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Phylogenetic placement of the ectomycorrhizal genus *Cenococcum* in Gloniaceae (Dothideomycetes)

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Abstract: *Cenococcum* is a genus of ectomycorrhizal Ascomycota that has a broad host range and geographic distribution. It is not known to produce either meiotic or mitotic spores and is known to exist only in the form of hyphae, sclerotia and host-colonized ectomycorrhizal root tips. Due to its lack of sexual and asexual spores and reproductive structures, it has proven difficult to incorporate into traditional classification within Ascomycota. Molecular phylogenetic studies of ribosomal RNA placed *Cenococcum* in Dothideomycetes, but the definitive identification of closely related taxa remained elusive. Here we report a phylogenetic analysis of five nuclear loci (SSU, LSU, *TEF1*, *RPB1*, *RPB2*) of Dothideomycetes that placed *Cenococcum* as a close relative of the genus *Glonium* of Gloniaceae (Pleosporomycetidae incertae sedis) with strong statistical support. *Glonium* is a genus of saprobic Dothideomycetes that produces darkly pigmented, carbonaceous, hysteriate apothecia and is not known to be biotrophic. Evolution of ectomycorhizae, *Cenococcum* and Dothideomycetes is discussed.

Key words: *Cenococcum*, Dothideomycetes, ectomycorrhizae, fungi, Gloniaceae, phylogenetics, systematics

Introduction

Cenococcum is one of the most frequently encountered genera of ectomycorrhizal (EcM) fungi (LoBuglio 1999). It has been documented to associate with a broad diversity of host plants, including angiosperms and gymnosperms, in numerous habitats, environments and geographic regions (Trappe 1964, 1969; Tedersoo et al. 2010). Its EcM are black and carbonaceous with darkly pigmented, wiry hyphae emanating from root tips (Fig. 1). No definitive sexual or asexual spore-producing structures are known, although it does produce vegetative hyphae and abundant sclerotia (Fig. 1). Cleistothecia putatively associated with *C. geophilum* recently were described and considered to be the teleomorph but no molecular or culture data were collected and a definitive connection remains untested (Fernández-Toirán and Águeda 2007). Thus one of the most common and globally abundant genera of EcM fungi is also one of the most poorly characterized phylogenetically and biologically.

One of the long-standing ecological questions associated with *Cenococcum* was how could such an ecologically common, asexual EcM fungus be distributed globally. The dispersal of sclerotia (Massicotte et al. 1992) and spread of nursery stock (Trappe 1964) were hypothesized to be potential mechanisms. Studies focusing on *Cenococcum* population structure and fine-scale diversity however revealed considerable variation consistent with recombination (Douhan et al. 2007b, Jany et al. 2002, LoBuglio and Taylor 2002, Wu et al. 2005) and potentially multiple phylogenetic species (Douhan and Rizzo 2005; Douhan et al. 2007a, b). Thus it seems more plausible that population and phylogenetic variation as well as the widespread distribution of the genus were more likely due to unobserved sexual reproduction and phylogenetic divergence.

The lack of known sexual and asexual reproductive structures has impeded the incorporation of *Cenococcum* into the classification and evolutionary hypotheses of Ascomycota. The traditional classification of Ascomycota is based on characters associated with ascomata, asci and ascospores and correlating traits of mitotic reproduction. Data and analyses associated with molecular phylogenetics provide an independent test of these morphological systems and, it is important to note, allow for the incorporation of taxa such as *Cenococcum* (Hibbett et al. 2007).

Phylogenetic studies by LoBuglio et al. (1996) did not support a close phylogenetic relationship between *Cenococcum* and other genera of ectomycorrhizal Ascomycota (e.g. *Tuber* of Pezizales), and in particular

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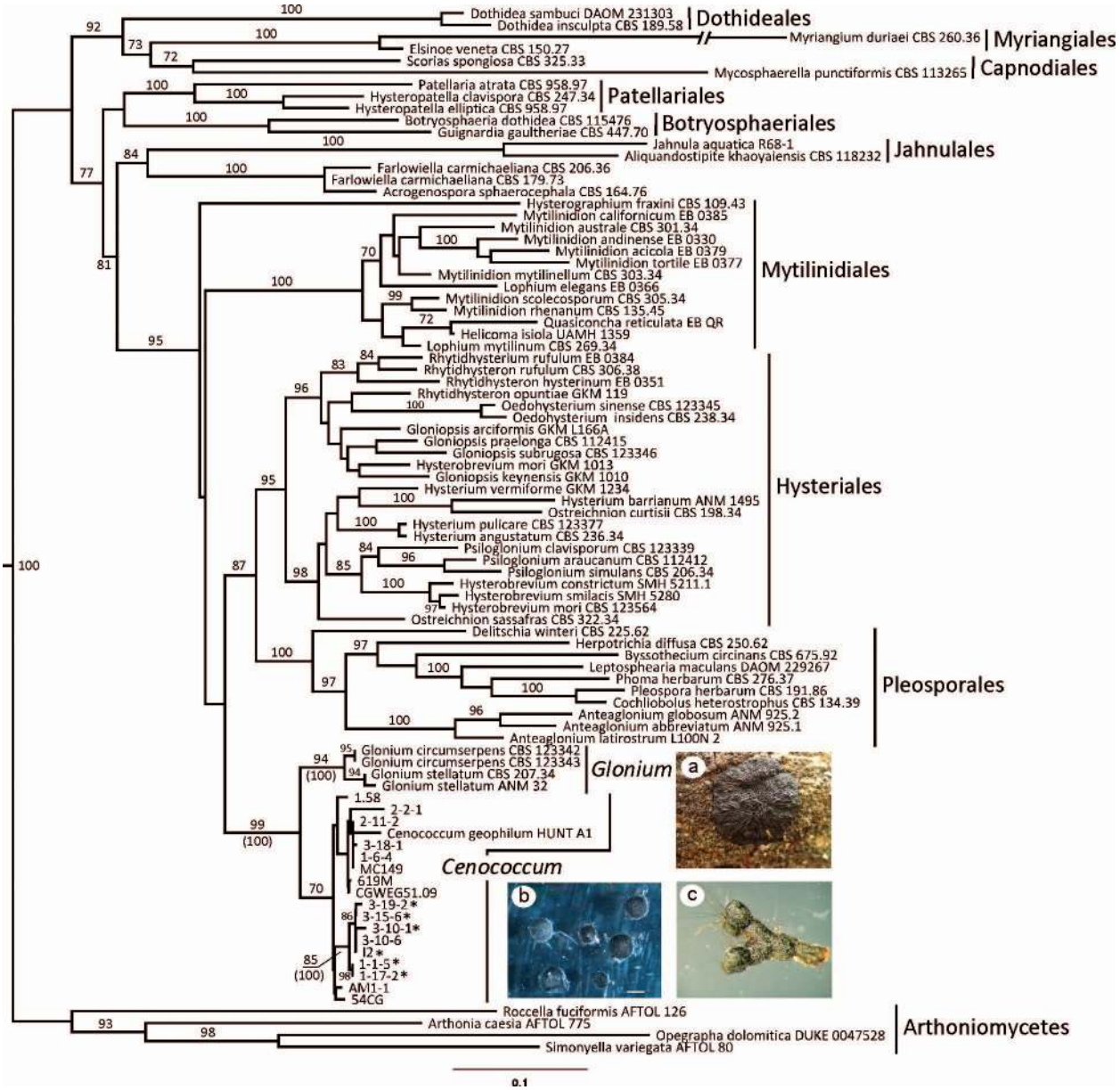

FIG. 1. RAxML tree of Dothideomycetes highlighting the placement of *Cenococcum*. The multigene alignment included 3764 nucleotide positions (911 SSU, 931 LSU, 727 TEF, 575 RPB1, 620 RPB2). Arthoniomycetes is designated as the outgroup. Bootstrap partitions greater than or equal to 70% are shown above corresponding branches for the complete supermatrix and in parentheses below corresponding branches for selected branches asterisk for missing *Cenococcum* data analyses. a. Image of *Glonium stellatum* ascostroma courtesy of Jason Karakehian. b. Image of *Cenococcum* sclerotia courtesy of Martina Peter. c. Image of ectomycorrhizae courtesy of Dan Luoma and Joyce Eberhart.

it did not support the hypothesis of a teleomorph link with the EcM genus *Elaphomyces* (Trappe 1971). Instead molecular analyses inferred a phylogenetic relationship between *Cenococcum* and Dothideomycetes (= Loculoascomycetes), which contained no known EcM taxa. Sampling at that time was limited to the small subunit rDNA (SSU) and relatively few taxa and its relationship to Dothideomycetes as well as the monophyly of the class were not well supported. Liu and Hall (2004) included one specimen of *Cenococcum* in a phylogenetic study using DNA sequences from the second largest subunit of the RNA polymerase II genes. Their phylogeny placed *Cenococcum* within Dothideomycetes but still outside any of the main orders. Tsui and Berbee (2006) in a study of helicosporous fungi demonstrated a phylogenetic relationship between *Cenococcum geophilum* and *Helicoma isiola*, an enigmatic species that did not

group with the majority of *Helicoma* species in the Tubeufiaceae (Pleosporales). But like similar studies the relationships of the Dothideomycetes clades remained unresolved and the taxa potentially closely related to *Cenococcum* remained undersampled.

Since the study of LoBuglio et al. (1996) multigene phylogenetics with broad taxon sampling has significantly advanced the field of fungal systematics and has helped to clarify many classifications within the fungi (Hibbett et al. 2007). Dothideomycetes is now well supported and recognized as one of the largest and most ecologically diverse classes of Ascomycota (Schoch et al. 2006, 2009a). Phylogenetic studies of the class have integrated morphologically (e.g. ascomata, asci) and ecologically (e.g. pathogens, saprobes, aquatic) diverse taxa into a common phylogeny and advanced our understanding of important evolutionary events within the class (Schoch et al. 2009b). As part of phylogenetic investigations of Dothideomycetes an expansion of sampling of fungi traditionally classified in Hysteriales occurred (Boehm et al. 2009a, b). These fungi produce darkly pigmented, carbonaceous, hysteriate apothecia (hysterothecia) and thick-walled fissitunicate asci with pigmented, septate ascospores. Boehm et al. (2009a, b) demonstrated that Hysteriales were not a monophyletic group, with species occurring in multiple clades throughout Dothideomycetes. By combining nearly all the available data suitable for a class phylogenetic analysis of the Dothideomycetes Schoch et al. (2009b) serendipitously discovered that *Cenococcum* was strongly supported as a close relative to *Glonium*, one of the genera of the hysteriate Dothideomycetes.

Glonium includes an estimated 87 species that are characterized by hysterothecia that are progressively dichotomously branched and form radiating flabelliform or pseudostellate composites (FIG. 1). The hamathecium comprises persistent narrow cellular pseudoparaphyses, the asci are clavate to cylindrical and fissitunicate and the ascospores are hyaline to lightly pigmented with a single conspicuous septum (Boehm et al. 2009b). Ecologically species of *Glonium* are saxicolous, terricolous or lignicolous but none are known to be biotrophic. Here we expand upon that sampling with additional isolates of *Cenococcum* that represents the known diversity of the genus (Douhan et al. 2007a) and demonstrate that *Cenococcum* is a close relative of *Glonium*. The effect of these findings on the evolution of nutritional modes within Dothideomycetes and the evolutionary origin of one of the most common forms of ectomycorrhizae are discussed.

MATERIALS AND METHODS

Data.—A total of 18 isolates of *Cenococcum* were included in these analyses. Data for one isolate, *Cenococcum geophilum* HUNT A1 (LoBuglio et al. 1996), was obtained from GenBank (L76616). For the remaining 17 isolates (TABLE I) regions of five nuclear loci, including the small (SSU) and large (LSU) subunits of the ribosomal RNA repeat unit, translation elongation factor 1-α (*TEF1*) and the first (*RPB1*) and second (*RPB2*) largest subunits of RNA polymerase II were amplified with the primers described in Schoch et al. (2009b). PCR amplification of all genes was performed with PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with 2 μL of each primer, 20 μL water and 1 uL DNA per reaction. Reactions were conducted on a Bio-Rad MyCylcer (Bio-Rad, Hercules, California) with this protocol: 94 C for 3 min; 10 cycles of 94 C for 30 s, 55 C for 1 min and 72 C for 2 min; 35 cycles of 94 C for 3 min, 50 C for 1 min and 72 C for 2 min; 72 C for 3 min. A total of 66 sequences were determined as part of this study; sequencing reactions were performed at University of Washington High-Throughput Sequencing Solutions (Seattle, Washington). Based on the findings of Boehm et al. (2009a), *Cenococcum* sequences were combined with data from 66 isolates representing nine orders and 18 families of Dothideomycetes with an emphasis on Pleosporomycetidae, Hysteriales and Mytilinidiales. Four Arthoniomycetes species were included as outgroup taxa. No phylogenetic conflicts were detected among these sequences as reported in Schoch et al. (2009b). New sequences were edited and assembled in CodonCode Aligner 2.0.6 (Dedham, Massachusetts) appended to existing single-gene alignments in MUSCLE (Edgar 2004) and refined by direct examination. Single-gene alignments were concatenated into a five-gene superalignment with a customized Perl script and exported in Phylip format for phylogenetic analyses. A summary of sampling and GenBank accession numbers for all isolates sequenced is provided (TABLE I).

Phylogenetic analyses.—All sequences were analyzed as DNA and ambiguously aligned regions of individual gene alignments were identified and masked with GBlocks 0.91b with these settings: maximum number of contiguous nonconserved positions allowed = 4; minimum length of a block allowed = 10 (Castresana 2000). Maximum likelihood analyses were performed with RAxML 7.2.6 (Stamatakis 2006) with the superalignment divided into 11 partitions, including SSU, LSU and the three codon positions for each of three the protein-coding genes and a unique GTRGAMMA model of evolution assigned to each partition. Branch support was assessed through 1000 bootstrap partitions (BP) with the rapid bootstrap option. Two sets of analyses were performed to assess the effect of missing data on the placement of *Cenococcum*, one on the complete supermatrix that included 18 *Cenococcum* isolates and one on the supermatrix with the six *Cenococcum* isolates with no missing data (TABLE I).

RESULTS

Gblocks excluded 3022 ambiguously aligned positions from the initial alignments, creating a final dataset that consisted of 88 OTUs and 3764 nucleotide

TABLE I. Origin and GenBank accession information of *Cenococcum* isolates sequenced for this study. All other data are from Boehm et al. (2009a), LoBuglio et al. (1996) and Tsui and Berbee (2006)

Isolate	Location and potential host	Reference or source	GenBank accession numbers				
			SSU	LSU	TEF	RPB1	RPB2
1-1-5	Browns Valley, California, USA; *Quercus douglassi*	Douhan and Rizzo 2005	JN860120	JN860134	JN860113	JN860099	JN860087
1-6-4	‘‘	‘‘	JN860122	JN860136	_	JN860101	JN860089
1-17-2	‘‘	‘‘	JN860121	JN860135	JN860114	JN860100	JN860088
2-2-1	‘‘	‘‘	JN860124	JN860138	JN860115	JN860103	_
2-11-2	‘‘	‘‘	JN860123	JN860137	_	JN860102	JN860090
3-10-1	‘‘	‘‘	JN860125	JN860139	JN860116	JN860104	JN860091
3-10-6	‘‘	‘‘	_	JN860140	_	_	_
3-15-6	‘‘	‘‘	JN860126	JN860141	JN860117	JN860105	JN860092
3-18-1	‘‘	‘‘	JN860127	JN860142	_	_	_
3-19-2	‘‘	‘‘	JN860128	JN860143	JN860118	JN860106	JN860093
54CG	Holland; unknown	Francis Martin	JN860129	JN860144	_	JN860107	JN860094
619M	Portugal; *Quercus ilex*	Susana Goncalves	_	JN860145	_	JN860108	JN860095
AM1-1	France; *Fagus sylvania*	Francis Martin	_	JN860146	_	JN860109	JN860096
I-2	Oregon, USA; *Qeurcus garyana*	Douhan and Rizzo 2005	JN860130	JN860147	JN860119	JN860110	JN860097
MC149	Washington, USA; *Pseudotsuga menziesii*	Kathy LoBuglio	JN860133	_	_	_	_
CGWEG 51.09	Switzerland; unknown	LoBublio et al. 1991	JN860131	JN860148	_	JN860111	_
1.58	Switzerland; *Pinus sylvestris*	Martina Peter	JN860132	JN860149	_	JN860112	JN860098

positions (911 SSU, 931 LSU, 727 TEF, 575 RPB1 and 620 RPB2; TreeBASE S12009). PCR and sequencing were not successful for all loci and the final alignment included 14 SSU, 16 LSU, 7 TEF, 14 RPB1 and 12 RPB2 sequences for the 18 *Cenococcum* isolates included in these analyses (TABLE I). In addition we sequenced the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA repeat unit for 13 isolates for their potential use in studies involving environmental sequencing and barcoding efforts (GenBank JN943882–JN943894). The best scoring RAxML tree from this alignment is illustrated (FIG. 1) with corresponding BP branch support and midpoint rooting between Arthoniomycetes and Dothideomycetes. The topology of this tree is consistent with that from the work of Boehm et al. (2009a, b) and Schoch et al. (2009b) with respect to the resolution of Dothideomycetidae, Pleosporomycetidae and branches supported by BP of greater than 70%. Notably the monophyly of Mytilinidiales (Gloniaceae and Mytilinidiaceae), which was weakly supported in Schoch et al. (2009b) and Boehm et al. (2009a), was not resolved.

Cenococcum was resolved as a monophyletic taxon with a 70% BP and as sister to *Glonium* with a BP of 99–100% (FIG. 1). As in studies of *gpd* and ITS rDNA (Douhan and Rizzo 2005, Douhan et al. 2007a), phylogenetic variation was detected within the *Cenococcum* clade. Four groups of *Cenococcum* isolates were resolved with weak to moderate BP branch support but with branch lengths that were comparable to interspecies diversity detected in other closely related genera (e.g. *Glonium*, *Hysterium* etc.; FIG. 1). Group 1: *C. geophilum* HUNT A1, CGWEG51.09, 619M, 2-2-1, 2-11-2, 3-18-1, 1-6-4, and MC149; Group 2: 1.58; Group 3: AM1-1 and 54CG; and Group 4: 3-10-1, 3-10-6, I2, 3-15-6, 3-19-2 and 1-17-2. Phylogenetic analyses of *Cenococcum* isolates with no missing data did not result in any topological differences and had only moderate effect on BP (FIG. 1).

DISCUSSION

Dothideomycetes phylogenetics.—The placement of *Cenococcum* as a close relative of the genus *Glonium* was demonstrated in this study (FIG. 1). The original placement of *Cenococcum* within or close to Dothideomycetes (LoBuglio et al. 1996) was surprising in that the class primarily contains plant pathogens and plant saprobes, but no known examples of EcM taxa and its membership within the class previously remained a point of debate (Tedersoo et al. 2010). A study by Liu and Hall (2004) using DNA sequence comparisons from *RPB2* provided additional evidence for placement of *Cenococcum* in Dothideomycetes and good support for its close relationship with Pleosporales. Taxon sampling within Dothideomycetes remained relatively sparse however, and this latter study also found mixed support for the monophyly of ''loculoascomycetes'' sensu Barr (1987), a relationship that was not recovered in more recent multigene studies (e.g. Schoch et al. 2009a). Molecular phylogenetic evidence for the monophyly of Dothideomycetes remained elusive until the widespread development of multigene phylogenies (Schoch et al. 2006, 2009a).

Schoch et al. (2009b) produced the most comprehensive phylogenetic study of Dothideomycetes to date. The result was a heightened understanding of subclass phylogenetic relationships, including tests of monophyly of numerous orders and families, and improved the development of evolutionary hypotheses concerning the evolution of novel ecologies (e.g. marine, Suetrong et al. 2009), unique environmental niches (e.g. rock-inhabiting, Ruibal et al. 2009) and symbioses (e.g. lichens, Nelson et al. 2009). Dothideomycetes is now understood to be one of the largest classes of fungi and one of the most ecologically diverse. It contains two subclasses, Pleosporomycetidae and Dothideomycetidae, that are consistent with the presence and absence of pseudoparaphyses and associated hamathecial tissues, respectively (Schoch et al. 2006), but several families remain to be incorporated into the current phylogenetic classification. Dothideomycetes ancestral ecological (or nutritional mode) character state is hypothesized to be a plant saprobe with multiple independent derivations of obligate biotrophs (e.g. plant pathogens, lichens; Schoch et al. 2009b).

Origin of Cenococcum *EcM among Dothideomycetes.*—As the only known mycorrhizal example of Dothideomycetes the placement of *Cenococcum* within the class clarifies one of the independent origins of EcM within Ascomycota. The other two origins of EcM within Ascomycora are *Elaphomyces* of Eurotiales (Geiser et al. 2006) and numerous lineages of Pezizales (Hansen and Pfister 2006). *Cenococcum* is a close relative of the genus *Glonium*, which is not known to be mycorrhizal. *Glonium* is a saprobic genus that produces darkly pigmented, carbonaceous ascomata on bark, wood or soil. It is a poorly studied genus primarily because it not an economically important plant pathogen and because its ascomata are relatively inconspicuous. *Glonium* is the sole genus of Gloniaceae and is classified as ordo Pleosporomycetidae incertae sedis, although recent phylogenies suggested a close relationship with Mytilinidiales (Boehm et al. 2009b). However subsequent molecular phylogenetic analyses did not find increased support for shared monophyly with Mytilinidiales, although a close relationship cannot be ruled out (FIG. 1; Boehm et al. 2009a).

The relationship between *Cenococcum* and *Glonium* is well supported by these analyses (BP 98, FIG. 1) and consistent with the recognition of *Cenococcum* as a member of Gloniaceae. As such *Cenococcum* represents an independent origin of ectomycorrhizae within a saprobic lineage and provides another line of evidence supporting the hypothesis that EcM fungi are derived from saprobic lineages (Matheny et al. 2007, Binder and Hibbett 2006). It also is reminiscent of the placement of *Elaphomyces* among Eurotiomycetes (LoBuglio et al. 1996, Geiser et al. 2006) and like *Elaphomyces*, *Cenococcum* is a common and ecologically abundant taxon that is a member of a class where the origin and diversification of EcM taxa has been limited compared to other EcM rich clades (e.g. Pezizales in Ascomycota, Agaricomycetes in Basidiomycota).

Search for the teleomorph.—*Cenococcum* is one of the most frequently encountered taxa in morphological and molecular studies of EcM fungi (LoBuglio 1999, Tedersoo et al. 2010). Its ecological distribution and common occurrence has long been at odds with its presumably asexual mode of reproduction (LoBuglio et al. 1999). The amount of phylogenetic diversity detected within *Cenococcum* however supports the hypothesis that it is sexually reproducing in nature (FIG. 1; Douhan and Rizzo 2005; Douhan et al. 2007a, b; Jany et al. 2002). Furthermore the close phylogenetic relationship between *Cenococcum* and *Glonium* provides some clues as to where the sexually reproducing stage might be found and what its morphology might be. Based on these results we predict that the teleomorph of *Cenococcum* occurs on soil or downed wood in a manner similar to other EcM fungi (e.g. *Tomentella*, Lilleskov and Bruns 2005) and that the fruiting bodies are darkly pigmented, hysteriate ascomata. Whether the sexual stage of *Cenococcum* is equally distributed as is its mycorrhizae and sclerotia is debatable because many fungi exhibit an epidemic population structure with sexual reproduction geographically isolated and rarely observed (e.g. *Aspergillus fumigatus*, O'Gorman et al. 2009).

Conclusion.—We provide strong multigene phylogenetic evidence that *Cenococcum* is a member of Gloniaceae (ordo incertae sedis, Pleosporomycetidae, Dothideomycetes). It represents an independent origin of EcM within a family of saprobic Ascomycota, providing an additional evolutionary sampling point of transitions from saprobic to biotrophic nutritional modes. We propose that its close phylogenetic affinity with *Glonium* provides predictive value that could lead to the discovery of its sexually reproductive state and is at odds with the hypothesis that *Cenococcum* produces cleistothecia as reported by Fernández-Toirán and Águeda (2007). The genome sequence of *Cenococcum*, which is produced from isolate 1.58 (TABLE I) also undoubtedly will prove to be an important resource for understanding the reproductive biology of the genus and will make it one of the prime candidates for population genomic studies on a global scale.

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