Mycoscience (2012) 53:373–380
DOI 10.1007/s10267-012-0181-4

FULL PAPER

Freshwater ascomycetes: *Coniochaeta gigantospora* sp. nov. based on morphological and molecular data

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Received: 20 September 2011 / Accepted: 16 January 2012 / Published online: 23 February 2012

Abstract *Coniochaeta gigantospora* collected from submerged wood in a freshwater habitat in France is described and illustrated as a new species in the family Coniochaetaceae (Coniochaetales, Ascomycota). This placement is based on morphology and phylogenetic analyses of partial nuclear ribosomal 28S large subunit and complete internal transcribed spacer DNA sequence data. *Coniochaeta gigantospora* is distinguished from other *Coniochaeta* species in possessing unusually large, ellipsoid, nearly equilateral, olivaceous to olivaceous-brown ascospores.

Keywords Aquatic fungi · ITS · LSU · Sordariomycetes · Submerged wood

Electronic supplementary material The online version of this article (doi:10.1007/s10267-012-0181-4) contains supplementary material, which is available to authorized users.

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Introduction

Coniochaeta (Sacc.) Cooke is a highly diversified genus that occurs in the Coniochaetaceae (Malloch and Cain 1971) within the order Coniochaetales in the subclass Sordariomycetidae, class Sordariomycetes (Huhndorf et al. 2004; Garcia et al. 2006). It is typified by *C. ligniaria* (Grev.) Massee (Clements and Shear 1931; von Arx and Müller 1954) and characterized by dark-brown to black, setose ascomata, which may be pyriform and ostiolate or globose and non-ostiolate; inamyloid asci; and ellipsoid, dark-brown, smooth ascospores with a longitudinal germ slit (Garcia et al. 2006). Currently 54 species are accepted in *Coniochaeta* (Asgari et al. 2007); however, according to online databases such as Index Fungorum (http:\\www.indexfungorum.org, May 2011) and Mycobank (Crous et al. 2004), up to 88 or 92 names, respectively, may be included in the genus. About 70 species and six synonyms are included in *Coniochaeta* according to Garćia et al. (2006), while Kirk et al. (2008) include about 65 species in the genus.

Garćia et al. (2006) report the anamorphic genera *Cladobotryum* Nees, *Lecythophora* Nannf., *Paecilomyces* Bainier, and *Verticillium* Nees as belonging to the genus *Coniochaeta.* A phialidic anamorph belonging to the genus *Lecythophora* is associated with many *Coniochaeta* species (Weber 2002; Weber et al. 2002; Garcia et al. 2006), but a *Nodulisporium*-like anamorph has also been reported for a few *Coniochaeta* species (Hawksworth 1978; Asgari and Zare 2006; Asgari et al. 2007). Members of *Lecythophora* are phialidic hyphomycetous fungi with the phialide often reduced to a collarette laterally positioned on a hyphal cell (see Gams 2000; Weber 2002; Weber et al. 2002; Seifert et al. 2011), while *Nodulisporium* Preuss is characterized by long, mononematous conidiophores with holoblastic,

polyblastic hyaline conidia in a sympodial sequence, which gives rise to the characteristic nodulose appearance of the conidiogenous cell (Jong and Rogers 1972; Seifert et al. 2011). Currently, within the Coniochaetales, 17 *Coniochaeta* and one species of *Barrina* A.W. Ramaley form *Lecythophora* anamorphs (Damm et al. 2010). Recent phylogenetic work based on nuclear ribosomal DNA sequences of the D1–D2 divergent domains of the large subunit (LSU) and the complete internal transcribed spacer (ITS) region, however, suggest that taxa with *Nodulisporium*-like anamorphs are phylogenetically unrelated to *Coniochaeta* but instead show phylogenetic affinities with the Xylariales (Zare et al. 2010; Asgari and Zare 2011; see Seifert et al. 2011).

During investigations of freshwater ascomycetes in France (Zhang et al. 2008, 2009; Fournier and Lechat 2010; Fournier et al. 2010; Gardiennet 2010; Réblová et al. 2010), an ascomycetous fungus with features characteristic of *Coniochaeta* was collected on submerged wood in a lotic freshwater habitat. We could not assign the fungus to any of the species currently accepted in the genus using species keys (Hawksworth and Yip 1981; Mahoney and LaFavre 1981; Checa et al. 1988; Romero et al. 1999; Asgari et al. 2007). The fungus from France is unique in having an evenly setose, thick-walled ascoma and unusually large single-celled, multiguttulate, smooth ascospores with a short longitudinal germ slit. Molecular phylogenetic analyses of LSU and ITS sequences also confirm this species as being distinct from other species of *Coniochaeta* for which sequence data are currently available in GenBank. A new species, *C. gigantospora*, is therefore proposed, described and illustrated herein.

Materials and methods

Morphological study

The methods for collection, morphological characterization, and illustration are outlined in Fournier and Lechat (2010). We were unable to isolate the fungus in pure culture because of the limited amount of material available for study. The holotype specimen is deposited in the Illinois Natural History Survey Fungarium (ILLS).

Molecular study

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

Total genomic DNA was extracted directly from ascomata, PCR amplified and sequenced following procedures in Promputtha and Miller (2010). The entire ITS region (ITS1-5.8S-ITS2) and D1–D2 variable domains of the LSU were amplified with ITS1F and LR3 and subsequently sequenced using ITS primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993) and LSU primers LROR and LR3 (Vilgalys and Hester 1990; Rehner and Samuels 1995).

Taxon sampling and phylogenetic analyses

Three datasets were assembled for phylogenetic analyses: (1) an expanded LSU dataset that consisted of 59 taxa representing the orders Xylariales (Xylariaceae, Diatrypaceae, Coniocessiaceae) and Coniochaetales (Coniochaetaceae) with taxon sampling based on a recent study by Asgari and Zare (2011); (2) a limited LSU dataset that consisted of 44 taxa belonging to various species of *Coniochaeta* and its *Lecythophora* anamorph; and (3) an ITS dataset from GenBank consisting of 53 taxa belonging to *Coniochaeta* and *Lecythophora* species. GenBank accession numbers are shown in Supplementary Fig. 1; Figs. 1 and 2. All three datasets were aligned initially with the multiple alignment program MUSCLE (Edgar 2004), using default parameters as implemented in Seaview 4.1 (Gouy et al. 2010). Alignments were optimized by visual examination and manually corrected using MacClade 4.08 (Maddison and Maddison 2000). After the datasets were aligned and prior to the running of maximum likelihood (ML) and Bayesian analyses (BA), ambiguous regions were excluded using Gblocks with the program default parameters in effect (Castresana 2000; Talavera and Castresana 2007). A section of nucleotides from the 5′ and 3′ ends was excluded in all three datasets due to missing characters in most taxa.

Maximum likelihood analyses were conducted on all three datasets after Modeltest 3.7 (Posada and Crandall 1998) was used to obtain the best-fit model of nucleotide evolution. The Akaike information criterion (AIC) (Posada and Buckley 2004) as implemented in Modeltest selected the Tamura-Nei model (Trn+I+G) (Tamura and Nei 1993) for the expanded LSU dataset; the transition model (TIM+I+G) for the limited LSU dataset; and the symmetrical model (SYM+I+G) (Zharkikh 1994) for the ITS dataset. Maximum likelihood analyses were then conducted using the selected models including invariable sites and a discrete gamma shape distribution with 1000 ML bootstrap (BS) replicates with the combined nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) tree search option in effect using the program PHYML (Guindon and Gascuel 2003). We consider clades with a BS of $\geq$70% as significant and strongly supported (Hills and Bull 1993).

Bayesian analyses were performed on the datasets with MrBayes 3.12 (Huelsenbeck et al. 2001; Huelsenbeck and

Ronquist 2005) using the CIPRES Portal 2.0 (Miller et al. 2010) to assess clade support. Mr. Modeltest 2.2 (Nylander 2004) and PAUP 4.0b10 (Swofford 2003) were used with the implementation of AIC to select the best-fit model of evolution. The general time reversible model (GTR+I+G) (Rodríguez et al. 1990) was selected for the two LSU datasets and the SYM+I+G model was selected for the ITS dataset. The selected model was implemented and constant characters were included in the BA which were run using 10 million generations with trees sampled every 1000th generation, resulting in 10000 total trees. The online program AWTY (Nylander et al. 2008) was used to compare the split frequencies of different runs to insure that the stationary phase was reached. The program TRACER v.1.5 was used to plot the log-likelihood scores against generation time to confirm that the log-likelihood values reached a stable equilibrium (Huelsenbeck et al. 2001). Based on the results from TRACER, 1000 trees that extended beyond the burn-in were discarded, and the remaining 9000 trees were used to calculate the posterior probability (PP) in each analysis. Consensus trees were generated and viewed in PAUP 4.0b10 (Swofford 2003). For each dataset, the BA were run twice, starting from a different random tree to ensure that trees from the same tree space were being sampled. We consider clades with a PP of ≥95% as significant and strongly supported.

Results

Molecular study

Expanded LSU dataset

The expanded LSU alignment originally consisted of 59 taxa and 1386 characters. After portions of the 5′ and 3′ ends, introns, and ambiguous regions were excluded, the final alignment consisted of 507 characters. PHYML analysis of the LSU produced a single most likely tree ($\ln(L) = -3105.15$), which shows that *C. gigantospora* clearly belongs in the monophyletic Coniochaetales with strong PP support (Supplementary Fig. 1). It occurred on an independent branch within *Coniochaeta* and was placed basal to the clade containing isolates of the type species, *C. ligniaria* (Supplementary Fig. 1).

Limited LSU dataset

Taxon sampling in the limited LSU dataset focused within the Coniochaetales and consisted of 44 taxa, which included several *Coniochaeta* strains available in GenBank along with their *Lecythophora* anamorphs. The original alignment comprised 1435 characters, but after the removal of portions of the 5′ and 3′ ends and ambiguous regions, the final alignment consisted of 524 characters. PHYML analysis produced a single most likely tree ($\ln(L) = -2130.45$) (Fig. 1). The Coniochaetales occurs as a well-supported monophyletic group with strong PP (≥95%) and BS (100%) support. *Coniochaeta gigantospora* occurs in an unsupported clade with *C. malacotricha* (Auersw. ex Niessl) Traverso and *C. ostrea* (Malloch & Cain) Dania García, Stchigel & Guarro (Fig. 1).

ITS dataset

The ITS dataset consisted of 53 taxa including species of *Coniochaeta* and *Lecythophora*. The original ITS alignment consisted of 1272 characters, which were reduced to 522 characters in the final alignment after the removal of ambiguous regions and missing data. PHYML analysis generated a single most likely tree ($\ln(L) = -3344.45$) (Fig. 2). The Coniochaetales is again well supported and occurs as a monophyletic group with strong PP (≥95%) and BS (100%) support. *Coniochaeta gigantospora* occurred near the base of the Coniochaetales on an independent branch basal to a large unsupported clade containing *Coniochaeta* and *Lecythophora* species (Fig. 2).

Alignments of all three datasets are deposited in TreeBase (S12255).

Taxonomy

Coniochaeta gigantospora J. Fourn., Raja & A.N. Mill. sp. nov. Figs. 3–14

MB 563245

Ascomata 500–550 × 380–420 µm (dry specimen 330–460 × 290–380 µm; mean = 420 × 340 µm; $n = 20$), scattered, solitary or in small groups, superficial with the base slightly immersed in the substrate, subglobose to ovoid, black, leathery, with short setae at sides and apex; setae 10–35 × 2–4 µm, dark brown, thick-walled, one-septate, straight to contorted, with blunt to acute ends. Ascomatal apex black, rounded, papillate, bearing setae up to 35 µm high. Peridium pseudoparenchymatous, 30–40 µm thick at sides and base, up to 50 µm thick at apex, distinctly two-layered; outer layer of *textura angularis*, 25–35 µm thick, composed of small, thick-walled, brown cells; inner layer of *textura prismatica*, 5–15 µm thick, composed of large, flattened, less pigmented cells. Asci 170–190 × 15–19 µm (dry specimen 150–160 × 14–16 µm; mean = 155 × 15 µm; $n = 20$), cylindrical, unitunicate, stipitate (15–25 µm long), with a refractive, inamyloid apical ring 1.5 µm high × 5 µm broad, with eight obliquely uniseriate ascospores. Paraphyses copious, filiform, slightly moniliform at base, 4–8.5 µm broad,

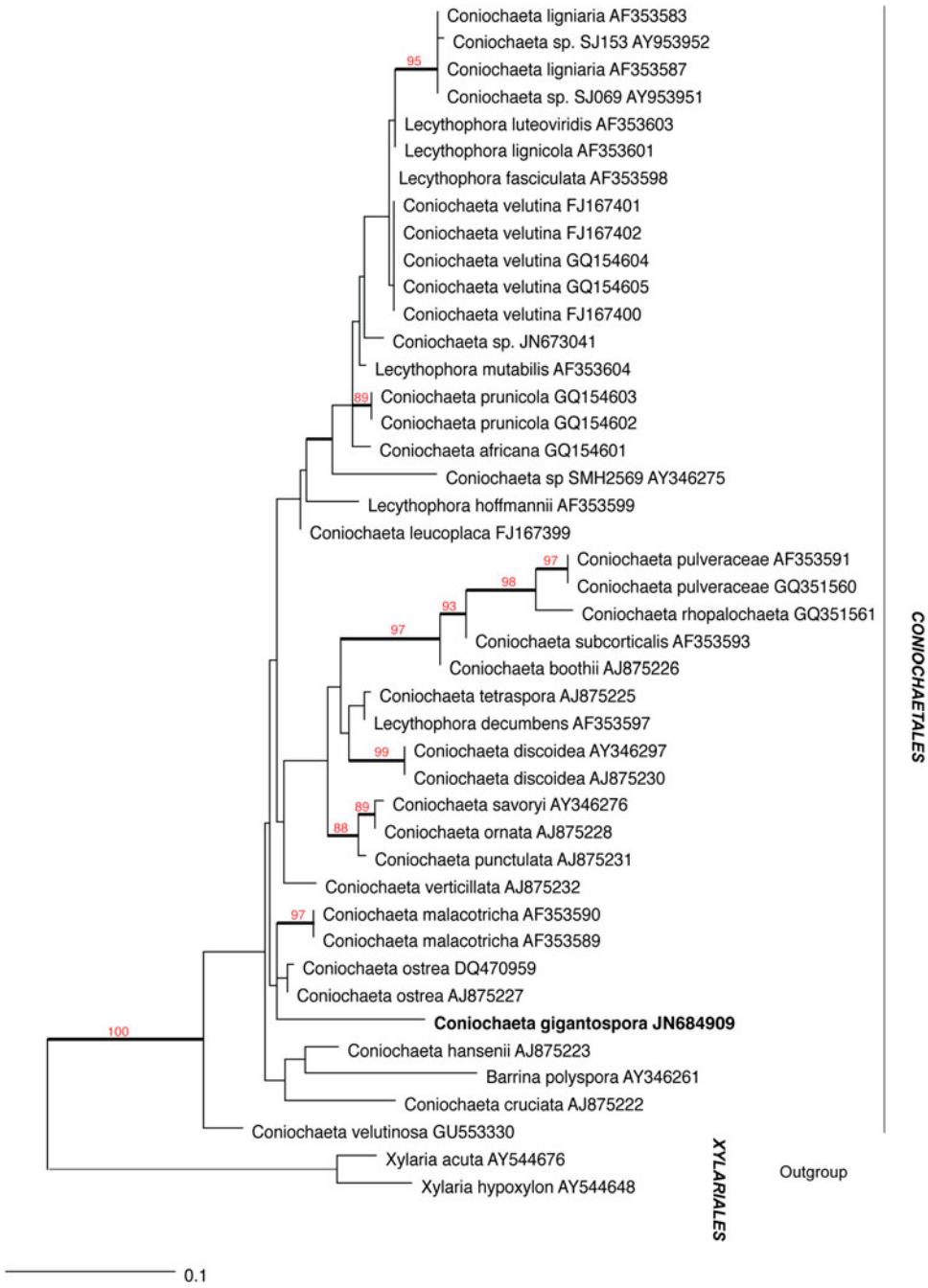


Fig. 1 Phylogram of the most likely tree (ln$L = -2130.45$) using PHYML based on 44 large subunit (LSU) nrDNA sequences of taxa in the Coniochaetales. Maximum likelihood bootstrap support values ≥70% are shown above the branches. Thickened branches indicate Bayesian posterior probabilities ≥95%. The new species is in *bold*. Members of the Xylariales were used as outgroup taxa (nr = nuclear)

septate. Ascospores 23–30 × 11–13 µm (dry specimen 21–26 × 10–13 µm; mean = 27 × 11 µm; $n = 30$), ellipsoid, nearly equilateral with broadly rounded ends, olivaceous to olivaceous-brown, smooth, one-celled, densely multiguttulate, with a short unilateral germ slit 8.5–12 µm long, median to eccentric; germination through the germ slit was observed on released ascospores.

Comments: Microscopic examination of fresh material indicated that the ascomycete fungus from France fits well within the genus *Coniochaeta,* but the combination of large and evenly setose, thick-walled ascomata, as well as unusually large, olivaceous brown ascospores with a short, longitudinal germ slit suggests this fungus is unique, and it is therefore described as a new species of *Coniochaeta.*

Etymology: From the Greek "gigas" = giant

Specimen examined: France, Vendée: L'Orbrie, Sauvaget, the river Vendée, 15 m a.s.l., 13 May 2009, on submerged wood of *Fraxinus excelsior*, associated with a *Cosmospora* sp., leg & det Jacques Fournier, *J. Fournier 09098* (**HOLOTYPE designated here, ILLS 60816**).

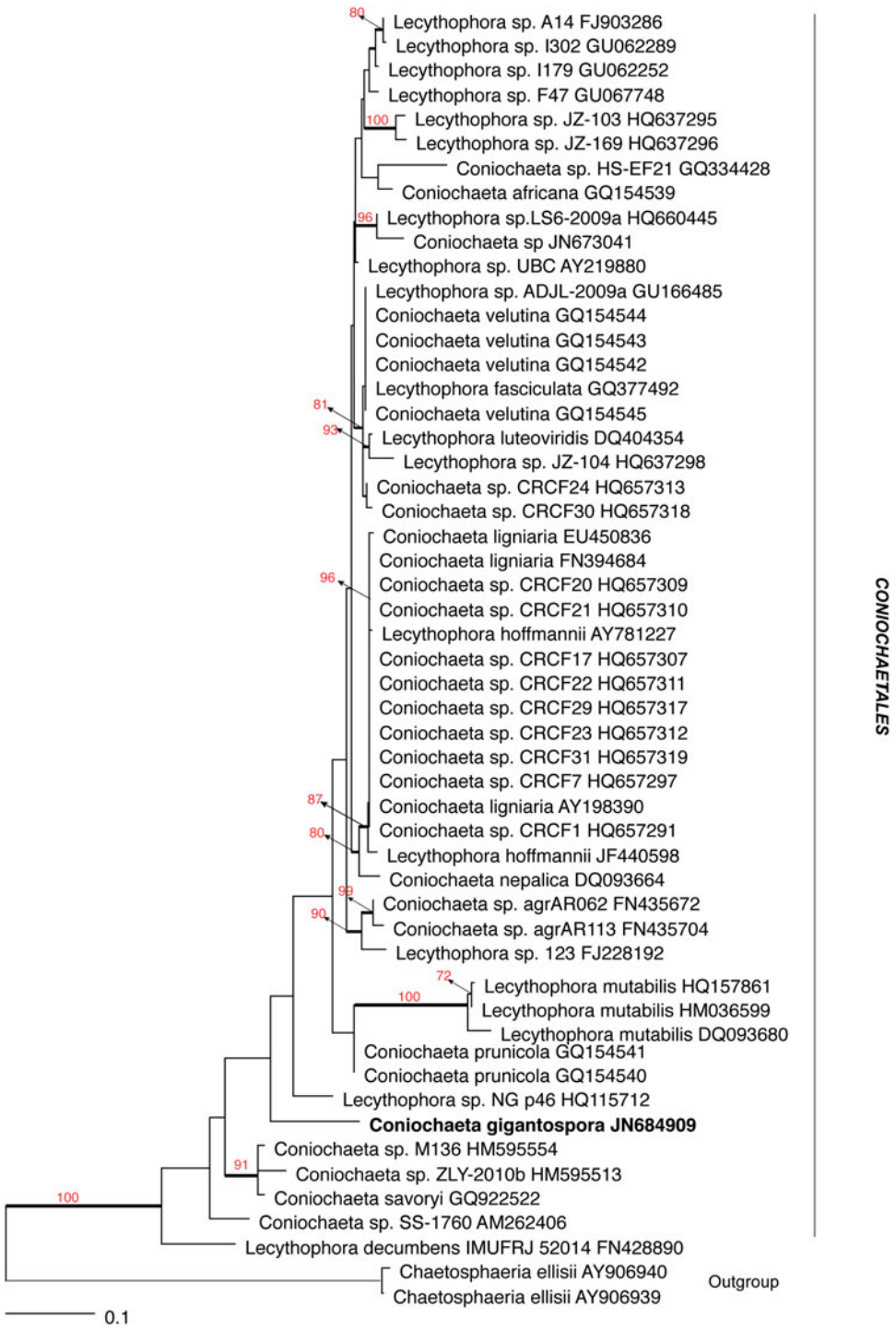
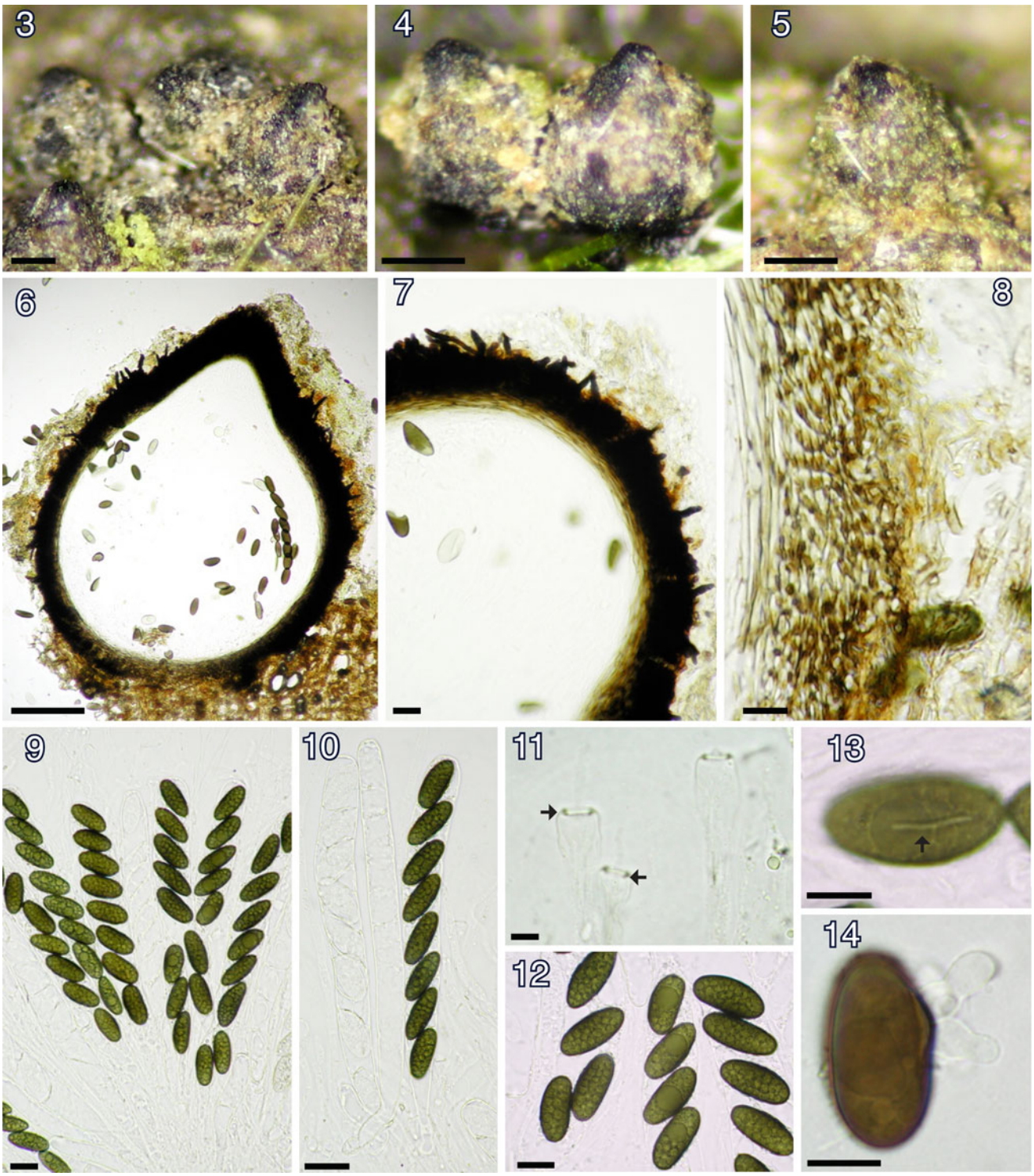


Fig. 2 Phylogram of the most likely tree ($\ln L = -3344.45$) using PHYML based on 53 internal transcribed spacer (ITS) nrDNA sequences of taxa in the Coniochaetales. Support values as in Fig. 1. The new species is in *bold*. Members of the Chaetosphaeriales were used as outgroup taxa

Discussion

The results of our study provide strong support for the placement of *C. gigantospora* in the Coniochaetales; however, its relationship to other species in the genus remains ambiguous because it occurred on an independent branch in all analyses (Supplementary Fig. 1; Figs. 1, 2). When compared morphologically with the terrestrial species occurring on wood, dung, soil, etc. reported in the literature (Hawksworth and Yip 1981; Mahoney and LaFavre 1981; Checa et al. 1988; Romero et al. 1999; Asgari et al. 2007), *C. gigantospora* is distinguished in having larger ascomata and ascospores than any other species of *Coniochaeta*. It also has wider ascospores than those of other *Coniochaeta* species reported in the literature. Among the previously described species of



Figs. 3–14 *Coniochaeta gigantospora* from the holotype (ILLS 60816). **3–5** Erumpent ascomata on wood with dense algal coating. **6** Longitudinal section through the ascoma. **7–8** Longitudinal section through the ascomal wall, showing setae. **9–10** Asci. **11** Ascus apical apparatus (*arrows*). **12** Multiguttulate, brown ascospores. **13** Ascospore showing a longitudinal germ slit, indicated with an *arrow*. **14** Ascospore showing germ tubes. *Bars* **3–5** 250 μm, **6** 100 μm, **7–12** 20 μm, **13–14** 10 μm

Coniochaeta, *C. ovata* Matsush. (Matsushima 1971) and *C. caryotae* R. Rao (Rao 1970) have large ascospores (17–23 × 6–7.5 μm in *C. ovata*, 20–26 × 7–10 μm in *C. caryotae*), but they are not as long and wide as those found in *C. gigantospora* (23–30 × 10–13 μm). *Coniochaeta gigantospora* also differs from the above taxa in

having larger ascomata and asci. In addition, *C. gigantospora* occurred on submerged wood of *Fraxinus excelsior* L. in fresh water, whereas *C. ovata* was isolated from soil (Matsushima 1971) and *C. caryotae* was found on terrestrial dead stems and rachides of leaves of *Cayota urnes* L. (Rao 1970). *Coniochaeta magniquadrispora* Matsush. also has large ascospores (17–20 × 9–11 μm), but differs from *C. gigantospora* in having four, smaller ascospores per ascus (Matsushima 1996).

Coniochaeta gigantospora occurred as a sister group to *C. malcotricha* and *C. ostrea* in the limited LSU analyses (Fig. 2); however, this relationship was not supported based on ML bootstrap analyses and Bayesian PP values. Based on morphological characters, *C. gigantospora* can be distinguished from both *C. malacotricha* and *C. ostrea* in having larger ascospores. In addition, *C. gigantospora* ascospores are ellipsoid to nearly equilateral with broadly rounded ends, whereas C. *malacotricha* has millstone-shaped ascospores that are broadly elliptical in face view and narrowly elliptical in side view (Munk 1957; Kobayashi and Katzu 1970; Rogers and Grand 1971; Asgari et al. 2007). Ascospores of *C. ostrea* differ from those of *C. gigantospora* in being bivalved in shape (Malloch and Cain 1971).

The ascomata of *C. gigantospora* were covered with an algal coating (Figs. 3, 4). The presence of algal coatings might be attributed to the fact that *C. gigantospora* was collected during the summer (May 2009) during a time when algal photosynthesis might peak due to the presence of nutrients and an increase of day length (DeWreede 2001). To the best of our knowledge, algal coatings have not been observed or reported from previously described species of *Coniochaeta,* including the species that have been described or reported from freshwater habitats.

Thus far, seven species of *Coniochaeta* have been reported from freshwater habitats (Shearer 1993; Shearer and Raja 2011), including *C. renispora* Crane & Shearer (Shearer and Crane 1995). The aquatic taxon, *C. renispora*, however, differs from *C. gigantospora* in having much smaller, kidney-shaped ascospores (11–13.3 × 6.7–8.9 μm). *Coniochaeta gigantospora* differs from the other six species reported from fresh water viz: *C. kellermanni* (Ellis & Everh.) Munk, *C. leucoplaca* (Berk. & Ravenel) Cain, *C. ligniaria* (Grev.) Massee, *Coniochaeta* sp. 4022, *C. tetraspora* Cain, and *C. velutina* (Fuckel) Cooke in having larger ascospores. Molecular data (this paper) also indicate that *C. gigantospora* is distinct from the aforementioned taxa (Supplementary Fig. 1; Figs. 1, 2).

Acknowledgments Financial support from the National Science Foundation (NSF Grant No. 0844722) is greatly appreciated. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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