INTER- AND INTRASPECIFIC VARIATION WITHIN *CYLINDROCARPON* INFERRED FROM MITOCHONDRIAL SMALL SUBUNIT rDNA SEQUENCES

by

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ABSTRACT

Mitochondrial small subunit (mitSSU) rDNA sequences were used to study inter- and intraspecific variation within the fungal genus *Cylindrocarpon*. For analysis of interspecific variation, 12 isolates (seven species) of *Cylindrocarpon* were subjected to phylogenetic analysis with *Fusarium* sp. and *Tubercularia* sp. as outgroups. Maximum parsimony analyses were performed with various combinations of indel coding schemes and transition:transversion biases. DNA sequence divergence among the twelve *Cylindrocarpon* isolates was 2.3 - 7.4%, and this was sufficient to resolve the interspecific relationships in the genus. There were three well-supported, monophyletic groups in the phylogenies. These groups were congruent in all taxa with *Cylindrocarpon* groupings based on morphological and cultural characteristics described in the literature, thus suggesting that mitSSU rDNA region is appropriate for phylogenetic analysis of this genus. Furthermore, results presented here have helped to resolve the inconsistent placement of *Nectria coronata* (anamorph: *C. coronatum*) in different *Nectria* groups. For analysis of intraspecific variation, the ingroup consisted of 21 isolates of *C. destructans* from British Columbia and three isolates from Ontario, and the outgroup included was *C. cylindroides*. Three different analytical methods, i.e., maximum parsimony, distance method, and the method developed by Templeton *et al.* (1992) were used to construct phylogenetic trees for this study. Very low sequence divergence (< 1.1%) was detected among the isolates studied. Nevertheless, three variants of mitSSU rDNA could be separated in the analysis. Evidence for association between the variants and either localities or host plant species was not obtained, but all isolates obtained from Douglas-fir belonged to one mitSSU rDNA variant. The clonal nature of *C. destructans* may be one explanation for the low level of mitSSU rDNA variation; however,

results from genetic variation analysis using nuclear DNA-based markers are needed in the future to confirm this speculation.

DEDICATION

To:

My lovely wife Carla for your everlasting prayers, love, patience, support and encouragement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

General

Cylindrocarpon is an anamorphic genus of fungal species whose members are generally considered as weak or minor pathogens; nevertheless, these fungi may have a significant economic impact. A survey conducted at forest nurseries in Quebec in 1994 revealed that root rot caused by *Cylindrocarpon* spp., together with *Cylindrocladium floridanum* Sobers and Seymour, and *Fusarium* spp, damaged more than two million trees at six nurseries (Hall, 1994). In Ontario nurseries, the fungi *Cylindrocarpon* sp. and *Fusarium* sp. were associated with 12% mortality of black spruce, and damaged red pine and jack pine (Hall, 1994). *Cylindrocarpon* spp. have also been shown to cause seedling losses of orchard trees, and apple replant disease (Hall, 1994; Braun 1991). Moreover, a few species of *Cylindrocarpon*, especially *C. heteronemum* (anamorph: *N. galligena*), are known to cause canker diseases on orchard and forest trees (Agrios, 1988; Sinclair *et al.*, 1987). Mention has been made in the literature that *Cylindrocarpon* spp. can be found on a wide range of unrelated host plants and occur in the temperate, semi-temperate and tropical regions (Booth, 1966).

Identification of *Cylindrocarpon* species is based primarily on growth characteristics and spore morphology and has always been difficult (Samuels, pers. comm.). This difficulty results from the limited number of morphological characteristics available for species identification and the variability of these characteristics. Booth (1966) delineated four morphological groups within *Cylindrocarpon* based upon the presence or absence of microconidia and/or mycelial chlamydospores formed in culture.

However, Brayford and Samuels (1993) argued that formation of abundant microconidia and/or mycelial chlamydospores are species characteristics and do not delineate natural groups. They then used a holomorph approach for identifying *Cylindrocarpon*. Unfortunately, their approach has not been very successful. Due to intergradation between morphological characteristics used in identification of *Cylindrocarpon* in its teleomorph state, *Nectria*, some species are sometimes included in different *Nectria* groups (Brayford and Samuels, 1993; Samuels *et al.*, 1990).

Cylindrocarpon destructans (Zinnsm.) Scholten is a common, soil borne fungus with a wide distribution. The plant pathology literature contains many records of its involvement in diseases, most often as the cause of seedling blights, basal rot of bulbs and root rot of diverse plants (Booth, 1966; Domsh *et al.*, 1980). In addition, *C. destructans* has also been identified as the causal agent of a new mold problem of apricot, peach, and pear trees in cold storage (Traquair and White, 1992). Furthermore, Dugan and Grove (1994) found that *C. destructans*, together with *C. didymum and C. magnusianum*, was pathogenic on apple seedlings that had been in storage at 2-7 °C for 4 months.

Knowledge of the amount and distribution of genetic variability in plant pathogen populations is a prerequisite to studies of gene flow, natural selection, and host-pathogen co-evolution in agroecosystems (Adachi *et al.*, 1993). Genetic variability of pathogenic fungi at the population level, in particular, is of great interest in the field of plant pathology because it is a window into the process of speciation. The genetic population structure of pathogens may indicate their potential for development of pathogenic specialization and fungicide resistance (Bruns *et al.*, 1991).

Diversity within *C. destructans* has not received much attention. Samuels and Brayford (1990) found that strains of *C. destructans* from different sources and localities showed variation in morphology of their cultures and conidia. However, since the fungus has relatively few distinctive morphological traits, and those available tend to be plastic, it is difficult to interpret the observed patterns of variation (Samuels and Brayford, 1990).

The broad geographic range and host origins and morphological variation of *C. destructans* suggest that the species is composed of strains. If this hypothesis is correct, genetic variation between strains should be correlated with one or more distinguishing adaptations (e.g. geographical origin or host specificity). Alternatively, *C. destructans* may not have a history of substantial intraspecific adaptive divergence and each isolate may have the capacity to infect a wide range of hosts and persist under a variety of environmental conditions. In the latter case, groups of isolates defined by genetic variation would not correlate with groups based on ecological data.

In recent years, DNA-based molecular methods have been used to differentiate genera, species, subspecies, races, and strains of fungi as well as to identify individuals or clones within fungal populations in ecological studies. These methods include restriction fragment length polymorphism (RFLP) analysis, polymerase chain reaction (PCR) based methods and DNA sequencing (see literature review). DNA sequence comparisons of the mitochondrial small sub-unit rDNA and nuclear rDNA regions have been shown to be useful in determining relationships between fungal genera and species (Bruns *et al.*, 1992; LoBuglio *et al.*, 1993; O'Donell, 1992).

Research objectives

The primary objectives of my research project were threefold: a) to infer the phylogenetic relationships among *Cylindrocarpon* spp. based on mitSSU rDNA sequences, b) to test the congruence between morphological groups of *Cylindrocarpon* outlined by Booth (1966) and the grouping based on mitSSU rDNA sequences, and c) to assess genetic diversity among isolates of *C. destructans*.

LITERATURE REVIEW

Taxonomy and characteristics of *Cylindrocarpon* spp.

The generic name *Cylindrocarpon* was introduced by Wollenweber in 1913 for anamorphs of *Nectria* Fr. Section Willkommiotes Wollenw., which would today be equated with the *Nectria coccinea* group (Booth, 1959). Wollenweber later expanded his concept of *Cylindrocarpon* to include chlamydospore-forming taxa. Booth (1966) redescribed further species of *Cylindrocarpon* that were anamorphs of *Nectria* taxa.

Cylindrocarpon spp. belong to the family Tuberculariaceae Fr., order Tubercularíales, which is a member of Class Hyphomycetes in Subdivision Deuteromycetes (Subramanian, 1983). They are similar to *Fusarium* spp. in that they have teleomorphs in the genus *Nectria* (where known) and they produce slimy, multicelled conidia (macroconidia) often accompanied by single-celled microconidia and chlamydospores.

Conidia of *Cylindrocarpon* spp. are slimy phialospores formed from phialides in basipetal succession, generally not adhering in chains (see Figure 1). Macroconidia are always present, hyaline, straight or curved, cylindrical to fusoid but with rounded ends and without a distinctive foot cell characteristic of *Fusarium* spp. (Zoutman and Sigler, 1991), with 1-10 transverse septa. Most species of *Cylindrocarpon* form microconidia (Booth, 1966). They are hyaline, oval to ellipsoid, 0-1 septate. Phialides are simple, with single apical pore bearing a collar, formed laterally on hyphae, terminally on simple lateral branches or singly or in groups as termination to branches of penicillately branched conidiophores. There is no continuation of conidiophore axis into a sterile

appendage characteristic of *Cylindrocladium* spp. (Holliday, 1980). Chlamydospores are present or absent, hyaline to brown, globose, formed singly, in chains or clumps, intercalarily or terminally or on lateral branches, or singly or in chains in cells of the macroconidia. Mycelia of *Cylindrocarpon* spp. in culture are beige, orange brown to purple, floccose to felted. Sterile stromatic pustules or sporodochia may be present or absent (Booth, 1966).

Booth (1966) outlined four morphological groups within *Cylindrocarpon* based upon the presence or absence of microconidia and/or mycelial chlamydospores formed in culture. Species of the first group form microconidia abundantly but lack mycelial chlamydospores. Species of the second group lack both microconidia and mycelial chlamydospores. The third *Cylindrocarpon* group is characterized by formation of both microconidia and mycelial chlamydospores. Species of the fourth group have mycelial chlamydospores but lack microconidia.

Like species of the genus *Fusarium*, members of the genus *Cylindrocarpon* commonly are cosmopolitan soil fungi often associated with the roots of many host plants (Booth, 1966; Booth, 1967; Domsch *et al.*, 1980). Some species can also attack other parts of plant such as leaves, stems and fruit (Samuels and Brayford, 1990).

Cylindrocarpon destructans – Taxonomy and characteristics

Gerlach and Nilsson (1963) reported perithecia of an unidentified species of *Nectria* on leaves, peduncles, and bulbs of *Cyclamen persicum* L. infected with *Cylindrocarpon radicicola* Wollenw. in Sweden (Booth, 1966). The cultures derived from ascospores isolated from those perithecia were morphologically consistent with *C.*

radicicola. Gerlach and Nilsson (1963) described the *Nectria* as the new species, *N. radicicola*. Booth (1966) later concluded that the name *C. radicicola* was preceded by *C. destructans* (Zinnsm.) Scholten.

Perithecia of *N. radicicola* are red to reddish-brown, 170-350 X 150-320 μm, formed sparsely and occur superficially on woody or herbaceous tissues (Booth, 1966). They are globose to subglobose, with a broadly conical papilla and a scaly to warted wall. Asci are cylindrical to subclavate, 53-85 X 4.5-10 μm, paraphysate, 8- or rarely 4-6 spored. Ascospores are ellipsoid, 1-septate, smooth, and colorless, 10-13 X 3-3.5μm. In these characters *N. radicicola* is not unique, but the perithecial anatomy of the fungus is distinctive (Samuels and Brayford, 1990). The perithecium is easily seen in whole mounts to possess an outer layer of globose cells 20-30 μm diameter and with thin (1-2 μm) walls. In section, these cells form a definite mantle over the perithecial wall through which the ostiolar region protrudes (Samuels and Brayford, 1990).

Perithecia of *N. radicicola* are not anatomically similar to any other known teleomorphs of *Cylindrocarpon*, which belong to the *Nectria mammoidea* and *N. coccinea* groups (*sensu* Booth, 1959). The only group of hypocrealean fungi having similar anatomy of the perithecial wall is that included by Rossman (1983) in *Calonectria* de Notaris. On the basis of perithecial anatomy, there is undoubtedly a close relationship between *Calonectria* species and *N. radicicola*. *Calonectria* is distinguished from *Nectria* primarily by its *Cylindrocladium* Morgan anamorphs, the shape of the ascospores and asci, and by the fact that the ascospores of most *Calonectria* are multi-septate (Samuels and Brayford, 1990).

The characteristic colony of *C. destructans* is relatively fast growing (30-70 mm diameter after 10 days at 20° on PDA). Aerial mycelia are floccose to felted, greyish-white becoming pale brown and later deep reddish-brown; underside of colonies is beige and generally becoming reddish-brown. Macroconidia formed in culture are hyaline, (1-)3(-5) septate (45-52 X 6.5-7.5 μm), cylindrical with rounded ends, straight or curved and narrowing slightly toward the base (see Figure 1), and often have a distinct, protuberant, flat, basal abscission scar (Booth, 1967; Samuels and Brayford, 1990). Microconidia are oval to elliptical, 6-10 X 3.5-4 μm. Distinct mycelial chlamydospores, either single or in chains or clusters, with thickened, golden-brown walls are abundant in mature colonies.

C. destructans occurs in temperate, semi-temperate and tropical regions, and it is distributed widely in Europe, North America, East and South Africa, Australia, New Zealand, South and Southeast Asia (Booth 1966; Booth, 1967). It is very widespread in its occurrence, frequently occurring in soil or associated with the roots or underground parts of a large number of woody and herbaceous hosts. Because of its abundance on the root surface, its niche is considered to be that of a pioneer colonizer (Matturi and Stenton, 1964). The fungus has been isolated from the roots of both healthy and diseased plants, and both pathogenic and non-pathogenic isolates occur (Dahm, 1989a; Dahm, 1989b; Dahm, 1990).

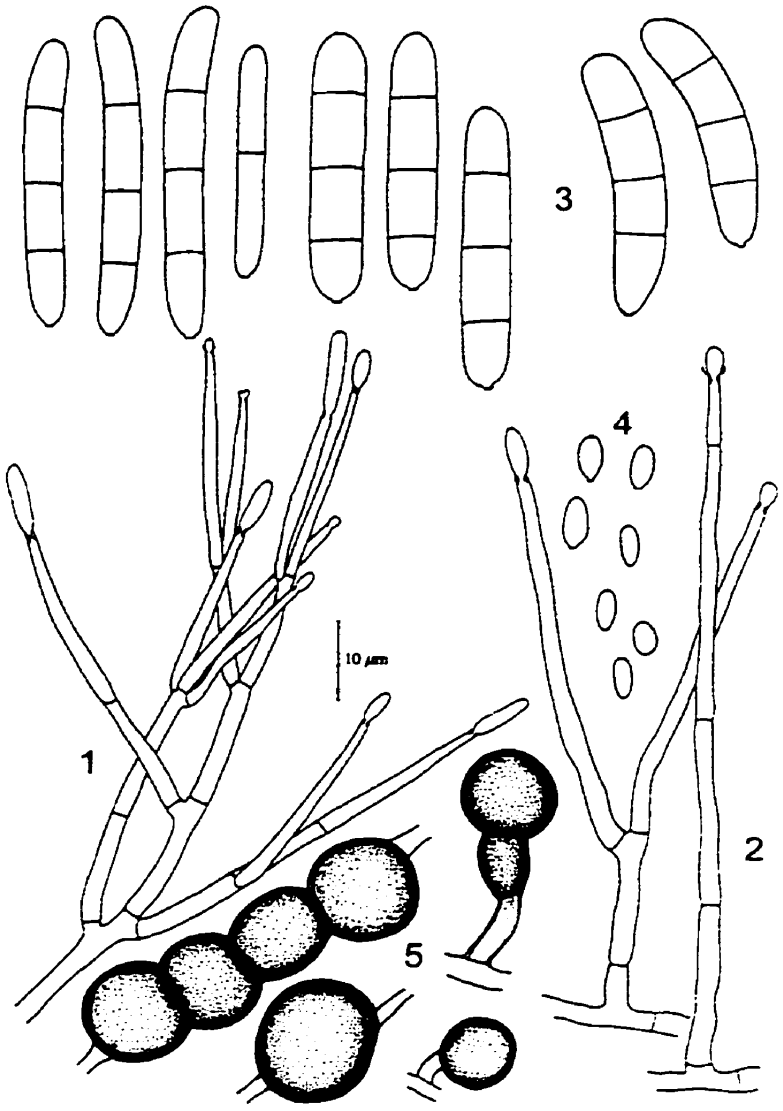

Figure 1. *Cylindrocarpon destructans*. 1) Sporodochial conidiophore with whorls of phialidic conidiogenous cells; 2) Sparsely branched conidiophores from aerial mycelium, with long phialidic conidiogenous cells; 3) Macroconidia; 4) Microconidia; and 5) Chlamydospores. From Samuels and Brayford (1990).

Cylindrocarpon destructans as the causal agent of plant diseases

Pathogenic properties of *C. destructans* have been described elsewhere. It has been reported as a common cause of root rot in many types of crop plants (Booth, 1966; Summerell *et al.*, 1990; Reeleder and Brammall, 1994; Blok and Bollen, 1995) and conifer seedlings (Unestam *et al.*, 1989; Dahm, 1989a; Dahm, 1990; Lilja *et al.*, 1992; Różycki *et al.*, 1990, Beyer-Ericson *et al.*, 1991), and has been identified as a factor responsible for poor natural regeneration in several plant species (Unestam *et al.*, .1989).

Typical symptoms of root damage caused by *C. destructans* to conifer seedlings are stunted growth, needle chlorosis, and browning beginning at the needle tips (Beyer-Ericson *et al.*, 1991). Fine roots are dead in most of the damaged root systems. Severe infestation can lead to death of root and seedlings (Unestam *et al.*, 1989).

On ginseng, *C. destructans* is pathogenic to seedlings and to mature plants (Seifert and Axelrood, 1998). On seedlings, the fungus causes a reddish-brown rot of roots and in some cases the root is completely destroyed, leaving only the remains of the tap root (Reeleder and Brammall, 1994). On mature plants, this species is reported as causing rots of mature 3- to 4-year-old roots ("disappearing root rot") in North America (Hildebrand, 1935). The same disease on ginseng roots has previously been reported in Asia (Matuo and Miyazawa, 1984).

Cylindrocarpon destructans was also identified as the causal agent of a new mold problem of apricot (*Prunus armeniaca*), peach (*P. persica*), and pear (*Pyrus communis*) trees in cold storage. White or pinkish-white mold appeared on buds, leaf scars, and roots of stored, dormant, bare-rooted nursery stock. Inner bark and cambial tissues of infected

trees were dark brown and necrotic. The same symptoms were observed on inoculated trees (Traquair and White, 1992).

A number of environmental stresses and cultural practices are known to predispose plants to lethal invasion by *C. destructans*. These factors include light starvation (Chakravarty and Unestam, 1987), intensive exposure of roots to fungicides, and reduced gas exchange resulted from waterlogging (Unestam et al. 1989). Transplanting, root pruning, and shading associated with dense planting also enhance infection by *C. destructans* (Beyer-Ericson *et al.*, 1991).

The pathogenic behavior of *C. destructans* is not fully understood. The fungus inhabits the surface of healthy fine roots (Matturi and Stenton, 1964). Under favorable conditions, e.g., stressed seedlings incited by the above-mentioned environmental stresses, it invades the roots and produces a toxin, necrolide, that can kill the tissues (Evans *et al.*, 1967). The fungal toxin(s) also seem to have antibiotic effects on other fungi (Unestam *et al.*, 1989)

Molecular methods to analyze fungal diversity

Historically, fungal genetic variation and phylogeny have been studied based on various phenotypic characters, e.g. morphological, physiological and developmental characters, and/or chemical components such as secondary metabolites. The recent development of molecular techniques has enabled the derivation of fungal phylogenies based on the analyses of proteins or nucleic acids, e.g. isozyme analysis, DNA-DNA hybridization, restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), and DNA sequencing. However, these molecular

techniques have been difficult to apply to obligate parasites from which only small amounts of fungal materials are available, or to rare taxa which are available only as herbarium specimens. The polymerase chain reaction (PCR) developed in the mid 1980s has made possible the amplification of particular nucleotide sequences from very small amounts of starting materials (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Mullis and Fallona, 1987). In addition, relatively impure DNA extracts can be used for PCR.

Of the five molecular methods mentioned above, RAPD, RFLP and sequence analysis are the most popular in terms of frequency of publication in fungal phylogenetics. Isozyme analysis allows rapid analysis of large numbers of samples and is usually sufficiently sensitive to detect within-population variation. However, isozyme markers are subject to phenotypic variation depending upon growth conditions, age, and type of tissues, are limited to coding regions of DNA, and are sometimes not sufficiently variable for population analysis (Franke, 1973). On the other hand, the use of DNA-DNA hybridization method has been restricted to cross-hybridization measurements to help define species (Bruns *et al.*, 1991).

Restriction fragment length polymorphism (RFLP) analyses have been used extensively for the investigation of genetic variability and phylogenetic relationships among several genera of phytopathogenic fungi (Michelmore and Hulbert, 1987; Bruns *et al.*, 1991). Differences in banding patterns, called RFLPs, result primarily from single base mutations within the recognition sequence of the endonuclease, or from insertions and deletions within or flanking regions homologous with the probe. Comparison of RFLPs provides a crude estimate of sequence variation between isolates, based upon the assumption that restriction fragments of the same length code for homologous DNA

sequences. Where numbers of polymorphic bands are low this assumption is likely to be true, but as variation increases so does the probability that nonhomologous fragments will by chance be similar in length (Bruns *et al.*, 1991). Another problem of RFLP analysis is that insertion/deletion events will invalidate estimations of nucleotide divergence (Bruns *et al.*, 1991). RFLP of amplified fragments (PCR-RFLP) is now widely used for both fungal phylogeny and taxonomy (Chen, 1992; Liu and Sinclair, 1992; Bernier *et al.*, 1994; Hopple and Vilgalys, 1994; Appel and Gordon, 1995; Donaldson *et al.*, 1995; Erland, 1995; Harrington and Wingfield, 1995; Buscot *et al.*, 1996; Edel *et al.*, 1996; Guillemaud *et al.*, 1996; Leal *et al.*, 1997). PCR-RFLP is a simple and inexpensive method compared with traditional RFLP as it avoids the need for blotting and probing.

Random amplification of polymorphic DNA (RAPD; Williams *et al.*, 1990) or arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland, 1990) is a simple and rapid method for detecting genetic diversity. Genetic diversity is assessed by amplification at low stringency with a single short primer of arbitrary sequence. The technique has been used to detect genetic variation among strains or isolates within a species (Goodwin and Annis, 1991; Cooke *et al.*, 1996; Hseu *et al.*, 1996; Boyd and Carris, 1997; Jeng *et al.*, 1997; Maurer *et al.*, 1997; Pei *et al.*, 1997). However, Bruns *et al.* (1991) warned that some potential problems exist in the use of RAPDs, i.e., ambiguous scoring patterns and inconsistency of results. The authors suggested that amplification conditions need to be rigorously standardized in order to obtain reproducible and consistent results, as the fragment pattern obtained is highly sensitive to concentrations of Mg^{2+}, DNA polymerase, primers and template DNAs, and to cycling temperatures.

DNA sequence analysis is a powerful method for inferring phylogenetic relationships between organisms. Sequence data are preferable to other molecular methods for assessing evolutionary relatedness because they permit straightforward, quantitative interpretation (Pace *et al.*, 1986). The use of DNA sequences for evolutionary studies can overcome many of the problems associated with other molecular approaches (e.g., RFLP and RAPD analyses). Bruns *et al.* (1990) described the benefits of using DNA sequences for phylogenetic analyses as being the fact that 'the large number of characters compared can substantially increase the resolving power'. One can also observe the mode of sequence variation, that is, whether a change is a transversion or transition, silent or selected, and one can measure the degree of nucleotide bias; results from different laboratories can be compared directly, and the publication of sequences and their deposition in electronic databases (GenBank, EMBL, DDBJ) allows the confirmation of results and their application to other taxa without the need to obtain strains or clones, or to repeat experiments.

Before the advent of PCR, recombinant DNA techniques used to obtain sequence information were sufficiently difficult and laborious that the study of large numbers of species or individuals required exceptional efforts. Direct sequencing of amplified DNA fragments has circumvented the need for cloning and thus dramatically reduced the time and effort required for comparative sequencing studies.

Most fungal phylogenetic studies have used sequences from nuclear and mitochondrial rRNA genes clusters (Mitchell *et al.*, 1995). The nuclear rRNA genes (rDNA) of eukaryotes are arranged in tandemly repeated clusters with each cluster containing the genes for the small subunit (18S), 5.8S, and large subunit (25-28S) rRNA

(Gerbi, 1985). These genes show little sequence divergence between closely related species and are useful for phylogenetic studies among distantly related organisms (Bruns *et al.*, 1991; Taylor *et al.*, 1993). Within each repeat, the conserved regions are separated by two internal transcribed spacers (ITS), which show higher level of divergence (Ritland *et al.*, 1993). A third spacer regions, the intergenic spacers (IGS), evolve faster than the subunit genes and can be useful for studying closely related organisms, such as among species within a genus or among populations (Bruns *et al.*, 1991; Ritland *et al.*, 1993; O'Donnell, 1992; Hsiao *et al.*, 1995; Yan *et al.*, 1995). The mitochondrial rRNA (small and large subunits) genes appear to evolve much more rapidly than the nuclear rDNA, and so are also useful for studying closely related organisms (Avise *et al.*, 1987; Moritz *et al.*, 1987).

Fungal mitochondrial DNA genomes and variation

Mitochondrial DNA (mtDNA) has been widely used for evolutionary and population studies in fungi. In addition to its small size, ranging from 17.6 kb in *Schizosaccharomyces pombe* Lindner (Zimmer *et al.*, 1984) to 176 in *Agaricus bitorquis* (Quel.) Sacc. (Hintz *et al.*, 1985), and relative ease of extraction and purification (because of its high copy number), a few particular properties of the mitochondrial genome have led to the adoption of mitochondrial DNA variation in studies concerned with recent phylogenetic comparison. The rate of evolution of the mitochondrial genome exceeds that of single-copy fraction of the nuclear genome by a factor of about 10 (Brown *et al.*, 1979; Bruns and Szaro, 1992); therefore, mitochondrial DNA may be more sensitive measure of the level of variation between populations than some nuclear encoded

markers (Contolini *et al.*, 1992; Taylor, 1986). Also, no evidence exists for methylation of bases in mitochondrial DNA (Bruns *et al.*, 1991); consequently, a potentially confounding factor of nuclear DNA is avoided. Moreover, mitochondrial DNA is the best studied genomic element in fungi (Bruns *et al.*, 1991).

Usually, fungal mtDNA is a covalently closed circular (ccc) DNA. Only a few linear mtDNA species were demonstrated unequivocally, e.g., *Hansenula mrakii* which has 55 kb genome (Wesolowski and Fukuhara, 1981). Although there is a remarkable size polymorphism observed in fungal mtDNA genome, the coding capacity of fungal mtDNA is rather conserved (Bos, 1996). The typical fungal mitochondrial genome encodes eleven genes, representing the necessary subunits required during cellular respiration (cytochrome oxidase 1 - 3, apocytochrome b, NADH dehydrogenase 1 - 6, NADH dehydrogenase 4L), three genes coding the subunits of the ATP-synthetase complex (ATPase 6,8 and 9), two genes coding ribosomal RNAs (small and large subunits of ribosomal RNAs) and multiple tRNAs (Paquin *et al.*, 1997).

In fungi, in addition to considerable size diversity, the internal arrangement of the genes in mtDNA is extremely variable. This suggests that many insertion and deletion events and extensive sequence arrangements have been involved during the evolution of the fungal mitochondrial genome. The extent of this variation in fungi is demonstrated when the mitochondrial genomes in a range of yeast species are examined, though it should be emphasized that these species probably represent a wide range of taxonomic relationships. Among the yeasts, the size of the mtDNA ranges from about 19 kb in *Torulopsis glabrata* (Clark-Walker and Sriprakash, 1982) and *Schizosaccharomyces pombe* (Wolf *et al.*, 1982) to about 75 kb in *Saccharomyces cerevisiae* (Borst and Grivell,

1978), and over 100 kb in *Brettanomyces custerii* (McArthur and Clark-Walker, 1983). The gene order and arrangement within the genome of yeasts display very few features in common. The size differences can be accounted for by the presence of noncoding sequences between the genes and variation in the extent of the individual gene themselves, much of this being due to the presence or absence of introns (Hudspeth, 1992). One yeast, *Koeckera africana* (Clark-walker *et al.*, 1981), in common with the water mold, *Achyla bisexualis* (Hudspeth *et al.*, 1983), has been found to contain a duplication of the rRNA region. In the case of *Achyla*, the repeat is large and contains the functional rRNA genes, whereas in *K. africana* only a part of the large rRNA gene is involved.

In other mycelial fungi, where the gene order has been published, it is again found that there is considerable structural and size variation. The three species of which most information is available are *Aspergillus nidulans*, *Neurospora crassa*, and *Podospora anserina* where the sizes of the mitochondrial genomes are 32.4, 62, and 91 kb, respectively (Scazzocchio *et al.*, 1983; Macino, 1980; Kuck and Esser, 1982). Differences in the order of the genes between these three species are apparent, but if it is assumed that inversion has been involved in the divergence of these genomes, then only a small number of such events is required to explain those differences. However, it should be emphasized that the mechanism which led to these rearrangements is not understood at the present time.

In contrast with other organisms, mtDNA inheritance is complex in fungi and not necessarily maternal. Both uniparental maternal and paternal inheritance of mtDNA have been observed in fungi (Taylor, 1986). Biparental inheritance has not been detected in

fungi. However, in some Basidiomycetes colonies may contain a mosaic of mitochondrial genotypes because, although mitochondrial inheritance is maternal, both mating colonies may exchange nuclei through hyphal fusions (Baptista-Ferreira *et al.*, 1983; Casselton and Economou, 1985; May and Taylor, 1988; Hintz, *et al.*, 1988). Instances of mtDNA recombination are not unknown in higher fungi. In *Aspergillus nidulans* and *A. nidulans* var. *echinulatus*, mtDNA recombination was demonstrated following protoplast fusion and selection for extranuclear drug resistance (Earl *et al.*, 1981). *Coprinus cinereus* mtDNA has been shown to recombine in strains with mutant mtDNA crossed on selective media (Baptista-Ferreira *et al.*, 1983). In *Saccharomyces cerevisiae*, mtDNA recombination occurs without forced protoplast fusion or selection of recombinant mtDNA (Thomas and Wilkie, 1968; Fonty *et al.*, 1978). However, the significance of yeast mtDNA recombination to all Ascomycotina is not known.

Variation in mitochondrial DNA has been used in many studies to assess the level of intraspecific variation in fungi. Intraspecific variation was reported to be low compared to interspecific variation in *Sclerotinia* (Kohn *et al.*, 1988), *Armillaria* (Smith and Anderson, 1989), *Phytophthora megasperma* (Förster *et al.*, 1989) and *Agaricus* (Hintz *et al.*, 1988). In contrast, highly variable mitochondrial genomes, and cluster analysis of RFLP patterns correlated to geographic groupings, have been reported in *Pleurotus ostreatus* (Matsumoto and Fukumasa-Nakai, 1995), *Lentinula edodes* (Fukuda *et al.*, 1994), *Ophiostoma ulmi* (Bates *et al.*, 1993; Hintz *et al.*, 1993) and *Cryphonectria parasitica* (Milgroom and Lipari, 1993).

Restriction banding patterns of PCR-amplified mitochondrial large subunit (lsu) and small subunit (ssu) have also been used to separate species within the same genus,

and/or within strains (Kohn *et al.*, 1988; Li *et al.*, 1994; Chen, 1994). In their work, Li *et al.* (1994) suggested the use of a pair of primers (NMS1 and NMS2) which amplify a portion of mitochondrial small subunit rDNA gene. This pair of primers was a modification of another pair of primers (White *et al.*, 1990), designated MS1 and MS2, previously designed for the same locus. Primers NMS1 and NMS2 have been tested to eight fungal genera, i.e., *Aspergillus, Fusarium, Magnaporthe, Mycospharella, Neurospora, Saccharomyces, Sclerotinia* and *Verticillium*, and reproducibly yielded a fragment about 600 bp (Li *et al.*, 1994). The authors claimed that this pair of primers is not only useful for studying fungal cytoplasmic inheritance and for identifying DNA probes that are informative at or below species level, but is also useful for evolutionary and population studies of ascomycetes.

Phylogenetic analysis of DNA sequences

Molecular phylogenetics is the study of evolutionary relationships among organisms based on the comparison of molecular data. In phylogenetic studies, the evolutionary relationships among a group of organisms are illustrated by means of a phylogenetic tree or a phylogram. A phylogenetic tree is a graph composed of nodes and branches, in which only one branch connects any two adjacent nodes. The nodes represent the taxonomic units, and the branches define the relationships among the units in terms of descent and ancestry. The taxonomic units (taxa) represented by the nodes can be species, populations, individuals, or genes. The branching pattern of a tree is called the topology. The lengths of branches can either be scaled or unscaled, the former indicating lengths proportional to the number of molecular changes since divergence

from a common ancestor (Li and Graur, 1991). Phylogenetic trees can be either rooted or unrooted. In a rooted tree there exists a particular node, called a root, from which a unique path leads to any other node. The direction of each path corresponds to evolutionary time, and the root is the common ancestor of all the taxa under study. An unrooted tree is a tree that only specifies the relationships among the taxa and does not define the evolutionary path (Li and Graur, 1991).

A group of taxa is said to be monophyletic if they are derived from a single common ancestor but is said to be polyphyletic if they are derived from more than one common ancestor. A monophyletic group, called a clade, consists of an ancestor and all of its descendants. A statement that a group "appears" or "occurs" on a tree, or that a tree "contains" a group, implies that the group is monophyletic on the tree (Swofford, 1991). The ingroup is a set of taxa, often assumed monophyletic, designated as being the focus of interest, as compared to the outgroups, which are brought into the analysis to provide a broader phylogenetic context to aid in determining the root of the ingroup or ancestral states (Farris, 1972, 1982; Watrous and Wheeler, 1981; Maddison *et al.*, 1984).

There are many methods for constructing trees from molecular data. According to the type of data used, they can be divided into two categories: distance methods and discrete-character methods. In distance methods, evolutionary distance is computed for all pairs of taxa or DNA (or amino acid) sequences and a phylogenetic tree is constructed by considering the relationship among these distances. Once distance values are obtained, there are several ways of obtaining a tree. Distance methods include unweighted pair group method with arithmetic means (UPGMA; Sneath and Sokal, 1973), transformed distance (Farris, 1977), Fitch and Margoliash (FM; Fitch and

Margoliash, 1967), minimum evolution (Saitou and Imanishi, 1989), distance Wagner (Farris, 1972), neighborliness (Sattath and Tversky, 1977), and neighbor-joining (NJ; Saitou and Nei, 1987). In discrete-character methods, data with discrete character states such as nucleotide states in DNA sequences are used, and a tree is constructed by considering the evolutionary relationship of taxa or DNA sequences at each character or nucleotide position. Discrete-character methods include maximum parsimony (Fitch 1971), evolutionary parsimony (Lake, 1987) and maximum likelihood (Felsenstein, 1981). Some types of molecular data, e.g., DNA hybridization data, exist only as distance data. Therefore, phylogenetic trees for these data can be constructed only by distance methods. By contrast, discrete-character data can usually be converted into distance data, so that they can be analyzed either by distance methods or by discrete-character methods (Nei, 1991).

It has often been argued that character-state methods (e.g., maximum parsimony method) are more powerful than distance methods (Farris, 1981; Penny, 1982), because the raw data are a string of character states (e.g., the nucleotide sequence) and in transforming character-state data into distance matrices some information is lost. However, Li (1997) argued that this method is often less efficient than some distance matrix methods. The author reasoned that, while the maximum parsimony method indeed uses the raw data, it usually uses only a small fraction of the available data. This method is generally very effective if the number of informative sites (sites which differ for at least two of the taxa included) is large (Li, 1997).

Despite its drawbacks, the maximum parsimony method remains the most popular in terms of frequency of publication (Goldman, 1997). This can be attributed to two

main factors: the widespread availability of friendly computer programs (e.g., PAUP) which implement the method, and the speed of parsimony algorithm (Goldman, 1997). This method operates by selecting trees that minimize the total tree length: the number of evolutionary steps, i.e., transformations from one character state to another (Swofford *et al.*, 1996).

The consistency index (CI) has traditionally been used to evaluate the fit of character data on phylogenetic hypotheses (Kluge and Farris, 1969). The maximum value is 1, which corresponds to a complete fit; no homoplasy (a collection of phenomena that leads to similarities in character states for reasons other than inheritance from a common ancestor, i.e., convergence, parallelism, and reversal) is involved, and changes in any particular character (position) appear only once on the cladogram, so that no convergence, parallelisms or reversals are involved.

The bootstrap technique has been frequently used as a means to estimate the confidence level of phylogenetic hypothesis (Li, 1997). Bootstrapping methods are a general set of methods for creating pseudoreplicate data sets in situations where true resampling is impractical or impossible, as in the case of phylogenetics where evolutionary events cannot be replayed (Hillis *et al.*, 1996). In phylogenetic analyses, the pseudoreplicate data sets are generated by randomly sampling the original character matrix with replacement to create new character matrices of the same size as the original (Felsenstein, 1985). The frequency with which a given branch is found upon analysis of these pseudoreplicate data sets is recorded as the bootstrap proportion. These proportions can be used to assess the reliability of individual branches.

MATERIAL AND METHODS

Fungal isolation and storage

Fungal isolates for this study were made available through various sources and by isolation from the field. Isolates of *Cylindrocarpon destructans* obtained from Burnaby Mountain, British Columbia, were isolated by a modification of the method described by Lévesque (1990), as follows. Seedlings of trees and shrubs were washed under running tap water to remove soil from roots. The roots were excised by cutting the stems immediately above the crown and were cut into sections 2-3 cm in length with a sterile scalpel. A 90-mm autoclaved #1 Whatman filter paper was placed on top of a filter paper in a Buchner funnel. The funnel was filled with 20% bleach (Javex, Colgate-Palmolive Inc., Toronto). A three-way valve was placed in sequence after the funnel connecting into a vacuum source. The root sections were floated for 2 minutes during which time they were spread with a set of sterile forceps. After the 2-minutes sterilization time, the solution was quickly withdrawn. The procedure was repeated using a 50% EtOH solution at a 2-minutes sterilization time, followed by rinsing with sterile distilled water whilst under suction. The filter paper with the adhering root sections was then inverted onto a 90-mm petri dish of potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) amended with 250 mg ampicillin (Aldrich Chemical Co., Milwaukee, WI) per mL and 10 mg rifampicin (Sigma Chemical Co., St. Louis, MO) per mL . The paper and the root sections were pressed onto the agar with a sterile, bent glass rod. A small quantity of sterile distilled water was squirted onto the paper to cause the release of the root sections. The paper was then removed leaving the root sections spread on the agar. The cultures

were incubated at room temperature (23 + 3°C) in a 12 h light/dark cycle of fluorescent lamps. Fungi emerging from tissue sections were transferred to PDA and simple nutrient agar (SNA) (Nirenberg, 1981) and identified using taxonomic keys (Booth, 1966). For long-term storage, isolates were maintained on PDA at room temperature.

Isolates collected for this study originated in Canada, French Guinea, Jamaica, Puerto Rico, and United States, and were collected from a variety of host plants. The isolate designation, host, area, year of isolation and source of isolates are listed in Table 1. Cultures were maintained on PDA in 90-mm petri dishes and stored at room temperature.

DNA isolation

Initially, the nucleic acid was isolated using the protocol used in Dr. C.A. Lévesque's research laboratory. Isolates were grown in V-8 liquid medium in 250-mL flasks at room temperature under static, dark conditions. After 1-2 weeks growth, the mycelia of each fungus were harvested by vacuum filtration, frozen at –86°C and freeze-dried. Two to five grams of freeze-dried mycelium was ground to a fine powder, suspended in 10 mL of extraction buffer [20 mM ethylenediaminetetraacetic acid (EDTA); 50 mM Tris-HCl, pH 8; 2% sodium dodecyl sulfate (SDS)], and incubated at 65°C for 1 hour after the addition of proteinase K at final concentration of 200 µg/mL. Cell debris was removed by centrifugation at 15000 rpm for 20 minutes at 4°C, the supernatant was transferred to a fresh tube and 2-2.5 volumes of cold 95% EtOH added.

Table 1. Source, area and year of isolation, and host of isolates of *Cylindrocarpon* spp. included in this study.

Isolate	Source	Area and year of isolation	Substrate /Host
Cylindrocarpon candidulum (teleomorph *Nectria veiullotiana*)			
1. GJS 91-116	G. J. Samuels	Virginia, U.S.A., 1991	*Quercus*
Cylindrocarpon coronatum (teleomorph *N. coronata*)			
1. CTR 71-19	G. J. Samuels	Jamaica, ?	?
Cylindrocarpon cylindroides (teleomorph *N. neomacrospora*)			
1. P4c2n22ad	P. Axelrood	Pemberton, B.C., 1991	Douglas-fir (*Pseudotsuga mensiezii*)
2. P3p3n12cb	P. Axelrood	Pemberton, B.C., 1991	Douglas-fir (*P. mensiezii*)
3. C2cun2ab2	P. Axelrood	Chilliwack, B.C., 1991	Douglas-fir (*P. mensiezii*)
Cylindrocarpon destructans (teleomorph *N. radicicola*)			
1. JAT1378	J. Traquair	Harrow, Ont.,	Ornamental dogwood (*Cornus floridae*)
2. JAT1551	J. Traquair	Leamington, Ont.,	Peach (*Prunus persica*)
3. JAT 1901	J. Traquair	Leamington, Ont.,	*Lilium sp.*
4. P3p3n17c1	P. Axelrood	Pemberton, B.C., 1991	Douglas-fir (*P. mensiezii*)
5. C2cun1cc2	P. Axelrood	Chilliwack, B.C., 1991	Douglas-fir (*P. mensiezii*)
6. Ph29 283d2p	P. Axelrood	Pemberton, B.C., 1993	Douglas-fir (*P. mensiezii*)
7. Cr26 162bku	P. Axelrood	Reid Collins, B.C., 1993	Douglas-fir (*P. mensiezii*)
8. Cr1884bcp	P. Axelrood	Pemberton, B.C., 1993	Douglas-fir (*P. mensiezii*)
9. Cr21 152tbp	P. Axelrood	Surrey, B.C., 1993	Douglas-fir (*P. mensiezii*)
10. Cr26 15bcp	P. Axelrood	Reid Collins, B.C., 1993	Douglas-fir (*P. mensiezii*)
11. Cr18 81bbu	P. Axelrood	Peltons, B.C., 1993	Douglas-fir (*P. mensiezii*)
12. Ph29 234d1p	P. Axelrood	Pemberton, B.C., 1991	Douglas-fir (*P. mensiezii*)
13. C2cun9ae	P. Axelrood	Chilliwack, B.C., 1991	Douglas-fir (*P. mensiezii*)
14. C1cun5aa	P. Axelrood	Chilliwack, B.C., 1991	Douglas-fir (*P. mensiezii*)
15. RTDF14	R. Tanjung	Surrey, B.C., 1996	Douglas-fir (*P. mensiezii*)
16. RTP1	R. Tanjung	Surrey, B.C., 1996	Pine (*Pinus contorta*)
17. BCMAFFcdes1	V. Joshi	B.C.,?	*Trillium sp.*
18. BCMAFFcdes2	V. Joshi	B.C.,?	Ginseng (*Panax quinquefolius*)
19. FMa2.14	F. Mantiri	Burnaby, B.C., 1997	Salal (*Gaultheria shallon*)
20. FMc2.21	F. Mantiri	Burnaby, B.C., 1997	Western red cedar (*Thuja plicata*)
21. FMd2.1	F. Mantiri	Burnaby, B.C., 1997	Red alder (*Alnus rubra*)
22. FMe1.2	F. Mantiri	Burnaby, B.C., 1997	Thimbleberry (*Rubus parviflora*)
23. Fmi1.13	F. Mantiri	Burnaby, B.C., 1997	Willows (*Salix bebiana*)
24. FMh1.4	F. Mantiri	Burnaby, B.C.., 1997	Elderberry (*Sambucus racemosa*)

Table 1. continued from preceding page.

Isolate	Source	Area and year of isolation	Substrate /Host
Cylindrocarpon heteronemum (teleomorph *N. galligena*)			
1. JR0609B-2	J. Rahe	Surrey, B.C., 1996	Apple (*Malus pumila*)
2. GBA1	G. Braun	Nova Scotia, ?	Apple (*M. pumila*)
Cylindrocarpon ianothele var. *majus* (teleomorph *Nectria discophora*)			
1. GJS 91-116	G. J. Samuels	Puerto Rico, 1996	Bark of recently dead tree
Cylindrocarpon rugulosum (teleomorph *N. rugulosa*)			
1. GJS 86-222	G. J. Samuels	French Guinea, 1986	Bark of recently dead tree
Tubercularia sp. (teleomorph *N. cinnabarina*)			
1. GJS 91-111	G.J. Samuels	Virginia, U.S.A., 1991	Recently dead *Acer* branchlets.

The DNA was precipitated at –20°C for 4 hours, followed by centrifugation at 4°C for 20 minutes. The pellet was washed with cold 70% ethanol, resuspended in 400 μL of 1xTE (10 mM Tris, pH 7.5 with 10 MM EDTA), and transferred to a 1.5 mL microcentrifuge tube. Following the addition of 4 μL RNAse (1 μg/uL), the solution containing DNA was incubated at 37°C for 30 minutes. Extremely viscous pellet was additionally extracted once with TE saturated phenol, and twice with chloroform:isoamyl alcohol (24:1) to reduce polysaccharide contamination. DNA was precipitated from the aqueous layer by the addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of 95% cold ethanol, followed by incubation at –20 or –70°C for 20 minutes. The precipitated DNA was pelleted by centrifugation and washed with cold 70% ethanol and finally resuspended in 20 to 200 mL TE buffer, depending on the size of the pellet.

Later, a quicker method to prepare genomic DNA as templates for PCR was used (Walsh *et al.*, 1991). Mycelia were scraped from 7-week-old cultures using a sterile P-200 pipette tip, suspended in 1 mL 5% w/v Chelex 100 (100-200 mesh, Na+ form, Biorad) in sterile distilled water in a 1.5 mL microcentrifuge tube, and incubated at 57°C for 20 minutes or more. The sample was then vortexed vigorously for 10 seconds, boiled for 8 minutes, and vortexed again. Cell debris was removed by centrifugation at maximum speed for 3 minutes. The resulting supernatant was removed and used directly in PCR reactions.

DNA amplification with polymerase chain reaction (PCR)

A portion of the mitochondrial small subunit rRNA coding region was defined by primers NMS1 (5'CAGC AGTG AGGA ATAT TGGT CAATG) and NMS2 (5' GCGG ATCA TCGA ATTA AATA ACAT) (Li, 1994) and amplified by the polymerase chain reaction (PCR) (Mullis and Fallona, 1987; Saiki *et al.*, 1988). The PCR reaction (50 μL) included approximately 20 – 60 ng fungal genomic DNA as template, 200 μM each of the four deoxyribonucleotide triphosphates (dNTP) (Perkin-Elmer, Norwalk, CT), 1.5 mM $MgCl_2$, 10 μM of each primer, 1X reaction buffer [20 mM Tris-Hcl, pH 8.4, 50mM KCl (Gibco BRL, Burlington, ON), 2.5 U *Taq* DNA Polymerase (Gibco BRL, Burlington, ON)], and 50 μL light paraffin oil to overlay the total volume. A DNA-free control tube was included with each run as a safeguard against contamination. PCR was performed with a programmable DNA thermocycler (GTC Thermal Cycler). The initial cycle consisted of 3 min at 95 °C for DNA denaturation, 1 min at 56 °C for primer annealing and 1 min at 72 °C for primer extension; this was followed by 34 cycles consisting of 95°C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. The final step was a 4-min chain extension step at 72 °C. A portion (2 μL) of the amplified products was electrophoresed in 0.5% TBE buffer in an electrophoresis apparatus at 40 V for 2 hours. The gel was stained with ethidium bromide (0.5 μg/mL) for 10 min, examined under UV light and photographed. The PCR products were purified with NucleotraP®CR (Macherey-Nagel, Duren, Germany). The pure DNA sample was finally eluted with 50 μL sterile pure water and stored at –20 °C until required for sequencing.

DNA sequencing

The purified PCR products as described above were used as templates for sequencing using a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech, Inc., Cleveland, Ohio, USA). This kit combines two innovations for sequencing DNA using radioactive labels, i.e., the use of four [α-^{33}P] dideoxy-nucleotide (ddNTP) terminators (Evans and Read, 1992) and a thermostable enzyme called Thermo Sequenase DNA polymerase. The sequencing reactions were performed according to the protocol provided by the manufacturer. Both strands were sequenced using primers NMS1 and NMS2. Contiguous sequences were assembled by the ESEE software package (Cabot, 1990).

For automated DNA sequencing, the PCR products described above were sequenced on both strands employing the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA polymerase (FS), following the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA). Sequence reaction mixtures were run on a 4.75% acrylamide gel containing 8.3M urea in the ABI 373A DNA Sequencing/Reading System from the same manufacturer. All sequences were verified by sequencing both complementary strands utilizing both primers NMS1 and NMS2.

DNA sequence alignment and phylogenetic analyses

DNA sequences were aligned using the computer program MULTALIN version 5.3.3. (Corpet, 1988) on the internet (see http://www.toulouse.inra.fr/multalin.html). Alignment of all sequences was checked and optimized visually. Alignment gaps,

representing putative insertion-deletion (indel) sites, were coded either as "missing data" (gap=missing coding) or as character states, using a coding scheme adapted from Hibbett *et al.* (1995). Gap = missing coding is commonly employed (Hibbett and Vilgals, 1993) but has been criticized on the grounds that it ignores potentially informative sites (Baum *et al.*, 1994) or can lead to errors in phylogenetic estimates because of the way parsimony analyses (e.g., PAUP) may assign values where character states are unknown (Maddison, 1994). One alternative to treating gaps as missing data is to code all gaps as a fifth state (Swofford, 1993). However, under this coding, a single-insertion-deletion event that results in a multiple-base indel will be overweighted because adjacent nucleotide positions (with gaps) are coded as independent characters. Indels may also be coded as separate characters, with gaps coded as missing data in the body of the alignment. The indel coding used in this study (Hibbett *et al.*, 1995) is a hybrid of these approaches (Figure 2). Steps in coding for indels using PAUP 3.1s (Swofford, 1993) were as follows: 1) Indels were identified from the alignments and divided into two categories: single-base indels and multiple-base indels. 2) For each single-base indel, a separate character was created that reproduced the position with the indel, with nucleotides intact, except that the gap symbol (-) was replaced by 0 (e.g., a single-column in an alignment with AA--CC would be reproduced as AA00CC). These new characters were appended to the matrix at the end of the aligned sequences (Figure 2, row B, C). 3) A character set was defined that included all the positions in the body of the aligned sequences with single-base indels (the "single indel set"). 4) For each multiple-base indel, a separate binary characters (states = 0 or 1) was created and these were appended to the aligned sequences (Figure 2, row D, E). 5) A character set (the "all indel set") was designated

which combined the set of single- and multiple-base indel characters. The "all indels set" was set off at the end of the block of aligned sequences (as in Figure 2). To run an analysis under gap = missing coding the "all indels" character set was simply excluded (with the format command gapmode= missing data). To run an analysis under indel coding, the "all indels" character set was included, and the "single indels"character set was excluded (gapmode = missing data). In other words, under indel coding, nucleotide positions with single base indels would be scored as characters, with gaps as fifth state; multiple-base indels would be scored as binary characters; and single nucleotide positions aligned to multiple-base indel sites would be scored as additional characters with gaps as missing data. The goals of this coding scheme were to include potentially informative indels in the analyses, avoid overweighting single insertion-deletion events, and preserve phylogenetically informative nucleotide variation in sequences that aligned to gaps.

Parsimony analyses were performed using PAUP 3.1.1 (Swofford, 1993) running on Macintosh computers. The main analyses were performed with indel coding and gap = missing coding. Maximum parsimony trees were inferred using heuristic search algorithm. Support for the phylogenetic groupings was obtained with bootstrap analyses (Felsenstein, 1985). *Tubercularia* sp. and *Fusarium* sp. were used as the outgroups for constructing phylogenetic trees of fungal species within the genus *Cylindrocarpon*. The sequence for *Fusarium* sp. was obtained from the EMBL database (accession number U34519, deposited by O'Donnell *et al.*, 1997). These species were chosen as outgroups because their teleomorphic genus is *Nectria*, which is also the teleomorphic genus of all known *Cylindrocarpon* spp. For intraspecific analysis among isolates of *C. destructans*, *C. cylindroides* was chosen as the outgroup.

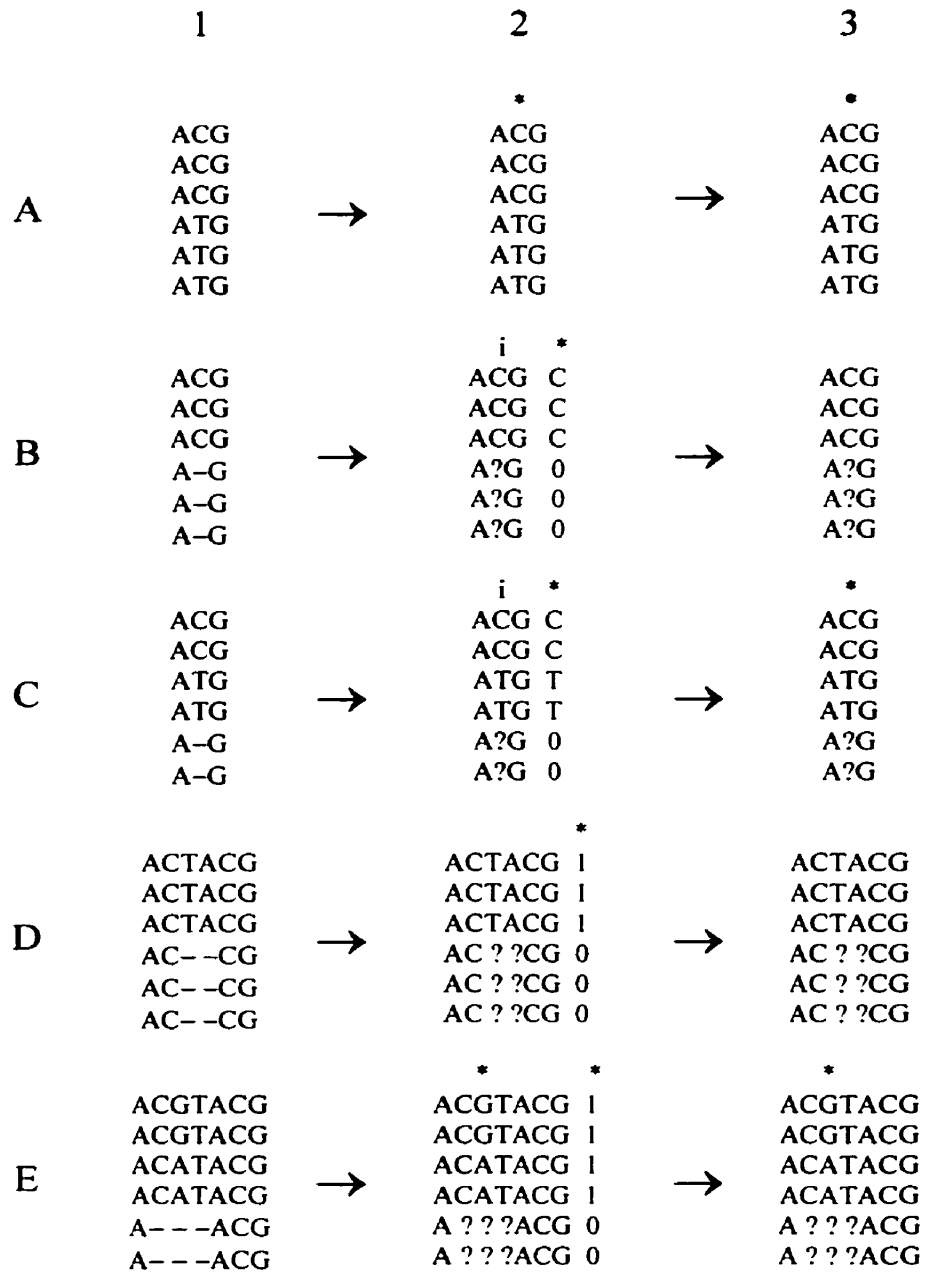

Figure 2. Comparisons of character coding from hypothetical DNA sequences under indel coding and gap = missing coding (Hibbett *et al.*, 1995). Column 1: aligned DNA sequences, with gaps indicated by dashes. Column 2: character coding under indel coding. Column 3: character coding under gap = missing coding. Potentially informative characters recognized under indel coding and gap = missing coding are indicated by asterisks. Positions that are omitted from analyses under indel coding are indicated by "i". Recoded indel characters under indel coding are appended to sequences in column 2. Rows A-E depict different classes of sequence variation. Row A: single base position with informative nucleotide variation and no gaps. Row B: single base position with gaps but without informative nucleotide variation. Row C: single base position with informative nucleotide variation and informative gaps. Row D: multiple base indel with no informative nucleotide variation. Row E: multiple base indel with informative nucleotide variation at one position.

In most analyses, all transformations were weighted equally. To explore the effects of incorporating a transition:transversion bias, a series of analyses with gap = missing coding and stepmatrices specifying transition and transversion biases of 1:3 and 1:10 were also run. The effects of transition:transversion bias were explored because the assumption that all nucleotide substitutions occur randomly is unrealistic in most cases (Li, 1997). Transitions (substitutions of a purine for a purine or a pyrimidine for a pyrimidine) are generally more frequent than transversions (substitutions of purines for pyrimidines or vice versa), and one might therefore give less weight to the former events than to the latter (Fitch and Ye, 1991).

Distance analyses were performed using PHYLIP 3.7c (Felsenstein, 1995), running on an IBM PC P200 computer. Aligned sequences in Nexus format were transformed into PHYLIP format manually. Pairwise distance matrices were generated from the aligned sequences using the DNADIST program of PHYLIP; the Kimura (1980) model of nucleotide substitution and a 2:1 transition:transversion bias was assumed. The Kimura model assumes equal frequencies of all nucleotides. Pairwise distances estimated with the Kimura model are based on nucleotide substitutions at all positions, and do not take indels into account. Phylogenies were estimated from distance matrices using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA, Sneath and Sokal, 1973) using the NEIGHBOR program. Bootstrapping was performed with Kimura and UPGMA analyses (1000 replicates, using the SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE programs). Only the phylogenies for intraspecific analysis of *C. destructans* isolates were generated using distance analysis.

A method developed by Templeton *et al.* (1992) was also used to construct phylogenetic trees for intraspecific analysis of *C. destructans*. This method was developed to estimate gene trees under assumption of low levels of divergence (i.e., intraspecific relationships), where few nucleotide substitutions separate operational taxonomic units (OTUs). This method has its greatest statistical power when the number of sequences shared among taxa is large and the number of differences is small, which is the opposite of more traditional methods such as maximum parsimony and distance analyses (Crandall, 1994). This power is obtained by conditioning the probability of a parsimonious connection between OTUs by the number of nucleotides shared between OTUs. Thus, for a pair of OTUs that differ by few nucleotides and share many, the probability of a parsimonious connection is greater than that between a pair of OTUs that differ by many nucleotides and share few (Crandall, 1994).

RESULTS

Interspecific variation among *Cylindrocarpon* species

PCR product and sequence alignment

By using primers NMS1 and NMS2 to amplify mitochondrial small subunit rDNA region, all the fungi tested yielded a single product (Figure 3 and 4). The amplified products ranged in size from 551 bp (*C. coronatum*) to 682 bp (*C. heteronemum*). From the multiple sequence alignment using MULTALIN version 5.3.3, followed by a manual adjustment (Figure 5), it was apparent that most of the length differences were due to insertions and deletions in two distinct variable regions that alternate with three relatively conserved regions, which nonetheless had a number of small length mutations scattered throughout. Nucleotide divergence among *Cylindrocarpon* species was 2.3-7.4% (Table 3).

Parsimony analysis

The aligned DNA sequence data matrix of seven *Cylindrocarpon* species (12 isolates) and two outgroup species (*Fusarium* sp. *and Tubercularia* sp.) under gap = missing coding comprised 756 characters of which 152 were variable and 73 were parsimony informative (Table 2). Under indel coding there were 780 characters of which 185 were variable and 100 were parsimony informative. The difference is due to two classes of characters that are ignored under gap = missing coding: 1) positions that have a potentially informative distribution of gaps, but no

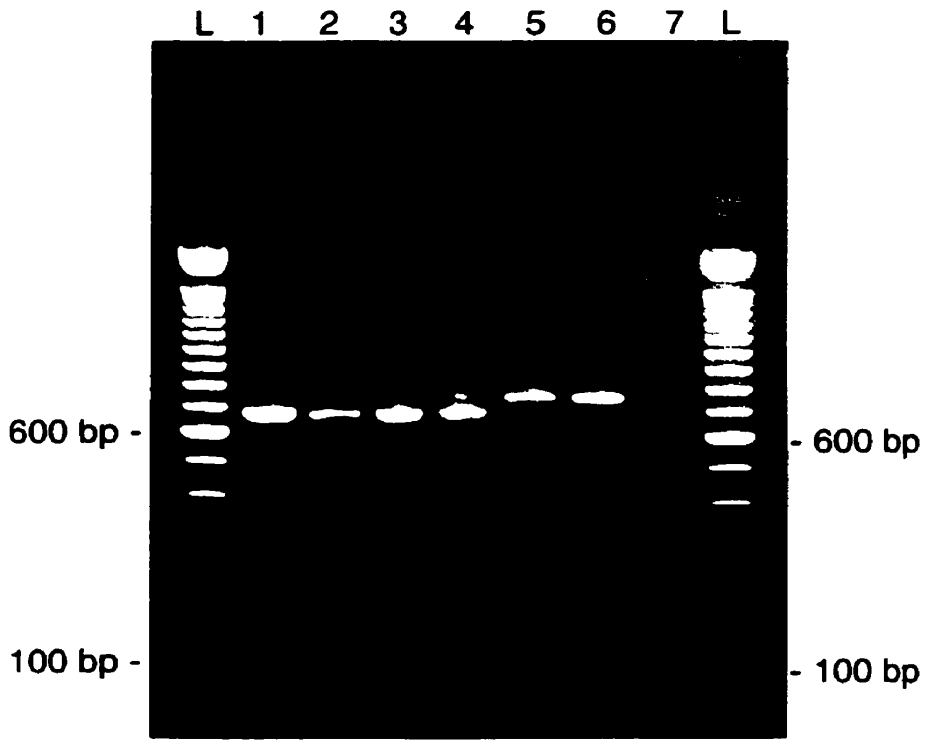

Figure 3. Agarose gel of PCR products of *Cylindrocarpon* spp. obtained with primers NMS1 and NMS2 (gel 1). Lanes L, 100 bp DNA ladder (BRL); lane 1, *Tubercularia* sp.; lane 2, *C. destructans* (JAT 1551); lane 3, *C. destructans* (RTDF14); lane 4, *C. destructans* (FMd2.1); lane 5, *C. heteronemum* (JR0609B-2); lane 6, *C. heteronemum* (GBA1); lane 7, negative control.

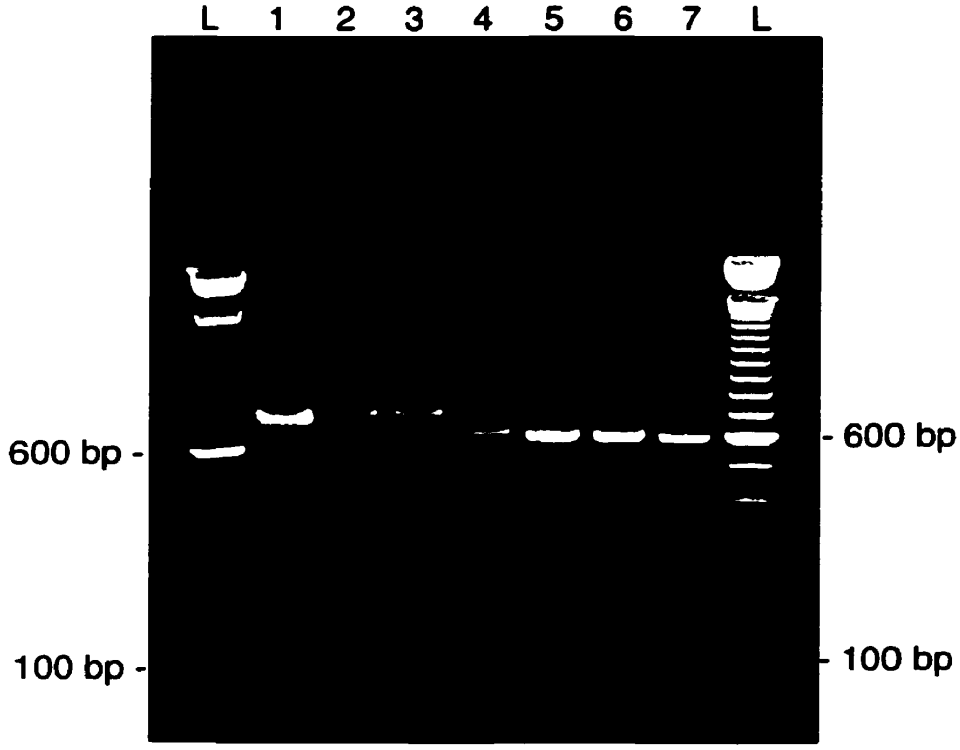

Figure 4. Agarose gel of PCR products of *Cylindrocarpon* spp. obtained with primers NMS1 and NMS2 (gel 2). Lanes L, 100 bp DNA ladder (BRL); lane 1, *C. cylindroides* (P4c2n22ad); lane 2, *C. cylindroides* (P3p3n12cb); lane 3, *C. cylindroides* (C2cun2ab2); lane 4, *C. candidulum*; lane 5, *C. rugulosum*; lane 6, *C. coronatum*; lane 7, *C. ianothele* var. *majus*.

Table 2. Distribution of different classes of characters between the two coding schemes

Coding schemes	Number of characters	Number of variable characters	Number of informative characters
Missing coding	756	152	73
Indel coding	780	185	100

informative nucleotide variation (five characters; e.g., Figure 2, row B) and, 2) multiple indels (21 characters; e.g. Figure 2, row D).

Parsimony analysis of the entire data set under gap = missing with no transition:transversion bias coding using the heuristic search algorithm yielded 3 equally parsimonious trees. The 50% majority rule consensus tree (216 steps, CI = 0.903) of the three most parsimonious trees along with the bootstrap values is shown in Figure 6. Analyses with gap = missing coding and transition:transversion bias weighted at 1:3 and 1:10 each yielded trees with identical topologies with those of produced under gap = missing coding with no transition:transversion bias. Under indel coding, parsimony analysis also yielded three equally parsimonious trees. The 50% majority rule consensus tree (269 steps, CI = 0.862) generated under this coding scheme along with the bootstrap values is shown in Figure 7.

The inferred phylogenies resolved the interspecific relationships in the genus. Under gap = missing coding, the monophyly of *Cylindrocarpon* was supported by 74 % bootstrap frequency and there were three well-supported groups in *Cylindrocarpon* as measured by bootstrapping. These clades have been designated groups I-III (Figure 6 and 7). Group I (bootstrap 82%) includes five isolates (two species; i.e., *C. heteronemum* and *C. cylindroides*). Group II

(bootstrap 95%) includes four isolates (four species; i.e., *C. coronatum*, *C. ianothele* var. *majus*, *C. candidulum*, and *C. rugulosum*). Group III (bootstrap 98%) includes three isolates of *C. destructans*. Although phylogenetic analysis under indel coding produced trees with slightly different topologies, these three well-supported groups were also evident under this coding scheme. Group I and II received stronger bootstrap support under indel coding than under gap = missing coding, whereas group III received slightly weaker bootstrap support than under gap = missing coding. In addition, under indel coding, monophyly of *Cylindrocarpon* spp. could not be supported statistically.

Figure 5. Aligned sequences of mitochondrial SSU rDNA of *Cylindrocarpon* spp. Positions identical to reference sequence (*Fusarium* sp.) indicated by dots. Gaps, representing putative insertion-deletion sites, indicated by dashes. Final block of characters, after aligned sequences, are recoded indel characters . Numbers in parentheses are isolate numbers (see Table 1).

```
                                10         20         30         40         50
                                .          .          .          .          .
Fusarium sp.                    -GCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTCTTCCAGTATG
Tubercularia sp.                GC.....................................AT.A.T--
C. heteronemum (2)              GC.....................................T..AT.A.T--
C. heteronemum (1)              GC.....................................T..AT.A.T--
C. cylindroides (2)             GC.....................................T..AT.A.T--
C. cylindroides (1)             GC.....................................T..AT.A.T--
C. cylindroides (3)             GC.......A.............................T..AT.A.T--
C. destructans (15)             GC.....................................T..AT.A.T--
C. destructans (21)             GC.....................................T..AT.A.T--
C. destructans (2)              GC................GC...................T..AT.A.T--
C. candidulum                   GC.............................T....T..AT.ACC--
C. rugulosum                    GC.....................................T..AT.A.T--
C. coronatum                    GC.....................................T..AT.A.T--
C. ianothele                    GC...TG.CG.......................T.....T..AT.ACT--
var. majus

                                60         70         80         90         100
                                .          .          .          .          .
Fusarium sp.                    GGGAGCAAAACAGCTATGGGTCAAGTCCG-------ATATCTTTAGGAGA
Tubercularia sp.                --------TCT..TAT.A...----------------..T...A.------
C. heteronemum (2)              --------..T..TA...AAGGCGCAAGC-------TCT...A.------
C. heteronemum (1)              --------..T..TA...AAGGCGCAAGC-------TCT...A.------
C. cylindroides (2)             --------..T..TA..TAA.----------------..T...A.------
C. cylindroides (1)             --------..T..TA..TAA.----------------..T...A.------
C. cylindroides (3)             --------..T..TA..TAA.----------------..T...A.------
C. destructans (15)             --------..T..TA...AA.----------------..T...A.------
C. destructans (21)             --------..T..TA...AA.----------------..T...A.------
C. destructans (2)              --------..T..TA..TAA.----------------..T...A.------
C. candidulum                   --------..T..A..CTTA.GCGC.G.TTCGTCAG.G....A.------
C. rugulosum                    --------..T..TA..TTA.----------------..T...A.------
C. coronatum                    --------..T..TA..TTA.----------------..T...A.------
C. ianothele                    ---------.T.TT.-----------------------.T.T...------
var. majus

                                110        120        130        140        150
                                .          .          .          .          .
Fusarium sp.                    AGTCTTATTGTGAGGGCGAGTTATATAACACCATAGGACTGGCCGTCCCA
Tubercularia sp.                --------------------A.T.....----------------------
C. heteronemum (2)              --------------------..T.....----------------------
C. heteronemum (1)              --------------------..T.....----------------------
C. cylindroides (2)             --------------------..T.....----------------------
C. cylindroides (1)             --------------------..T.....----------------------
C. cylindroides (3)             --------------------..T.....----------------------
C. destructans (15)             --------------------..T.....----------------------
C. destructans (21)             --------------------..T.....----------------------
C. destructans (2)              --------------------..T.....----------------------
C. candidulum                   --------------------..T.....----------------------
C. rugulosum                    --------------------..T.....----------------------
C. coronatum                    --------------------..T.....----------------------
C. ianothele                    --------------------..T..A..----------------------
var. majus
```

```
                                160       170       180       190       200
                                .         .         .         .         .
Fusarium sp.                    TATGAAAAGATTATATTAGAATTGAATGAAGCTTTGTTTATATATTGATA
Tubercularia sp.                --------....C.....................................
C. heteronemum (2)              --------....C.....................................
C. heteronemum (1)              --------....C.....................................
C. cylindroides (2)             --------....C.....................................
C. cylindroides (1)             --------....C.....................................
C. cylindroides (3)             --------....C.....................................
C. destructans (15)             --------A...C.....................................
C. destructans (21)             --------A...C.....................................
C. destructans (2)              --------A...C..... ...............................
C. candidulum                   --------A...C.....................................
C. rugulosum                    --------A...C.....................................
C. coronatum                    --------A...C.....G...............................
C. ianothele                    --------A...C.....................................
var. majus

                                210       220       230       240       250
                                .         .         .         .         .
Fusarium sp.                    ATGACAGTATATATATCGTGTCTTGACTAATTGCGTGCCAGCAGTCGCGG
Tubercularia sp.                ..................................................
C. heteronemum (2)              ..................................................
C. heteronemum (1)              ..................................................
C. cylindroides (2)             ..................................................
C. cylindroides (1)             ..................................................
C. cylindroides (3)             ..................................................
C. destructans (15)             ..................................................
C. destructans (21)             ..................................................
C. destructans (2)              ..................................................
C. candidulum                   ..........................C....A..................
C. rugulosum                    ..........................C....A..................
C. coronatum                    .........................G.C....A..........C.....
C. ianothele                    ..........................C....A.......CG........
var. majus

                                260       270       280       290       300
                                .         .         .         .         .
Fusarium sp.                    TAATACGTAAGAGACTAGTGTTATTCATCTTAATTAGGTTTAAAGGGTAC
Tubercularia sp.                ..................................................
C. heteronemum (2)              ..................................................
C. heteronemum (1)              ..................................................
C. cylindroides (2)             ..................T...............................
C. cylindroides (1)             ..................................................
C. cylindroides (3)             ..................................................
C. destructans (15)             ..................................................
C. destructans (21)             ..................................................
C. destructans (2)              ..................................................
C. candidulum                   ..................................................
C. rugulosum                    ..................................................
C. coronatum                    ..................................................
C. ianothele                    ..................................................
var. majus
```

```
                              310         320         330         340         350
                               .           .           .           .           .

Fusarium sp.                  CCAGACGGTCAATATAGCTTATAAAATGTTAGTACTTGACTAGAGTTTTA
Tubercularia sp.              ...A.....A.....C..T.........................
C. heteronemum (2)            ..................C..C.........................
C. heteronemum (1)            ..................C..T.........................
C. cylindroides (2)           ..................C..T.........................
C. cylindroides (1)           ..................C..T.........................
C. cylindroides (3)           ..................C..T.........................
C. destructans (15)           ...............T...C..T.........................
C. destructans (21)           ...............T...C..T.........................
C. destructans (2)            ...............T...C..T.........................
C. candidulum                 ..................C..G.........................
C. rugulosum                  ..................C..T.........................
C. coronatum                  ..............C...C..G.........................
C. ianothele var. majus       ...........A.....C..G.....G....T.............

                              360         370         380         390         400
                               .           .           .           .           .

Fusarium sp.                  TGTAAGAGGGCAGTACTTGAGGAGGAGAGATGAAATTTCGTGATACCAAA
Tubercularia sp.              ....................................CTA.........
C. heteronemum (2)            ........................................................
C. heteronemum (1)            ........................................................
C. cylindroides (2)           ........................................................
C. cylindroides (1)           ........................................................
C. cylindroides (3)           ........................................................
C. destructans (15)           ........................................................
C. destructans (21)           ........................................................
C. destructans (2)            ........................................................
C. candidulum                 ....................................C...................
C. rugulosum                  ....................................C...................
C. coronatum                  ....................................C...................
C. ianothele var. majus       .A.G....T..............A............C...................

                              410         420         430         440         450
                               .           .           .           .           .

Fusarium sp.                  GGGACTCTGTAAAGGCGAAGGCAGCCCTCTATGTAAAAACTGACGTTGAA
Tubercularia sp.              ..............................A.....................
C. heteronemum (2)            ...................................G.....................
C. heteronemum (1)            ...................................G.....................
C. cylindroides (2)           ........................................................
C. cylindroides (1)           ........................................................
C. cylindroides (3)           ........................................................
C. destructans (15)           ...................................G.....................
C. destructans (21)           ...................................G.....................
C. destructans (2)            ...................................G.....................
C. candidulum                 .......A.....................................T.....................
C. rugulosum                  .......G.....T.................G.....................
C. coronatum                  .......A.....................................T.....................
C. ianothele var. majus       .......G.................A.........TT.....................
```

```
                                460       470       480       490       500
                                .         .         .         .         .
Fusarium sp.                    GGACGAAGGCACAGAGAACAAACAGGATTAGATACCCAAGTAGTCTTTGC
Tubercularia sp.                ..........T....TC.............................CG
C. heteronemum (2)              ...........................................
C. heteronemum (1)              ...........................................
C. cylindroides (2)             ...........................................
C. cylindroides (1)             ...........................................
C. cylindroides (3)             ...........................................
C. destructans (15)             ...........................................
C. destructans (21)             ...........................................
C. destructans (2)              ...........................................
C. candidulum                   ....................................T.A.....
C. rugulosum                    ....................................T.A.....
C. coronatum                    ....................................T.A.....
C. ianothele                    ..............T.....................T.A.....
var. majus

                                510       520       530       540       550
                                .         .         .         .         .
Fusarium sp.                    AGTAAATGATGAATGCCATAGGTCAGATAACCAGTTAATGTTTATAG--T
Tubercularia sp.                ..........................GT.AG.T......G.AG...GCC
C. heteronemum (2)              ..........................CTAT........C.-C.GCGG.
C. heteronemum (1)              ..........................CTAT........C.-C.GCGG.
C. cylindroides (2)             ..........................CTAT........C.-C.GCGG.
C. cylindroides (1)             ..........................CTAT........C.-C.GCGG.
C. cylindroides (3)             ..........................CTAT........C.-C.GCGG.
C. destructans (15)             ..........................CTAA.T..C.........TAT.
C. destructans (21)             ..........................CTAA.T.............AT.
C. destructans (2)              ..........................CTAA.T.............AT.
C. candidulum                   .......................T......--------------------
C. rugulosum                    .......................T......--------------------
C. coronatum                    ............................--------------------
C. ianothele                    ............................T-------------------
var. majus

                                560       570       580       590       600
                                .         .         .         .         .
Fusarium sp.                    CTAATAGGGTTAGCCTAG-------CAAACTAATGACATAGACTAT----
Tubercularia sp.                GGCT..TT.CATAG.G.AAA------...ACTG-.CT.AG.CTCG-----
C. heteronemum (2)              GA..ACAT.CC..GA.T.AATCTAT.G...CTG..CT.G.TTT.T.TGTC
C. heteronemum (1)              GA..ACAT.CC..GA.T.AATCTAT.G..ACTG..CT.G.TTT.T.TGTC
C. cylindroides (2)             G...ACAA.CC..TA.T.AATCTAT....ACTG..CTTGCT.A.T.TTTT
C. cylindroides (1)             G...ACAA.CC..TA.T.AATCTAT....ACTG..CTTGCT.A.T.TTTT
C. cylindroides (3)             G...ACAA.CC..TA.T.AATCTAT....ACTG..CTTGCT.A.T.TTTT
C. destructans (15)             A...A..C.CCT.AA.T.--------GC.ACTT..CA.A.A.T...----
C. destructans (21)             A...A..C.CCT.AA.T.--------GC.A.TT..CA.A.A.T...----
C. destructans (2)              A...A..C.CCTTAA.T.--------GC.ACTT..CA.A.A.T...----
C. candidulum                   ----------------------------------------------------
C. rugulosum                    ----------------------------------------------------
C. coronatum                    ----------------------------------------------------
C. ianothele                    ----------------------------------------------------
var. majus
```

```
                          610        620        630        640        650
                           .          .          .          .          .

Fusarium sp.              ----------------------------------------------CCATT
Tubercularia sp.          ----------------------GACTACACAT----------------.A
C. heteronemum (2)        TGCTGGATCTTTCGAAACATGCTATAAAAAATAAGAAGCCTGTATA....
C. heteronemum (1)        TGCTGGATCTTTCGAAACATGCTATAAAAAATAAGAAGCCTGTATA....
C. cylindroides (2)       TG-TGTATTTAT----ACAT-CGAAAAGATAT-----GCCTGTATA....
C. cylindroides (1)       TG-TGTATTTAT----ACAT-CGAAAAGATAT-----GCCTGTATA....
C. cylindroides (3)       TG-TGTATTTAT----ACAT-CGAAAAGATAT-----GCCTGTATA....
C. destructans (15)       --------------------------AAATAT----------------.C
C. destructans (21)       --------------------------AAATAT----------------.A
C. destructans (2)        --------------------------AAATAT----------------.A
C. candidulum             -------------------------------------------------.
C. rugulosum              -------------------------------------------------.
C. coronatum              --------------------------------------------------
C. ianothele              --------------------------------------------------
var. majus

                          660        670        680        690        700
                           .          .          .          .          .

Fusarium sp.              AAAAATATTTGGTCTATAAATGAAAGTGTAAGCATTTCACCTCAAGAGTA
Tubercularia sp.          ..C.T.....................................................
C. heteronemum (2)        ....TA....................................................
C. heteronemum (1)        ....TA....................................................
C. cylindroides (2)       ....TA....................................................
C. cylindroides (1)       ....TA....................................................
C. cylindroides (3)       ....TA....................................................
C. destructans (15)       ....T.....................................................
C. destructans (21)       ....T.....................................................
C. destructans (2)        ....T.....................................................
C. candidulum             CT..T.....................................................
C. rugulosum              CT..T.....................................................
C. coronatum              ----T.G.C.................................................
C. ianothele              ---T.AT..............T.....................................
var. majus

                          710        720        730        740        750
                           .          .          .          .          .

Fusarium sp.              ATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGACACCAGTAG
Tubercularia sp.          ..........C...............................................
C. heteronemum (2)        ...........................................................
C. heteronemum (1)        ...........................................................
C. cylindroides (2)       ...........................................................
C. cylindroides (1)       ...........................................................
C. cylindroides (3)       ...........................................................
C. destructans (15)       ...........................................................
C. destructans (21)       ...........................................................
C. destructans (2)        ...........................................................
C. candidulum             ...........................................................
C. rugulosum              ...........................................................
C. coronatum              ........................................A..................
C. ianothele              ...........................................................
var. majus
```

		760	770	780	790
		.	.	.	.
Fusarium sp.	TGAAG-	01A110A111T1T10100C1T1T0000000001T1A0			
Tubercularia sp.	TGAAGT	G0T100A001A1A1111001010000001100OT1AT			
C. heteronemum (2)	TGAAGT	G0A110T00101011111C1T1T111GC11111T1AT			
C. heteronemum (1)	TGAAGT	G0A110T00101011111C1T1T111GC11111T1AT			
C. cylindroides (2)	TGAAGT	G0A100A00101011111C1T1T1010C11011T1AT			
C. cylindroides (1)	TGAAGT	G0A100A00101011111C1T1T1010C11011T1AT			
C. cylindroides (3)	TGAAGT	G0A100A00101011111C1T1T1010C11011T1AT			
C. destructans (15)	TGAAGT	G0A100A001T1T1110001T1T0000001000T1AT			
C. destructans (21)	TGAAGT	G0A100A001T1T1110001T1T0000001000T1AT			
C. destructans (2)	TGAAGT	G0A100A001T1T1110001T1T0000001000T1AT			
C. candidulum	TGAAGT	G0A111A000000000000000000000000001AT			
C. rugulosum	TGAAGT	G0A100A000000000000000000000000001AT			
C. coronatum	TGAAGT	G0A100A00000000000000000000000000T			
C. ianothele var. *majus*	TGAAGT	G000000000000000000000000000000000TT			

Table 3. Pairwise genetic distances among the different *Cylindrocarpon* mitochondrial SSU rDNA sequences analyzed under gap = missing coding. Below diagonal: absolute distances (base substitutions between two sequences). Above diagonal: mean distances (adjusted for missing data) Numbers in parentheses next to the species name are isolate numbers of corresponding species (see Table 1)

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *Fusarium* sp.	-	0.112	0.100	0.101	0.085	0.083	0.085	0.080	0.075	0.083	0.068	0.056	0.062	0.087
2. *Tubercularia* sp.	68	-	0.104	0.101	0.099	0.098	0.099	0.100	0.097	0.099	0.076	0.059	0.065	0.090
3. *C. heteronemum* (2)	62	65	-	0.003	0.038	0.036	0.038	0.053	0.053	0.058	0.055	0.040	0.044	0.070
4. *C. heteronemum* (1)	63	63	2	-	0.035	0.033	0.035	0.050	0.050	0.055	0.055	0.038	0.044	0.070
5. *C. cylindroides* (2)	52	62	25	33	-	0.002	0.003	0.053	0.053	0.055	0.045	0.031	0.038	0.070
6. *C. cylindroides* (1)	51	61	24	22	1	-	0.002	0.052	0.052	0.053	0.043	0.029	0.036	0.068
7. *C. cylindroides* (3)	52	62	25	23	2	1	-	0.053	0.053	0.055	0.045	0.031	0.038	0.068
8. *C. destructans* (15)	49	62	33	31	33	32	33	-	0.006	0.011	0.045	0.032	0.034	0.070
9. *C. destructans* (21)	46	60	33	31	33	32	33	4	-	0.008	0.045	0.032	0.034	0.070
10. *C. destructans* (2)	51	61	36	34	34	33	34	7	5	-	0.047	0.034	0.036	0.074
11. *C. candidulum*	38	42	31	31	25	24	25	25	25	26	-	0.023	0.029	0.062
12. *C. rugulosum*	31	33	22	21	17	16	17	18	18	19	13	-	0.024	0.062
13. *C. coronatum*	34	36	24	24	21	20	21	19	19	20	16	13	-	0.064
14. *C. ianothele* var. *majus*	47	49	38	38	38	37	37	38	38	40	34	34	35	-

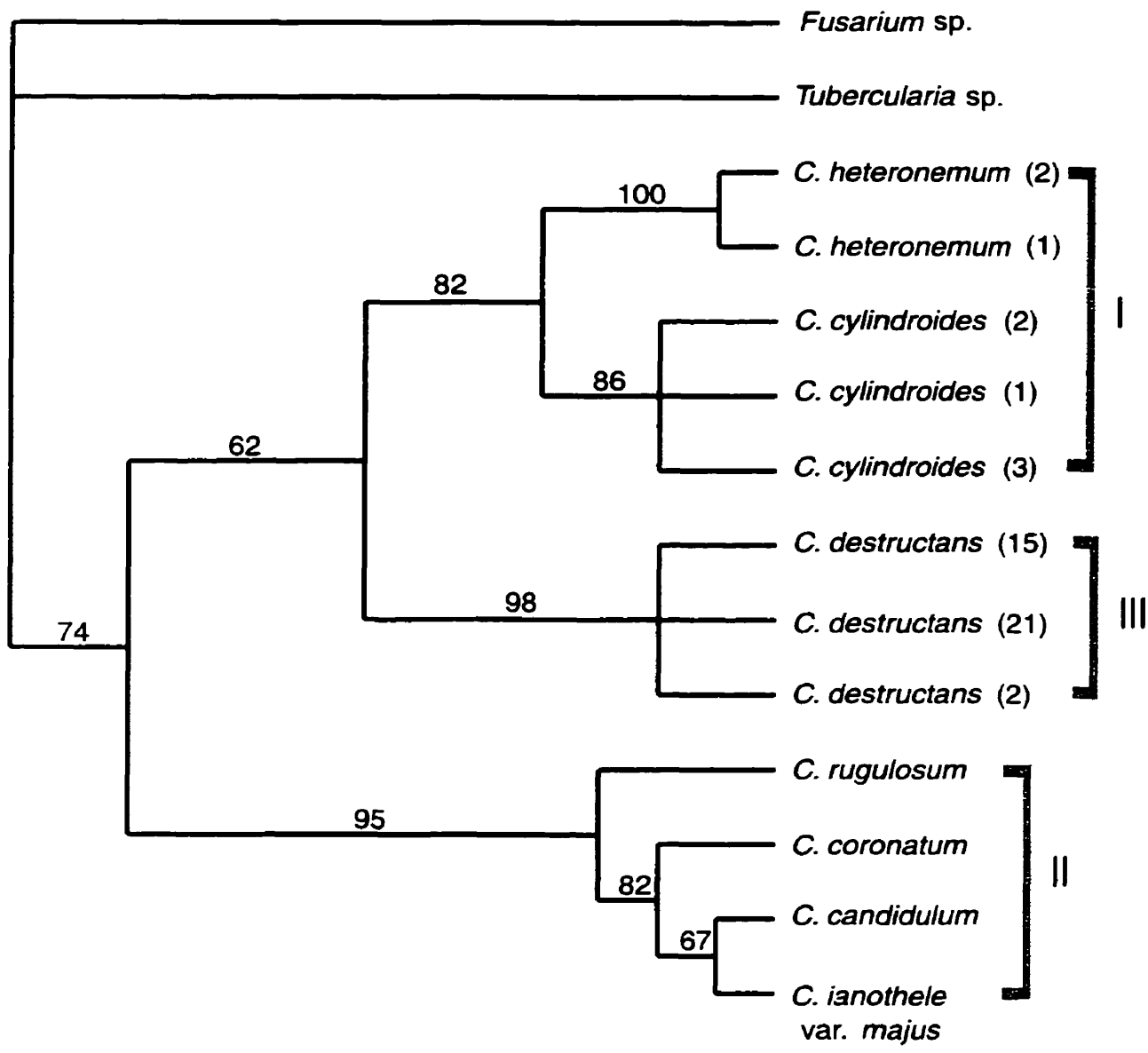

Figure 6. Majority-rule consensus tree (216 steps, CI = 0.903) resulting from 100 bootstrap replications of maximum parsimony analysis of *Cylindrocarpon* data set using the heuristic search algorithm of PAUP 3.1.1 under gap = missing coding. Bootstrap values above 50% are given adjacent to the corresponding node. Numbers in parentheses next to species name are isolate numbers (see Table 1). Bracketed groups I - III are discussed in text.

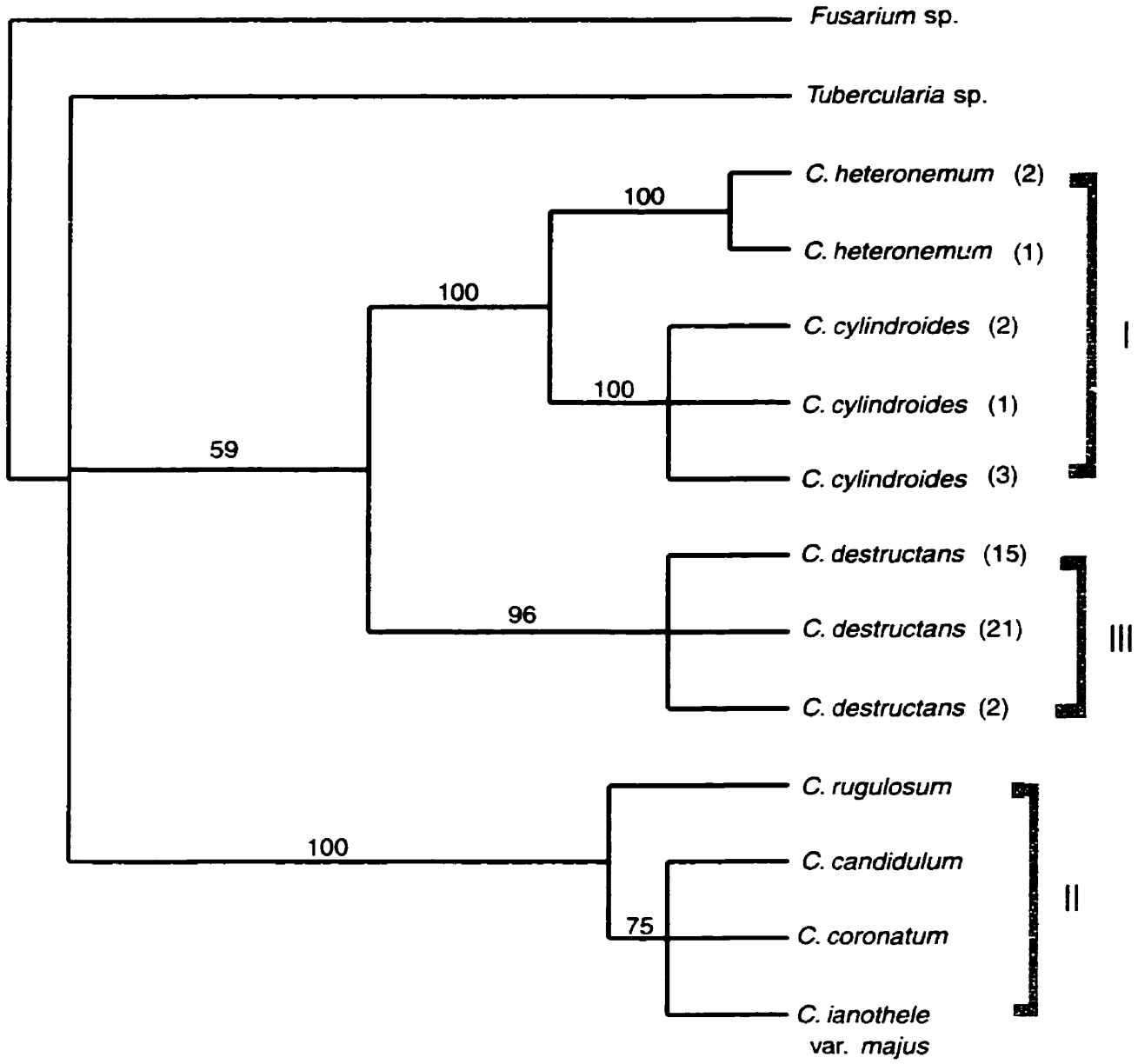

Figure 7. Majority-rule consensus tree (269 steps, CI = 0.862) resulting from 100 bootstrap replications of maximum parsimony analysis of *Cylindrocarpon* data set using the heuristic search algorithm of PAUP 3.1.1 under indel coding. Bootstrap values above 50% are given adjacent to the corresponding node. Numbers in parentheses next to species name are isolate numbers (see Table 1). Bracketed groups I - III are discussed in text.

Intraspecific variation among isolates of *Cylindrocarpon destructans*

Fungal isolation

All but one isolate of *C. destructans* obtained from Douglas-fir that were used in this study were kindly provided by Dr. P. Axelrood. To obtain isolates from other host plant species, I selected six host plant species that grew within Burnaby Mountain area. Initially, attempts to isolate *C. destructans* were both laborious and time consuming because, even after surface sterilization, a large number of colonies needed to be plated and screened. After 1 week, the number of plates was reduced by selecting isolates with cultural characteristics of *C. destructans*. Typical characteristics of *C. destructans* on PDA were initially white colonies, becoming cream-colored to pale brown, and 15 to 25 mm diameter after 1 week. Aerial mycelia were cottony or tuft-like. Cream-colored to tan slimy conidial masses covered the surface of mycelial tufts, especially toward the centre of the colonies. By 5 to 6 weeks, the underside of colonies become yellowish-brown to dark brown. Based on cultural characteristics, *Trichoderma* sp., *Penicillum* sp., *Verticillium* sp., and *Fusarium* sp. were among other fungi commonly observed growing from surface-sterilized roots.

The task of isolating *C. destructans* became easier when Dr. P. Axelrood provided me with a recipe for special nutrient agar (SNA). Using this medium, conidiation of *C. destructans* could be observed as early as 4 days after inoculation. Under a dissecting microscope the fungus was easily differentiated by the presence of characteristic microconidia borne on lateral or terminal phialides. Putative isolates of *C. destructans* were selected based on this observation after comparing with positive isolates provided by Dr. P. Axelrood. Hyphae from the putative colonies of *C. destructans* were transferred to PDA medium for further microscopic examinations.

Under the compound microscope, all putative isolates showed morphological characteristics of *C. destructans* as described by Booth (1966). Macroconidia were abundant on 6-week-old cultures and were hyaline, one to three septate and cylindrical in shape with rounded ends, and measured 23 to 30 by 4 to 6 μm. Microconidia were oval to elliptical, sometimes slightly curved, hyaline, and measured 6 to 9 by 3 to 4 μm. Chlamydospores were observed within and on the surface of agar and could be easily seen even under a dissecting microscope. They were globose to elliptical with brown walls, terminal or in intercalary chains, and measured 8 to 12 by 7 to 11 μm.

Isolates of *C. destructans* were found on the roots of all six of the selected host plant species from Burnaby Mountain, B.C. For each host plant species, several isolates from individual plants were obtained. However, preliminary sequence and RFLP analyses (data not included) revealed no differences among individual isolates obtained from each of several individual plants of a species studied. Therefore, only one isolate from each host plant species was chosen for sequence analysis.

PCR product and sequence alignment

PCR experiments conducted on the mitochondrial small subunit rDNA region of 24 isolates of *C. destructans* reproducibly amplified a fragment of 621 bp (Figure 8 to 10). Multiple sequence alignment using MULTALIN 5.3.3 among *C. destructans* isolates indicated extremely low levels of polymorphism (Figure 11), with sequence divergence ranging from 0 to 1.1 % (Table 4).

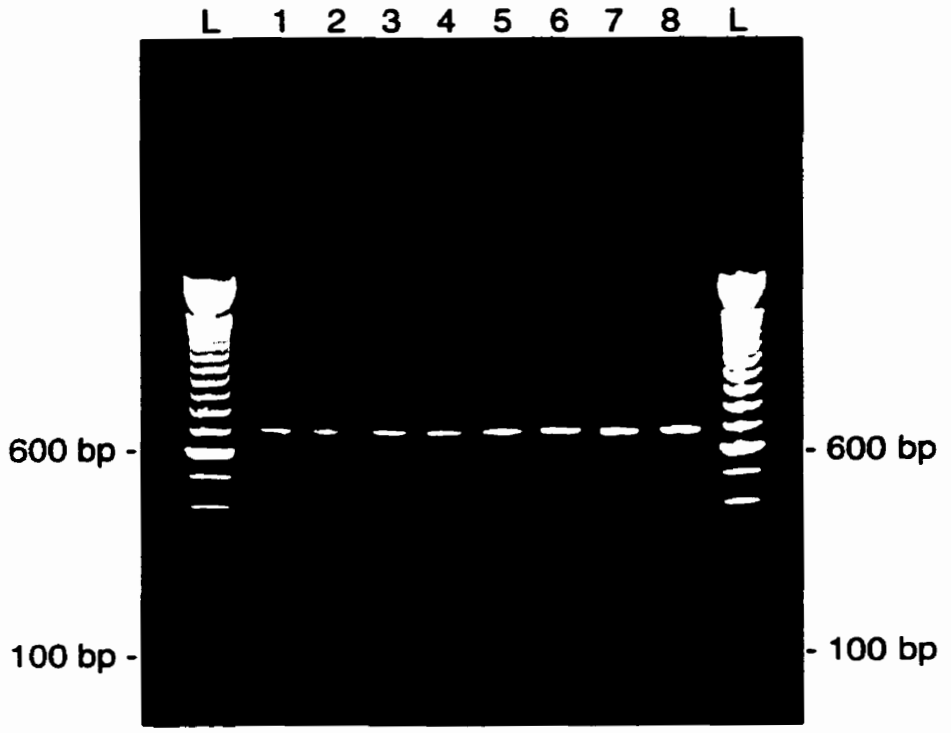

Figure 8. Agarose gel of PCR products of isolates of *Cylindrocarpon destructans* obtained with primers NMS1 and NMS2 (gel 1). Lanes L, 100 bp DNA ladder (BRL); lane 1, JAT 1378; lane 2, JAT 1551; lane 3, JAT 1901; lane 4, P3p3n17c1; lane 5. C2cun1cc2; lane 6, Ph29 283d2p; lane 7, Cr26 162bku; lane 8, Cr18 84bcp.

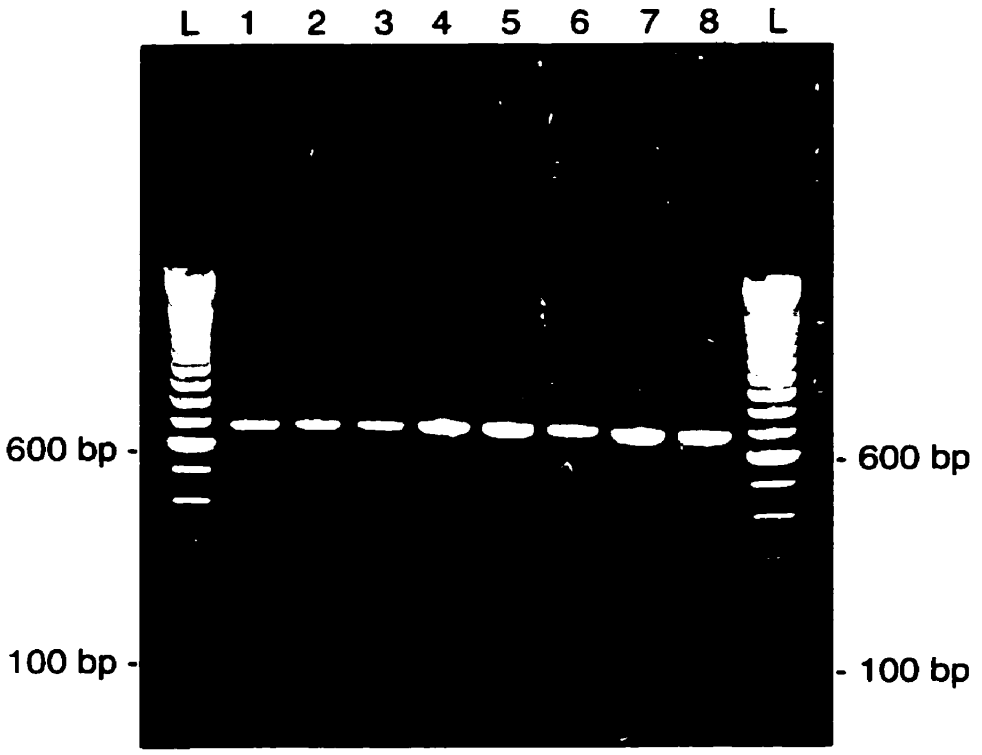

Figure 9. Agarose gel of PCR products of isolates of *Cylindrocarpon destructans* obtained with primers NMS1 and NMS2 (gel 2). Lanes L, 100 bp DNA ladder (BRL); lane 1, Cr21 152tbp; lane 2, Cr26 15bcp; lane 3, Cr18 81bbu; lane 4, Ph29 234d1p; lane 5, C2cun9ae; lane 6, c1cun5aa; lane 7, RTDF14; lane 8, RTP1.

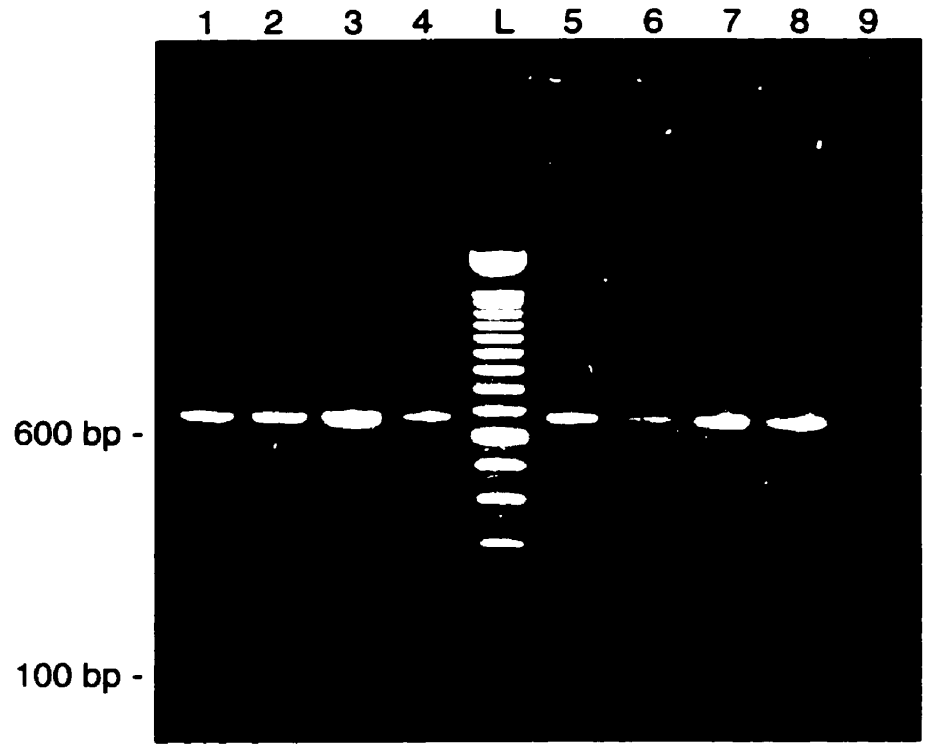

Figure 10. Agarose gel of PCR products of isolates of *Cylindrocarpon destructans* obtained with primers NMS1 and NMS2 (gel 3). Lanes L, 100 bp DNA ladder (BRL); lane 1, BCMAFFCdes1; lane 2, BCMAFFCdes2; lane 3, FMa2.14; lane 4, FMc2.21; lane 5, FMd2.1; lane 6, FMe1.2; lane 7, FMi1.13; lane 8, FMh1.4; lane 9, negative control.

Phylogenetic analyses

The aligned DNA sequence data matrix of 24 isolates of *C. destructans* (excluding the outgroup, *C. cylindroides*) contained only six phylogenetically informative characters. Parsimony analysis using the heuristic search algorithm of PAUP 3.1.1 yielded 300 equally parsimonious trees (45 steps, CI = 0.933). As can be seen in Figure 12, there is no resolution within any of the isolates in the 50% majority-rule consensus tree as measured by bootstrapping.

Similarly, the phylogeny generated using distance method (UPGMA) showed very low resolution within the isolates. Although the phylogeny suggested the existence of five variants (four variants for isolates from British Columbia and one variant from Ontario), only one cluster of isolates in one putative variant received more than 50% bootstrap support (Figure 13).

The phylogenetic tree generated using the algorithm of Templeton *et al.* (1992) showed a better resolution for the clustering of the isolates (Figure 14). In the phylogeny there were three variants. Three isolates from Ontario (obtained from ornamental dogwood, peach and easter lily) belong to one variant. The second variant includes isolates obtained from western red cedar, red alder, pine, *Trillium*, and ginseng which originated from various locations in British Columbia. The third variant includes all isolates obtained from Douglas-fir which originated from various location in B.C. and four isolates originated from Burnaby Mountain (obtained from salal, elderberry, willows, and thimbleberry).

Figure 11. Aligned sequences of mitochondrial SSU rDNA of isolates of *Cylindrocarpon destructans*. Positions identical to reference sequence (*C. cylindroides*) indicated by dots. Gaps, representing putative insertion-deletion sites, indicated by dashes. Numbers in parentheses are isolate numbers (see Table 1).

```
                                10        20        30        40        50
                                .         .         .         .         .
C. cylindroides          GCCTAACGGATGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
C. destructans (4)       .........C.....................................
C. destructans (12)      .........C.....................................
C. destructans (9)       .........C.....................................
C. destructans (22)      .........C.....................................
C. destructans (5)       .........C.....................................
C. destructans (11)      .........C.....................................
C. destructans (6)       .........C.....................................
C. destructans (13)      .........C.....................................
C. destructans (15)      .........C.....................................
C. destructans (14)      .........C.....................................
C. destructans (19)      .........C.....................................
C. destructans (24)      .........C.....................................
C. destructans (23)      .........C.....................................
C. destructans (7)       .........C.....................................
C. destructans (8)       .........C.....................................
C. destructans (10)      .........C.....................................
C. destructans (20)      .........C.....................................
C. destructans (21)      .........C.....................................
C. destructans (16)      .........C.....................................
C. destructans (18)      .........C.....................................
C. destructans (17)      .........C.....................................
C. destructans (1)       .........C.....................................
C. destructans (3)       .........C.....................................
C. destructans (2)       .........C........GC...........................

                                60        70        80        90        100
                                .         .         .         .         .
C. cylindroides          TAGTAATTAATATTTCTATTTTATAAGATTCTATTAGAATTGAATGAAG
C. destructans (4)       ..............................A.....................
C. destructans (12)      ..............................A.....................
C. destructans (9)       ..............................A.....................
C. destructans (22)      ..............................A.....................
C. destructans (5)       .......G......................A.....................
C. destructans (11)      .......G......................A.....................
C. destructans (6)       .......G......................A.....................
C. destructans (13)      .......G......................A.....................
C. destructans (15)      .......G......................A.....................
C. destructans (14)      .......G......................A.....................
C. destructans (19)      .......G......................A.....................
C. destructans (24)      .......G......................A.....................
C. destructans (23)      .......G......................A.....................
C. destructans (7)       ..............................A.....................
C. destructans (8)       ..............................A.....................
C. destructans (10)      ..............................A.....................
C. destructans (20)      .......G......................A.....................
C. destructans (21)      .......G......................A.....................
C. destructans (16)      .......G......................A.....................
C. destructans (18)      .......G......................A.....................
C. destructans (17)      ..............................A.....................
C. destructans (1)       ..............................A.....................
C. destructans (3)       ..............................A.....................
C. destructans (2)       ..............................A.....................
```

```
                                  110        120        130        140        150
                                   .          .          .          .          .
C. cylindroides          CTTTGTTTATATATTGATAATGACAGTATATATATCGTGTCTTGACTAAT
C. destructans (4)       ...........................................................
C. destructans (12)      ...........................................................
C. destructans (9)       ...........................................................
C. destructans (22)      ...........................................................
C. destructans (5)       ...........................................................
C. destructans (11)      ...........................................................
C. destructans (6)       ...........................................................
C. destructans (13)      ...........................................................
C. destructans (15)      ...........................................................
C. destructans (14)      ...........................................................
C. destructans (19)      ...........................................................
C. destructans (24)      ...........................................................
C. destructans (23)      ...........................................................
C. destructans (7)       ...........................................................
C. destructans (8)       ...........................................................
C. destructans (10)      ...........................................................
C. destructans (20)      ...........................................................
C. destructans (21)      ...........................................................
C. destructans (16)      ...........................................................
C. destructans (18)      ...........................................................
C. destructans (17)      ...........................................................
C. destructans (1)       ...........................................................
C. destructans (3)       ...........................................................
C. destructans (2)       ...........................................................

                                  160        170        180        190        200
                                   .          .          .          .          .
C. cylindroides          TGCGTGCCAGCAGTCGCGGTAATACGTAAGAGACTAGTGTTATTCATCTT
C. destructans (4)       ...........................................................
C. destructans (12)      ...........................................................
C. destructans (9)       ...........................................................
C. destructans (22)      ...........................................................
C. destructans (5)       ...........................................................
C. destructans (11)      ...........................................................
C. destructans (6)       ...........................................................
C. destructans (13)      ...........................................................
C. destructans (15)      ...........................................................
C. destructans (14)      ...........................................................
C. destructans (19)      ...........................................................
C. destructans (24)      ...........................................................
C. destructans (23)      ...........................................................
C. destructans (7)       ...........................................................
C. destructans (8)       ...........................................................
C. destructans (10)      ...........................................................
C. destructans (20)      ...........................................................
C. destructans (21)      ...........................................................
C. destructans (16)      ...........................................................
C. destructans (18)      ...........................................................
C. destructans (17)      ...........................................................
C. destructans (1)       ...........................................................
C. destructans (3)       ...........................................................
C. destructans (2)       ...........................................................
```

```
                                 210        220        230        240        250
                                  .          .          .          .          .
C. cylindroides          AATTAGGTTTAAAGGGTACCCAGACGGTCAATATAGCTTCTATAATGTTA
C. destructans (4)       ....................................T.............
C. destructans (12)      ....................................T.............
C. destructans (9)       ....................................T.............
C. destructans (22)      ....................................T.............
C. destructans (5)       ....................................T.............
C. destructans (11)      ....................................T.............
C. destructans (6)       ....................................T.............
C. destructans (13)      ....................................T.............
C. destructans (15)      ....................................T.............
C. destructans (14)      ....................................T.............
C. destructans (19)      ....................................T.............
C. destructans (24)      ....................................T.............
C. destructans (23)      ....................................T.............
C. destructans (7)       ....................................T.............
C. destructans (8)       ....................................T.............
C. destructans (10)      ....................................T.............
C. destructans (20)      ....................................T.............
C. destructans (21)      ....................................T.............
C. destructans (16)      ....................................T.............
C. destructans (18)      ....................................T.............
C. destructans (17)      ....................................T.............
C. destructans (1)       ....................................T.............
C. destructans (3)       ....................................T.............
C. destructans (2)       ....................................T.............

                                 260        270        280        290        300
                                  .          .          .          .          .
C. cylindroides          GTACTTGACTAGAGTTTTATGTAAGAGGGCAGTACTTGAGGAGGAGAGAT
C. destructans (4)       ...........................................................
C. destructans (12)      ...........................................................
C. destructans (9)       ...........................................................
C. destructans (22)      ...........................................................
C. destructans (5)       ...........................................................
C. destructans (11)      ...........................................................
C. destructans (6)       ...........................................................
C. destructans (13)      ...........................................................
C. destructans (15)      ...........................................................
C. destructans (14)      ...........................................................
C. destructans (19)      ...........................................................
C. destructans (24)      ...........................................................
C. destructans (23)      ...........................................................
C. destructans (7)       ...........................................................
C. destructans (8)       ...........................................................
C. destructans (10)      ...........................................................
C. destructans (20)      ...........................................................
C. destructans (21)      ...........................................................
C. destructans (16)      ...........................................................
C. destructans (18)      ...........................................................
C. destructans (17)      ...........................................................
C. destructans (1)       ...........................................................
C. destructans (3)       ...........................................................
C. destructans (2)       ...........................................................
```

```
                                  310        320        330        340        350
                                   .          .          .          .          .
C. cylindroides                   GAAATTTCGTGATACCAAAGGGACTCTGTAAAGGCGAAGGCAGCCCTCTA
C. destructans (4)                ....................................................
C. destructans (12)               ....................................................
C. destructans (9)                ....................................................
C. destructans (22)               ....................................................
C. destructans (5)                ....................................................
C. destructans (11)               ....................................................
C. destructans (6)                ....................................................
C. destructans (13)               ....................................................
C. destructans (15)               ....................................................
C. destructans (14)               ....................................................
C. destructans (19)               ....................................................
C. destructans (24)               ....................................................
C. destructans (23)               ....................................................
C. destructans (7)                ....................................................
C. destructans (8)                ....................................................
C. destructans (10)               ....................................................
C. destructans (20)               ....................................................
C. destructans (21)               ....................................................
C. destructans (16)               ....................................................
C. destructans (18)               ....................................................
C. destructans (17)               ....................................................
C. destructans (1)                ....................................................
C. destructans (3)                ....................................................
C. destructans (2)                ....................................................

                                  360        370        380        390        400
                                   .          .          .          .          .
C. cylindroides                   TGTAAAAACTGACGTTGAAGGACGAAGGCACAGAGAACAAACAGGATTAG
C. destructans (4)                ....G...............................................
C. destructans (12)               ....G...............................................
C. destructans (9)                ....G...............................................
C. destructans (22)               ....G...............................................
C. destructans (5)                ....G...............................................
C. destructans (11)               ....G...............................................
C. destructans (6)                ....G...............................................
C. destructans (13)               ....G...............................................
C. destructans (15)               ....G...............................................
C. destructans (14)               ....G...............................................
C. destructans (19)               ....G...............................................
C. destructans (24)               ....G...............................................
C. destructans (23)               ....G...............................................
C. destructans (7)                ....G...............................................
C. destructans (8)                ....G...............................................
C. destructans (10)               ....G...............................................
C. destructans (20)               ....G...............................................
C. destructans (21)               ....G...............................................
C. destructans (16)               ....G...............................................
C. destructans (18)               ....G...............................................
C. destructans (17)               ....G...............................................
C. destructans (1)                ....G...............................................
C. destructans (3)                ....G...............................................
C. destructans (2)                ....G...............................................
```

```
                                410        420        430        440        450
                                .          .          .          .          .
C. cylindroides                 ATACCCAAGTAGTCTTTGCAGTAAATGATGAATGCCATAGGTCAGATCTA
C. destructans (4)              ....................C.............................
C. destructans (12)             ....................C.............................
C. destructans (9)              ....................C.............................
C. destructans (22)             ....................C.............................
C. destructans (5)              ....................C.............................
C. destructans (11)             ....................C.............................
C. destructans (6)              ....................C.............................
C. destructans (13)             ....................C.............................
C. destructans (15)             ....................C.............................
C. destructans (14)             ....................C.............................
C. destructans (19)             ....................C.............................
C. destructans (24)             ....................C.............................
C. destructans (23)             ....................C.............................
C. destructans (7)              ....................C.............................
C. destructans (8)              ....................C.............................
C. destructans (10)             ....................C.............................
C. destructans (20)             ....................C.............................
C. destructans (21)             ....................C.............................
C. destructans (16)             ....................C.............................
C. destructans (18)             ....................C.............................
C. destructans (17)             ....................C.............................
C. destructans (1)              ....................C.............................
C. destructans (3)              ....................C.............................
C. destructans (2)              ....................C.............................

                                460        470        480        490        500
                                .          .          .          .          .
C. cylindroides                 TAGTTAATGCT-CTGCGGTGTAAACAAGCCAGTATTGAATCTATCAAAAC
C. destructans (4)              A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (12)             A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (9)              A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (22)             A.T......T.TA.ATAT.A....GGC...T.A....-------------
C. destructans (5)              A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (11)             A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (6)              A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (13)             A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (15)             A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (14)             A.T..C...T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (19)             A.T......T.TA.ATAT.A....GGC...T.A....-------------
C. destructans (24)             A.T......T.TA.ATAT.A....GGC...T.A....-------------
C. destructans (23)             A.T......T.TA.ATAT.A....GGC...T.A....-------------
C. destructans (7)              A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (8)              A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (10)             A.T......T.TA.ATAT.A....AGC...T.A....-------------
C. destructans (20)             A.T......T.TA.AGAT.A....AGC...T.A....-------------
C. destructans (21)             A.T......T.TA.AGAT.A....AGC...T.A....-------------
C. destructans (16)             A.T......T.TA.AGAT.A....AGC...T.A....-------------
C. destructans (18)             A.T......T.TA.AGAT.A....AGC...T.A....-------------
C. destructans (17)             A.T......T.TA.AGAT.A....AGC...T.A....-------------
C. destructans (1)              A.T......T.TA.AGAT.A....AGC...TTA....-------------
C. destructans (3)              A.T......T.TA.AGAT.A....AGC...TTA....-------------
C. destructans (2)              A.T......T.TA.AGAT.A....AGC...TTA....-------------
```

```
                                510        520        530        540        550
                                .          .          .          .          .
C. cylindroides                 TGTGCTTGCTAATTTTTTTTGTGTATTTATACATCGAAAAGATATGCCTG
C. destructans (4)              -------..A.C...G------------------.A....AT...AAA.A
C. destructans (12)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (9)              -------..A.C...G------------------.A....AT...AAA.A
C. destructans (22)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (5)              -------..A.C...G------------------.A....AT...AAA.A
C. destructans (11)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (6)              -------..A.C...G------------------.A....AT...AAA.A
C. destructans (13)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (15)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (14)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (19)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (24)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (23)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (7)              -------..A.C...G------------------.A....AT...AAA.A
C. destructans (8)              -------..A.C...G------------------.A....AT...AAA.A
C. destructans (10)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (20)             -------..A.T...G------------------.A....AT...AAA.A
C. destructans (21)             -------..A.T...G------------------.A....AT...AAA.A
C. destructans (16)             -------..A.T...G------------------.A....AT...AAA.A
C. destructans (18)             -------..A.C...G------------------.A....AT...AAA.A
C. destructans (17)             -------..A.T...G------------------.A....AT...AAA.A
C. destructans (1)              -------..A.C...G------------------.A....AT...AAA.A
C. destructans (3)              -------..A.C...G------------------.A....AT...AAAGA
C. destructans (2)              -------..A.C...G------------------.A....AT...AAA.A

                                560        570        580        590        600
                                .          .          .          .          .
C. cylindroides                 TATACATTAAAATAATTTGGTCTATAAATGAAAGTGTAAGCATTTCACCT
C. destructans (4)              .-----.C.....T.....................................
C. destructans (12)             .-----.C.....T.....................................
C. destructans (9)              .-----.C.....T.....................................
C. destructans (22)             .-----.C.....T.....................................
C. destructans (5)              .-----.C.....T.....................................
C. destructans (11)             .-----.C.....T.....................................
C. destructans (6)              .-----.C.....T.....................................
C. destructans (13)             .-----.C.....T.....................................
C. destructans (15)             .-----.C.....T.....................................
C. destructans (14)             .-----.C.....T.....................................
C. destructans (19)             .-----.C.....T.....................................
C. destructans (24)             .-----.C.....T.....................................
C. destructans (23)             .-----.C.....T.....................................
C. destructans (7)              .-----.A.....T.....................................
C. destructans (8)              .-----.A.....T.....................................
C. destructans (10)             .-----.A.....T.....................................
C. destructans (20)             .-----.A.....T.....................................
C. destructans (21)             .-----.A.....T.....................................
C. destructans (16)             .-----.A.....T.....................................
C. destructans (18)             .-----.A.....T.....................................
C. destructans (17)             .-----.A.....T.....................................
C. destructans (1)              .-----.A.....T.....................................
C. destructans (3)              .-----.A.....T.....................................
C. destructans (2)              .-----.A.....T.....................................
```

```
                                610       620       630       640       650
                                .         .         .         .         .
C. cylindroides                 CAAGAGTAATGTGGCAACGCAGGAACTGAAATCACTAGACCGTTTCTGAC
C. destructans (4)              .....................................................
C. destructans (12)             .....................................................
C. destructans (9)              .....................................................
C. destructans (22)             .....................................................
C. destructans (5)              .....................................................
C. destructans (11)             .....................................................
C. destructans (6)              .....................................................
C. destructans (13)             .....................................................
C. destructans (15)             .....................................................
C. destructans (14)             .....................................................
C. destructans (19)             .....................................................
C. destructans (24)             .....................................................
C. destructans (23)             .....................................................
C. destructans (7)              .........C...........................................
C. destructans (8)              .....................................................
C. destructans (10)             .....................................................
C. destructans (20)             .....................................................
C. destructans (21)             .....................................................
C. destructans (16)             .....................................................
C. destructans (18)             .....................................................
C. destructans (17)             .....................................................
C. destructans (1)              .....................................................
C. destructans (3)              .....................................................
C. destructans (2)              .....................................................


                                660
                                .
C. cylindroides                 ACCAGTAGTGAAGT
C. destructans (4)              ..............
C. destructans (12)             ..............
C. destructans (9)              ..............
C. destructans (22)             ..............
C. destructans (5)              ..............
C. destructans (11)             ..............
C. destructans (6)              ..............
C. destructans (13)             ..............
C. destructans (15)             ..............
C. destructans (14)             ..............
C. destructans (19)             ..............
C. destructans (24)             ..............
C. destructans (23)             ..............
C. destructans (7)              ..............
C. destructans (8)              ..............
C. destructans (10)             ..............
C. destructans (20)             ..............
C. destructans (21)             ..............
C. destructans (16)             ..............
C. destructans (18)             ..............
C. destructans (17)             ..............
C. destructans (1)              ..............
C. destructans (3)              ..............
C. destructans (2)              ..............
```

Table 4. Pairwise genetic distances of mitochondrial SSU rDNA sequences of *C. destructans* isolates. Below diagonal: absolute distances (base substitutions between two sequences). Above diagonal: mean distances (adjusted for missing data). Numbers in parentheses are isolate numbers (Table 1).

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. .*C. destructans* (4)	-	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.005	0.005	0.005	0.005	0.005	0.003
2. *C. destructans* (6)	1	-	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.002
3. *C. destructans* (9)	1	1	-	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.002
4. *C. destructans* (22)	2	1	1	-	0.003	0.003	0.003	0.003	0.003	0.005	0.002	0.002	0.002	0.005	0.003
5. *C. destructans* (5)	2	1	1	2	-	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.005	0.003
6. *C. destructans* (11)	2	1	1	2	0	-	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.005	0.003
7. *C. destructans* (12)	2	1	1	2	0	0	-	0.000	0.000	0.002	0.002	0.002	0.002	0.005	0.003
8. *C. destructans* (13)	2	1	1	2	0	0	0	-	0.000	0.002	0.002	0.002	0.002	0.005	0.003
9. *C. destructans* (15)	2	1	1	2	0	0	0	0	-	0.002	0.002	0.002	0.002	0.005	0.003
10. *C. destructans* (14)	3	2	2	3	1	1	1	1	1	-	0.003	0.003	0.003	0.006	0.005
11. *C. destructans* (19)	3	2	2	1	1	1	1	1	1	2	-	0.000	0.000	0.006	0.005
12. *C. destructans* (24)	3	2	2	1	1	1	1	1	1	2	0	-	0.000	0.006	0.005
13. *C. destructans* (23)	3	2	2	1	1	1	1	1	1	2	0	0	-	0.006	0.005
14. *C. destructans* (7)	3	2	2	3	3	3	3	3	3	4	4	4	4	-	0.002
15. *C. destructans* (8)	2	1	1	2	2	2	2	2	2	3	3	3	3	1	-
16. *C. destructans* (10)	2	1	1	2	2	2	2	2	2	3	3	3	3	1	0
17. *C. destructans* (20)	5	4	4	5	3	3	3	3	3	4	4	4	4	4	3
18. *C. destructans* (21)	5	4	4	5	3	3	3	3	3	4	4	4	4	4	3
19. *C. destructans* (16)	5	4	4	5	3	3	3	3	3	4	4	4	4	4	3
20. *C. destructans* (18)	4	3	3	4	2	2	2	2	2	3	3	3	3	3	2
21. *C. destructans* (17)	4	3	3	4	4	4	4	4	4	5	5	5	5	3	2
22. *C. destructans* (1)	4	3	3	4	4	4	4	4	4	5	5	5	5	3	2
23. *C. destructans* (3)	5	4	4	5	5	5	5	5	5	6	6	6	6	4	3
24. *C. destructans* (2)	6	5	5	6	6	6	6	6	6	7	7	7	7	5	4
25. *C. cylindroides*	31	30	30	30	31	31	31	31	31	32	31	31	31	31	30

Table 4. *Continued from preceding page.*

Isolates	16	17	18	19	20	21	22	23	24	25
1. .*C. destructans* (4)	0.003	0.008	0.008	0.008	0.006	0.006	0.006	0.008	0.009	0.047
2. *C. destructans* (6)	0.002	0.006	0.006	0.006	0.005	0.005	0.005	0.006	0.008	0.045
3. *C. destructans* (9)	0.002	0.006	0.006	0.006	0.005	0.005	0.005	0.006	0.008	0.045
4. *C. destructans* (22)	0.003	0.008	0.008	0.008	0.006	0.006	0.006	0.008	0.009	0.045
5. *C. destructans* (5)	0.003	0.005	0.005	0.005	0.003	0.006	0.006	0.008	0.009	0.047
6. *C. destructans* (11)	0.003	0.005	0.005	0.005	0.003	0.006	0.006	0.008	0.009	0.047
7. *C. destructans* (12)	0.003	0.005	0.005	0.005	0.003	0.006	0.006	0.008	0.009	0.047
8. *C. destructans* (13)	0.003	0.005	0.005	0.005	0.003	0.006	0.006	0.008	0.009	0.047
9. *C. destructans* (15)	0.003	0.005	0.005	0.005	0.003	0.006	0.006	0.008	0.009	0.047
10. *C. destructans* (14)	0.005	0.006	0.006	0.006	0.005	0.008	0.008	0.009	0.011	0.048
11. *C. destructans* (19)	0.005	0.006	0.006	0.006	0.005	0.008	0.008	0.009	0.011	0.047
12. *C. destructans* (24)	0.005	0.006	0.006	0.006	0.005	0.008	0.008	0.009	0.011	0.047
13. *C. destructans* (23)	0.005	0.006	0.006	0.006	0.005	0.008	0.008	0.009	0.011	0.047
14. *C. destructans* (7)	0.002	0.006	0.006	0.006	0.005	0.005	0.005	0.006	0.008	0.047
15. *C. destructans* (8)	0.000	0.005	0.005	0.005	0.003	0.003	0.003	0.005	0.006	0.045
16. *C. destructans* (10)	-	0.005	0.005	0.005	0.003	0.003	0.003	0.005	0.006	0.045
17. *C. destructans* (20)	3	-	0.000	0.000	0.002	0.002	0.005	0.006	0.008	0.047
18. *C. destructans* (21)	3	0	-	0.000	0.002	0.002	0.005	0.006	0.008	0.047
19. *C. destructans* (16)	3	0	0	-	0.002	0.002	0.005	0.006	0.008	0.047
20. *C. destructans* (18)	2	1	1	1	-	0.003	0.003	0.005	0.006	0.047
21. *C. destructans* (17)	2	1	1	1	2	-	0.003	0.005	0.006	0.045
22. *C. destructans* (1)	2	3	3	3	2	2	-	0.002	0.003	0.047
23. *C. destructans* (3)	3	4	4	4	3	3	1	-	0.005	0.048
24. *C. destructans* (2)	4	5	5	5	4	4	2	3	-	0.050
25. *C. cylindroides*	30	31	31	31	31	30	31	32	33	-

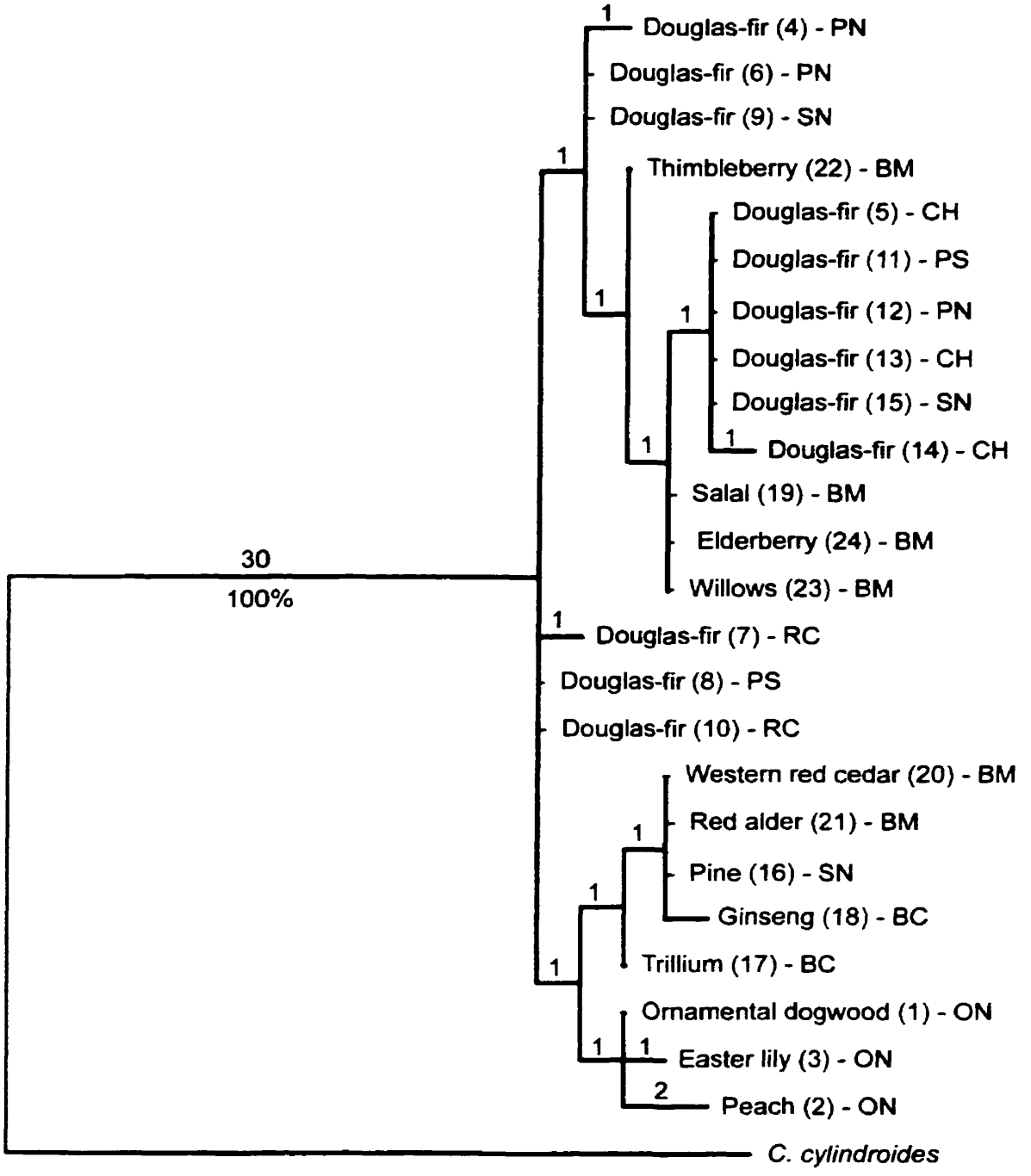

Figure 12. Phylogram depicting majority rule consensus tree of 300 trees (45 steps, CI = 0.933) resulting from maximum parsimony analysis of isolates of *Cylindrocarpon destructans* data set using heuristic search algorithm of PAUP 3.1.1. Numbers above branches are number of character state changes along branch. Bootstrap values above 50% (100 replications) are given under branches. Isolates are presented according to their host plants. Numbers in parentheses are isolate numbers (Table 1). BC = British Columbia; BM = Burnaby Mountain; CH = Chilliwack; ON = Ontario; PN = Pemberton; PS = Peltons; RC = Reid Collins Nursery; SN = Surrey Nursery.

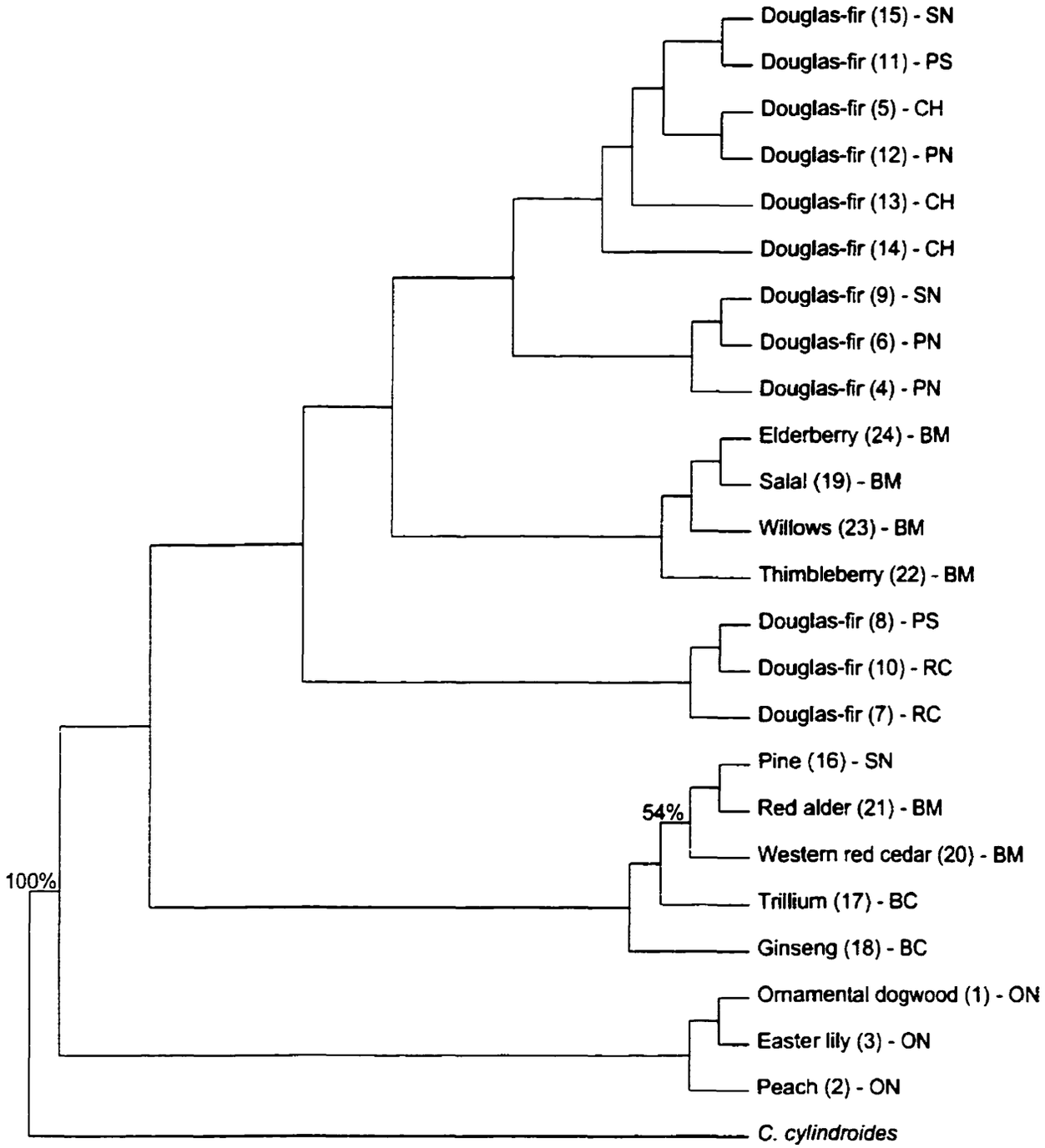

Figure 13. Bootstrapped (1000) UPGMA cladogram computed from mitSSU rRNA gene region among isolates of *Cylindrocarpon destructans* from different host plants and different localities in B.C. and Ontario. Bootstrap values above 50% are shown at the respective nodes. Isolates are presented according to their host plants. Numbers in parentheses are isolate numbers (Table I). BC = British Columbia; BM = Burnaby Mountain; CH = Chilliwack; ON = Ontario; PN = Pemberton; PS = Peltons; RC = Reid Collins Nursery; SN = Surrey Nursery.

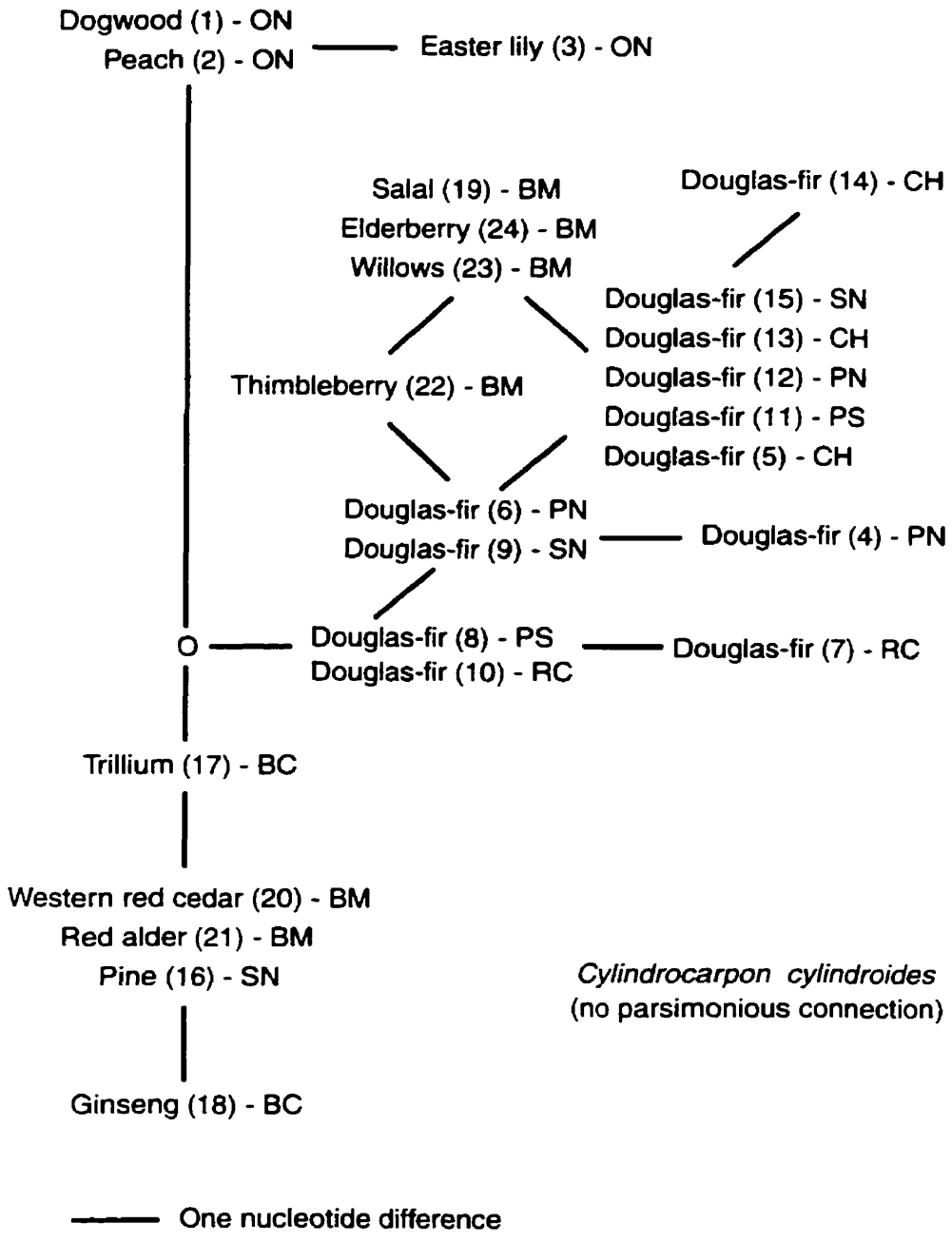

Figure 14. Cladogram computed from mitSSU rRNA gene region among isolates of *Cylindrocarpon destructans* from different host plants and different localities in B.C. and Ontario. The cladogram was constructed using the algorithm of Templeton *et al.* (1992). Isolates are presented according to their host plants. Numbers in parentheses are isolate numbers (Table 1). BC = British Columbia; BM = Burnaby Mountain; CH = Chilliwack; ON = Ontario; PN = Pemberton; PS = Peltons; RC = Reid Collins Nursery; SN = Surrey Nursery.

DISCUSSION

This study represents, to my knowledge, the first attempt to resolve relationships within the genus *Cylindrocarpon* and genetic variation among isolates of *C. destructans* using DNA sequence analysis of mitochondrial small subunit rDNA. Phylogenies presented here are not only useful for revealing inter- and intraspecific relationships and variation within *Cylindrocarpon*, but are also important for molecular phylogenetic studies of the Red Nectria complex (part of the Nectriaceae). Very little information from this complex is available pertaining to the phylogenetic study of the Hypocreales (Samuels, pers. comm.).

The various analytical methods that were used for the mitSSU rDNA sequence data in this study, i.e. maximum parsimony, distance method, and the method developed by Templeton *et al.* (1992), each have unique tree-building algorithms and optimization criteria. Steps for analyzing these methods are briefly described below.

To analyze sequence data using the maximum parsimony approach of PAUP 3.1.1 (Swofford, 1993), the sequence data for taxa under study must first be aligned. This initial step is crucial, as all subsequent analyses are dependent on the final alignment. In some instances, sequence alignment is easy and can be done by eye, without the aid of an alignment algorithm. On the other hand, many kinds of sequences vary so much across taxa that computer-assisted alignment is essential to minimize the differences among them. Most computer procedures (e.g., CLUSTAL and MULTALIN) use some measure of similarity (or dissimilarity) to search for the best alignment for a given pair of sequences. Different pairwise comparisons are then combined to produce the final overall result (see Figure 5 for an example). The next step is to convert the final alignment to a format that can be read by PAUP, i.e. NEXUS format (see Appendix A for

an example). Any editors that can output files of type "TEXT" or "ASCII", e.g., word processors, spreadsheet programs, and text editors, can be used for that purpose. Any assumptions and optimizations that need to be made for a particular data set can be made either by adding commands at the end of the data block (see Appendix A) or by using the OPTION menu. The final step is to search for optimal trees. The goal of a search is to find all of the equally parsimonious trees that exist for a particular data set under the chosen assumptions (Swofford, 1993). PAUP provides two basic types of methods for searching for optimal trees, namely, exact and heuristics methods. Exact methods, which include exhaustive and branch-and-bound algorithm, guarantee to find the optimal tree(s) but may require a prohibitive amount of computer time for medium- to large-sized data sets. Heuristic methods do not guarantee optimality but generally require far less computer time. Bootstrap replications can be done to estimate statistical support for internal branches in the tree(s).

The first step in analyzing nucleotide data using the distance methods included in the PHYLIP software package (Felsenstein, 1995) is to align the sequences against one another. This step is the same as described under PAUP above. Then, the final multiple alignment has to be converted to the PHYLIP format (see Appendix B for an example). The next step is to do bootstrap replications using the SEQBOOT program. This is then followed by conversion of sequence data to distance data using the DNADIST program. Using data matrices created in the previous step, the optimal trees are searched using the NEIGHBOR program, which includes UPGMA as an option for tree-searching algorithm. Finally, the majority rule consensus tree, i.e., the tree which consists of all groups that occur more than 50% of all the input trees searched using the NEIGHBOR program, is constructed using the CONSENSE program. The above-

mentioned programs are subsets of 30 programs included in PHYLIP software package (Felsenstein, 1995).

The tree-building algorithm developed by Templeton, Crandall, and Sing (1992) is a relatively new one. This algorithm was specifically designed to estimate intraspecific gene trees under conditions in which the levels of divergence among operational taxonomic units (OTUs) are low. Crandall (1994) showed that this method is more accurate than more traditional methods (e.g., maximum parsimony) for inferring evolutionary relationships when few characters are available. The estimation procedure is based on a parsimony criterion with a statistical procedure to evaluate the limits of the parsimony assumptions. Unfortunately, the algorithm has not been written in the form of a user-friendly program and currently is not accessible publicly. The analysis for intraspecific variation of *C. destructans* in this study was kindly done by Dr. K.A. Crandall himself.

Interspecific variation among *Cylindrocarpon* species

In parsimony analyses, I explored the effects of indels and transition:transversion biases on phylogenetic estimates. Throughout these analyses, certain aspects of the topologies remained constant and I take these as the best-supported estimate of phylogenetic relationships among *Cylindrocarpon* species (Figure 6 and 7). Under gap = missing coding with no, 1:3, and 1:10 transition:transversion biases all *Cylindrocarpon* species under study were readily separated from *Fusarium* sp. as well as *Tubercularia* sp. and formed a monophyletic group with moderate bootstrap support (74%). This result is not unexpected because, although *Fusarium* sp., *Tubercularia* sp., and *Cylindrocarpon* spp. belong to *Nectria sensu lato*, they are

morphologically distinctive in both anamorph and teleomorph (Samuels and Seifert, 1987; Samuels and Seifert, 1995).

In contrast, under indel coding, monophyly of the specified ingroup (*Cylindrocarpon* spp.) could not be supported statistically. This difference is not surprising considering the fact that the size of samples in the analyses was small. The small sample size increases the probability of stochastic variation along the branches (Ruedi *et al.*, 1998). It is therefore inappropriate to test the monophyly of *Cylindrocarpon* in this study. More samples of *Cylindrocarpon* (or *Nectria*) and samples of species from other genera in Hypocreales as well as outgroups from another order within Pyrenomycetes (e.g., Sphaeriales) are needed to test the monophyly of *Cylindrocarpon*.

In both coding schemes, all recognized groups, i.e., group I, II, and, III, were consistently supported by over 82% of the bootstrap replicates, thus suggesting the monophyly of these groups. Support for group I in parsimony analyses was sensitive to the choice of character coding scheme. Group I received only moderate support under gap = missing coding (82%), whereas under indel coding it received very strong support (100%). This indicates that the indel characters provide considerable additional support for group I. However, the consistency index (CI) of the phylogeny under indel coding is lower (0.862) than that of under gap = missing coding (0.903), which means that the inclusion of the indel characters under indel coding increased the level of homoplasy by 0.041. Since the greater the level of homoplasy, the less confidence we can have in a phylogenetic estimate, gap = missing coding is possibly better than indel coding for mitSSU sequence data set used in this study. The increased level of homoplasy under indel coding may be due to several ambiguous alignments in the two hypervariable regions (Figure 5) which generated a number of indel characters (especially multiple base indels such as

row E Fig. 2) that probably do not represent true indel events. This could be one of the explanations for the difference of the topology of trees generated under both coding schemes. Therefore, the use of indel coding should probably be avoided when using DNA regions with considerable size diversity, e.g., mitochondrial small subunit, and limited to DNA regions with low size diversity, e.g., nuclear coding regions and 5.8S rDNA.

Congruence between the grouping of Cylindrocarpon *spp. by mitSSU rDNA sequences and the groupings based on morphological and cultural characteristics*

Traditional fungal classifications have been constructed using morphological and cultural characters, but several limitations were encountered. One major obstacle was the difficulty of implementing the recognition criteria of homology - position, quality of resemblance, and continuance of similarity - when working with morphology at high taxonomic levels (Wiley, 1981). Another significant impediment was the lack of morphological characters for microscopic fungi. An additional complication was imposed by species having an anamorphic stage. Molecular data are now generally promoted as useful tools to infer phylogenetic relationships at higher taxonomic levels (Eernisse and Kluge, 1993). Early arguments for the advantages of molecular data over morphological data included a larger number of characters and characters that were generally free from nonheritable variation (Hillis, 1987). However, it has become apparent that there are problems with molecular data that include difficulties with alignment and scoring of missing sites. In addition, there is not necessarily a one-to-one correspondence between phylogenetic trees based upon genes and the genealogical relationships of the organisms bearing those genes (Eernise and Kluge, 1993). Since fungal systematists are primarily interested in species/population phylogenies rather than gene phylogenies, many believe that both molecular data and morphological data should be examined to estimate

organismal phylogenies (Eernise and Kluge, 1993; Miyamoto, 1985; Nixon and Carpenter, 1996). In addition to gaining a better idea of the robustness of a given phylogenetic hypothesis, congruence between morphological and molecular data sets becomes a criterion for determining if a particular data set of molecular characters can be used to estimate a species/population phylogeny for a given set of taxa (Nixon and Carpenter, 1996).

In this study, the grouping of *Cylindrocarpon* species by mitochondrial small subunit rDNA sequences and grouping on the basis of morphological and cultural characteristics as described by Booth (1966) were congruent for all taxa (see Table 5). This strongly indicates that the mitSSU rDNA sequence data set is a good tool for estimating phylogenetic relationships among *Cylindrocarpon* species.

Group I, which received 82 to 100 % bootstrap support, includes *C. cylindroides* Wollenw. and *C. heteronemum* (Berk. and Br.) Wollenw. This group is congruent with group 1 of *Cylindrocarpon* species delineated by Booth (1966). Species within this group are separated from other species in *Cylindrocarpon* by the presence of both micro- and macroconidia and the absence of chlamydospores in the mycelia. Other species in this group include *C. wilkommii*, *C. hederae*, *C. candidum*, *C. coprosmae*, *C. album*, and *C. faginatum*. *C. cylindroides* and *C. heteronemum* [*C. mali* (Allesch.) Wollenw.] were the only species originally included in the genus when Wollenweber (1913) erected *Cylindrocarpon* for the conidial states of *Nectria*s in the Willkommiotes section (Booth, 1966). Nucleotide divergence between these two species was less than 3.8 % (Table 3). However, several morphological and cultural characteristics clearly distinguish between the two species. *C. cylindroides* produces significantly bigger microconidia (8-14 x 4-6 μm) than *C. heteronemum* (4-8 x 2-3 μm) (Booth, 1966). On PDA, *C. heteronemum* grows faster (approx. 6 cm diameter after 14 days) than *C. cylindroides* (approx. 2 cm diameter

Table 5. The groupings of *Cylindrocarpon* species based on morphological characters (Booth, 1966) and mitSSU rDNA sequences

Cylindrocarpon species	Grouping based on morphological characters	Grouping based on mitSSU rDNA sequences
1. *C. heteronemum*	group 1	group I
2. *C. cylindroides*	group 1	group I
3. *C. rugulosum*	n/a	group II
4. *C. coronatum*	n/a	group II
5. *C. candidulum*	group 2	group II
6. *C. ianothele* var. *majus*	group 2	group II
7. *C. destructans*	group 3	group III

after 14 days). In addition, swollen hyphae but no true chlamydospores are observed in *C. cylindroides*, whereas in *C. heteronemum* such structures are not found (Booth, 1966).

Group II, with very strong bootstrap support (95 to 100 %), includes *C. rugulosum* Brayford *et* Samuels, *sp. nov.* (Samuels and Brayford, 1994), *C. coronatum* Brayford *et* Samuels, *sp. nov.* (Brayford and Samuels, 1993), *C. candidulum* (Sacc.) Wollenw., and *C. ianothele* Wollenw. var. *majus* Wollenw. This group is congruent with group 2 of *Cylindrocarpon* species outlined by Booth (1966) which is characterized by lack of both microconidia and mycelial chlamydospores. Of the four species included in Group II, only *C. candidulum* and *C. ianothele* var. *majus* were described in Booth's work. *C. rugulosum* and *C. coronatum* were recently described as the anamorphs of *N. rugulosa* and *N. coronata*, respectively (Samuels and Brayford, 1994; Brayford and Samuels, 1993). *C. rugulosum* showed morphological characteristics that are consistent with those of group 2 of *Cylindrocarpon*, i.e., lack of both microconidia and mycelial chlamydospores (Samuels *et al.*, 1990). Similarly, microconidia were absent in *C. coronatum*, and although chains of swollen, globose cells with granular, vacuolate cytoplasm

occurred in hyphae of some old cultures, wall thickening was not apparent (Brayford and Samuels, 1993). Although nucleotide sequence divergence among species in this group was relatively low (2.3 - 6.4%), several morphological and cultural characters clearly distinguish species in Group II.

Group III includes three isolates of *C. destructans*. This group received strong bootstrap support (96 to 98%), but there were no other species in this group. This group corresponds to group 3 of *Cylindrocarpon* species described by Booth (1966). Group 3 is characterized by formation of both micro- and macroconidia as well as mycelial chlamydospores. Included in this group, in addition to *C. destructans*, are *C. didymum* and *C. obtusisporum*. Sequence divergence among the three isolates of *C. destructans* in this group was 0.6 - 1.1%. Intraspecific variation among isolates of *C. destructans* will be discussed separately later.

While classification of deuteromycetes based on the morphology of asexual reproductive structures might not always represent the biological relationships among this diverse group of fungi, the results presented here indicate that *Cylindrocarpon* species that I studied appear to be closely related and the few morphological and cultural characters that have been used to classify these fungi are reliable, since they coincide with the molecular characters in every case. Molecular studies, representing true genetic characters, especially of those fungi belonging to Deuteromycotina, can provide valuable taxonomic and genetic information that can lead to a more thorough understanding of the relationships and the biology of these fungi.

Anamorphic-teleomorphic relationships

Based upon perithecial anatomy, the species of *Nectria* Fr. with *Cylindrocarpon* anamorphs can be distributed amongst several different groups of *Nectria* (Brayford and

Samuels, 1993). To date, there are five major groups of *Nectria* that have *Cylindrocarpon* anamorph (Samuels, pers. comm.) namely, *N. discophora* (mammoidea) group, *N. coccinea/galligena* group, *N. radicicola* group, *N. cinnamomea* group, and *N. veuillotiana* group. However, intergradation exists between these groups such that some species are sometimes included in different groups.

Correspondence between anamorphic taxa within the monophyletic groups of *Cylindrocarpon* species in this study and teleomorphic grouping in *Nectria* was congruent for some taxa (see Table 6). In groups I and III, relationships of the anamorphic taxa within the monophyletic groups appeared to be correlated with teleomorph state. In contrast, the placement of *Cylindrocarpon* species in group II to *Nectria* groups was inconsistent.

In group I, *N. galligena* and *N. neomacrospora* (anamorph: *C. heteronemum* and *C. cylindroides*, respectively) share close morphological characters and are included in *N. coccinea/galligena* group (Samuels, pers. comm.). This group is primarily temperate in distribution (Booth, 1966) and most easily recognized by the red perithecia that are formed in large aggregates with many stages of development in each aggregate. The perithecial wall of fungi in this group is KOH positive, up to 25 μm wide or more, relatively thick, and comprised of two distinct regions. The outer region consists of mainly spherical cells with walls 1.5 - 2.5 μm thick (Booth, 1966; Samuels *et al.*, 1990). Ascospores are ellipsoidal, 1-septate, smooth, and colorless.

The teleomorph of *C. destructans*, the only species in group III, is *N. radicicola*, and is included in the *N. radicicola* group. Morphological characteristics of *N. radicicola* have been described in the literature review. Samuels *et al.* (1990) state that perithecial wall anatomy of this species is very different from that of other *Cylindrocarpon*-forming *Nectria* groups. This

Table 6. Relationships between *Cylindrocarpon* species and their teleomorphic species (*Nectria* spp.)

Cylindrocarpon species	*Cylindrocarpon* group[1]	*Nectria* species	*Nectria* group[2]	References
1. *C. heteronemum*	group I	*N. galligena*	*N. coccinea/galligena*	Samuels *et al.* (1990)
2. *C. cylindroides*	group I	*N.* neomacro-*spora*	*N. coccinea/galligena*	Booth and Samuels (1981)
3. *C. coronatum*	group II	*N. coronata*	*N. coccinea/galligena*	Samuels *et al.* (1990)
			N. veuillotiana	Samuels and Brayford (1993)
4. *C. rugulosum*	group II	*N. rugulosa*	*N. coccinea/galligena*	Samuels *et al.* (1990)
5. *C. candidulum*	group II	*N. veuillotiana*	*N. discophora/ mammoidea*	Booth (1959)
			N. veuillotiana	Samuels and Brayford (1993)
6. *C. ianothele var. majus*	group II	*N. discophora*	*N. discophora/ mammoidea*	Samuels *et al.* (1990)
7. *C. destructans*	group III	*N. radicicola*	*N. radicicola*	Samuels *et al.* (1990)

[1]Based on both morphological characters (Booth, 1966) and mitSSU rDNA sequences
[2]Mainly based on perithecial wall anatomy

anatomy is similar to perithecial wall anatomy in *Calonectria*, which leads Samuels and Rossman to suspect a close relationship between *N. radicicola* and species of *Calonectria* (Rossman, 1983; Samuels *et al.*, 1990). In their work, Samuels and Rossman classify all *Nectria* that have *Cylindrocarpon* anamorph in a new genus, namely *Neonectria*, except for *N. radicicola* (Samuels, pers. comm.). They are inclined to place this species in another genus. However, their classification is not supported by the phylogenetic analyses presented here.

Group II includes species of *Cylindrocarpon* whose teleomorphs were inconsistently placed in different *Nectria* groups. In Samuels *et al.* (1990), *N. rugulosa* and *N. coronata* were included in *N. coccinea/galligena* group, and *N. dischopora* (anamorph: *C. ianothele* var. *majus*) was included in *N. discophora/mammoidea* group. However, in 1993, Brayford and Samuels

erected a new *Nectria* group with a *Cylindrocarpon* anamorph, namely the *N. veuillotiana* group. This group, centered on *N. veuillotiana* (anamorph: *C. candidulum*), includes *N. coronata* in addition to several other *Nectria* species. *N. veuillotiana* itself were previously included in the *N. discophora/mammoidea* group (Booth, 1959), but the two groups can be distinguished by their respective perithecial morphology and anatomy, and by their anamorphs (Brayford and Samuels, 1993). Results presented here do not support the placement of *N. coronata* and *N. rugulosa* in the *N. coccinea/galligena* group. Phylogenetic analyses and anamorphic characters of *N. coronata* reinforce the inclusion of this species in the *N. veuillotiana* group. In the case of *N. rugulosa*, it is most likely that this species also belongs to the *N. veuillotiana* group. The placement of *N. rugulosa* in this group is supported by the phylogenies and the level of nucleotide divergence. Alternatively, judging by its separation from the other three species in this group in phylogenetic analysis (Figure 6 and 7), *N. rugulosa* may belong to another group, other than the *N. coccinea/galligena* group, the *N. discophora/mammoidea* group, or the *N. Veuillotiana* group. More study is therefore needed to solve this nomenclature problem.

To conclude, relationships of anamorphs and teleomorphs in *Cylindrocarpon* appear challenging. In the taxonomy of fungi having an anamorphic stage, the trend, on both morphological and molecular observations, has been toward correlating teleomorphic genera with anamorphs (Samuels and Rossman, 1974; Samuels and Seifert, 1987). This is because, although the teleomorphic states have provided a meaningful taxonomic framework, there are still many nomenclature problems that cannot be resolved by analyzing teleomorphic states alone. As shown in this study, molecular studies are useful in the attempts to correlate teleomorphs with their anamorphs. In addition, phylogenetic studies based on molecular data can expose weaknesses in interpretation of morphological characters. Therefore, to solve

nomenclature problems in *Cylindrocarpon*, an integration of morphological (in both anamorph and teleomorph) and molecular data will be necessary.

Intraspecific variation among isolates of *Cylindrocarpon destructans*

Intraspecific variation in nuclear and mitochondrial rDNA has been correlated with geographical location and/or host preference of some phytopathogenic fungi. For instance, polymorphism in rDNA of *Colletotrichum gloeosporioides* has been linked to host source within geographical locations (Hodson *et al.*, 1993), whereas isolates of *Gaeumannomyces graminis* with the same preferred cereal host generally had few polymorphisms irrespective of geograpical origins of the isolates (O'Dell *et al.*, 1992). In this study, 24 isolates of *C. destructans* from different localities and host plants were analyzed for evidence of intraspecific genetic variation correlated with ecological and morphological traits. If *C. destructans* is composed of strains, then it is expected that the analyses will show that genetically related isolates share some of these traits. Alternatively, if *C. destructans* is not divided into strains then genetic relatedness will not show this correlation.

The almost complete lack of mitSSU rDNA variation observed in isolates of *C. destructans* in the present study is reflected in the phylogenies of those isolates (Figure 12 - 14). In phylogenetic analysis using maximum parsimony and distance methods, there was no resolution in the phylogenetic trees generated. Thus, all isolates under study showed no significant difference statistically. However, although statistical support for internal branches was virtually lacking, the trees suggested the existence of mitSSU rDNA variants among the isolates. This was supported by the phylogeny generated using the method developed by Templeton *et al.* (1992), which revealed the presence of three mitSSU rDNA variants. The first

variant was represented by three isolates from Ontario. This variant was quite different from the other two variants, based on the number of mutational steps required from an imaginary common ancestor (represented by O in the cladogram) to reach the present state. The second variant was represented by five isolates from different localities in British Columbia. The third variant was the most common, and included 16 isolates from different localities in British Columbia.

The three mitSSU rDNA variants identified, however, showed no apparent association with the host or geographic origin. Such lack of concordance was also observed in *C. destructans* when nuclear genes were used as molecular markers. By using the internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) subunit as a marker, Hamelin *et al.* (1996) found that, although there were three ITS variants identified within 11 isolates of *C. destructans*, there was no apparent association between ITS variants and host or geographical origin. In another case, Axelrood (pers. comm.) used Histone 3 DNA region as a molecular marker in RFLP analysis of *C. destructans* from different nurseries and reforestation sites and found no correlation between the variants and the origins of the isolates.

Despite the virtually non-existent association between mitSSU rDNA variants and host or geographical origin, it is interesting to notice that all isolates of *C. destructans* obtained from Douglas-fir (originated from 5 populations) that were included in this study could be grouped in one variant. Although this variant also includes isolates obtained from four unrelated host plant species, this may suggest a very early development of host specificity by a variant of *C. destructans*. Alternatively, this finding may also suggest recent gene flow between the five populations. Human intervention is a possible cause for the recent dispersal events between these populations, perhaps by distribution of Douglas-fir seedlings and their associated fungal flora.

The low polymorphism indicated by sequence analysis of mitSSU rDNA is characteristic of root fungi that exist as vegetative clones, such as *Armillaria* (Smith *et al.*, 1990), which are spread by mycelial growth and root contact. This condition is also seen in *Phytophthora cactorum*, in which a single clone was speculated to cause crown rot of strawberry in Western Europe (Hantula *et al.*, 1997). Based on the very low nucleotide divergence among the isolates of *C. destructans* (less than 1.1%), it can be speculated that the extant variants of mitSSU rDNA identified in the present study may be the result of mutations that occurred on an ancestral mitSSU rDNA type in a single clonal genotype of *C. destructans*. This argument is supported by the fact that some isolates from different localities (isolates number 5, 11, 12, 13, and 15) have identical mitSSU rDNA sequences, and many of the sequences differ by only one or two nucleotides. The clonality of *C. destructans*, however, should be studied more thoroughly before it can be considered completely resolved.

Such homogeneity in mitSSU DNA variation can potentially be explained by the uniparental mode of inheritance of most, if not all, fungal mitochondria. Uniparental inheritance reduces the possibility of heteroallelism at mitochondrial gene loci, since progeny inherit one parental mitochondrial type. Also, while recombination of mtDNA has been observed in several Ascomycotina after asexual fusion (anastomosis), the field clones of these fungi were found to be uniform for mtDNA types. It was observed that heteroplasmy that resulted from recombination of mtDNA was a transient state due to rapid segregation of mitochondrial types in relatively few generations (Smith, 1990). If it is assumed that there is an advantage of maintaining a successful organelle intact (perhaps certain mitochondrial-encoded subunits works best in concert), then it would follow that the most successful progeny would inherit one parent's entire organelle genome (Taylor, 1986).

RECOMMENDATIONS FOR FUTURE RESEARCH

Phylogenetic relationships among *Cylindrocarpon* spp.

Further research is necessary to clarify phylogenetic relationships of *Cylindrocarpon* species. Results presented here have shown a congruence between mitochondrial small subunit rDNA sequence data and morphological as well as cultural characteristics of three groups of *Cylindrocarpon* species as outlined by Booth (1966). However, there is still one group, i.e., the fourth group which is characterized by formation of mycelial chlamydospores but lack microconidia, that was not represented by any isolate in this study. The inclusion of species which belong to this fourth group, as well as some more species from the first three groups, in the phylogenetic analysis will therefore be needed to gain a thorough understanding of phylogenetic relationships within the genus. Moreover, this would also allow the examination of relationships between *Cylindrocarpon*-forming *Nectria* groups and other nectrioid fungi, in addition to the testing of monophyly of *Cylindrocarpon*.

Genetic variation of *Cylindrocarpon destructans*

The phylogenies inferred here will serve as a basis for further analysis of genetic variation of *C. destructans*, using other informative and polymorphic molecular markers. There are a few good candidates of molecular markers that have been shown to be more informative and polymorphic than mitochondrial or nuclear rDNA, namely random amplification of polymorphic DNA (RAPD), microsatellites, and random amplified microsatellite (RAMS). The use of RAPD in fungal population studies has been discussed in the literature review. Microsatellites are segments of DNA with tandem repeats of short-sequence motifs (1 - 6 bases

long). They have also been shown to be useful for intraspecific analyses of fungi (Bonfante *et al.*, 1997; Sastry *et al.*, 1996). Yet another marker originally described by Zietkiewicz *et al.* (1994), called random amplified microsatellite (RAMS), has been shown to be applicable to fungi (Hantula and Müller, 1997; Hantula *et al.*, 1997). This marker combines most of the benefits of RAPD and microsatellites, and is therefore promising for studies of genetic variation. In RAMS analysis, the DNA between the distal ends of two closely located microsatellites is amplified and the resulting PCR products are separated electrophoretically (Hantula *et al.*, 1996). In addition to gaining more information about genetic variation of *C. destructans*, the use of these nuclear DNA-based markers would also allow a thorough examination of the clonal nature of the fungus. This can be done by comparing the distribution of mitochondrial DNA variants presented here and that of nuclear DNA variants that result from analyses using the above-mentioned markers. If the nuclear and mitochondrial distributions of the variants were found to be perfectly correlated and linked, it would support the hypothesis of clonal nature of *C. destructans*, and vice versa.

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APPENDIX A

An example of a data set in Nexus format for maximum parsimony analysis using PAUP. This data set was used for interspecific analysis of *Cylindrocarpon* in this study.

```
#NEXUS;
[mitSSU rRNA alignment of 14 species of fungi]
BEGIN DATA;
DIMENSIONS  NTAX=14 NCHAR=793;
FORMAT DATATYPE=DNA  MISSING=? GAP=-  SYMBOLS="01"  INTERLEAVE ;

MATRIX
Fusarium     -GCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTC TTCCAGTATG
cinnabar     GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTC TTATAATT--
heteroneON   GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
heteroneBC   GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
cylindrop3   GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
cylindrop4   GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
cylindroc2   GCCTAACGGA TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
destrucpa17  GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
REDCEDAR     GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
JAT1551      GCCTAACGGC TGAACTGGGC ACTTGGAGAA GTGGCAAGTT TTATAATT--
veiullotia   GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGTAAGTT TTATAACC--
jungneri     GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
coronata     GCCTAACGGC TGAACTGGCA ACTTGGAGAA GTGGCAAGTT TTATAATT--
discophora   GCCTATGGCG TGAACTGGCA ACTTGGAGAA GTGTCAAGTT TTATAACT--

Fusarium     GGGAGCAAAA CAGCTATGGG TCAAGTCCG- ------ATAT CTTTAGGAGA
cinnabar     --------TC TAGTATTAGG T--------- ------ATTT CTAT------
heteroneON   --------AA TAGTAATGAA GGCGCAAGC- ------TCTT CTAT------
heteroneBC   --------AA TAGTAATGAA GGCGCAAGC- ------TCTT CTAT------
cylindrop3   --------AA TAGTAATTAA T--------- ------ATTT CTAT------
cylindrop4   --------AA TAGTAATTAA T--------- ------ATTT CTAT------
cylindroc2   --------AA TAGTAATTAA T--------- ------ATTT CTAT------
destrucpa17  --------AA TAGTAATGAA T--------- ------ATTT CTAT------
REDCEDAR     --------AA TAGTAATGAA T--------- ------ATTT CTAT------
JAT1551      --------AA TAGTAATTAA T--------- ------ATTT CTAT------
veiullotia   --------AA TAGATACTTA TGCGCTGCTT CGTCAGAGAT CTAT------
jungneri     --------AA TAGTAATTTA T--------- ------ATTT CTAT------
coronata     --------AA TAGTAATTTA T--------- ------ATTT CTAT------
discophora   ---------A TATTT----- ---------- -------TTT TTTT------

Fusarium     AGTCTTATTG TGAGGGCGAG TTATATAACA CCATAGGACT GGCCGTCCCA
cinnabar     ---------- ---------- ATTTATAA-- ---------- ----------
heteroneON   ---------- ---------- TTTTATAA-- ---------- ----------
heteroneBC   ---------- ---------- TTTTATAA-- ---------- ----------
cylindrop3   ---------- ---------- TTTTATAA-- ---------- ----------
cylindrop4   ---------- ---------- TTTTATAA-- ---------- ----------
cylindroc2   ---------- ---------- TTTTATAA-- ---------- ----------
destrucpa17  ---------- ---------- TTTTATAA-- ---------- ----------
REDCEDAR     ---------- ---------- TTTTATAA-- ---------- ----------
JAT1551      ---------- ---------- TTTTATAA-- ---------- ----------
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veiullotia  ---------- ---------- TTTTATAA-- ---------- ----------
jungneri    ---------- ---------- TTTTATAA-- ---------- ----------
coronata    ---------- ---------- TTTTATAA-- ---------- ----------
discophora  ---------- ---------- TTTTAAAA-- ---------- ----------

Fusarium    TATGAAAAGA TTATATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
cinnabar    --------GA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
heteroneON  --------GA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
heteroneBC  --------GA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
cylindrop3  --------GA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
cylindrop4  --------GA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
cylindroc2  --------GA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
destrucpa17 --------AA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
REDCEDAR    --------AA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
JAT1551     --------AA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
veiullotia  --------AA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
jungneri    --------AA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA
coronata    --------AA TTCTATTAGG ATTGAATGAA GCTTTGTTTA TATATTGATA
discophora  --------AA TTCTATTAGA ATTGAATGAA GCTTTGTTTA TATATTGATA

Fusarium    ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
cinnabar    ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
heteroneON  ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
heteroneBC  ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
cylindrop3  ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
cylindrop4  ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
cylindroc2  ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
destrucpa17 ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
REDCEDAR    ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
JAT1551     ATGACAGTAT ATATATCGTG TCTTGACTAA TTGCGTGCCA GCAGTCGCGG
veiullotia  ATGACAGTAT ATATATCGTG TCTTGACCAA TTACGTGCCA GCAGTCGCGG
jungneri    ATGACAGTAT ATATATCGTG TCTTGACCAA TTACGTGCCA GCAGTCGCGG
coronata    ATGACAGTAT ATATATCGTG TCTTGGCCAA TTACGTGCCA GCAGCCGCGG
discophora  ATGACAGTAT ATATATCGTG TCTTGACCAA TTACGTGCCA CGAGTCGCGG

Fusarium    TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
cinnabar    TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
heteroneON  TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
heteroneBC  TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
cylindrop3  TAATACGTAA GAGACTAGTT TTATTCATCT TAATTAGGTT TAAAGGGTAC
cylindrop4  TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
cylindroc2  TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
destrucpa17 TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
REDCEDAR    TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
JAT1551     TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
veiullotia  TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
jungneri    TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
coronata    TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC
discophora  TAATACGTAA GAGACTAGTG TTATTCATCT TAATTAGGTT TAAAGGGTAC

Fusarium    CCAGACGGTC AATATAGCTT ATAAAATGTT AGTACTTGAC TAGAGTTTTA
cinnabar    CCAAACGGTC AAAATAGCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
heteroneON  CCAGACGGTC AATATAGCTT CTACAATGTT AGTACTTGAC TAGAGTTTTA
heteroneBC  CCAGACGGTC AATATAGCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
cylindrop3  CCAGACGGTC AATATAGCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
cylindrop4  CCAGACGGTC AATATAGCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
cylindroc2  CCAGACGGTC AATATAGCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
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destrucpa17 CCAGACGGTC AATATATCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
REDCEDAR    CCAGACGGTC AATATATCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
JAT1551     CCAGACGGTC AATATATCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
veiullotia  CCAGACGGTC AATATAGCTT CTAGAATGTT AGTACTTGAC TAGAGTTTTA
jungneri    CCAGACGGTC AATATAGCTT CTATAATGTT AGTACTTGAC TAGAGTTTTA
coronata    CCAGACGGTC AATATACCTT CTAGAATGTT AGTACTTGAC TAGAGTTTTA
discophora  CCAGACGGTC AAAATAGCTT CTAGAATGTG AGTATTTGAC TAGAGTTTTA

Fusarium    TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
cinnabar    TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTCTA TGATACCAAA
heteroneON  TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
heteroneBC  TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
cylindrop3  TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
cylindrop4  TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
cylindroc2  TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
destrucpa17 TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
REDCEDAR    TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
JAT1551     TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTTCG TGATACCAAA
veiullotia  TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTCCG TGATACCAAA
jungneri    TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTCCG TGATACCAAA
coronata    TGTAAGAGGG CAGTACTTGA GGAGGAGAGA TGAAATTCCG TGATACCAAA
discophora  TATGAGAGTG CAGTACTTGA GGAGAAGAGA TGAAATTCCG TGATACCAAA

Fusarium    GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAAAAAC TGACGTTGAA
cinnabar    GGGACTCTGT AAAGGCGAAG GCAGCCCTCT AAGTAAAAAC TGACGTTGAA
heteroneON  GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAGAAAC TGACGTTGAA
heteroneBC  GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAGAAAC TGACGTTGAA
cylindrop3  GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAAAAAC TGACGTTGAA
cylindrop4  GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAAAAAC TGACGTTGAA
cylindroc2  GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAAAAAC TGACGTTGAA
destrucpa17 GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAGAAAC TGACGTTGAA
REDCEDAR    GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAGAAAC TGACGTTGAA
JAT1551     GGGACTCTGT AAAGGCGAAG GCAGCCCTCT ATGTAGAAAC TGACGTTGAA
veiullotia  GGGACTCAGT AAAGGCGAAG GCAGCCCTCT ATGTATAAAC TGACGTTGAA
jungneri    GGGACTCGGT AAATGCGAAG GCAGCCCTCT AGGTAAAAAC TGACGTTGAA
coronata    GGGACTCAGT AAAGGCGAAG GCAGCCCTCT ATGTATAAAC TGACGTTGAA
discophora  GGGACTCGGT AAAGGCGAAG GCAGCACTCT ATGTATTAAC TGACGTTGAA

Fusarium    GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
cinnabar    GGACGAAGGC TCAGATCACA AACAGGATTA GATACCCAAG TAGTCTTTCG
heteroneON  GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
heteroneBC  GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
cylindrop3  GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
cylindrop4  GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
cylindroc2  GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
destrucpa17 GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
REDCEDAR    GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
JAT1551     GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCAAG TAGTCTTTGC
veiullotia  GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCTAA TAGTCTTTGC
jungneri    GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCTAA TAGTCTTTGC
coronata    GGACGAAGGC ACAGAGAACA AACAGGATTA GATACCCTAA TAGTCTTTGC
discophora  GGACGAAGGC ACAGATAACA AACAGGATTA GATACCCTAA TAGTCTTTGC

Fusarium    AGTAAATGAT GAATGCCATA GGTCAGATAA CCAGTTAATG TTTATAG--T
cinnabar    AGTAAATGAT GAATGCCATA GGTCAGAGTA AGATTTAATG GTAGTAGGCC
heteroneON  AGTAAATGAT GAATGCCATA GGTCAGATCT ATAGTTAATG CT-CTGCGGT
heteroneBC  AGTAAATGAT GAATGCCATA GGTCAGATCT ATAGTTAATG CT-CTGCGGT
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cylindrop3  AGTAAATGAT GAATGCCATA GGTCAGATCT ATAGTTAATG CT-CTGCGGT
cylindrop4  AGTAAATGAT GAATGCCATA GGTCAGATCT ATAGTTAATG CT-CTGCGGT
cylindroc2  AGTAAATGAT GAATGCCATA GGTCAGATCT ATAGTTAATG CT-CTGCGGT
destrucpa17 AGTAAATGAT GAATGCCATA GGTCAGATCT AAATTTCATG TTTATATATT
REDCEDAR    AGTAAATGAT GAATGCCATA GGTCAGATCT AAATTTAATG TTTATAGATT
JAT1551     AGTAAATGAT GAATGCCATA GGTCAGATCT AAATTTAATG TTTATAGATT
veiullotia  AGTAAATGAT GAATGCCATA GGTTAGATAA ---------- ----------
jungneri    AGTAAATGAT GAATGCCATA GGTTAGATAA ---------- ----------
coronata    AGTAAATGAT GAATGCCATA GGTCAGATAA ---------- ----------
discophora  AGTAAATGAT GAATGCCATA GGTCAGATAT ---------- ----------

Fusarium    CTAATAGGGT TAGCCTAG-- -----CAAAC TAATGACATA GACTAT----
cinnabar    GGCTTATTGC ATAGCGAAAA ------AAAA CTG-GCTAAG GCTCG-----
heteroneON  GAAAACATGC CAGGATTGAA TCTATCGAAC CTGTGCTAGA TTTTTTTGTC
heteroneBC  GAAAACATGC CAGGATTGAA TCTATCGAAA CTGTGCTAGA TTTTTTTGTC
cylindrop3  GTAAACAAGC CAGTATTGAA TCTATCAAAA CTGTGCTTGC TAATTTTTTT
cylindrop4  GTAAACAAGC CAGTATTGAA TCTATCAAAA CTGTGCTTGC TAATTTTTTT
cylindroc2  GTAAACAAGC CAGTATTGAA TCTATCAAAA CTGTGCTTGC TAATTTTTTT
destrucpa17 ATAAAAGCGC CTGAATTG-- ------GCAA CTTTGCAAAA AATTAT----
REDCEDAR    ATAAAAGCGC CTGAATTG-- ------GCAA TTTTGCAAAA AATTAT----
JAT1551     ATAAAAGCGC CTTAATTG-- ------GCAA CTTTGCAAAA AATTAT----
veiullotia  ---------- ---------- ---------- ---------- ----------
jungneri    ---------- ---------- ---------- ---------- ----------
coronata    ---------- ---------- ---------- ---------- ----------
discophora  ---------- ---------- ---------- ---------- ----------

Fusarium    ---------- ---------- ---------- ---------- -----CCATT
cinnabar    ---------- ---------- --GACTACAC AT-------- --------TA
heteroneON  TGCTGGATCT TTCGAAACAT GCTATAAAAA ATAAGAAGCC TGTATACATT
heteroneBC  TGCTGGATCT TTCGAAACAT GCTATAAAAA ATAAGAAGCC TGTATACATT
cylindrop3  TG-TGTATTT AT----ACAT -CGAAAAGAT AT-----GCC TGTATACATT
cylindrop4  TG-TGTATTT AT----ACAT -CGAAAAGAT AT-----GCC TGTATACATT
cylindroc2  TG-TGTATTT AT----ACAT -CGAAAAGAT AT-----GCC TGTATACATT
destrucpa17 ---------- ---------- ------AAAT AT-------- --------TC
REDCEDAR    ---------- ---------- ------AAAT AT-------- --------TA
JAT1551     ---------- ---------- ------AAAT AT-------- --------TA
veiullotia  ---------- ---------- ---------- ---------- ---------T
jungneri    ---------- ---------- ---------- ---------- ---------T
coronata    ---------- ---------- ---------- ---------- ----------
discophora  ---------- ---------- ---------- ---------- ----------

Fusarium    AAAAATATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
cinnabar    AACATTATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
heteroneON  AAAATAATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
heteroneBC  AAAATAATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
cylindrop3  AAAATAATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
cylindrop4  AAAATAATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
cylindroc2  AAAATAATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
destrucpa17 AAAATTATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
REDCEDAR    AAAATTATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
JAT1551     AAAATTATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
veiullotia  CTAATTATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
jungneri    CTAATTATTT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
coronata    ----TTGTCT GGTCTATAAA TGAAAGTGTA AGCATTTCAC CTCAAGAGTA
discophora  ---TAATTTT GGTCTATAAA TTAAAGTGTA AGCATTTCAC CTCAAGAGTA

Fusarium    ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
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cinnabar     ATGTGGCAAC CCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
heteroneON   ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
heteroneBC   ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
cylindrop3   ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
cylindrop4   ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
cylindroc2   ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
destrucpa17  ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
REDCEDAR     ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
JAT1551      ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
veiullotia   ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
jungneri     ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG
coronata     ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTA ACACCAGTAG
discophora   ATGTGGCAAC GCAGGAACTG AAATCACTAG ACCGTTTCTG ACACCAGTAG

Fusarium     TGAAG-  01A110A111T1T10100C1T1T0000000001T1A0
cinnabar     TGAAGT  G0T100A001A1A1111001010000001100OT1AT
heteroneON   TGAAGT  G0A110T00101011111C1T1T111GC11111T1AT
heteroneBC   TGAAGT  G0A110T00101011111C1T1T111GC11111T1AT
cylindrop3   TGAAGT  G0A100A00101011111C1T1T1010C11011T1AT
cylindrop4   TGAAGT  G0A100A00101011111C1T1T1010C11011T1AT
cylindroc2   TGAAGT  G0A100A00101011111C1T1T1010C11011T1AT
destrucpa17  TGAAGT  G0A100A001T1T1110001T1T0000001000T1AT
REDCEDAR     TGAAGT  G0A100A001T1T1110001T1T0000001000T1AT
JAT1551      TGAAGT  G0A100A001T1T1110001T1T0000001000T1AT
veiullotia   TGAAGT  G0A111A00000000000000000000000000001AT
jungneri     TGAAGT  G0A100A00000000000000000000000000001AT
coronata     TGAAGT  G0A100A00000000000000000000000000000T
discophora   TGAAGT  G000000000000000000000000000000000TT

;

END;

begin paup;

      exclude 1 59 87 543 576 584 596 603 621 622 649 654 756 /only;


endblock;
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APPENDIX B

An example of a data set in PHYLIP format for distance analysis using PHYLIP software package. This data set was used for intraspecific analysis of *C. destructans* in this study.

```
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PEMBERTONB GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
NURSERY1.1 GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
THIMBLEBER GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
CHILLIWAKA GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
PELTONSB   GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
PEMBERTONC GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
CHILLIWAKB GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
NURSERY1.2 GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
CHILLIWAKC GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
SALAL      GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
ELDERBERRY GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
WILLOWS    GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
NURSERY2.1 GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
PELTONSA   GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
NURSERY2.2 GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
REDCEDAR   GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
REDALDER   GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
PINE       GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
GINSENG    GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
TRILLIUM   GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
JAT1378    GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
JAT1901    GCCTAACGGCTGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA
JAT1551    GCCTAACGGCTGAACTGGGCACTTGGAGAAGTGGCAAGTTTTATAATTAA
CYLINDROID GCCTAACGGATGAACTGGCAACTTGGAGAAGTGGCAAGTTTTATAATTAA

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