversely, recent molecular phylogenetic data resulted in the synonymy of two orders: Triblidiales under Rhytismatales (Karakehian et al. 2019) and Erysiphales under Helotiales (Johnston et al. 2019). Apart from Cyttariales and Erysiphales, all of these orders are basal to Helotiales within Leotiomycetes, although the placement of Lahmiales (no sequence data available) and Medeolariales (only ITS and LSU sequences, tentatively basal within Helotiales, unpubl. data) remains unknown or poorly resolved.

Following the broad concept for Helotiales sensu Johnston et al. (2019), representatives are usually apothecial, small (< 2 mm diam.), variable in the presence of a stalk (from sessile to long-stalked), variable in color, and superficial or erumpent. Some taxa form cleistothecia, such as *Amorphotheca* (Amorphothecaceae), *Bicornispora* (Rutstroemiaceae), *Connersia*, *Pleuroascus* (Pleuroascaceae), and

Fig. 2 The best-scoring ML tree (-lnL=676,456.342151) of Leotiomycetes, reconstructed from the concatenated 15 locus dataset. ML bootstraps (if ≥ 70) are presented above or in front of the branch leading to each node. Names for the collapsed family-level clades are those accepted in Johnston et al. (2019) and in this paper. The labels for taxa that are not included in one of the family-level clades include the voucher specimen from which the sequences were obtained, the type status of the specimen (whether it is the ex-type specimen of the type species, annotated with ^T, or whether it has been identified as the type species, annotated with “TypeSpecies”), and the source of the genome data for those that have had their genome sequenced (*JGI* joint genome initiative, *MWLR* Manaaki Whenua—Landcare Research, *NCBI* National Center for Biotechnology Information)



members of Erysiphaceae and Myxotrichaceae, whereas others form perithecia (Loramycetaceae and *Unguicularia* in Hyaloscyphaceae). *Cyttaria* is apothecial, like the majority of described Helotiales members, although unique in forming spherical ascomata that consist of sterile stroma with many apothecial cavities. Many members of Helotiales are saprotrophs; some are associated with living organisms as parasites, pathogens, or mutualists (e.g., Peterson and Pfister 2010; Stenroos et al. 2010; Jaklitsch et al. 2016; Haelewaters et al. 2018; Tanney and Seifert 2020). Members of Cyttariaceae and Erysiphaceae, now both included in Helotiales, are plant parasitic.

Pärtel et al. (2017) already found strong support for a broad concept of Helotiales including both Cyttariales and Erysiphales. This was based on the phylogenetic reconstruction of a dataset with SSU, LSU, *RPB1*, *RPB2*, and *TEF1* sequences, although both Cyttariales and Erysiphales were only represented by rDNA sequence data (and two *TEF1* sequences for *Cyttaria hariatii*). Peterson and Pfister (2010) found support for *Cyttaria* being sister to Cordieritidaceae, both placed sister to Chlorociboriaceae. This was based on the phylogenetic reconstruction of a four-locus Pezizomycotina-wide dataset. Our 15-locus phylogenetic tree including eight loci of *Cyttaria nigra* confirms a close (but weakly supported) relationship with Chlorociboriaceae. This phylogenetic relatedness is reflected macromorphologically; both *Chlorociboria* and *Cyttaria* produce stromata from which one or multiple apothecia and/or anamorphic pycnidia arise (Gamundí 1971; Peterson and Pfister 2010). However, morphology of the amyloid ascus ring, often predictive of phylogenetic relationships within Leotiomycetes (e.g., Baral et al. 2015), is quite distinct between *Chlorociboria* (Baral et al. 2015), *Cyttaria* (Mengoni 1986; Fig. 1), and *Polydesmia* (Hosoya 2009).

If orders such as Cyttariales and Erysiphales were retained for their unique morphological or ecological characteristics (A.H. Ekanayaka and K.D. Hyde in Wijayawardene et al. 2020), this would render Helotiales a paraphyletic taxon. We prefer to avoid paraphyletic taxa as they mislead about relationships. A way to resolve the paraphyly would be to elevate many families currently accepted in Helotiales to order level. However, under this scenario, the majority of these new orders would be monotypic (with only one family). In other words, the additional taxonomic level would add little additional information.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11557-021-01736-2>.

Author contribution D.P. and P.R.J. generated sequence data. D.H. performed phylogenetic analyses. D.H. and P.R.J. wrote the first draft of the manuscript. All authors read and approved the final version of the manuscript.

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Data availability The datasets generated and analyzed during this study are available as Supplemental Materials S1 (data matrix) and S2 (partitions file with best-fit models).

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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