*Mycologia,* 95(3), 2003, pp. 457–466.


# Filamentous ascomycetes inhabiting the rhizoid environment of the liverwort *Cephaloziella varians* in Antarctica are assessed by direct PCR and cloning

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***Abstract:*** We molecularly assesed the ascomycetous fungal communities inhabiting the rhizoid environment of *Cephaloziella varians*, collected at Rothera Point on the western Antarctic Peninsula. The RFLP-phenotyped and cloned PCR products of a partial small subunit of the ribosomal RNA gene were sequenced and analyzed with neighbor joining (NJ) and maximum parsimony (MP). Both analyses identified four bootstrap-supported groups: (i) a sister group to Onygenales, (ii) a well-supported clade with *Phialocephala fortinii*, (iii) a large group of clones nested within Chaetothyriales, and (iv) a group nested within Eurotiales with a likely affinity to the genus *Aspergillus*. An additional marginally supported clade, including helotialean *Hymenoscyphus fructigenus*, was detected in the NJ analysis. Placement of one clone (possibly helotialean) was not supported by either analysis. We included *Hymenoscyphus ericae* (Helotiales) in our analyses to test for its presence in clone libraries. None of our clones showed strongly supported affinity to *H. ericae*. The culture-independent technique proved useful for assessing the composition of rhizoid fungal communities, although it remains unknown whether any of these fungi colonize *C. varians* tissues. Direct-community assays of this kind might be best combined with traditional isolation techniques to get a more holistic view of fungi occupying plant tissues.

***Key words:*** ascomycete, *Cephaloziella varians*, fungal community structure, fungi, liverwort, rhizoids, rhizosphere

Accepted for publication October 1, 2002.

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## INTRODUCTION

Roots and closely associated soil—the rhizosphere—form an environment distinct from bulk soil (Lynch and Whipps 1990). This environment provides a microhabitat where soil and root-inhabiting microbes overlap. Many fungi in this environment might be opportunistic, using root exudates, while others might be either facultative or obligate symbionts of plant roots. Rhizosphere fungal communities are thought to have many functions. The benefits of root-associated fungi to their hosts include improved nutrient and water uptake, increased fitness and potential protection from pathogenic or parasitic microorganisms (Newsham et al 1995, Smith and Read 1997, Jumpponen 2001). The function of opportunistic rhizosphere organisms is less well understood, but these organisms also have been proposed as agents that affect plant health and performance, as well as plant community dynamics (Shivanna et al 1994, Jarosik et al 1996, Westover et al 1997, Holah and Alexander 1999, Packer and Clay 2000, Westover and Bever 2001).

As with roots of higher plants, fungi also are known to be associated with the rhizoids of hepatics (Pocock and Duckett 1985a, Duckett and Clymo 1988, Duckett and Renzaglia 1988). These fungi primarily are ascomycetous, although basidiomycetes (Pocock and Duckett 1985b, Lignore and Duckett 1990) and zygomycetous arbuscular mycorrhizal fungi (Pocock and Duckett 1984, Lignore and Lopez 1989) have been shown to colonize the rhizoids of liverworts in different ecosystems. It also has been shown that rhizoids of hepatics in Antarctica are colonized by ascomycetous fungi (Williams et al 1994). These fungi, which possess septate hyphae, frequently are recorded in the basal walls of rhizoids and adjacent axis cells of Antarctic leafy liverworts belonging to the genus *Cephaloziella*, which occur in moist habitats in continental and maritime Antarctica (Williams et al 1994). These plants, referred to as *Cephaloziella exiliflora* (Taylor) Steph., in previous studies (Williams et al 1994, Chambers et al 1999), now are referred to as *C. varians* (Gottsche) Steph. (Bednarek-Ochyra et al 2000). Recent research has shown that fungi colonizing *C. varians* from the Bailey Peninsula, continental Antarctica, are conspecific with *Hymenoscyphus ericae*, the typical mycorrhizal associate of ericaceous plants

(Chambers et al 1999). This raises the possibility that the liverwort benefits from endophytic colonization of its rhizoids. However, the presence of endophytes in the rhizoids of *C. varians* from other locations in the Antarctic has not been confirmed. The rhizoids and surrounding soil of *C. varians* are likely to host additional fungi, and these broader fungal associates of the liverwort have hitherto remained unstudied.

In this study, we PCR-amplified a partial small subunit (SSU) of the ribosomal RNA gene (rDNA) and cloned PCR amplicons to characterize fungal communities inhabiting the rhizoid environment, which we define here as rhizoids and closely associated soil and organic matter, of *C. varians* from the western Antarctic Peninsula. The techniques allowed an assessment of the fungal communities without potential biases resulting from pure culture techniques and media. We chose to target the conserved SSU of the rDNA to allow a broad taxonomic placement of several potentially unknown taxa for which other genes or rDNA regions might be unavailable. We used a primer set with a known strong bias toward ascomycetes (Smit et al 1999) to get a culture-independent cross section of ascomycetes in the rhizoid environment. To specifically test whether *H. ericae* was present in our tissue samples, we also sequenced this taxon for our analyses.

## MATERIALS AND METHODS

*Sampling and DNA extraction.*—Rhizoid tissues from eight discrete colonies (labeled A–H in Tables and Figures) of *C. varians* were collected at Rothera Point on the Wright Peninsula, Adelaide Island, western Antarctic Peninsula (67° 34′ S, 68° 07′ W) in January 2000 (FIG. 1). Rothera Point is sparsely vegetated with a vascular plant and cryptogamic flora. The nearest vegetated land is on Anchorage Island, 4.5 km southwest of Rothera Point.

*C. varians* tissues were dried at 55 C for 48 h and stored until further processing. To avoid airborne contamination, rehydration and DNA extraction were performed under sterile conditions in a biosafety cabinet. Approximately 50 μg of dry rhizoid tissues were transferred into sterile Eppendorf tubes, rehydrated with double distilled water ($ddH_2O$), and surface sterilized with 10% domestic bleach (active ingredient 0.6% sodium hypochlorite). The surface sterilized tissues were rinsed three times with $ddH_2O$ to remove residual bleach and homogenized with a micropestle in lysis buffer. DNA was extracted following the protocol provided by the extraction kit manufacturer (Plant DNeasy, Qiagen, Valencia, California). Although this procedure would have inactivated fungi present on rhizoid surfaces it might have failed to denature and remove DNA of these organisms.

*PCR parameters.*—A partial sequence of the SSU of the fungal rDNA was amplified in 50 μl PCR reaction mixtures containing final concentrations or absolute amounts of reagents as follows: 300 nM of each of the forward and reverse primers (EF4 and fung5—Smit et al 1999), 2 μL of the extracted template DNA, 200 μM of each deoxynucleotide triphosphate, 1.7 mM $MgCl_2$, 2 units of Taq DNA polymerase (Promega, Madison, Wisconsin), and 5 μL of manufacturer's PCR buffer. PCR cycle parameters consisted of an initial denaturation at 94 C for 3 min, then 40 cycles of denaturation at 94 C for 1 min, annealing at 48 C for 1 min and extension at 72 C for 3 min, followed by a final extension step at 72 C for 10 min. The PCR reactions were performed in a Hybaid OmniCycler (Hybaid Ltd., Middlesex, U.K.). Possible PCR amplification of airborne and reagent contaminants was determined with two different controls; (i) a blank sample run through the extraction protocol simultaneously with the actual samples; (ii) a negative PCR control in which the template DNA was replaced with $ddH_2O$.

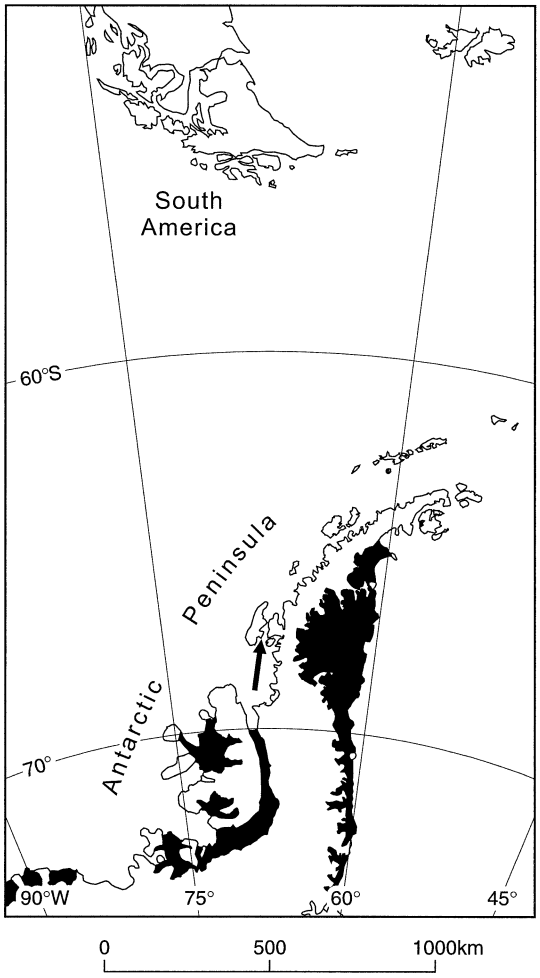


FIG. 1. Map shows the sampling location (Rothera Point; arrow) on Adelaide Island, western Antarctic Peninsula. Infilled areas are permanent ice sheets.

*Small-subunit rDNA clone library construction.*—Due to the specific primers and stringent PCR conditions, we observed amplicons of the expected size (ca 550 bp) when the PCR

products were seen on 1.5% agarose gels. The mixed populations of PCR products were ligated into a linearized pGEM-T vector (Promega, Madison, Wisconsin). The circularized plasmids were transformed into competent JM109 cells (Promega, Madison, Wisconsin) by heat shock, and the putative positive transformants were identified by α-complementation (Sambrook 1989).

*Analysis of rDNA clone libraries.*—All positive transformants in each of the clone libraries were confirmed by PCR amplification of the target product in 15 μL reaction volumes under the same reaction conditions as described above. To select different plasmids for sequencing, PCR products were digested with endonucleases (*HinfI, AluI*; New England BioLabs, Beverly, Massachusetts) and resolved on 3% agarose gels (Gardes and Bruns 1996). The approach using PCR and restriction fragment-length polymorphisms (RFLP), in addition to allowing efficient screening of clone libraries, made it possible to select different RFLP phenotypes for sequencing. An approximately 550 bp sequence was obtained from one or two representatives of each RFLP phenotype in all clone libraries in one direction, using fluorescent dideoxy-terminators (ABI Prism® BigDye™; Applied Biosystems, Foster City, California) and an automated ABI Prism® 3700 DNA Analyzer (Applied Biosystems, Foster City, California) at the DNA Sequencing and Genotyping Facility at Kansas State University. In addition to the cloned PCR amplicons from *C. varians* rhizoids, we sequenced this region of the rDNA from *H. ericae* (isolate HMER100 maintained at the Forestry Sciences Laboratory in Corvallis, Oregon). All sequences were determined with primer EF4 (GenBank accession Nos. AF462168–AF462186). Vector contamination was removed with the automated vector trimming function in Sequencher (GeneCodes, Ann Arbor, Michigan). The similarities to existing rDNA sequences in the GenBank database were determined at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/—Altschul et al 1997) using standard nucleotide BLAST (version 2.2.1) without limiting queries and Sequence Match (version 2.7) at the Ribosomal Database Project (RDP—http://rdp.cme.msu.edu/html/—Maidak et al 1999).

A total of 16 sequences obtained from *C. varians* rhizoids, 49 from GenBank, and one from *H. ericae*, were aligned in 843 positions with Sequencher (GeneCodes Inc., Ann Arbor, Michigan) and manually adjusted to maximize conservation. The alignment included a large, 290-bp insert in *H. ericae* at approximately Position 516, as reported for other ascomycetes in Caliciales and Lecanorales (Gargas et al 1995). The *H. ericae* insertion did not show close homology to others available at the GenBank, as judged by BLAST searches. The taxonomic relationships among the fungal sequences were inferred with the neighbor-joining (NJ) and maximum-parsimony (MP) analyses in PAUP* (Swofford 2001). Four species of ascomycetous yeasts belonging to the Saccharomycetales were selected as outgroup because the clone libraries exclusively contained filamentous ascomycetes as a result of our choice of primers with a strong bias in favor of these fungi. In the NJ analyses, rates for variable sites were assumed equal and no sites were assumed invariable. Data matrices were corrected using Jukes-Cantor correction. Sites with missing data—ambiguous nucleotides or gaps—were ignored for the affected pairwise comparisons. The most-parsimonious trees were obtained using a branch swapping algorithm with tree bisection reconnection. The number of equiparsimonious trees was expected to be high, attributable to several closely related sequences in the clone libraries. As a result, the maximum number of retained trees was restricted to 1000. The robustness of inferred NJ and MP topologies was tested by 1000 bootstrap replicates. Because of the large number of possible equiparsimonious trees in MP analyses, stepwise heuristic searches were used in the bootstrapping.

Chimeric sequences might be a frequent phenomenon in environmental samples with diverse, mixed populations of competing templates. To check for possible chimeric sequences, all sequenced clones were analyzed by the Chimera Check program of the RDP database (version 2.7; Maidak et al 1999).

## RESULTS

We were able to obtain target-size ascomycetous amplicons (ca 550 bp) from dried *C. varians* rhizoid tissues (TABLE I). Seven of the eight samples produced visible amplicons; one failed despite repeated attempts. The two controls included in the PCR reactions consistently failed to produce visible amplicons, indicating that the PCR-products indeed were amplified from the surface-sterilized rhizoid tissues, not from air- or reagent-borne contaminants.

As determined by BLAST (NCBI), our sequence data showed 96–99% similarity to ascomycetous sequences representing Chaetothyriales, Eurotiales and Helotiales in GenBank (TABLE I); analyses using Sequence Match (RDP) identified the same fungal sequences. Accordingly, we present only the results of the BLAST searches. Most of the fungal sequences obtained from *C. varians* were distributed among five ascomycete clades (FIGS. 2 and 3). (i) Three clones from three different *C. varians* samples (A–C) formed a well-supported clade (Group I in FIGS. 2 and 3) as a sister group of the Onygenales in NJ analysis. This grouping also was detected in MP analysis but received no bootstrap support. The cloned sequences were identical, showing no sequence divergence within the cloned sequence and might represent a conspecific grouping of an abundant rhizosphere fungus. (ii) One clone (E9) formed a strongly supported clade (Group II in FIGS. 2 and 3) with *Phialocephala fortinii*. This clone also was found to be 99% similar to *P. fortinii* in the BLAST searches (TABLE I). (iii) A total of nine clones obtained from five of the eight different samples (A, D–F and H) nested within the Chaetothyriales (Group IV in FIGS. 2 and 3) and matched *Coniosporium* sp. in BLAST searches

TABLE I. Cloned PCR products obtained from *Cephaloziella varians* rhizoids from Rothera Point on the western Antarctic Peninsula. Accession numbers of the cloned products and their closest BLAST matches are in parentheses

| Clone | BLAST | Species | Order | Chimer Sequence[a] |
|---|---|---|---|---|
| A2 (AF462170) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |
| A3 (AF462171) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |
| A7 (AF462172) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |
| A11 (AF462169) | 98% | *Phacidium coniferarum* (AF203467) | Helotiales | 410–560 |
| B3 (AF462174) | 98% | *Phacidium coniferarum* (AF203467) | Helotiales | 1–140 |
| B4 (AF462175) | 98% | *Phacidium coniferarum* (AF203467) | Helotiales | 1–140 |
| B12 (AF462173) | 98% | *Phacidium coniferarum* (AF203467) | Helotiales | 1–140 |
| C1 (AF462176) | 98% | *Phacidium coniferarum* (AF203467) | Helotiales | 1–140 |
| C4 (AF462177) | 99% | *Aspergillus fumigatus* (M55626) | Eurotiales | 1–70 |
| D5 (AF462178) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |
| E5 (AF462180) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |
| E9 (AF462181) | 99% | *Phialocephala fortinii* (L76626) | In certae sedis | 1–150 |
| E12 (AF462179) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |
| F3 (AF462182) | 99% | *Phacidium coniferarum* (AF203467) | Helotiales | — |
| F4 (AF462183) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |
| F5 (AF462184) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |
| H7 (AF462185) | 99% | *Phacidium coniferarum* (AF203467) | Helotiales | — |
| H8 (AF462186) | 96% | *Coniosporium* sp. (Y11712) | Chaetothyriales | 410–560 |

[a] = Based on Chimera Check (version 2.7) at RDP (Maidak et al 1999).

(96% similarity). It remains unclear from our data whether these sequences represent a conspecific group. These clones formed a single, well-supported clade with >85% bootstrap support in both NJ and MP analyses and had sequence divergence ranging only up to 1.8%, equivalent to 10 bp over the entire cloned sequence. (iv) One clone (C4) formed a well-supported clade (Group V in FIGS. 2 and 3) nested within the Eurotiales and matched *Aspergillus fumigatus* and several other aspergilli with 99% similarity in BLAST searches. We estimate that these four groups approximate order-level resolution in our analyses. Finally, one clone (F3) was grouped with *H. fructigenus* in the NJ analyses with marginal (55%) bootstrap support (Group III in FIG. 2); this grouping was not supported by MP analysis. In one case we were unable to establish a grouping supported by an appreciable bootstrap value in either of the analyses. This clone (H7) was similar to that grouped with *H. fructigenus* in NJ analysis (F3). Both of these clones were very similar (99%) to helotialean *Phacidium coniferarum* in BLAST searches (TABLE I), suggesting an affinity to the Helotiales. Our data analyses did not support monophyletic Helotiales.

To specifically test whether we would be able to detect *H. ericae* in the sampled *C. varians* individuals, we sequenced this species (GenBank Accession No. AF462168) for our analyses. *Hymenoscyphus ericae* contained an approximately 290 bp intron within the sequenced region. This intron was excluded from the analyses because no similar introns were observed in our clone libraries or among the sequences from GenBank. Although five of our clones were 98–99% similar to a member of the Helotiales (*P. coniferarum*; TABLE I), none of them grouped with *H. ericae* in NJ or MP analyses. One clone (F3) grouped with *H. fructigenus* in the NJ analyses with low-bootstrap support, but this grouping was not supported by MP analyses. Another clone (H7) showed no clear affinity to any of the Helotialean fungi included in these analyses. The remaining three clones (A11, B3, C1) with a BLAST-match within Helotiales formed a sister group with the Onygenales in both MP and NJ analyses. Bootstrapping only in NJ analysis supported this grouping.

The Chimera Check of the RDP indicated that the majority of sequences in our rDNA libraries possibly were chimeric (TABLE I). Among our sequences, two main-break points of chimeras were found: the first of these was at Position 140 and the second at Position 410 of our approximately 550 bp sequence. To test if these data affected our conclusions, we repeated our NJ analyses after we excluded the portions of the sequence that might have originated from another organism. Exclusion of positions 1–140 resulted in the collapse of groups IV and V (FIG. 2), but no new groupings were observed. Exclusion of positions 410–560 resulted in topologies closely similar to that shown in FIG. 2; four groups (I, II, IV and V) were present and no new rearrangements were observed. In summary, possible chimeric regions do not affect

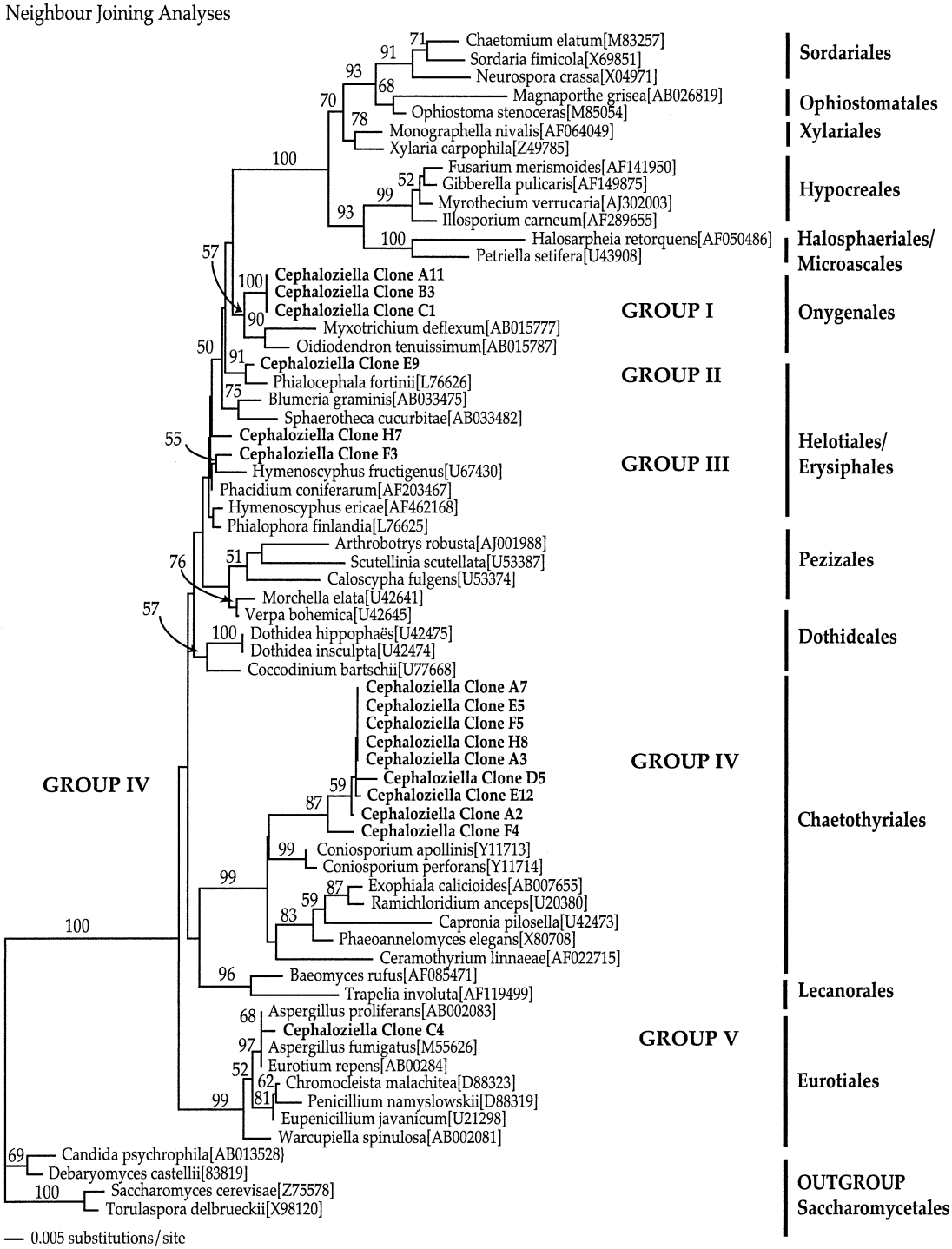


FIG. 2. This is a neighbor-joining phylogram of ascomycetes in the rhizoids of the leafy liverwort *Cephaloziella varians* at Rothera Point on the western Antarctic Peninsula. Branch lengths correspond to pairwise distances from PAUP (Swofford 2001). The PCR-amplified and cloned rhizoid-inhabiting fungi are shown in boldface. Accession numbers of GenBank-obtained sequences are shown in parentheses. Accession numbers of the rhizoid-inhabiting fungi are shown in TABLE I. Numbers on clades refer to proportion (occurrence ≥50%) of bootstrap replicates containing that clade.

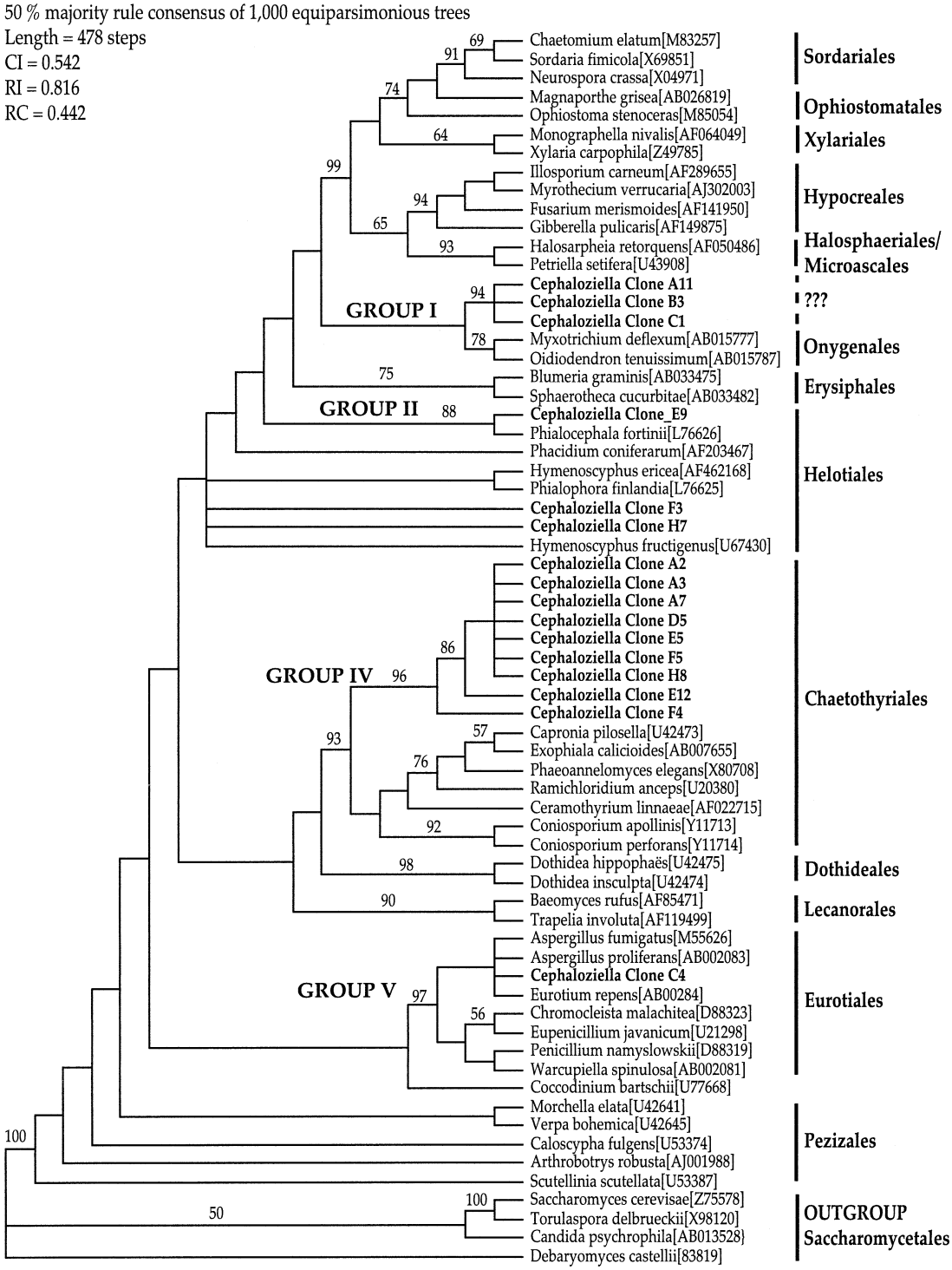


FIG. 3. Analyses revealed 50% majority rule consensus of 1000 retained equally parsimonious trees of ascomycetes in the rhizoids of the leafy liverwort *Cephaloziella varians* at Rothera Point on the western Antarctic Peninsula. PCR-amplified and cloned rhizoid-inhabiting fungi are shown in boldface. Accession numbers of GenBank-obtained sequences are shown in parentheses. Accession numbers of the rhizoid-inhabiting fungi are shown in TABLE I. Numbers on clades refer to proportion (occurrence ≥50%) of bootstrap replicates containing that clade.

our conclusions on the affinities of the fungal sequences in our rDNA clone libraries.

## DISCUSSION

Our attempt to assess the ascomycetous fungal communities present in the rhizoid environment of Antarctic *C. varians* by direct PCR and cloning proved to be successful. Seven of the eight samples produced visible amplicons, which could be further analyzed. We also carefully controlled potential air- and/or reagent-borne contamination by two negative controls, which remained free of any visible PCR amplification.

Based on earlier studies (Williams et al 1994, Chambers et al 1999), we expected to detect ascomycetes in the rhizoid environment of *C. varians*. Our analyses confirmed that ascomycetes indeed are present in this environment: Sequence data showed 96–99% similarity to ascomycetous sequences representing the Chaetothyriales, Eurotiales and Helotiales. In many cases the results of BLAST searches and bootstrapping analyses produced similar results. For example, clone E9 was 99% similar to *P. fortinii* in BLAST searches and it formed a strongly supported clade with *P. fortinii* in our analyses. In contrast, results from NJ or MP analyses often did not support the results of BLAST searches. For example, two clones (F3 and H7), which were not supported by bootstrap analyses, were highly (99%) similar to *P. coniferarum* in BLAST searches. In the light of our results and analyses, BLAST searches appear to provide a useful starting point for determination of unknown taxa in environmental DNA samples, but the reliability of the inferences on relationships among unknown and known taxa need to be tested further.

The majority of our clones from the rhizoid environment of *C. varians* were placed in three ascomycetous groups that received bootstrap support >50% in MP and NJ analyses. The functional significance of these groups remains unclear but some of the recorded fungi are frequent inhabitants of roots and rhizosphere soil. One of our clones was nested within the Eurotiales and possibly is related to various aspergilli, which are cosmopolitan and commonly encountered in soil and the rhizosphere. Members of the Eurotiales, including aspergilli and penicilli, have been isolated from plant seeds (Yamaji et al 1999, 2001, Sharma and Champawat 2000) and rhizosphere samples (Summerbell 1989, Shivanna et al 1994), and, although many Eurotialean fungi might be saprotrophs in the rhizosphere environment, recent evidence suggests that some species of *Aspergillus* colonize host root tissues inter- and intracellularly (Barrow and Osuna 2002). Five of the eight samples in this study produced clones that nested within the Chaetothyriales. Affinity to the Chaetothyriales is not surprising because many members of this order are either saprobes or plant parasites. However, we were unable to relate these clones more closely to any known taxa: Our clones were only 96% similar to *Coniosporium* sp. in BLAST searches. These results hence underline the importance of updating and appending ribosomal databases such as that maintained by RDP (Maidak et al 1997, Maidak et al 1999). *Phialocephala fortinii*, to which one of the clones in our study showed high affinity, is one of the dark septate endophytes ubiquitous in the roots of higher and lower plants in arctic, tropical, boreal and temperate habitats (Jumpponen and Trappe 1998, Jumpponen 2001). *Phialocephala fortinii* has been shown to improve the growth of a cool-temperate plant species through enhancement of nutrient uptake from rhizosphere soil (Jumpponen et al 1998), which raises the possibility that at least some of the septate fungi colonizing *C. varians* tissues in the Antarctic are beneficial to host growth. Some of the clones in our root samples hence are similar or related to fungi known from functional root symbioses, and might represent taxa that influence *C. varians* growth and performance. Others, however, might be casual, opportunistic inhabitants of the rhizoid environment.

Earlier studies, which relied on isolation and subsequent identification by ITS (internal transcribed spacer) sequence comparisons among unknown and authenticated isolates of rhizoid-inhabiting fungi, putatively identified *H. ericae* from *C. varians* rhizoids from the Bailey Peninsula, continental Antarctica (Chambers et al 1999). To test whether populations of *C. varians* from Rothera Point on Adelaide Island hosted helotialean *H. ericae*, we included two *Hymenoscyphus* species in our analyses. We found little evidence to support the ubiquitous presence of *Hymenoscyphus* spp. in our liverwort samples; only one clone formed a marginally supported group with *H. fructigenus* in NJ analysis. These data corroborate the observation that hyphal coils are not found in rhizoid bases of *C. varians* from Rothera Point and adjacent islands (K. Newsham pers obs).

We offer several possible explanations for the differences between our observations and those of Chambers et al (1999). First, it is possible that *H. ericae* has a heterogeneous distribution in the Antarctic. Our samples were collected at Rothera Point on Adelaide Island in the maritime Antarctic, more than 5000 km from the location at which Chambers et al (1999) collected material. It is recognized that there is little overlap in the species of Acari inhabiting maritime and continental Antarctica (Pugh and Convey 2000), and the same might be true of fungal endophytes inhabiting pan-Antarctic plant species such as

*C. varians*. Second, our sampling was rather limited. With more extensive sampling, either covering a greater geographical range or a greater number of plants, we might have detected a greater diversity of fungi, including *H. ericae*. Third, endophyte colonization might vary within a growing season (Duckett and Read 1995). Our sampling represents a snapshot in time, and it is possible that we missed the peak of colonization by *H. ericae* in *C. varians* rhizoids. However, the seasonal succession of fungi in the tissues of plants exposed to the cold in a short growing season is unlikely to be rapid, and hence this is probably not a plausible explanation for our observations. Fourth, it is possible that an intron might have been present in the small subunit of the ribosomal RNA gene of *Hymenoscyphus* spp. in *C. varians* rhizoids, as was observed for the *H. ericae* isolate that was sequenced in our study. Such introns could have interfered with amplification of fungal DNA from rhizoids. Type I introns frequently are present in ericoid mycorrhizal fungi (Perotto et al 2000) and might be common in other ascomycetes (Holst-Jensen et al 1999, Borneman and Hartin 2000). However, we were able to amplify and sequence DNA from an isolate of *H. ericae* using the primers that also were used to amplify environmental DNA. This suggests that if strains conspecific or closely related to *H. ericae* were abundant in our samples, it is likely that we would have detected them. Even in the absence of introns at the priming sites, it is possible that longer templates were discriminated against in the presence of a mixed population of shorter templates. None of the sequences in our rDNA clone libraries contained insertions similar to those observed in *H. ericae*. Finally, additional factors that might have influenced the assessment of microbial diversity in the environmental samples have been reported. These include PCR and DNA extraction biases, different copy numbers of ribosomal RNA genes, primer specificity, template concentration, chimeric sequences, and/or amplification of contaminants (Kopczynski et al 1994, von Wintzingerode et al 1997, Wilson 1997, Hugenholtz et al 1998, Becker et al 2000, Martin-Laurent et al 2001, Ranjard et al 2001). The results regarding the apparent absence of *H. ericae* from one location on the western Antarctic Peninsula remain to be confirmed with more widespread sampling and fungal isolation from *C. varians* rhizoids.

In summary, this study indicates that direct PCR and downstream cloning might be useful tools to assess the ascomycetous fungal communities in the rhizosphere environment of an Antarctic plant species. Using these techniques we tested for the presence of *H. ericae* in the rhizoid environment of *C. varians* but were unable to confirm its presence in our samples. However, the presence of other fungi with possible beneficial effects on plant growth, such as *P. fortinii*, was demonstrated with this approach. The function and ecological role of these fungi remain unclear in this study but will be a focus of forthcoming work. Although direct PCR and cloning appears to be a useful option to estimate organismal diversity (Pace 1997, Hugenholtz et al 1998, Smit et al 1999, Borneman and Hartin 2000, van Elsas et al 2000), it might be necessary to include assessments of culturable organisms as well. Culturing organisms from environmental samples would provide useful reference data to compare with unknown sequences obtained from the same samples.

## ACKNOWLEDGMENTS

This work was supported by Kansas State University BRIEF program, National Science Foundation EPSCoR Grant No. 9874732, matching support from the State of Kansas (to AJ) and the British Antarctic Survey's Terrestrial and Freshwater Biodiversity project (to KKN). We thank Doni McKay of U.S.D.A. Forest Service at Forestry Sciences Laboratory in Corvallis, Oregon, for providing the *H. ericae* isolate. We are grateful to Cameron Cooley for his assistance in the sequence alignment and BLAST searches. Drs. Carolyn Ferguson, Charles L. Kramer and James M. Trappe provided helpful comments on early drafts of this manuscript. Anna MacArthur edited and revised the manuscript.

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