

# Phylogenetic Origins of the Asexual Mycorrhizal Symbiont *Cenococcum geophilum* Fr. and Other Mycorrhizal Fungi among the Ascomycetes

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The phylogenetic relationship of the asexual mycorrhizal fungus *Cenococcum geophilum* Fr. among sexual ascomycetes was examined by phylogenetic analysis of nucleotide sequence data from the nuclear small subunit (18S) ribosomal RNA genic region. A specific focus of this study was to test the hypothesis that the genus *Elaphomyces* is the closest sexual relative of *C. geophilum*. Thus nucleotide sequence data of five *C. geophilum* isolates, three *Elaphomyces* species, and 44 additional genera of ascomycetes were included in the phylogenetic analyses. The percentage of similarity among the 18S rDNA sequences of the *C. geophilum* isolates examined was 99.8 to 100%, indicating that *C. geophilum* is monophyletic. Percent similarity of nucleotide sequence among the three *Elaphomyces* species was also high and ranged from 99.4 to 99.5%. DNA parsimony and distance analysis of the sequence data separated these 2 genera on distant clades when sequence from 44 additional genera of ascomycetes was included. Parsimony and distance analyses positioned *C. geophilum* as a basal, intermediate lineage between the two Loculoascomycete orders, the Pleosporales and the Dothidiales, and strongly supported *Elaphomyces* to be of Plectomycete origin. Among the sexual Ascomycetes examined, which included representative taxa from four classes of filamentous Ascomycetes (Plectomycetes, Pyrenomycetes, Discomycetes, and Loculoascomycetes), no close sexual relative to *C. geophilum* was identified. At least four independent lineages of mycorrhizal fungi were identified among the ascomycetes examined. © 1996 Academic Press, Inc.

## INTRODUCTION

The mycorrhizal fungus *Cenococcum geophilum* Fr. is cosmopolitan and is the most widespread ectomycorrhizal fungus. It has an extremely broad host and habitat range, a pioneering capability, and can be the dominant mycorrhizal fungus in forests of arctic, temperate,

or subtropical environments (Read and Haselwandter, 1981; Vogt *et al.*, 1981, Molina and Trappe, 1982, 1984; Trappe, 1962, 1962a, 1964). *C. geophilum* exists as a sterile mycelium and thus lacks the sexual or asexual spores that are important characters in the classification of fungi. Positive identification of *C. geophilum* relies primarily on mycelium morphology and mycorrhizal characteristics (Chilvers, 1968; Trappe, 1962a; Miller *et al.*, 1983). Its reduced morphological characters and lack of a mating system have obscured its phylogenetic position, making it a monotypic form-genus in the Agonomycetales (mycelia sterilia).

The worldwide distribution of *C. geophilum* (Mikola, 1948; Trappe, 1964) seems at odds with its lack of spores, but the spread of isolates on nursery stock (Trappe, 1964) and dispersal via sclerotia may compensate for this absence (Trappe, 1969; LoBuglio *et al.*, 1991, Massicotte *et al.*, 1992). The puzzle of *C. geophilum*'s widespread persistence in the absence of mitotic spore production might be solved should a sexual state for *C. geophilum* be identified. However, the possibility exists that this fungus is truly asexual, resembling the endomycorrhizal Glomales which appear to have thrived since the Devonian with only relatively large mitotic propagules and without sexual reproduction (Simon *et al.*, 1993).

Since the mid 1800s, the genus *Elaphomyces* (a hypogeous ascomycete most recently classified in the Elaphomycetales by Trappe, 1979) has been considered the sexual state (teleomorph) of *C. geophilum* (hence *C. geophilum* would be the asexual state or anamorph of *Elaphomyces*) based on morphological and ecological traits (early work summarized by Ferdinandsen and Winge, 1925). *Elaphomyces* and *C. geophilum* are both mycorrhizal, and *Cenococcum* mycorrhizae are often found embedded in peridia of *Elaphomyces* ascomata (Trappe, 1971; Miller and Miller, 1984; Lihnell, 1942). In addition, the surface hyphae of the mycorrhizal mantle and sclerotia of *C. geophilum*, and the dark (dematiaceous) hyphae on the outer peridial layer of *Elaphomyces* species with blackish peridia, both appear in

tangential section as a “. . . mosaic of stellate hyphal clusters . . .” (Trappe, 1971). A definitive anamorph–teleomorph connection between these two morphs, however, is precluded by the absence of a sexual state in cultures of *C. geophilum* and the possibility of morphological homoplasy. For example, *Elaphomyces* ssp. are not the only ascomycetes with “peridium walls composed of plates of radiating cells originating from a number of meristematic regions,” known as cephalothecoid peridia (Hawksworth and Booth, 1974); other genera have cephalothecoid peridia and could be closely related to *C. geophilum*.

Phylogenetic analysis of nucleotide sequence data has been useful in the integration of asexual (mitotic) fungal species with sexual (meiotic) species of fungi (Guadet *et al.*, 1989; Berbee and Taylor, 1992; LoBuglio *et al.*, 1993, 1994; O'Donnell, 1993; Rehner and Samuels, 1994; Berbee, 1996; Geiser *et al.*, 1996; Taylor, 1995). To further assess the hypothesis that *Elaphomyces* is the teleomorph of *C. geophilum*, or that *C. geophilum* has lost its sexual cycle but is of *Elaphomyces* origin (Trappe, 1971), nucleotide sequence data was obtained from the 18S rRNA genic region of five *C. geophilum* isolates and three *Elaphomyces* species and analyzed with sequences from other ascomycetes in the classes Plectomycetes, Pyrenomycetes, Discomycetes, and Loculoascomycetes. Representatives of several loculoascomycetes, including *Zopfia rhizophila* Rabenh., *Neotestudina rosatii* Segretain & Destombes, and *Phaeotrichum* sp., all of which have cephalothecoid peridia (Hawksworth, 1986), have also been included in this study to elucidate whether possession of a cephalothecoid hyphal arrangement is of phylogenetic significance among these taxa.

## MATERIALS AND METHODS

### Source of Fungal Isolates

Herbarium specimens of *Elaphomyces* species were received from M. A. Castellano of the Forest Research laboratory, Oregon State University, Corvallis, OR, and are listed in Table 1. Ascomata of a yet undescribed *Elaphomyces* species, *Elaphomyces* 100A, with a peridium consisting of black encrusting hyphae (identified as *Elaphomyces* by S. O. Miller, M. A. Castellano, and J. M. Trappe) were collected by K. F. LoBuglio at the State University of New York, College of Environmental Science and Forestry, Huntington Wildlife Refuge, Newcomb, New York (Table 1). The source of the five *C. geophilum* isolates has been cited (LoBuglio *et al.*, 1991).

### DNA Extraction and PCR Amplification of Ribosomal RNA Regions

DNA was isolated from *C. geophilum* isolates as previously described (LoBuglio *et al.*, 1991). Isolation of

TABLE 1

### List of Species for Which 18S rDNA Nucleotide Sequence Was Obtained and Their GenBank Accession Numbers

Taxon	Isolate number and source	GenBank accession numbers
<i>Cenococcum geophilum</i>	349/M. D. Coleman	L76614
<i>Cenococcum geophilum</i>	149/M. A. Castellano	L76615
<i>Cenococcum geophilum</i>	HUNT-A1/K. F. LoBuglio	L76616
<i>Cenococcum geophilum</i>	CGMONT/F. LeTacon	L76617
<i>Cenococcum geophilum</i>	010/R. K. Antibus	L76618
<i>Elaphomyces</i> sp.	100A/K. F. LoBuglio	L76619
<i>Elaphomyces leveillei</i>	646/M. A. Castellano	L76620
<i>Elaphomyces virgatosporus</i>	6205/M. A. Castellano	L76621
<i>Zopfia rhizophila</i>	207.26/CBS <sup>a</sup>	L76622
<i>Neotestudina rosatii</i>	427.62/CBS	L76623
<i>Phaeotrichum benjaminii</i>	24076/ATCC <sup>b</sup>	L76624
<i>Phialophora finlandia</i>	FAG15/S. K. Harney & C. J. K. Wang	L76625
<i>Phialocephala fortinii</i>	FAP7/S. K. Harney & C. J. K. Wang	L76626

<sup>a</sup> CBS, Centraalbureau voor Schimmelcultures.

<sup>b</sup> ATCC, American Type Culture Collection.

DNA from the *Elaphomyces* species was according to the procedure of Rogers and Bendich (1985) and the spore crushing technique of Swann *et al.* (1991). The nuclear small subunit rDNA region (18S gene) was amplified in two portions, each approximately 1000 bp, using the primer pairs NS1 and NS4 (which amplified the 5' end of the 18S rRNA gene) and NS3 and NS8 (which amplified the 3' end of the 18S rRNA gene) (White *et al.*, 1990). The DNA was amplified by PCR as in LoBuglio *et al.* (1993) using an automated temperature cycling device (Perkin–Elmer Cetus) and the following parameters: 2 min initial denaturation at 95°C, followed by 30 cycles of 1 min primer annealing at 50° to 55°C, 45 s extension at 72°C, 1 min denaturation at 95°C, and a final extension period of 10 min at 72°C.

### DNA Sequencing

The 18S rDNA region was sequenced from double-stranded template obtained by PCR. To eliminate primers and concentrate PCR products, samples were washed three times with 350 µl of sterile water using “Ultrafree-MC filter units,” 30,000 NMWL low-binding PLTK membrane (Millipore, Marlborough, MA). Samples were brought up in sterile water at half the volume of the original reaction (25 µl). The PCR products were sequenced following the 377 DNA sequencing system developed by Applied Biosystems (Foster City, CA) using the dye-labeled terminator chemistry. In addition to the external primers NS1, NS4, NS3, and NS8, the following internal primers were used in sequencing reactions to obtain nucleotide sequences in both directions: NS19, NS21, NS23, NS7, NS22, NS2 (White *et*

*al.*, 1990; Gargas and Taylor, 1992) and the 3' primer (NS20.KL) 5'-TTC TCA GGC TCC CTC TCC GGA GTT G-3' (which replaced NS20). Two additional primers were designed specifically for *C. geophilum* to improve sequencing results, the 5' primer (NS876.KL) 5'-GAA GAC TAA CTA CTG CGA AAG C-3' (which replaced NS21) and the 3' primer (NSS1602.KL), 5'-GCC ATT CAA TCG GTA GTA GC-3' (which replaced NS6).

### Data Analysis

DNA sequences were aligned using the DNA and protein sequence comparison software "Sequence Navigator" by Applied Biosystems along with visual optimization (alignment is available upon request). Both parsimony methods, available in the Phylogenetic Analysis Using Parsimony program (PAUP 3.1, Swofford, 1993), and distance methods, available in the Phylogenetic Inference computer Package (PHYLIP 3.56c, Felsenstein, 1994), were used to analyze the sequence data. The most parsimonious tree was found using the random-addition-sequence option, 1000 replications with one tree held at each step, in the heuristic maximum parsimony algorithms of PAUP. Distance-based trees were generated with the "Neighbor-Joining" option of PHYLIP 3.56c (Felsenstein, 1994) using either the Jukes-Cantor or Kimura two-parameter algorithm. The Kimura two-parameter algorithm was chosen because a transition:transversion ratio of 1.8:1.0, determined from MacClade version 3 (Maddison and Maddison, 1992), was observed in this data set. There was, however, no difference in topology between trees based on the Jukes-Cantor or Kimura two-parameter algorithm. The significance of the branches in both the parsimony and neighbor-joining trees were tested by bootstrap analysis using 500 bootstrap replications (Felsenstein, 1985). The Maximum Likelihood program DNAML from PHYLIP 3.56c (Felsenstein, 1994) was used to compare the likelihood of the data for trees representing alternative phylogenetic hypotheses using the method suggested by Kishino and Hasegawa (1989).

## RESULTS

### Phylogenetic Position of *Cenococcum* and *Elaphomyces* among the Ascomycetes

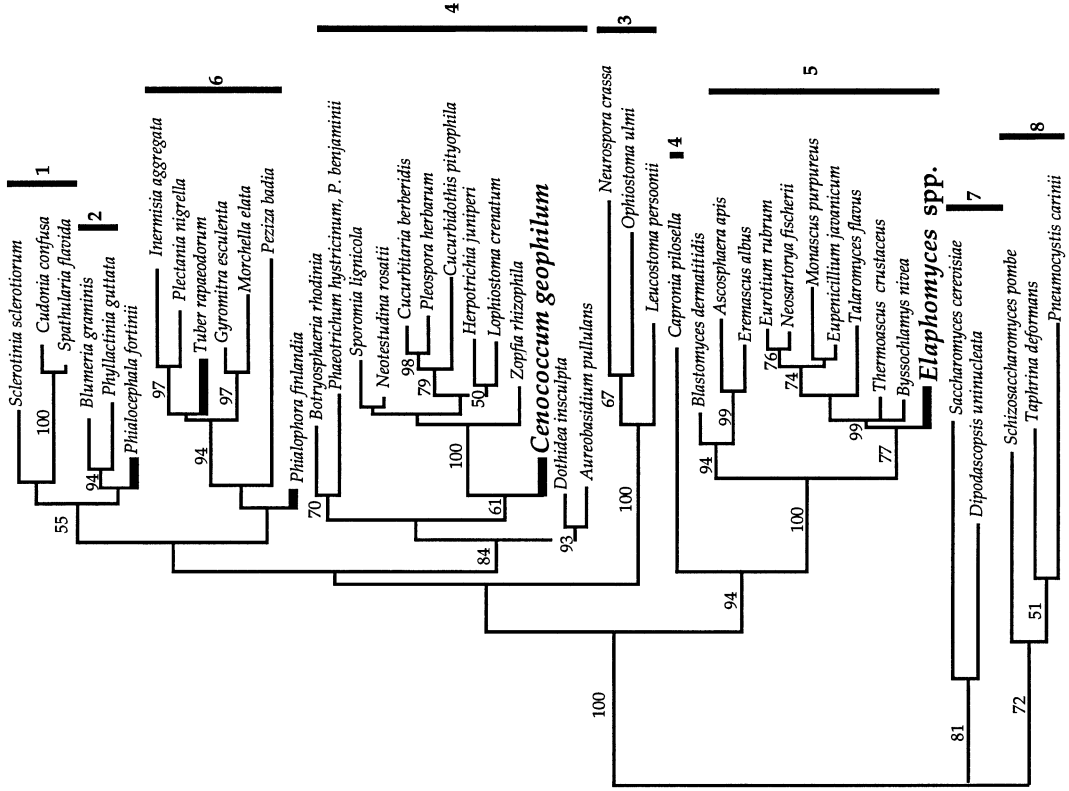
As might be expected from the wide range of host species and site conditions in which *C. geophilum* is found, restriction enzyme analysis of the rDNA repeat revealed considerable genetic variation among 71 isolates of *C. geophilum* (LoBuglio *et al.*, 1991). These results suggested that *C. geophilum* is either an extremely heterogeneous species or it has the diversity expected of a broader taxonomic rank. Nucleotide sequences for five *C. geophilum* isolates were obtained from the 18S rRNA gene. The isolates were selected as representing

the range of RFLP variation observed in the entire rDNA repeat among 71 *C. geophilum* isolates (LoBuglio *et al.*, 1991) and nucleotide sequence variation of the Internal Transcribed Spacer rDNA region (Shinohara, 1994). The percentage of similarity among the 18S rDNA sequences for the 5 *C. geophilum* isolates was very high and ranged from 99.8 to 100%. Ascomycetes with identical 18S rDNA sequences are typically conspecific, suggesting that *C. geophilum* is monophyletic and that rDNA RFLPs among different isolates represents variation in a single clade (LoBuglio *et al.*, 1991). The percentage of similarity of nucleotide sequence among the three *Elaphomyces* species sequenced in this study, *Elaphomyces* sp. 100A, *E. leveillei* Tul., and *E. virgatosporus* Holl., was also high and ranged from 99.4 to 99.5%. The sequence of *E. leveillei* was identical to a partial sequence of a different individual of the same species determined at the University of Umea, Umea Sweden (Sara Landvik, personal communication).

Parsimony and distance analysis of 18S rDNA sequences for the five *Cenococcum* isolates, three *Elaphomyces* species, and 44 genera of ascomycetes place *Cenococcum* and *Elaphomyces* on different lineages (Fig. 1). Both methods of phylogenetic analysis suggest that the *C. geophilum* lineage has evolutionary origins within the Loculoascomycetes (Fig. 1). In the parsimony analysis, heuristic searches yielded four most parsimonious trees each requiring 1610 nucleotide substitutions. Differences among the trees can be attributed to varying positions of *Herpotrichia juniperi* (Duby) Petr., *Lophiostoma crenatum* (Pers.:Fr.) Fuckel, *Cucurbitaria berberidis* (Pers.:Fr.) S.F. Gray, *Pleospora herbarum* (Fr.) Rabenh. ex Ces. de Not, and *Cucurbitodhis pityophila* Petrak (Fig. 1A) within the Pleosporales, Loculoascomycete clade. All parsimony trees, as well as the neighbor-joining tree, positioned *C. geophilum* as a basal, intermediate lineage between the two Loculoascomycete orders, the Pleosporales and Dothidiales. Although the placement of *C. geophilum* was always consistent, bootstrapping did not strongly support the Pleosporales and Dothidiales as a monophyletic group which included *C. geophilum*. Neighbor-joining and parsimony analyses of 500 bootstrapped data sets clustered *C. geophilum* within the Loculoascomycetes in 84 and 54%, respectively, of all replicates. Among the sexual ascomycetes examined, no close sexual relative to *C. geophilum* was identified.

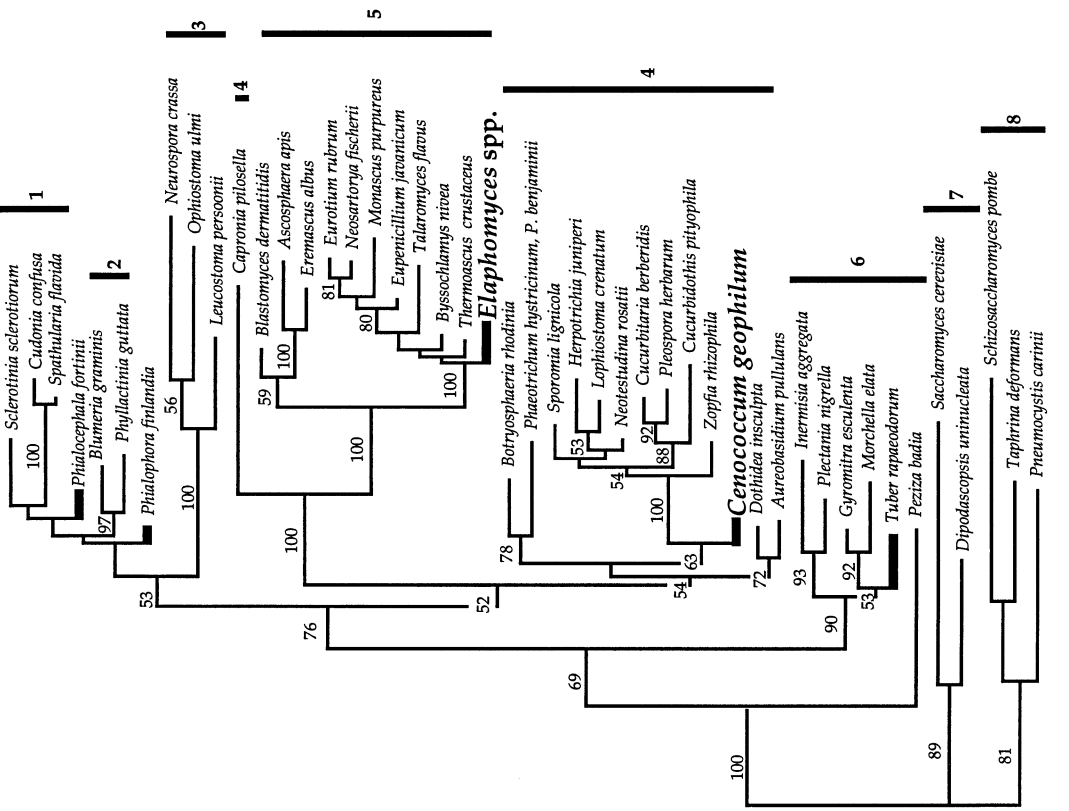
The three *Elaphomyces* species (shown as *Elaphomyces* spp. in Fig. 1) clustered together among cleistothecial ascomycetes within the Plectomycetes clade as defined by a basal branch supported by 100% of the neighbor-joining and parsimony bootstrapped data sets. Both analyses position the Onygenales and Ascospaerales as a sister group to the Eurotiales and strongly support the Plectomycete origin of *Elaphomyces*. Parsimony and neighbor-joining trees position *Elaphomyces* spp. at the base of the Eurotiales, which

**B**



— = 1 Nucleotide change per 100 positions

**A**



— = 10 Nucleotide Substitutions

comprises pectinate divergences of species of *Thermoascus*, *Byssoschlamys*, and *Talaromyces*, ending in a clade containing *Eurotium*, *Neosartorya*, *Eupenicillium*, and *Monascus* (Fig. 1). With parsimony analysis, a clade of *Elaphomyces* spp. plus the Eurotiales was strongly supported (100%), but less so with neighbor-joining (77%).

Three alternative hypotheses were compared to the results obtained from parsimony and distance analyses using the statistical test Maximum Likelihood from PHYLIP 3.56c (Felsenstein, 1994). The first hypothesis examined the likelihood of *C. geophilum* and *Elaphomyces* as each other's closest relatives. Through parsimony analysis (PAUP 3.1) a constraint tree was produced which forced *C. geophilum* and *Elaphomyces* to be monophyletic. This hypothesis tree required 1654 nucleotide substitutions (44 steps longer than the most parsimonious trees) and had a log likelihood (-12398.15, SD = 41.70) which was more than 4 standard deviations lower than the likelihood of the parsimony tree (log likelihood = -12209.54). This indicates that the fit of the data to the parsimony tree was significantly better than the fit of the data to the tree forcing *C. geophilum* and *Elaphomyces* to be monophyletic. Thus, the hypothesis that *Elaphomyces* has teleomorphic or ancestral origins with *C. geophilum* can be rejected.

The second hypothesis tested, that *Elaphomyces* is phylogenetically related to *Tuber* in the Peziziales, required a tree 33 steps longer than the most parsimonious tree. The log likelihood (-12351.33, SD = 34.99) of

the constraint tree was 4 standard deviations lower than the likelihood of the parsimony tree and thereby permits rejection of this second hypothesis.

The third hypothesis examined the evolution of the mycorrhizal symbiosis among the ascomycetes. Our results indicate that several independent lineages in the ascomycetes have given rise to mycorrhizal species (Fig. 1). Forcing the four mycorrhizal species analyzed in this study (*C. geophilum*, *Elaphomyces*, spp., *Tuber*, and *P. finlandia*) to be monophyletic produced a tree requiring 43 more steps than the most parsimonious tree and had a log likelihood (-12503.49 SD = 43.11) which was more than 6 standard deviations worse than the fit of the data to the unconstrained or parsimony tree. Therefore, the hypothesis that mycorrhizal ascomycetes arose as a monophyletic group can be rejected as well.

## DISCUSSION

Molecular phylogenetic analysis of the 18S rRNA gene phylogeny conflicts with the hypothesis that the genus *Elaphomyces* is the sexual state (teleomorph) of *C. geophilum* and indicates two distinct evolutionary origins for these two taxa; *Elaphomyces* is allied with the Plectomycetes while *C. geophilum* is most closely associated with the Loculoascomycetes.

In the phylogenies presented in Fig. 1, *C. geophilum* is positioned as a basal or intermediate lineage in between the Pleosporales and Dothidiales with no close

**FIG. 1.** Phylogenetic relationship of *Cenococcum geophilum* and *Elaphomyces* species among 44 genera of ascomycetes. The *C. geophilum* lineage is represented by 5 isolates: 349, 149, HUNT-A1, 010, and CGMONT. The *Elaphomyces* lineage is represented by three species: *Elaphomyces* 100A, *E. leveillei*, and *E. virgatosporus*. The phylograms are based on nucleotide sequence from the 18S rRNA gene using *Dipodascopsis uninucleata* (Biggs) Batra & Millner, *Saccharomyces cerevisiae* Meyen ex Hansen, *Pneumocystis carinii* Chagas, *Taphrina deformans* (Berk.) Tul., and *Schizosaccharomyces pombe* Lindner to root the trees. (A) One of four equally most parsimonious trees of 1610 steps found using the random-addition-sequence option, 1000 replications with one tree held at each step, in the heuristic maximum parsimony algorithms of PAUP 3.1s (Swofford, 1993). Branch lengths correspond to the total nucleotide changes assigned to each branch by PAUP 3.1 (Swofford, 1993). (B) Neighbor-joining tree generated from PHYLIP 3.56c (Felsenstein, 1994). Branch lengths correspond to pairwise distances from PHYLIP 3.56c (Felsenstein, 1994). In both trees the percentages above the branches are the frequencies with which a given branch appeared in 500 bootstrap replications. Bootstrap values below 50% are not displayed. Bold lines within each phylogram indicate mycorrhizal lineages. Vertical bold lines with adjacent numbers represent the following classifications: 1, Inoperculate Discomycetes; 2, Erisyphales; 3, Pyrenomycetes; 4, Loculoascomycetes; 5, Plectomycetes; 6, Operculate Discomycetes; 7, Hemiascomycetes; and 8, Archiascomycetes. GenBank Accession numbers are as follows: *Sclerotinia sclerotiorum* (Libert) de Bary, L37541; *Cudonia confusa* Bres., Z30240; *Spathularia flavida* Pers., Z30239; *Phialocephala fortinii*, L76626; *Blumeria graminis* (DC.) Speer, L26253; *Phyllactinia guttata* (Wallr.:Fr.) Lev., G. Saenz personal communication; *Phialophora finlandia*, L76625; *Neurospora crassa* Shear and Dodge, X04971; *Ophiostoma ulmi* (Buism.) Nannf., M83261; *Leucostoma persoonii* Hohn, M83259; *Capronia pilosella* (Karsten) Muller, U42473; *Blastomyces dermatitidis* Gilchrist & Stokes, X55624; *Ascospaera apis* (Maasen ex Claussen) Olive & Spiloir, M83264; *Eremascus albus* Eidam, M83258; *Eurotium rubrum* Konig & al., U00970; *Neosartorya fischerii* (Wehmer) Malloch & Cain, U21299; *Monascus purpureus* Went, M83260; *Eupenicillium javanicum* (van Beyma) Stolk & Scott, U21298; *Talaromyces flavus* (Klocker) Stolk & Samson, M83262; *Byssoschlamys nivea* Westling, M83256; *Thermoascus crustaceus* (Apinis and Chesters) Stolk, M83263; *Elaphomyces* sp. No. 100, L76619; *Elaphomyces leveillei*, L76620; *Elaphomyces virgatosporus*, L76621; *Botryosphaeria rhodinia* (Berkeley & Curtis) von Arx, U42476; *Phaeotrichum hystricinum* Cain and Barr, M. Berbee personal communication; *Phaeotrichum benjaminii* Malloch & Cain, L76624; *Sporomium lignicola* Phill. & Plowr., U42478; *Herpotrichia junipera*, U42483; *Lophiostoma crenatum*, U42485; *Neotestudina rosatii* Segretain & Destombes, L76623; *Cucurbitaria berberidis*, U42481; *Pleospora herbarum*, U05201; *Cucurbitodithis pityophila*, U42480; *Zopfia rhizophila*, L76622; *Cenococcum geophilum* No. 349, No. 149, HUNT-A1, CGMONT, 010, L76614, L76615, L76616, L76617, L76618; *Dothidea insculpta* Wallr., U42474; *Aureobasidium pullulans* (de Bary) Arnaud, M55639; *Inermisia aggregata* (Berk. & Broome) Svrek, Z30241; *Gyromitra esculenta* (Pers.:Fr.) Fr., Z30238; *Morchella elata* Fr.:Fr., L37537; *Tuber rapaeodorum*, Z49755; *Peziza badia* Pers., L37539; *Saccharomyces cerevisiae*, J01353; *Dipodascopsis uninucleata*, U00969; *Schizosaccharomyces pombe*, X54866; *Taphrina deformans*, U00971; *Pneumocystis carinii*, X12708.

sexual relative among the taxa examined. The present study includes the additional Loculoascomycetes, *Z. rhizophila* Rabenh., *N. rosatii* Segretain & Destombes, and *Phaeotrichum* sp., because we hypothesized that *C. geophilum* might have close phylogenetic origins with these taxa due to their sharing the characters of a cephalothecoid peridia (Hawksworth, 1986) and, as in the genus *Phaeotrichum*, long appendages on the ascocarps. These characters are represented in *C. geophilum* by the hyphal pattern and setae found on the surface of both the mycorrhizae and sclerotia. In addition, species such as *Z. rhizophila* are associated with plant roots and so the possibility was considered that this habitat might have some evolutionary relationship with *C. geophilum*'s mycorrhizal nature. However, as depicted in Fig. 1, a close evolutionary relationship between *C. geophilum* and these three genera was not observed. As previously classified on morphological grounds (Hawksworth and Booth, 1974), *Zopfia* and *Neotestudina* cluster within the Pleosporales. The two *Phaeotrichum* species, which have been classified in the Dothidiales (Hawksworth *et al.*, 1983), have closest evolutionary ties with *Botryosphaeria*. Thus, our results support the view of Malloch and Cain (1970) that possession of a cephalothecoid hyphal arrangement does not necessarily reflect common ancestry but may rather be indicative of similarities in the ecology of these fungi.

Over the years, alternate classification schemes have been considered for the genus *Elaphomyces*. As summarized by Alexopolous and Mims (1979) and Benny and Kimbrough (1980), mycologists have classified *Elaphomyces* either in the Tuberales because of its hypogaeal, mycorrhizal habitat, and ascospore size and shape (Fischer, 1938; Gilkey, 1939; Fennell, 1973; Korf, 1973), as a member of the Plectomycetes because of its irregularly arranged asci and cleistothecial ascocarps (Dodge, 1929; Bessey, 1950; Hawker, 1954; Werdermann, 1954; and Gaumann, 1964), or as a new monotypic order, the Elaphomycetales (Trappe, 1979). Recently, Landvik and Erikson (1994a) examined the phylogenetic position of *Elaphomyces* and *Tuber* from cladistic analysis of 18S rDNA and found that these two genera may be nested within the Pezizales. However, upon reevaluation of their findings, these results were determined to be inconclusive (Landvik and Erikson, 1994b; Landvik, personal communication). The strongly supported clustering of *Elaphomyces* among the Plectomycetes in the present study concurs with the ideas of mycologists such as Dodge (1929). Apparently the development of a closed fruiting body and irregularly arranged asci in ascocarps of *Elaphomyces* have a common evolution with members of the Plectomycete orders the Eurotiales and Onygenales.

*Phialophora finlandia* (Wang & Wilcox) and *Phialocephala fortinii* (Wang & Wilcox), also included in this study, were first isolated by H. E. Wilcox as sterile dark

mycelia from *Pinus sylvestris* L. roots in Finland in 1975 and later described by Wang and Wilcox (1985) upon induction of conidiation. Since then these fungi have frequently been encountered in different environmental habitats (Wang and Wilcox, 1985; O'Dell and Trappe, 1992; O'Dell *et al.*, 1993; Stoyke and Currah, 1990; Stoyke *et al.*, 1992; and Harney, 1994). *P. finlandia* and *P. fortinii* are similar to *C. geophilum* in that they are dematiaceous, asexual fungi and are commonly associated with plant roots, the former being ecto- or ectendomycorrhizal (Wilcox and Wang, 1987a) and the latter reported as both mycorrhizal and pathogenic depending on the environmental conditions and host plant association (Wilcox and Wang, 1987b; Stoyke *et al.*, 1992; and Harney, 1994).

The phylogenetic position of these two taxa could not be resolved in this study, but it is clear that they are not closely related to *C. geophilum*. Parsimony and distance analysis positioned *P. fortinii* near the inoperculate Discomycetes, the Helotiales (Fig. 1). *P. finlandia* is positioned with the Helotiales by parsimony analysis but distance analysis suggests a closer alliance with the operculate Discomycetes, the Pezizales.

Some insight into the evolution of the mycorrhizal symbiosis among the ascomycetes can be made from the results presented. It appears that the mycorrhizal symbiosis has arisen at least four times among the ascomycetes: within the Plectomycetes as represented by *Elaphomyces*, within the Loculoascomycetes as represented by *C. geophilum*, within the Discomycetes as represented by *Tuber*, and from a fourth lineage represented by *P. finlandia* whose phylogenetic position is not yet resolved. This is similar to the multiple independent lineages of symbiosis observed among mycorrhizal basidiomycetes (Bruns, 1995) and the lichenized fungi (Gargas *et al.*, 1995).

If *C. geophilum* is a Loculoascomycete, it is the first mycorrhizal Loculoascomycete. The absence of a close sexual relative to *C. geophilum* could very well be due to our sampling too few taxa; however, the taxa sampled do cover the range of phenotypes known in the Loculoascomycetes, and still *C. geophilum* lies at the base. Alternatively *C. geophilum* could represent an old and successful asexual lineage dating back to more than 150 million years when the Loculoascomycete lineage diverged from among the filamentous Ascomycetes (Berbee and Taylor, 1993). Other studies examining the evolution of asexual species have found them to be short, lineages recently derived from sexual species (LoBuglio *et al.*, 1993; Geiser *et al.*, 1996). The absence of sexual reproduction among fungi in field collections or in cultivation is no guarantee that the population structure in nature will be clonal, as has recently been found for another putatively asexual ascomycete (Burt *et al.*, 1996). Presently we are investigating the hypothesis that *C. geophilum* is truly asexual. Polymorphic loci are being obtained from *C. geophilum* pop-

ulations, and population genetic analyses will test for a clonal or recombining population structure. Results will determine if *C. geophilum* is asexual and provide insight into the success of asexual reproduction among fungi.

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