MOLECULAR TAXONOMY OF *CERATOCYSTIS SENSU LATO*

by

Georg Hausner

A thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Note to the Reader:

This thesis begins with general Introduction, Literature Review, and Materials and Methods sections, but the remainder of this dissertation is then organized into seven chapters followed by concluding remarks. Each of the chapters has its own introduction, a short summary of materials and methods used, and a results/discussion section.

ABSTRACT

Both restriction fragment analysis of the ribosomal DNA and ribosomal DNA sequence analysis were applied in the grouping of isolates and species of Ceratocystis sensu lato. Restriction fragment analysis, althougth useful in grouping closely related species, failed to provide data appropriate for establishing generic limits within this group of fungi. However, phylogenetic analysis of partial rDNA sequences from both the small and large subunit genes of species of Ceratocystis s.l., and those from other fungi included to serve as outgroups, supports the contention that Ceratocystis species that lack Chalara anamorphs, are resistant to cycloheximide, and have rhamnose in their cell walls should be assigned to Ophiostoma, whereas only species with Chalara anamorphs should be accommodated in Ceratotysis s.s.. Ribosomal DNA sequence data also placed species of Ceratocystiopsis within Ophiostoma, therefore Ceratocystiopsis is reduced to synonymy with Ophiostoma.

This study also demonstrates that yeast-like genera that produce galeate (hat-shaped) ascospores and similar-spored members of the Ophiostomatales, do not form a monophyletic group. Therefore the belief that the Ophiostomataceae should be included within an expanded concept of the Endomycetaceae is not supported. This investigation indicates that for now, Ophiostoma should remain the sole genus of the Ophiostomataceae, and the

latter should be the sole family within the Ophiostomatales, whereas Ceratocystis s.s. would be best disposed within the Microascales.

Analysis of a partial LSrDNA dataset that included sequences from 55 species assignable to Ophiostoma, failed to support the strict subdivision of the genus based on either ascospore characters or the nature of the anamorph.

The taxonomic position of Ceratocystiopsis falcata and Sphaeronaemella fimicola was not resolved, although the rDNA data clearly excludes both from either Ceratocystis or Ophiostoma. Similar findings were made for Ceratocystiopsis alba, Ophiostoma roraimense, and Ceratocystis autographa which appear to have little relationships to either Ceratocystis or Ophiostoma. Ceratocystiopsis proteae, on the other hand, should be made the type species of a new genus that would be aligned with Ceratocystis within the Microascales.

Sph. fimicola was further distinguished from other members of Ceratocystis s.l. by its unusually compact ribosomal RNA gene cluster. Molecular systematics techniques were also applied to species of Togninia and Beauveria to evaluate taxonomic decisions made to designate new species based on morphological criteria.

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CHAPTER 1

INTRODUCTION

Ceratocystis Ell. & Halst. *sensu lato*, which according to some authorities includes the genera *Ceratocystis* Ell. & Halst. *sensu stricto*, *Ophiostoma* H. & P. Sydow and *Ceratocystiopsis* Upadh. & Kendrick (Benny and Kimbrough 1980; de Hoog and Scheffer 1984; Barr 1990), is important because it contains many insect-associated plant pathogens and wood-staining species. In countries where forestry is a vital part of the economy, the latter cause significant loss in timber value due to their staining of the wood. This results from either the penetration of their dark-coloured hyphae into the wood, or the diffusion therein of dark-coloured pigments produced by the fungus. The most significant wood-staining fungi belong to the genus *Ceratocystis* *s.l.*, which includes over 100 species (Wolfaardt *et al*. 1992b). As pathogens, species of *Ceratocystis* *s.l.* are also of great economic importance: e.g. *Ceratocystis fagacearum* (Münch) Bakshi causes oak wilt; *Ceratocystis fimbriata* Ell. & Halst. causes rot in sweet potatoes and wilt in coffee and rubber trees; and *Ophiostoma novo-ulmi* Brasier is the causative agent of the new aggressive form of Dutch elm disease (Brasier 1991).

Ceratocystis *s.l.*, which generally has been placed in the family Ophiostomataceae, is characterized by asci that are produced randomly within the base of the darkly

pigmented ascocarp, and these asci deliquesce upon maturation of the ascospores. Once they have deliquesced, the ascospores are extruded in a slimey droplet from the ostiole at the tip of the perithecial neck. And as such fruiting structures of both saprophytic and parasitic species of Ceratocystis s.l., as well as their anamorphic states, are often found in bark beetle galleries on the host trees, and emergent beetles may serve as the spore dispersal agents.

The taxonomic history of the species of Ceratocystis s.l. is very complex and, as a consequence, the generic limits and the disposition of the genus within the Ascomycotina is unresolved. There has also been difficulty stemming from the repeated controversial attempts that have been made to divide its species into more homogeneous groupings based on characters which have not always been accepted as being significant. Such groupings have been attempted employing: ascospore morphology (Griffin 1968; Olchowecki and Reid 1974; Upadhyay and Kendrick 1975; Upadhyay 1981); chemical composition of the cell wall (Rosinski and Campana 1964; Smith et al. 1967; Spencer and Gorin 1971; Jewell 1974; Weijman and de Hoog 1975; de Hoog and Scheffer 1984); anamorph characters (Nannfeldt in Melin and Nannfeldt 1934; Hunt 1956; Mathiesen-Kaarik 1960; de Hoog and Scheffer 1984); tolerance to cycloheximide in culture (Harrington 1981; de Hoog and Scheffer 1984); and the lack of an ostiole (Parker 1957; von Arx 1974; von Arx

and van der Walt 1987). But even when such studies appear to be partially successful, i.e. de Hoog and Scheffer (1984) presented convincing arguments for the segregation of species with Chalara anamorphs (and other characteristics) into Ceratocystis s.s., they have failed to resolve other major problems. This is a common failing with many of the studies, described above.

Recently centrum morphology and ascospore development have also been used in an attempt to define species relationships (Van Wyk and Wingfield 1991a, 1991b; Van Wyk et al. 1991), but these too have left many unanswered questions.

Historically, systematic studies of the species of Ceratocystis s.l. have been hampered by a lack of truly definitive taxonomic characters, as well as cases in which the teleomorphic and anamorphic characters suggest contradictory placement. But it seems generally agreed that members of Ceratocystis s.l. are very likely polyphyletic, and thus more traditional morphological and physiological data have led to confusing and often contradictory classification systems for the ophiostomataceous fungi (Redhead and Malloch 1977; Benny and Kimbrough 1980; Upadhyay 1981; von Arx and van der Walt 1987; Barr 1990).

Since ophiostomataceous fungi are of economic importance, a revision of this group which could result in a more natural classification system is desirable, and the use of molecular criteria may prove to be the means to this end.

Molecular systematics has the potential to provide data which can be used to evaluate objectively the phylogenetic significance of morphological features; in addition it can permit phylogenetic reconstruction independent of morphological criteria. In order to assess the generic limits proposed for the members of Ceratocystis s.l., and relationships between genera deemed assignable or related to this group of fungi, the 5'terminal sequence of the large subunit ribosomal gene (LSrDNA) was determined for about 100 strains representing 70 different species. In addition, partial sequences were obtained from the small subunit ribosomal gene (SSrDNA) for about 50 different species that represent various aspects of previous groupings proposed within the Ophiostomatales.

Overall this investigation attempted to answer the following questions:

(i) Do members of the Endomycetaceae and Ophiostomataceae with galeate-ascospores share a common phylogeny?

(ii) Does the segregation of members of Ceratocystis s.l. into Ophiostoma, Ceratocystiopsis, and Ceratocystis s.s. result in a more natural classification?

(iii) Do members of the Ophiostomataceae and Microascaceae share a common phylogeny?

(iv) Are morphological characters such as ascospore shape, and features of the anamorph and centrum characteristics useful in proposing phylogenetic relationships for the ophiostomataceous fungi?

LITERATURE REVIEW

GENERIC CONCEPTS WITHIN *Ceratocystis* s.l.

The rather complex nomenclatural history of the genus *Ceratocystis*, and that of its type species *Ceratocystis fimbriata* Ell. and Halst. have been reviewed by Hunt (1956), Upadhyay (1981), and Hutchison (1984). The genus *Ophiostoma*, with *Ophiostoma piliferum* (Fr.) H. and P. Sydow as the type species, was formally proposed by Sydow and Sydow (1919), but both Bakshi (1951) and Hunt (1956) considered *Ophiostoma* to be a synonym of *Ceratocystis*. Hunt (1956) was the first to attempt to monograph species assigned to *Ceratocystis*. He used their production of deliquescent asci and the irregular arrangement of the asci within the perithecia as the main taxonomic criterion to delimit this genus. Based on features of the anamorphs he recognized three sections. However, the three types of conidial states may not be as distinct as Hunt believed, because modern taxonomy of the Hyphomycetes is primarily based on the exact method of conidiogenesis and not on the structures supporting the conidiogenous cells (Hughes 1953; Kendrick 1962; Barron 1968).

Over the years as the generic concept of *Ceratocystis* was expanded to include species with two-celled ascospores (e.g. *Ceratocystis falcata* Wright and Cain 1961), species with hyaline perithecia (e.g. *Ceratocystis alba* DeVay, Davidson and Moller 1968), species with very short necks (e.g. *Ceratocystis minima* Olchowecki and Reid 1974), and

species in which the perithecial base is different in colour from that of the neck (e.g. Ceratocystis bicolor (Davids. & Wells) Davids. 1958), a greater importance was placed on ascospore morphology as a taxonomic character (Wright and Cain 1961; Griffin 1968; Olchowecki and Reid 1974; Upadhyay 1981).

Griffin (1968) recognized four ascospore types in species of Ceratocystis, and Olchowecki and Reid (1974) used Griffin's ascospore types, with some modifications, to divide Ceratocystis into four groups: the Fimbriata, Pilifera, Ips, and Minuta groups. Upadhyay and Kendrick (1975) argued that Ceratocystis species with falcate ascospores, essentially those of Olchowecki and Reid's Minuta group, were morphologically discrete because no (or few) intermediate shapes were known to exist between falcate and crescent-shaped ascospores. Thus they erected the genus Ceratocystiopsis (type: Ceratocystiopsis minuta (Siem.) Upadh. and Kendrick) to segregate the Minuta spore group of Olchowecki and Reid from Ceratocystis. Upadhyay and Kendrick (1975) also proposed that the genus Europhium Parker should be considered a synonym of Ceratocystis. Parker (1957) had erected Europhium (type: Europhium triancriforme Parker) for those Ceratocystis-like species that produce cleistothecioid ascocarps. Since the presence or absence of an ostiolum is a variable character that may be dependent on environmental conditions (von Arx 1973; Upadhyay 1981), this genus was not accepted by either de Hoog (1974) or Upadhyay (1981); it was

maintained by von Arx (1974, 1987) for practical reasons.

In his monograph of Ceratocystis and Ceratocystiopsis, Upadhyay (1981) recognized 75 species in Ceratocystis and 15 in Ceratocystiopsis. Within Ceratocystis he recognized four sections based on ascospore morphologies similar to those of Olchowecki and Reid (1974). Upadhyay's sections Ips and Ophiostoma are comparable to Olchowecki and Reid's Ips and Pilifera groups, and sections Ceratocystis and Endoconidiophora are contained within the Fimbriata group of Olchowecki and Reid (1974). Upadhyay (1978) also considered Sphaeronaemella P. Karst. to be a synonym of Ceratocystis, and he formally transferred species of Sphaeronaemella to Ceratocystis section Ophiostoma (Upadhyay 1981). This latter synonymy has not been generally accepted (Barr 1990), and de Hoog and Scheffer (1984) particularly disagreed with this decision. Sphaeronaemella species have oblate ascospores with narrow germ slits, fleshy, pink to reddish white ascomata, and Gabarnaudia-like anamorphs, this led de Hoog and Scheffer (1984) to place the genus in the Hypocreaceae.

As noted earlier, the synonymization of Ophiostoma with Ceratocystis has not been universally accepted, and currently species of Ceratocystis s.l. with enteroblastic-phialidic anamorphs are viewed as being unrelated to Ophiostoma species that have holoblastic anamorphs with percurrent or sympodial proliferation (de Hoog 1974; von Arx 1974; Weijman and de Hoog 1975; Samuels and Müller 1979; Harrington 1981, 1987; de Hoog and Scheffer 1984; Wolfaardt

et al. 1992b). Following their detailed reevaluation, de Hoog and Scheffer (1984) concluded that Ophiostoma should be maintained for those species with anamorphs other than Chalara, with rhamnose and cellulose in their cell walls (Rosinski and Campana 1964; Spencer and Gorin 1971; Jewell 1974; Weijman and de Hoog 1975), and insensitivity to cycloheximide (Harrington 1981). Conversely, species of Ceratocystis s.s. appear to have a more typical ascomycete hyphal wall chemistry (Jewell 1974) and are sensitive to cycloheximide. De Hoog and Scheffer (1984) did accept the genus Ceratocystiopsis, but Harrington (1987, 1988) and Wingfield et al. (1988) felt that species of Ceratocystiopsis should be accommodated within Ophiostoma.

The final disposition of members of the genus Ceratocystis s.l. within the Ascomycotina remains unresolved. They have been variously disposed in the orders Plectascales (Nannfeldt 1932; Hunt 1956), Microascales (Luttrell 1951; Upadhyay 1981; Barr 1990), Ophiostomatales (Benny and Kimbrough 1980; von Arx and van der Walt 1987), and the Sphaeriales (Müller and von Arx 1973). Those who believe that Ceratocystis s.s. is not closely related to Ophiostoma have placed Ceratocystis s.s. within the Lasiosphaeriaceae (Sordariales) (Barr 1990) or the Pyxidiophoraceae (Order Ophiostomatales sensu von Arx and van der Walt 1987). And in broadening the discussion, Redhead and Malloch (1977) proposed that yeasts with galeate (helmet- or hat-shaped) ascospores were related to the

genera within the Ophiostomataceae. They felt that galeate ascospores did not have multiple originations, and, being homologous, serve to link Ceratocystis and Ophiostoma species with galeate-ascospored members of the Endomycetaceae. However Weijman (1976), Samuels and Müller (1979), and Benny and Kimbrough (1980) felt that many features mitigate against a close relationship of the Ophiostomataceae with the Endomycetaceae.

MOLECULAR SYSTEMATICS

Systematics, taxonomy and nomenclature are often defined differently by various authors. However, one most useful approach for defining the relationships between these subjects is to say that systematics is the sum of the two components nomenclature and taxonomy. Nomenclature deals with the laws and principles involved in the correct application of scientific names to taxa, and with the placement of taxa within a particular nomenclatural system (Talbot 1971). Taxonomy concerns itself with the classification of organisms using a variety of criteria, e.g. morphological, physiological, anatomical, genetical or ecological features which are identified and then compiled. The data are analyzed, and characters which appear to be useful either in describing an organism, or that suggest a common descent (or relatedness) with other taxa, are defined, and these become taxonomic characters. Ultimately more and more inclusive groups are defined by more and more generally shared taxonomic characters; thus taxa are arranged in a hierarchy of consecutive ranks, i.e. species to genera, genera to families, etc. But as Luttrell (1958) has pointed out, the taxa arranged, and their names, are incidental to the wider task of classifying information about fungi.

The objective of phylogenetic classification is to group organisms according to their evolutionary history.

This type of classification system may allow one to predict biochemical and physiological properties of members within taxa, since common ancestry would suggest that there should be a similarity in genetic background. However, any phylogenetic classification is an ideal that is difficult to achieve, since phylogeny can only be inferred from the similarities and differences of contemporaneous organisms.

Traditionally, classification systems have been based on morphological and developmental features in conjunction with fossil data. For the fungi fossil data is scarce, and often a lack of consistently distinctive differences render the developmental and morphological approaches problematic. In addition, it is often extremely difficult to differentiate between homologous characters that are derived due to common descent, and analogous characters that are products of strong selection for certain morphological features. Phylogenetic reconstructions are based on morphological features that are assumed to be homologous. Consequently, the use of molecular characters as an alternative source of taxonomic characters has developed in recent years (Bruns et al. 1991; Kohn 1992).

Molecular data are considered as an alternative to morphological data when organisms have only simple or potentially convergent morphologies. From a more practical viewpoint, it is also hoped that molecular techniques will generate datasets independent of morphology, then the molecular characters could be used to evaluate objectively

the phylogenetic significance of the various morphological features. This would permit a critical evaluation of existing classification systems. For example those shown to be based on analogous morphological characters could be revised based on the characters which were shown to be homologous by independent molecular analysis. But even if useful morphological characters are shown to be completely lacking, molecular systematics alone could be an alternative if the molecular method employed generated sufficient independent characters to allow a phylogenic inference that could be statistically evaluated.

A variety of biochemical approaches has been tested in an attempt to identify molecular characters suitable for use in fungal systematics. But, those investigated have proven to be useful only for testing existing classifications at higher levels of hierarchy, e.g. polyol patterns (Pfyffer et al. 1986) and cell wall composition data (Bartnicki-Garcia 1968, 1970), or they have had only limited taxonomic 'resolving power' at the intraspecific level, e.g. whole-cell fatty acid analysis (Johnk and Jones 1992), protein electrophoresis including allozyme analysis (May and Royse 1988; Otrosina et al. 1992) and isozyme analysis (Zambino and Harrington 1989; Vogler et al. 1991; Chen et al 1992b; Liu and Sinclair 1992). Isozyme characterization in particular is gaining in popularity as a source of non-morphological characters either for resolving taxonomic

problems within a species, or for delimiting closely related species (Stasz et al 1989; Zambino and Harrington 1992).

The application of isozyme analysis in fungal taxonomy has been reviewed by Micales et al. (1986), and the limitations and potential artifacts of protein electrophoretic techniques have been discussed by Ferguson (1980). Protein electrophoresis is only an indirect method of assessing genetic (phylogenetic) relatedness, because amino acid differences which are compensatory in their contribution towards the overall charge and molecular weight of a particular protein will go undetected. In addition, due to the degeneracy of the genetic code changes at the DNA sequence level are not necessarily detected by analysing the products of genes.

Biochemical characteristics derived from protein electrophoresis or lipid content are potentially useful only for estimating genetic variability within a species. Therefore these characters have not been useful for establishing phylogenetic classification systems for a diverse group of organisms. Phylogenetic systems at higher taxonomic levels require macromolecules that are found in all organisms and contain regions that are conserved in structure and function. Therefore most of the literature on fungal molecular systematics concerns itself with techniques that involve the comparison of nucleic acids between different fungal strains.

A variety of methodologies has been developed to characterize DNA differences between fungal strains. Cytological karyotyping (Hansen 1987) and electrophoretic karyotyping (Miao et al. 1991) have rarely been used, but the few studies available suggest that in fungi there is a high level of intraspecific and even population level karyotype variability (Kohn 1992). Estimation of nuclear DNA content also appears to yield data that are potentially useful for resolving relatedness at the specific or intraspecific level (Motta et al. 1986; Eilam et al. 1992). However in general the methodologies employed to determine nucleic acid relatedness for applications in fungal taxonomy are as follows: (1) DNA base composition; (2) DNA relatedness (DNA-DNA hybridizations); (3) restriction enzyme analysis; and (4) DNA sequence analysis. Some or all of these methodologies to study DNA relatedness between fungi have been reviewed by Kurtzman (1984, 1985a, 1985b, 1987); Blanz and Unseld (1987); Hillis and Moritz (1987); Michelmore and Hulbert (1987); Jahnke (1987); Bruns et al. (1991); and Kohn (1992).

(1) DNA base composition (G+C content)

Nuclear DNA base composition is the simplest molecular technique for obtaining molecular characters. Most reported G+C values have been determined using either thermal denaturation profiles of nuclear DNA (Kurtzman 1985a), or by cesium chloride buoyant density gradient ultracentrifugation

methods (de Hoog and Guého 1984; Martini and Kurtzman 1988). Overall, G+C content is of little value for taxonomic purposes because ranges in G+C content from 30 to 70% have been reported for individual fungal species, and an overlap in values obtained for unrelated taxa is inevitable (Storck 1965, 1967; Kurtzman 1985a). Even in studies where closely related species are examined, G+C content can be misleading; e.g. Saccharomyces dairensis and Saccharomyces servazzii have identical %G+C values, but DNA cross-hybridization between these two species indicates only 13% DNA relatedness (Martini and Kurtzman 1988). Thus G+C values could imply identity for species that actually share little genetic similarity.

(2) DNA relatedness

DNA/DNA reassociation methodologies for both total DNA extracts, and nuclear DNA extracts for fungal genomes have been described (Kurtzman 1985b; Jahnke 1987). In general, DNA complementarity values for fungi have been obtained by determining the percentage of cross-hybridization between total DNA extracts. Most DNA relatedness studies have had some usefulness in systematic studies of ascomycetous and basdiomycetous yeasts (Lachance et al. 1986; Kurtzman and Phaff 1987; Martini and Kurtzman 1988; Kurtzman 1990), as well as in a more limited number of studies of filamentous fungi (Kurtzman 1985b; Vilgalys 1988). Generally nuclear DNA relatedness comparisons resolve taxonomic questions only at

the genetic sibling species level (Kurtzman 1985b; Kurtzman and Pfaff 1987; Kurtzman 1990). This is due to the observation that the DNA relatedness between closely related species is typically less than 20%, whereas between individuals belonging to the same species values greater than 90% are observed (Kurtzman 1985a, 1986b; Bruns et al. 1991).

Amongst the yeasts, it has been noted that an almost linear relationship generally exists between levels of DNA relatedness and interfertility, although some exceptions both in yeasts and filamentous fungi have been recorded (Ellis 1985; Kurtzman 1985; Vilgalys and Johnson 1987). Kurtzman argued that exceptions to this linear relationship between DNA relatedness and interfertility could be explained by such genomic factors as major chromosomal rearrangements or differences in ploidy levels; those could result in the genetic isolation of strains that would otherwise appear to belong to one biological species.

In some studies, to increase the level of taxonomic resolution obtained using DNA complementarity at the species or even the genus level, only the conserved components of the genome have been analyzed, e.g. the nuclear RNA genes (Baharaeen and Vishniac 1984; Kwok et al. 1986). However this approach has not become popular since new methods have become available that permit the rapid determination of rRNA sequences (Lane et al. 1985) or rDNA sequences (White et al 1990); these are thought to be superior both for

establishing the hierarchy of species within genera, and phylogenetic relationships above the species level.

(3) Restriction enzyme analysis

Restriction enzyme analysis is one of the approaches used most commonly to characterize various components within the fungal genome. DNA can be cleaved at specific target sequences with type II restriction endonucleases, and the DNA restriction products can then be separated by size using agarose gel electrophoresis to yield informative restriction patterns. In most instances the DNA fragments are transferred to, and immobilized on, a filter, and then hybridized to a labelled DNA probe which permits the visualization of specific DNA fragments by autoradiography. Restriction fragment length polymorphisms (RFLPs) are the result of differences in the DNA sequence that affect either the location or the nucleotide sequence of restriction endonuclease target sites. Therefore RFLPs can result from single base-pair substitutions, insertions and deletions, transposition events, and the inversion or translocation of DNA fragments. The assumption made with RFLP analysis is that closely related species will show similar hybridization patterns when their restriction fragments are hybridized to an appropriate probe, whereas species thought to be more distantly related are expected to exhibit significantly different hybridization patterns (Olsen et al. 1986).

In general, the RFLP patterns that result from a

particular restriction enzyme-probe combination from different strains are compared, and phylogenetic relatedness is estimated directly or indirectly by the number of restriction fragments shared (Kozlowski and Stepien 1982; Egger et al. 1991; Gardes et al. 1991a). But increasingly, the entire RFLP pattern of one restriction enzyme-probe combination is being viewed as one discrete character (Garber and Yoder 1984; Kohn et al. 1988; Jacobson and Gordon 1990; Jeng et al. 1991). Therefore RFLP phenotypes for a large number of restriction endonucleases should be generated to obtain sufficient discrete characters to permit phylogenetic reconstruction (Dowling et al. 1990).

Generally, restriction enzyme analysis is a relatively fast technique which permits simultaneous examination of a fairly large number of isolates, and it is a popular approach used to find useful molecular markers in studies of population genetics (Förster et al. 1989; Jacobson and Gordon 1990; Correll et al. 1992; Gordon and Okamoto 1992; Gray and Hepburn 1992; Kohli et al 1992), evolutionary relationships (Natvig et al. 1987; Taylor and Natvig 1989; Smith and Anderson 1989), and DNA fingerprinting of economically important fungal strains (Michelmore and Hulbert 1987; Bruns et al. 1991). However, it has been noted that RFLP analysis probably has only limited application for phylogenetic reconstruction because of length mutations (insertions and deletions) which appear to occur at high frequency in fungal genomes; these result in an

overestimation of evolutionary distances between fungal strains (Swofford and Olson 1990; Bruns et al. 1991; Kohn 1992). The taxonomic resolution of RFLP analysis is therefore limited. In addition, as pointed out by Bruns et al. (1991), restriction fragments should not be viewed as independent characters; thus data analysis is limited to phenetic methods, as cladistic approaches should be based on independent character states.

Restriction site mapping is an alternative to simply analyzing restriction pattern phenotypes. Physical mapping of restriction sites determines the physical relationships of restriction fragments, and therefore length mutations can be identified or localized. And as restriction endonucleases basically sample nucleotide sequences, the larger the variety of endonucleases utilized for restriction mapping, the more reliable the data will be in reflecting the actual sequence divergence between various species. But restriction mapping is very labor intensive, and it can be an error-prone process. In addition, detailed alignments of physical maps can be problematic; consequently some researchers argue that restriction maps are of limited value in phylogenetic studies of fungi (Bruns et al. 1991).

Several studies have utilized random-fragment hybridization analysis as a source of non-morphological characters (Natvig et al. 1987). In species of Verticillium, considerable molecular variation could be detected amongst some of its species with this approach (Carder and Barbara

1991). With Neurospora species it was demonstrated that random-fragment analysis, which included restriction fragment cataloging and restriction mapping, could be useful in evolutionary studies (Natvig et al. 1987). In general, anonymous probes consisting of repetitive DNA elements have been employed successfully in studying intraspecific relationships in Phytophthora megasperma f.sp. glycinea (Whisson et al. 1992), Sclerotinia sclerotiorum (Kohli et al. 1992), Mycosphaerella graminicola (McDonald and Martinez 1991). In addition, the complex hybridization patterns which result from these repetitive DNA probes could be very useful for "fingerprinting" strains (Levy et al. 1991; Kohn et al. 1991).

The most common targets for restriction enzyme analysis are the nuclear ribosomal DNA (rDNA) repeat unit and the mitochondrial genomes. In addition to its high copy number, small size (17-176 kb) (Cray 1982; Bruns et al. 1991) and relative ease of isolation (Garber and Yoder 1983), the mitochondrial genome appears to be functionally conserved in all eukaryotic organisms (Cray 1982; Wallace 1982); these features make it an appropriate target for studies in fungal evolutionary biology (Taylor 1986; Varma and Kwon-Chung 1989). However, as length mutations appear to be very common in fungal mtDNA (Taylor 1986; Taylor and Natvig 1989), effective use of restriction fragment analysis does require physical mapping of the mtDNA. This allows one to identify nucleotide substitutions, length mutations, rearrangements

(transposition events and inversions), and to assess their respective contribution towards the apparent genetic variability (Taylor 1986; Bruns et al, 1991). Overall, restriction analysis of mtDNA indicates that while mtDNA fragment patterns are very similar within biological species, they are quite dissimilar between different species (Borkhardt et al. 1987; Förster et al. 1988; Kohn et al. 1988; Smith and Anderson 1989; Taylor and Natvig 1989).

RFLP analysis of mtDNA has been used in attempts to assess phylogenetic relationships amongst species from many groups of fungi; these included Oomycetes (Förster et al. 1988; Förster et al. 1990b), the Ascomycotina (Kozlowski and Stepien 1982; Kohn et al. 1988; Suzuki et al. 1988; Taylor and Natvig 1989), and the Basidiomycotina (Jahnke et al. 1987; Gardes et al. 1991a; Weber et al. 1991). And most have focussed on finding RFLP markers either for economically important fungi (Gardes et al. 1991a; Jeng et al 1991; Gray and Hepburn 1992) or for resolving taxonomic questions at the intraspecific level.

A substantial number of these investigations have sought molecular markers that could be correlated with geographic origin (Garber and Yoder 1984; Vincent et al. 1986), host specificity (Förster et al. 1989; Egger et al. 1991), anamorph-teleomorph connections (Suzuki et al. 1988), pathogenicity (Jeng et al. 1991; Kim et al. 1992; Gordon and Okamoto 1992), speciation (Taylor et al. 1986), vegetative compatibility (Jacobson and Gordon 1990; Correll et al.

1992), or cytoplasmic inheritance (Hintz et al. 1985) of the fungi studied. Therefore mitochondrial DNA appears to be an appealing molecule for evolutionary studies. But results derived from mtDNA studies should be viewed with caution because the inheritance patterns, and the potentials for lateral transfer and structural rearrangements (i.e. recombinational events, degeneration rates of the mitochondrial genome during senescence, transposition events, and the possibilities for insertion of linear plasmid-like molecules) within this molecule are not yet fully understood (Nevzglyadova 1984; Bertrand et al. 1985; Taylor 1986).

The potential usefulness of both restriction patterns derived from the nuclear ribosomal DNA repeat unit, and rDNA physical maps have been explored for taxonomy and identification purposes in almost all lineages of life (Grimont and Grimont 1986; Hillis and Dixon 1991). And the application of such studies to our understanding of molecular evolution in both the fungi and members of other kingdoms have been reviewed by Olsen et al.(1986), Blanz and Unseld (1987), Bruns et al. (1991), and Hillis and Dixon (1991). Olsen et al. (1986) outlined some features of the rDNA/rRNA that appear to justify the great emphasis which many evolutionary studies have placed on rDNA/rRNA comparisons, while additional important characteristics of the rDNA have been noted by Bruns et al. (1991) and Kohn (1992). For example, the rDNA genes are universal and their

products are functionally and evolutionarily homologous in all organisms, while the ribosomal RNAs are ancient molecules whose secondary structures are highly conserved.

Although some portions of the rDNA sequences are quite variable, others are highly conserved across the primary Kingdoms. And these highly conserved rDNA components are suitable targets for hybridization against heterologous probes, primer directed sequencing techniques or *in vitro* primer dependent amplification protocols (polymerase chain reaction). Such conserved stretches facilitate restriction mapping because some restriction sites are, as a consequence, almost universal, and therefore these can be used as reference points. Conversely, restriction sites located in the more variable rDNA domains could become useful diagnostic molecular markers for either species identification or for delimiting fungal taxa. In addition, mapped restriction sites can be useful in orienting and positioning nucleotide sequences obtained from clones of the same genomic region.

Except for the 5s gene, which may or may not be a component of the rDNA repeat unit (Gerbi 1985), the order of fungal rDNA genes is conserved. In all true fungi, the rDNA exists as a tandomly repeated array of rDNA units consisting of the 16-18s, 5.8s, and 23-28s rRNA genes which are separated both by transcribed and non-transcribed spacers. But it can be argued that these rDNA repeat unit arrays behave like a single copy gene (Bruns *et al*. 1991) because

the multiple copies of the repeat unit appear to homogenize quickly via concerted evolution (Gerbi 1986).

The rRNAs and the rDNA constitute a significant component of the nuclear genome, therefore rDNA/RNA can be recovered from all types of organisms with reasonably high yields. And the rDNA genes, in particular the small subunit ribosomal gene (SSrDNA) and the large subunit ribosomal gene (LSrDNA), can provide sufficient sequence information to permit statistically relevant comparison. Overall, the conserved sequences of the rDNA offer the means of assessing affinities above the genus level, whereas the more variable domains of the rDNA such as the intergenic region (IGR, also referred to as the non-transcribed spacer or intergenic spacer) and internal transcribed spacers (ITS), could be useful for resolving inter- and intraspecific relationships (Hillis and Dixon 1991). Finally, the nuclear rDNA appears to lack the artifacts of lateral transfer between contemporaneous organisms.

Ribosomal DNA analysis has been very useful in the identification of economically important yeasts. Restriction mapping was applied in delineating species of the genus Clavispora (Lachance et al. 1986), while McGee et al. (1987) noted that EcoRI restriction maps could be an important taxonomic aid for the identification of Candida species. Verbeet et al. (1984) applied rDNA restriction mapping in studies of yeast evolution, and employed aligned rDNA physical maps from members of five different genera in their

investigation. Physical maps of rDNA have also been compared in other fungi which lack definitive morphological characters, and which were thus not amenable to traditional taxonomic techniques (Klassen et al. 1987). RFLP analysis of the rDNA has also been utilized to resolve taxonomic concerns at the intraspecific level (Specht et al. 1984; Vincent et al. 1986; Egger and Fortin 1990; Vilgalys and Gonzalez 1990; Lobuglio et al. 1991; Liyanage et al. 1992).

The polymerase chain reaction (PCR), which was developed by Mullis and Faloona (1987) and further refined by Saiki et al. (1988), makes possible in vitro amplification of specific DNA sequences by employing primers which flank a region of interest combined with a thermostable DNA polymerase.

The application of PCR technology to evolutionary studies has been reviewed by Kocher and White (1989) and White et al. (1990). The reaction is used to amplify regions of the genome that are deemed suitable for answering specific taxonomic questions. And as an appreciable number of rDNA genes have already been sequenced (Neefs et al. 1990; Hillis and Dixon 1991) oligonucleotide primers for the PCR reaction can be constructed based on the conserved stretches of rDNA genes. The PCR reaction requires very little genomic DNA (nanograms) as starting material, and many samples can be treated and analyzed simultaneously. Additionally, the yield of the amplification product is usually sufficient for direct visualization of DNA fragments

by staining gels with ethidium bromide and, as a consequence, the use of Southern hybridizations and autoradiography to visualize the position of DNA fragments are optional. They are necessary only intitially to confirm the identity of the PCR product. It is because of these benefits that restriction analysis of PCR amplification products is a methodology that has rapidly gained popularity.

White et al. (1990) demonstrated that sufficient genomic DNA for PCR analysis can be obtained from a single fungal spore. This is of great importance because many fungi grow poorly in culture, and thus it is difficult to obtain sufficient mycelium for DNA extraction and subsequent analysis. Bruns et al. (1990) showed that genomic DNA suitable for PCR analysis could be extracted from herbarium specimens. This opens up the possibility of obtaining DNA for molecular analysis directly from either the holotype or from other herbarium specimens of species which are no longer available as living cultures. Another significant advance was the demonstration by Gargas and Taylor (1992) and Gardes et al. (1991b) that taxon specific oligonuleotides could be constructed which allow PCR amplification of a specific target region in a single organism from mixed DNA derived from two or more organisms in a population. Thus fungi which are obligate symbionts or parasites, and cannot be grown in axenic culture, can still be studied using molecular techniques. This approach has

already been used in studies of ectomycorrhizal rootlets (Gardes et al. 1991b), and will likely be useful in studying the molecular evolution of lichenized fungi (Gargas and Taylor 1992).

Hibbet and Vilgalys (1991) used restriction analysis of enzymatically amplified rDNA to study species of the genus Lentinus. Their results led them to conclude that this genus is paraphyletic, with most of its members being intermediate between the Polyporaceae and Tricholomataceae. Vilgalys and Hester (1990) also obtained complex restriction phenotypes ("fingerprints") for species of Cryptococcus using RFLP analysis of enzymatically amplified rDNA, and from analysis of these they deduced the genetic relationships amongst the species they studied. Thus RFLP analysis based on rDNA genes can provide taxonomic resolution above the genus level.

By generating and analyzing PCR products that contained both of the internal transcribed spacers, Liu and Sinclair (1992) and Chen et al. (1992a) addressed taxonomic problems at the intra- and interspecific level. Liu and Sinclair (1992), Chen (1992), Chen et al. (1992a) also demonstrated that restriction mapping of highly variable domains within the rDNA allows for discrete character-based cladistic analysis of molecular data to resolve phyletic relationships at the intraspecific level. It is far more practical and economical to obtain detailed physical maps of PCR products than it is to map the entire rDNA repeat unit via extensive Southern hybridizations. Also, depending of the level of

taxonomic resolution required, the entire rDNA unit probably contains many non-informative segments, but PCR technology provides a tool whereby only the informative segments need to be analyzed.

It is also very likely that studies at the intraspecific level will also benefit from the application of another PCR methodology, random amplified polymorphic DNA (RAPD)(Welsh and McClelland 1990; Williams et al. 1990). The application of RAPD to studies of fungi has been reviewed by Williams et al. (1991).

Although restriction enzyme analysis is potentially very useful in finding markers that permit the discrimination of closely related organisms when morphological features suitable for this purpose are lacking, it provides very limited resolution between more distantly related organisms. The accumulation of length mutations in different lineages makes comparison of RFLP patterns problematic and, in addition, RFLP patterns from different studies cannot be directly compared. Even the alignment of restriction maps from different sources usually requires physical comparison of some restriction fragments. Therefore restriction fragment analysis is not useful in phylogenetic reconstruction above the genus level. Thus PCR and its applications to RFLP, RAPD, and fine structure mapping, will probably be most useful in providing sources of molecular markers for resolving intra- and interspecific taxonomic problems. Ultimately, however, DNA sequence

analysis of appropriate regions within the genome may well be the most useful procedure to aid in resolving phylogenetic relationships amongst fungi at all levels of the taxonomic hierarchy.

(4) Ribosomal DNA sequence analysis

The use of ribosomal RNA/DNA sequences to reconstruct the evolutionary relationships amongst fungi, and in other kingdoms, has been extensively reviewed by Olsen et al. (1986), Blanz and Unseld (1987), Hillis and Moritz (1990), Bruns et al. (1991), Hillis and Dixon (1991), Woese (1991), and Kurtzman (1992). The two most common approaches used to obtain rDNA sequences are direct sequencing of ribosomal RNA (Lane et al. 1985) and direct sequencing of amplified DNA fragments (PCR products) (White et al. 1990). Neither method requires the rather labour intensive procedure used to generate the DNA libraries necessary to eventually obtain clones of rDNA genes. Therefore comparative sequencing studies are quite feasible, and sequences of many taxa can be obtained per study. Sequencing of rDNA PCR products is becoming the more popular approach, since DNA sequencing is less prone to sequencing artifacts than is RNA sequencing (Bruns et al. 1991).

Sequences of the small subunit ribosomal gene (SSrDNA) that code for the 16-18s RNA, and those of the large subunit ribosomal gene (LSrDNA) that code for the 23-28s RNA, appear to contain enough sequence variability to be useful in

phylogenetic studies (Lane et al. 1985; Sogin et al. 1986a; Baroin et al. 1988; Qu et al. 1988). However, due to their small size and conservative secondary structure, the 5s and 5.8s rDNA/RNA sequences are not viewed as being very informative for resolving phylogenetic relationships (Bruns et al. 1991; Haylanych 1991). Nonetheless, the conservative nature and small size (ca. 120 nucleotides) of the 5s rRNAs have been used to estimate broad evolutionary relationships in the Basidiomycotina (Walker and Doolittle 1982, 1983; Huysman et al. 1983; Gottschalk and Blanz 1984) and in the Ascomycotina (Chen et al. 1984; Walker 1985). But as there is now considerable evidence that 5s RNA sequences are inappropriate for phylogenetic studies (Sytsma 1990; Haylanych 1991), conclusions based on 5s RNA analysis need to be confirmed by studies based on more reliable genes.

Sequences of the SSrDNA/RNA have been employed to deduce phylogenetic relationships amongst members of both the eukaryotic and prokaryotic Kingdoms (Hasegawa et al. 1985; Sogin et al. 1986a; Gunderson et al. 1987; Woese 1987, 1991; Cedergren et al. 1988; Field et al. 1988; Hendriks et al. 1991). The different methodologies available for the phylogenetic analysis of nucleotide sequences have been reviewed by Olsen et al. (1986), Nei (1987), Felsenstein (1988), Olsen (1988), and Swofford and Olsen (1990), but the three most common categories of methods are distance matrix methods, parsimony methods, and maximum likelihood methods.

Distance matrix methods construct phyletic estimates

based on a matrix of pairwise distances between all sequences tested, whereas parsimony methods attempt to reconstruct the course of events that led an assumed ancestral sequence to evolve into its various presumed descendent lineages. The overall assumption in parsimony analysis is that evolution takes the shortest route, so the phyletic estimate which requires the fewest changes (steps) going from the assumed ancestor to the given descendent sequences is considered to be the correct one. Maximum likelihood methods are computationally intense, and therefore less often used. The principles of maximum likelihood methods, which are based on specific assumptions about the evolution of sequences, have been reviewed by Felsenstein (1981) and Swofford and Olsen (1990).

Recently, great emphasis has been placed upon assessing the "reliability" (robustness) or level of confidence that can be placed upon phylogenies derived from molecular sequences. And statistical tests used to estimate the confidence of phyletic estimates obtained by various methods of inference have been reviewed by Felsenstein (1988). But bootstrapping (Felsenstein 1985), a procedure which will repeatedly randomly resample the data, is the most common approach used to assess the levels of confidence which can placed in branches within phylogenetic trees. The advantage of bootstrap analysis is that it can be applied to many different methods of tree construction and, depending on the computer facilities available, even large data sets can be

readily evaluated.

The nuclear SSrRNA/DNA gene has proven to be very valuable in phylogenetic reconstruction within the prokaryotes (Woese 1991), so it is not surprising that SSrRNA/DNA sequences have been used to reconstruct phylogenies within the fungi. And one question which has been addressed by such studies is: Are the fungi monophyletic?

Based on SSrRNA/DNA sequences, Gunderson et al. (1987) and Förster et al. (1990a) clearly showed that fungus-like protists such as the Oomycetes and the acellular slime molds, cannot be accommodated within the Kingdom Fungi. Förster et al. (1990a) concluded that there appear to be two fungal evolutionary lineages with the Oomycetes in one, within which they share a common ancestry with the Chrysophytes and diatoms. But the "true fungi", based on molecular data, include the Chytridiomycetes (Dore and Stahl 1991; Bowman et al. 1992a; Li and Heath 1992), Zygomycotina (Hendricks et al. 1991; Li and Heath 1992), Ascomycotina and the Basidiomycotina (Bruns et al. 1991; Bowman et al. 1992a). In addition, evolutionary studies based on the SSrDNA gene (Hendricks et al. 1991) failed to support the hypothesis that the red algae share a common ancestry with the true fungi (Demoulin 1975).

Within the fungi, rRNA/DNA sequence comparisons have been applied often to yeast classification. Barns et al. (1991) demonstrated that the asexual genus Candida is

heterogeneous and, in addition, they showed that pathogenic species such as Torulopsis glabrata (Candida glabrata) are closely related to nonpathogenic species such as S. cerevisiae. Furthermore, molecular studies of rRNA indicate that most ascomycetous yeasts are not reduced from filamentous forms (von Arx and van der Walt 1987), but instead they represent a monophyletic group that probably shares a common ancestry with the filamentous ascomycetes (Hendriks et al. 1992; de Peer et al. 1992).

The application of rRNA sequence comparisons to assessments of phylogenetic relationships amongst yeasts has been reviewed by Kurtzman (1992). Kurtzman and his coworkers utilized partial rDNA sequences of both the SSrDNA/RNA and LSrDNA/RNA to study phylogenetic relationships amongst species of a variety of ascomycetous and basidiomycetous yeasts (Guého et al. 1898; Kurtzman 1989; Fell and Kurtzman 1990; Guého et al. 1990; Peterson and Kurtzman 1991; Kurtzman and Robnett 1991). This was deemed appropriate since Lane et al. (1985) demonstrated that phylogenetic relationships derived from partial sequences are essentially the same as those calculated from complete sequences; therefore it is now quite common for evolutionary studies to employ only certain segments of the rDNA. The acceptability of partial sequences is fortunate, since obtaining complete rRNA sequences is time consuming and expensive. However, as stressed by Bruns et al. (1991), phylogenies based on partial rRNA sequences must be evaluated statistically.

Unfortunately, many phylogenetic studies lack statistical analysis (e.g. Yamada et al. 1989; Chang et al. 1991), thus the level of confidence which can be placed in the proposed phylogenetic relationship(s) is unknown.

There is also considerable interest in applying molecular taxonomic techniques to human pathogens, since many such fungi have evolved highly specialized life styles which make phylogenetic classification difficult. For example Pneumocystis carinii, an opportunistic pathogen in immunocompromised individuals, has evolved a life cycle that superficially resembles those seen in the Protozoa. However, rRNA sequences show that P. carinii is a true fungus (Edman et al. 1988), which very likely belongs to the Ascomycotina (Bowman et al. 1992b). In general, Bowman et al. (1992b) conclude that such pathogenicity has arisen more than once in different fungal lineages within the Ascomycotina. Thus studies on the molecular evolution of human pathogenic fungi clearly demonstrate that rDNA sequences can compensate for missing or confusing sexual reproductive morphologies (Barns et al. 1991; Berbee and Taylor 1992a; Bowman et al. 1992b). A case in point is Sporothrix schenckii; analysis of its SSrDNA sequences provided a statistically validated framework which allowed placement of this pathogen in the genus Ophiostoma (Berbee and Taylor 1992a). Thus phylogenetic trees inferred from rDNA have the potential to determine the nature of the affinities between the anamorphic fungi (Deuteromycotina) and teleomorphic genera,

thus circumscribing the holomorph. Arguments supporting the use of DNA sequence characters to place anamorphic (asexual) fungi within "holomorphic" genera have been presented by Reynolds and Taylor (1991).

The form of ascomata and the nature of their centra, i.e. the type and disposition of asci within the ascocarp, are important taxonomic characters upon which many ordinal and supraordinal classification schemes are based (Luttrell 1951; Barr 1990). Therefore it would be of great interest to study the evolution of such morphological characters using rDNA-based phylogenies. Recently, Berbee and Taylor (1992b) noted that rDNA analysis suggests that fungi with perithecioid fruiting bodies form a monophyletic group separate from that containing fungi with cleistothecioid ascocarps. Therefore their study which employed SSrDNA sequences of 11 different species, plus the sequence of Saccharomyces cerevisiae serving as an outgroup, provides some support for recognizing the classes Plectomycetes and Pyrenomycetes which, to some extent, are defined by the nature of their ascocarp. This conclusion will undoubtedly be controversial since many workers (e.g. Cain 1972; Malloch 1981) believe fruiting-body characters can be convergent characters, and therefore they are hesitant to accept the form of ascocarps as class-level characters. Clearly Berbee and Taylor's study requires expansion to include a far greater range of fungal genera; this would allow a more accurate assessment of the validity of fungal classes based

on fruiting-body characters.

Berbee and Taylor (1992b) also showed that Ophiostoma ulmi grouped with the pyrenomycetous fungi. But although most members of the genus Ophiostoma have perithecoid ascocarps, some authors (Nannfeldt 1932; Luttrell 1951; Benny and Kimbrough 1980) placed the genera of the Ophiostomataceae in the class Plectomycetes. They did so because in members of this family the asci are deliquescent, and are not organized into a single layer in the perithecial centrum. This suggests that the "plectomycetous type of centrum" has arisen independently in different fungal lineages, and therefore this type of centrum is not a reliable class-level character.

Berbee and Taylor (1992c) extended their work on ascomycete evolution and pursued the question of whether forcible ascospore discharge is an advanced condition in the pyrenomycetous fungi (Luttrell 1951) or an ancestral character. Their statistically supported rDNA based phylogeny showed that fungi with mechanisms for forcible ascospore discharge do not form a monophyletic group but, instead, group with those that lack forcible discharge mechanisms. This supports the view that the loss of forcible ascospore discharge mechanisms is probably an advanced character (Ingold 1971; Malloch 1981). And it also supports Cain and Weresub (1957) and Ingold (1971) who argued that the loss of forcible spore discharge evolved independently in different fungal lineages that adapted to insect

dispersal. Ultimately, studies similar to those of Berbee and Taylor (1992b, 1992c) should be of great value in indicating which morphological characters are suitable for defining taxonomic groups above the family level.

The nuclear LSrRNA gene has many divergent domains or expansion segments (Hassouna et al. 1984), which are potentially useful for reconstructing relatively recent events (Qu et al. 1988). In general, the LSrRNA gene may prove to be useful in separating organisms at the lower taxonomic level (Blanz and Unseld 1987; Hillis and Dixon 1991). Guadet et al. (1989) applied partial LSrRNA sequences to resolve the phylogeny of eight Fusarium species, and their results generally confirmed the traditional classification which was based on features of both the anamorphs and teleomorphs. Bruns et al. (1991) reanalyzed the partial LSrDNA data set of Guadet et al. (1989) using bootstrap analysis, and generally confirmed the conclusions the latter had reached. Partial LSrRNA sequences have also been used, with limited success, to find molecular markers useful to distinguish strains of Gibberella fujikuroi (Peterson and Logrieco 1991).

Ultimately, the highly variable ITS regions might prove to be more valuable for studying interspecific relatedness than are the more highly conserved rDNA gene sequences. This in spite of the fact that within the genus Armillaria, the ITS1 sequences were either too uniform to resolve relationships amongst the majority of the Northern

Hemisphere species, or too variable to permit reliable alignments between other Armillaria species tested (Anderson and Stasovski 1992). In Gibberella pulicaris, the ITS regions were noted to be quite variable, and therefore potentially useful in resolving intraspecific taxonomic concerns (O'Donnell 1992). However in isolates of Phytopthora species, the ITS sequences exhibited low intraspecific variability (Lee and Taylor 1992). And within the Boletaceae, the ITS regions also appear to be quite conserved. ITS sequences allowed Baura et al. (1992) to demonstrate that Gastrosuillus laricinus is a recent derivative of Suillus grevillei; indeed Baura et al. argued that G. laricinus should be viewed as but a local variant of S. grevillei. ITS1 sequences might also be potentially important in designing taxon-specific probes, confirming anamorph/teleomorph connections, and for resolving phylogenetic relationships between species that are morphologically very similar.

The most variable component of the rDNA repeat unit is the intergenic region (IGR)(Hillis and Dixon 1991), but too few studies are yet available to allow an estimate of the taxonomic resolution possible with IGR sequences. However it is expected to be useful in intraspecific studies (Kohn 1992). Anderson and Stasovski (1992) did show that the intergenic region between the LSrRNA and 5s gene could be useful in assessing phylogenetic relationships within northern hemisphere species of Armillaria.

The mt rRNA genes are also viewed as being informative in phylogenetic studies (Cummings et al. 1989). Bruns et al. (1989) analyzed partial mtLSrRNA sequences and demonstrated that false truffles evolved from a mushroom-like ancestor. In a more involved study, Bruns and Szaro (1992) analyzed both the nuclear SSrRNA and mtSSrRNA sequences in order to infer phylogenies for 10 members of the Boletales. They noted that the phylogenetic estimates determined independently for each gene by parsimony analysis and tested by bootstrap analysis, had identical topologies in all statistically significant branches. Phylogenies that can be supported by analyzing rDNA from two different components of the genome are clearly powerful tools in taxonomy.

The accumulation of rDNA sequences from a large variety of fungal taxa will undoubtedly lead to a better understanding of evolutionary trends within the fungi, for this should allow morphological characters which are truly homologous to be identified, and more natural classifications proposed. Historically, however, many problems have arisen in fungal taxonomy when conclusions were based on either too few or only a single character. But rDNA sequences can be analyzed by various tree building methods, and bootstrap analysis allows for an assessment if sufficient characters (or sequence divergence) are present to permit statistically valid phyletic estimates. However, since rDNA can be viewed as a single character, caution is

required when assessing the significance of rRNA/DNA analysis; this is particularly true when only a few isolates are included in a study.

The question arises whether rRNA sequences could be the "Rosetta Stone" of phylogenetics (Rothschild et al. 1986)? The attractive features of rRNA/DNA have already been discussed, but it remains to be determined whether rRNA sequences are reliable barometers of evolution. Many concepts in molecular evolution and phylogenetic reconstruction assume that there is a correlation between the rate of sequence divergence and divergence time, i.e. the "molecular clock" (Wilson et al. 1977; Kimura 1983). However Bhattacharya et al. (1991) and Rothschild et al. (1986) note that rDNA is not a perfect molecular chronometer. Their conclusions are based on the effect of: (1) compensatory slippage (Hancock and Dover 1990), i.e. the coevolution of repetitive motifs in expansion segments to maintain secondary structure; (2) compensatory base changes to maintain secondary structure (Lineares et al. 1991); and (3) structure-function coordination in the rDNA which probably set a limit to change beyond which convergence, back-mutations or parallel mutations "become as frequent as divergent mutations" (Meyer et al. 1986). Thus one should not expect an exact clock-like sequence divergence in all lineages and, more importantly, different lineages might evolve at different rates. Bruns et al. (1991) noted that with respect to nuclear ITS and mtSSrDNA sequences,

sordariaceous genera are no more divergent than are species of Laccaria or Suillus. This observation further illustrates the point that sequences have to be obtained from appropriate regions of the rDNA repeat unit, and for different taxa, different regions of the rDNA might have to be analyzed to obtain meaningful comparisons.

Overall, molecular data are preferable to morphological data when the organisms have either few distinctive morphological features or have frequently convergent morphologies. However, whenever possible, rDNA phylogenies should be evaluated with respect to other available data, i.e. morphological, genetic or biological characters (Kohn et al. 1992). The comparison of phylogenies based on two or more genes, thereby decreasing the chance of being misled by relying on a single gene, would clearly be desirable. But as molecular analysis is time consuming and expensive, it is very likely that individual studies will continue to be based on the analysis of one particular region within the genome.

MATERIALS AND METHODS

Culture methods

From the margin of a vigorous culture growing on malt extract agar, an agar plug (approx. 5 mm diameter) was transferred to a Roux bottle containing 100 mL - yeast extract - glucose medium (PYG) containing 3 g glucose, 1 g peptone, and 1 g Difco yeast extract per litre, and allowed to grow in standing culture in the dark for 2-4 days at 20°C; Ceratocystiopsis proteae required 28°C. Mycelium was then harvested by vacuum filtration onto Whatman No. 1 filter paper (Whatman Laboratory Products, Clinton, N.J.) and thoroughly washed with approximately 1 L distilled water. For yeast-like species, the cells were collected by centrifugation, washed twice with distilled water, then freeze dried. Mycelium harvested from two Roux bottles (100-300 mg dry weight) was generally sufficient for DNA extraction.

DNA extraction and purification

Initially, large scale DNA extraction was employed wherein 10-12 Roux bottles were harvested and, immediately after washing, the mycelium was extracted by grinding in a precooled mortar with pestle for 20 min in the presence of both liquid nitrogen and 25 to 30 g of 0.5 mm glass beads (Braun Melsungen AG, Melsungen, Germany). The DNA was then

purified by phenol extraction and CsCl-bisbenzimide density centrifugation (Garber and Yoder 1983). However, as this procedure was cumbersome and time consuming, a more rapid one was developed which also required less mycelium for extraction of suitable amounts of DNA.

A DNA "mini" preparation procedure based on the methods of Murray and Thompson (1980) and Kim et al. (1990) was used to extract "polymerase chain reaction grade" DNA (Saiki et al. 1988). Dried mycelium (100-200 mg) was added to sterile Falcon polystyrene conical tubes (Becton Dickinson Labware, Lincoln Park, N. J.), each of which contained 4 mL of ice cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris, pH 7.4), proteinase K (Sigma, St. Louis, Mo.), and 9 g of acid-washed and baked-dry 0.5 mm glass beads (Braun Melsungen). The mixtures were then vortexed for 2 to 3 min, and an additional 3 mL of lysis buffer added to each tube. Sodium lauryl sulfate (SLS; Fisher Scientific, Nepean, Ont.) was added to a final concentration of 1%, and the tubes then incubated at 55°C for at least 1 h. NaCl and hexadecyltrimethyl ammonium bromide (CTAB; Sigma) were added to the tubes to a final concentration of 1M and 1% respectively, and the tubes were then incubated for an additional 30 min at 55°C. Next the glass beads were pelleted by centrifugation at 2000 rpm for 2 min, and the supernatant transferred aseptically to sterile 15 mL glass Corex tubes (Canlab, Winnipeg, Man.), and the CTAB-protein complex and SLS were removed by two chloroform/isoamyl

alcohol (24:1, V:V) (Fisher Scientific) extractions. Approximately 50 to 100 µg of DNA was recovered from each strain by precipitation with 2.25 volumes of 95% ethanol (Fisher Scientific). This miniprep procedure was self-contained within separate sterile containers for each strain, thus cross contamination by even trace amounts of DNA from different samples was avoided. The DNA was redisolved in 150 to 500 µL of TE buffer (10 mM-Tris/HCL; 1 mM-EDTA; pH 7.6) and stored frozen at -20 °C. Although the quality (size range: 20-40 kb) and yield of the DNA was somewhat variable, one miniprep procedure yielded sufficient DNA from each isolate to permit genomic RFLP and PCR analysis.

DNA digestion and electrophoresis

Endonuclease digestions were performed using enzymes obtained from Pharmacia (Canada) Ltd. Dorval, Que. and BRL (Bethesda Research Laboratories Inc., Gaithersburg, Md.), according to the manufacturer's recommendations. Electrophoresis was carried out in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, ph 7.6) on 15 X 20 X 0.4 cm horizontal 0.8 or 1.6% agarose (Boehringer Mannheim Corporation, Indianapolis, Ind.) submarine gels at 2 V/cm for 14 to 20 h. Agarose gels at 1.6% were required for resolving restriction patterns obtained from treating genomic DNA with endonucleases that digest at sites

consisting of 4 nucleotides. In order to resolve mitochondrial restriction patterns, 15 X 25 X 0.4 cm horizontal 1.0% agarose submarine gels at 3 V/cm for 16 to 24 hours were employed. The BRL 1-kb (Bethesda Research Laboratories) ladder was the molecular weight standard used to estimate fragment size. Gels were stained for 15 min with ethidium bromide (0.5 µg/mL in TBE buffer) (Sigma) and illuminated with UV (310 nm) transillumination (preparative DNA transilluminator, Fotodyne Incorporated, Mississauga, Ont.), and photographed using Polaroid 667 film.

Southern hybridizations

pMF2 plasmid DNA, that contains the portion of the rDNA repeat unit of Neurospora crassa with the 18S, 5.8S, and 25S ribosomal RNA cistrons but little of the intergenic region (IGR, also called nontranscribed spacer), was prepared from Escherichia coli C600SF8 (Free et al. 1979) according to Birnboim and Doly (1979) and labelled with α-^{32}P-dATP (Dupont, NEN Research Products, Boston, Ma.) (Rigby et al. 1977; also see Maniatis et al. 1982). Blots were prepared using magnagraph nylon membrane (MSI, Westboro, Mass.) according to the manufacturer's instructions. Prehybridization of the blots was at 55°C for 2 h in 1 M NaCl (Fisher Scientific) and 1% SLS (Fisher Scientific) with constant agitation. The probe was denatured by boiling for 10 min, then added to the hybridization fluid and incubated

at 55°C with constant agitation for 12-14 h. Following hybridization, the membrane was washed twice in 2X sodium saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 5 min each, then three times in 2X SSC and 1% SDS at 55°C for 30 min each with constant agitation. Autoradiography employed Kodak X-Omat RP film with a Dupont Hi-Plus intensifying screen at -70°C for 48-96 h.

In order to assess the genetic variability within isolates of Ceratocystis ips originating from Scandinavia and New Zealand, restriction profiles were obtained by hybridizing total mtDNA against Southern blots of restricted genomic DNA. Total DNA was extracted from C. ips (isolate WIN(M) 58) and fractionated according to Garber and Yoder (1983) to obtain mtDNA that was used as the probe. Approximately 0.5-1.0 µg of mtDNA was restricted with 2 units of ClaI for 15 min, then the DNA was recovered by ammonium acetate/ethanol precipitation, pelleted by centrifugation, and dried. The DNA pellet was redissolved in 5 µL of TE buffer and immediately nick translated as described above. Prehybridization, hybridization and post hybridization washes were as described for pMF2 probing except the hybridization temperature was 65°C, and approximately 10 µg of C. ips (WIN(M) 58) nuclear DNA were denatured by boiling and added to the prehybridization solution.

Target sequences within the rDNA genes for amplification and sequencing

All oligonucleotide primers used for the polymerase chain reaction (PCR) amplifications and DNA sequencing are characterized in Table 1. Primers were obtained from the Department of Microbiology, University of Manitoba, where oligonucleotides were synthesized with the PCR-MATE (391 DNA synthesizer, Applied Biosystems, Cochrane, Alb.)

The SSrDNA is amongst the slowest evolving sequences known (Hillis and Dixon 1991), but as Lane et al. (1985) have shown the significance of partial rRNA sequences in phylogenetic analysis, only the relatively variable domains (Nickrent and Sargent 1991) the V3, V4 and V9 regions of this gene were analyzed. First the SSrRNA gene was amplified with primers SSJ and SST. Next, the V3 and V4 regions (Neefs et al. 1990), about 485 nucleotides, were sequenced with primers SSF and SSU in one direction, and with primers SSg and SSG in the other. Then the V9 region comprising about 200 nucleotides (Neefs et al. 1990), was sequenced in both directions with primers SS5 and SST.

The LSrDNA gene has many divergent domains or expansion segments (Hassouna et al. 1984) which are potentially useful for reconstructing relatively recent events (Qu et al. 1988). However, as domain 1 had been informative in reconstructing the phylogeny of Fusarium spp. (Guadet et al. 1989), the 5' end of the LSrRNA gene was amplified with

Table 1: Primers used to amplify and/or sequence regions within the SSrRNA gene and LSrRNA gene.

Primer	Position of the oligonucleotide	Sequence (5' to 3')
SSJ	4-23[a]	CTGGTTGATCCTGCCAGTAG
SSF	296-394[a]	GATTCCGGAGAGGGAGCC
SSU	576-596[a]	GTAATTCCAGCTCCAATAGC
SSg	621-636[a]	TCCAACTACCAGCTT
SSG	894-914[a]	CCAAGAATTTCACCTCTGAC
SS5	1522-1542[a]	GTGCTGGGGATAGAGCATTG
SST	1744-1764[a]	ACGGAACCTTGTTACGACT
LS1	21-39[b]	AGTACCCGCTGAACTTAAG
LSC	79-97[b]	GCCTTAGTAACGGCGAGTG
LS4	329-347[b]	TTGTGCGCTATCGGTCTC
LSD	1479-1496[b]	GGAACCTTTCCCCACTTC

[a]Based on the SSrRNA of S. cerevisiae (Rubstov et al. 1980).

[b]Based on the LSrRNA of S. cerevisiae (Gutell and Fox 1988).

primers LS1 and LSD; this yielded a segment of about 1.4 kb which contained domain 1. The 5' end of this segment was then sequenced in both directions with primers LSC and LS4.

In general, the majority of the sequences reported in this thesis were determined by sequencing in both directions. However, single-strand sequences were accepted when the data were clear and the sequence was identical to that of a strain that had already been analyzed in both directions.

Fragment amplification and preparation of sequencing templates

DNA fragments representing specific target sequences were amplified using the polymerase chain reaction (PCR) (Saiki et al. 1988) in a reaction mixture of 100 µL total volume containing the following: 10 µL 10X Taq DNA polymerase reaction buffer (Promega Corp., Madison, Wis.); 8 µL of deoxyribonucleotide triphosphates (dNTP, Pharmacia) mixture (stock concentration 2.5 mM with final concentration of each dNTP, 200 uM); 1 µL (32 pmol) of each primer; 1 µL template DNA (50-100 ng of DNA); 78.5 µL ultrapure water (HPLC grade, Fisher Scientific); and 0.5 mL (2.5 units) Taq polymerase (Promega). The reaction mixtures were overlaid with mineral oil (Paraffin oil, Fisher Scientific) and subjected to 25 cycles in a Perkin Elmer-Cetus DNA thermal cycler (Norwalk, Conn.) under the following temperature

regime: 1 min. at 93°C, 1 min at 55°C, and 2 min at 72 °C.

PCR products were purified by electrophoresis in 1% agarose followed by freeze-squeeze extraction of bands by a method similar to that of Tautz and Renz (1983), modified as follows. After staining with ethidium bromide, bands were cut out of the gel and frozen at -20°C. The gel plug was placed between two layers of Parafilm (American National Can, Greenwich, Conn.) and gently thawed by steady finger pressure. The expressed liquid was collected and made up to 1M NaCl and 1% CTAB. After incubation at 55°C for 10 min, two chloroform/isoamyl alcohol (25:1 v/v) extractions were done, followed by precipitation of DNA by the addition of 2.5 volumes of ethanol.

Sequencing of double stranded PCR products

Double-stranded templates were sequenced by a modification of a rapid denaturation-annealing-sequencing (RDAS) technique suggested by L.E. Pelcher (personal communication). Approximately 1 µg of lyophilized template DNA was dissolved in 3 µL of water and mixed with 12 µL of tricine buffer (0.6 M tricine (Sigma), 2% NP-40 (Sigma), 100mM $MgCl_2$ (Fisher Scientific)), 4 µL 0.6 N NaOH (Fisher Scientific), and 5 µL of primer solution. The standard amount of primer was 5 pmol, but this amount was adjusted to optimize sequencing for each of the primers used. The mixture was boiled for 3 min and then transferred

immediately to an ethanol bath at -70°C. The mixture was thawed on ice, and 4 units of Sequenase (0.5 µL) (United States Biochemical Co., Cleveland, O.) or T7 polymerase (Pharmacia) in 4 µL of Sequenase dilution buffer and 1 µL of 100 mM dithiothreitol (Sigma), and 2 uL of α-^{32}P-dATP (1 mCi in 100 µL; Dupont) were added. Of this mixture, 7.1 µL were added to each of the four sequencing termination mixes prepared as prescribed by the manufacturer for Sequenase (also see Sambrook et al. 1989). After incubation for 7 minutes at 37°C, the contents of each tube were diluted with 9 µL water and precipitated by the addition of 51 µL of ethanol (95% ethanol made to 0.12 M sodium acetate). The DNA was pelleted by centrifugation for 30 min in a tabletop centrifuge, the supernatant decanted, and the ethanol evaporated by heating the tube in a waterbath. The pellet was resuspended in TE buffer containing the sequencing stop solution (Pharmacia) and loaded on the sequencing gels.

Sequencing reaction products were separated by electrophoresis using 6% polyacrylamide (polyacrylamide stock solution: 97.5% acrylamide (Bio-Rad Laboratories, Richmond, Cal.) and 2.5% N,N'-methylene-bis-acrylamide, (Sigma)) and 48% urea (BRL) denaturing gels (Maniatis et al. 1982). The prepartion of sequencing gels, including the preparation of the acrylamide solution and the cleaning and taping of the sequencing gel plates, were as described by Sambrook et al. (1989). Two loadings were spaced approximately 2 to 2.5h apart; this allowed for the

determination of 250 to 280 nucleotide stretches. Gels were vacuum dried at 80°C and exposed for 1-4 days to Kodak X-OMAT film at room temperature.

Sequence alignment and phylogenetic analysis

The nucleotide sequences were initially aligned using CLUSTAL (PC-gene; IntelliGenetics, Inc., Mountain View, Cal.) and then the alignment was improved by eye with MASE (Multiple aligned sequence editor; Faulkner and Jurka 1988). Nucleotide sequences were also aligned with the alignment option within the CLUSTAL V package (Higgins and Sharp 1989). If required, alignment files from CLUSTAL V were transferred to MASE for modifications by the CLU2IG command. The READSEQ program was used to reformat files originating from MASE, thus alignments could be transferred from MASE to PHYLIP or CLUSTAL V. The partial rDNA sequence data were analyzed using the programs contained within PHYLIP (Version 3.4; Felsenstein 1991). As used herein, CLUSTAL V, MASE and PHYLIP were components of BIRCH (Biological Research Computer Hierarchy), a collection of programs set within the framework of the SUN Unix system at the University of Manitoba, compiled by Fristensky (1991).

Phylogenetic trees were obtained by parsimony and distance methods. Divergence (or distance) between two sequences was calculated by DNADIST using Kimura's (1980) two parameter model. The FITCH and KITSCH programs were used

to carry out Fitch and Margoliash's least-square method for estimating phylogenies from distance matrices. The FITCH program will fit a tree which has the branch lengths unconstrained, whereas the KITSCH program assumes the validity of an evolutionary clock, and thus creates a tree in which the branch lengths from the root of the tree to each tip are equal. For data sets containing more than 30 sequences, the NEIGHBOR program was used to generate unrooted phylogenetic distance networks based on the neighbor-joining method (Saitou and Nei 1987). Compared to NEIGHBOR (neighbor-joining option), both FITCH and KITSCH have relatively long execution times; therefore large data sets (over 30 sequences) were analysed by the NEIGHBOR program. In order to estimate the confidence in clusterings comprised of more than one species, bootstrap analysis was carried out. SEQBOOT was used to generate 1000 bootstrap replicates, and the distance matrices generated by DNADIST for each bootstrap replicate were analyzed by FITCH, KITSCH and/or NEIGHBOR, and a majority-rule consensus tree was constructed by the CONSENSE program. According to Felsenstein (1985), a consensus tree derived from a bootstrap analysis can be considered an overall estimate of the phylogeny.

The DNAPARS program was used to carry out unrooted parsimony on DNA sequences for estimating phylogenies. In order to estimate the confidence in all putatively monophyletic groups based on parsimony criteria, bootstrap

analysis was carried out by generating 1000 (sometimes 500, if more than 20 DNA sequences were involved) bootstrap replicates with SEQBOOT. Each bootstrap replicate was analyzed by DNAPARS, and a majority-rule consensus tree was generated by CONSENSE.

Cycloheximide sensitivity

The sensitivity of selected species towards cycloheximide was tested by aseptically removing approximately 4 mm^2 plugs from a one-week-old culture, and placing them centrally on the surface of malt extract agar (MEA; 15 g malt extract (Difco Laboratories, Detroit, Mi.); 15 g bacteriological agar (Gibco Laboratories, Madison, Wis.); L^{-1}) containing 100 mg/L^{-1} cycloheximide (Sigma), in 9 cm plastic non-vented Petri dishes. Harrington (1981) used this agar formulation and concentration of cycloheximide in testing the sensitivity of species of Ceratocystis s.l. to this antibiotic. Each test and control (MEA lacking cycloheximide) was replicated three times. Cultures were incubated in the dark at 25°C for seven days, at which time four perpendicular measurements of the colony diameters were made, and the average colony diameter determined for each species; from this value, the average colony areas were computed.

Because the strains of Cop. falcata (Ceratocystiopsis = Cop.) grow very poorly on MEA, these were tested on a

composite medium (CM) consisting of: 17 g corn meal agar, Difco; 10 g malt extract, Difco; 1 g yeast extract, Difco; 10 g bacteriological agar, Gibco; L^{-1}. All of the strains of Cop. falcata tested grow well and produce perithecia on CM.

The sensitivity of Cop. longispora was not tested. This organism grows so slowly in culture that measurable growth does not occur within seven days.

The results are reported as percentage inhibition of colony area growth of strains grown on the cycloheximide containing medium versus the area of control cultures. Although Harrington's criteria (1981, Table 1, footnote a) for determining cycloheximide sensitivity of species of Ceratocystis s.l. to this antibiotic suggest an "all or nothing" response, Fergus (1956, Table 1) had earlier shown that of the 11 Ophiostoma/Endoconidiophora species he recorded as being insensitive, seven actually showed some percentage of growth inhibition on media containing 100 mg/L^{-1} cycloheximide.

CHAPTER 1

RESTRICTION LENGTH POLYMORPHISMS IN RIBOSOMAL AND MITOCHONDRIAL DNAs OF *Ceratocystis ips* AND OTHER SPECIES OF *Ceratocystis s.l.*

INTRODUCTION

Assigning large numbers of isolates to a particular fungal species is often difficult due to the morphological variation that can be encountered. Ceratocystis ips is one of the more pleomorphic members of the genus Ceratocystis sensu lato, possessing at least three distinct synanamorphs which belong to the genera Acremonium, Graphilbum, and Hyalorhinocladiella. A large pool of C. ips isolates was available from New Zealand and Scandinavia, many of which exhibit considerable variation both in ascocarp morphology and culture characteristics when grown under standard conditions on common media (Hutchison 1984; Hutchison and Reid 1988). Observed variations included the ability to produce perithecia in culture, length of the perithecial necks, degree of pigmentation of perithecial bases, presence or absence of ostiolar hyphae, presence or absence of ornamenting hyphae on the perithecial bases, synanamorphs etc. Indeed, the only constant feature noted amongst the isolates studied was the size and shape of the ascospores when perithecia do form!

While variation such as that seen amongst these isolates does cause identification problems, the collection offered an ideal test case to investigate whether significant intraspecific differences existed at the ribosomal(r) or mitochrondrial(mt) DNA levels and, if they

occurred, whether such differences could be correlated with the cultural differences noted above. Such an investigation could also clarify whether some of the isolates represented new species, or at least distinct varieties of C. ips. Isolates of species reduced to synonymy with C. ips (Upadhyay 1981) were also included in an attempt to test the validity of such decisions.

As noted earlier, molecular taxonomy provides independent data sets which can be used objectively to evaluate the phylogenetic significance of morphological features. Using restriction lengths polymorphisms in mtDNA and rDNA, I sought to test the correctness of the hypothesis that ascospore characters are most important in assigning isolates to C. ips.

Mitochondrial DNA has also been employed with considerable success to investigate phylogenetic relationships between closely related organisms (Taylor 1986; Förster et al. 1988; Smith and Anderson 1989; Taylor and Natvig 1989). Mitochondrial polymorphisms appear to arise relatively quickly due to both deletions and insertions and, in addition, the lack of a mtDNA repair mechanism is estimated to cause mtDNA to evolve 10 fold more rapidly than nuclear DNA (Brown et al. 1979). Thus the use of mtDNA in taxonomy is limited to the phylogenetic study of very closely related species.

This study was later expanded to estimate the amount of genetic variability found within Ceratocystis s.l., for if

Ceratocystis s.l. is homogeneous (monophyletic), one would expect that RFLP analysis of the rDNA would be a useful approach for grouping members of this genus. However, should Ceratocystis s.l. represent a polyphyletic arrangement or a rapidly diverging group of fungi, then the sequence divergence might be too great for RFLP analysis to yield meaningful comparisons.

MATERIALS AND METHODS

Isolates employed in this investigation, and their sources, are listed in Table 1 under their designated catalogue names and accession codes. DNA was extracted from the strains by the miniprep procedure described previously. All other procedures relevant to this chapter have been described in the Material and Methods section.

Table 1: List of strains studied.

Species and Isolate No.	Source	Geographic origin
Cephaloascus fragrans Hanawa		
ATCC 36174	Not given	Japan
ATCC 60760	*Pseudotsuga menziesii* (Mirb.) Franco	Canada
Ceratocystiopsis falcata (Wright & Cain) Upadh.		
WIN(M) 82-23d	*Larix* sp.	New Zealand
Ceratocystiopsis minuta (Siem.) Upadh. & Kendrick		
CBS 145.59	Unknown	U.S.A. (?)
Ceratocystis adiposa (Butler) C. Moreau		
CBS 127.27	Unknown	Unknown
Ceratocystis adjuncta Davids.		
ATCC 34942	*Pinus ponderosa* Laws.	U.S.A.
Ceratocystis autographa Bakshi		
CBS 650.75	*Juniperus communis* L.	Not given
Ceratocystis coerulescens (Münch) Bakshi		
WIN(98)	*Picea abies* (L.) H. Karst	Norway
Ceratocystis fimbriata Ell. & Halst.		
DOAM 195303	*Kerria japonica* (L.) DC.	France
Ceratocystis hyalothecium Davids.		
ATCC 28825	*Pinus contorta* Douglas ex Loud.	U.S.A
Ceratocystis ips (Rumb.) C. Moreau		
WIN(M) 82-57b	*Pinus radiata* D. Don	New Zealand
WIN(M) 82-58	*P. radiata*	New Zealand
WIN(M) 82-70a	*P. elliotii*	New Zealand
WIN(M) 82-70b	*P. elliotii*	New Zealand
WIN(M) 82-77b	*P. radiata*	New Zealand
WIN(M) 82-83a	*P. radiata*	New Zealand
WIN(M) 82-83d	*P. radiata*	New Zealand
WIN(M) 82-85b	*P. radiata*	New Zealand
WIN(M) 82-87a	*P. radiata*	New Zealand
WIN(M) 88-100b	*P. radiata*	New Zealand
WIN(M) 88-105	*P. radiata*	New Zealand
WIN(M) 88-131	*P. radiata*	New Zealand
WIN(M) 88-134	*P. radiata*	New Zealand
WIN(M) 88-135	*P. radiata*	New Zealand
WIN(M) 88-138	*P. radiata*	New Zealand
WIN(M) 88-141	*P. radiata*	New Zealand
WIN(M) 88-185	*Pinus* sp.	New Zealand
WIN(M) 88-508	*P. contorta*	New Zealand
WIN(M) 92	*Pinus sylvestris* L.	Norway
WIN(M) 96	*P. sylvestris*	Norway
WIN(M) 114	*P. sylvestris*	Norway
WIN(M) 182	*P. sylvestris*	Norway
WIN(M) 391	Unknown	Norway (?)
Ceratocystis major (von Beyma) C. Moreau		
CBS 138.34	Isolated from air	Not given
Ceratocystis montia (Rumb.) Hunt		
CBS 151.78	*P. ponderosa*	U.S.A
C450	*P. contorta*	Canada
Ceratocystis ossiformis Olchow. & Reid		
WIN(M) 52	*Abies balsamea* (L.) Mill.	Canada
Ceratocystis paradoxa (Dade) C. Moreau		
CBS 107.22	*Cocos nucifera* L.	Not given
Ceratocystis penicillata (Gros.) C. Moreau		

CBS 211.67	P. abies	Sweden
Ceratocystis piceaperda (Rumb.) C. Moreau		
WIN(M) 82-14b	Pinus nigra	New Zealand
Ceratocystis pilifera (Fr.) C. Moreau		
WIN(M) 932	P. radiata	New Zealand
WIN(M) 22	Not given	Sweden
Ceratocystis radicicola (Bliss.) C. Moreau		
CBS 114.47	Phoenix dactylifera L.	Not given
Ceratocystis sp.		
DAOM 191892	Ulmus sp.	Canada (?)
Chalara sp.		
WIN(M) 82-141a	Podocarpus sp.	New Zealand
Endomyces decipiens (Tulasne) Reess.		
ATCC 11647	Not given	Not given
Europhium aureum Robinson-Jeffrey & R. W. Davidson		
CBS 438.69	Pinus contorta	Canada
Europhium clavigerum Robinson-Jeffrey & R. W. Davidson		
CBS 493.77	Pinus ponderosa	Unknown
Europhium triancriforme Parker		
CFB 527	Pinus monticola Douglas ex D. Don	Unknown
Neosartorya fischeri (Wehmer) Malloch & Cain var. fischeri		
CBS 525.65	Unknown	Unknown
Ophiostoma flexuosum Solheim		
NFRI 81-79/10	Picea abies	Norway
Sphaeronaemella fimicola Marchal		
WIN(M) 82-55a	P. radiata	New Zealand

ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.;
CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands;
CFB, Northern Forest Research Centre, Edmonton, Canada;
DAOM, Biosystematics Research Institute, Ottawa, Canada;
WIN(M), Culture collection of J. Reid, Department of Botany, Univerity of Manitoba, Winnipeg, Canada.

RESULTS

Ribosomal DNA restriction profiles were generated using endonucleases which recognize sites comprised of either six base pairs (six-cutters) or four base pairs (four-cutters), to investigate the intra- and interspecific variability to be found within C. ips isolates, and isolates of various closely related species.

The profiles generated by ClaI (a six-cutter) suggest that all of the tested C. ips isolates were identical in yielding a 1.2 kb and a 1.8 kb fragment from the coding regions (Figs. 1,2). However, the largest ClaI fragment produced was somewhat variable in size (Table 2), and from the restriction map (Fig. 1) it is evident that the large ClaI fragment contains the intergenic region.

With the exception of isolate 88-100B, all of the New Zealand isolates analyzed yielded a large 6.4 kb ClaI fragment, while the Scandinavian isolates always yielded a slightly smaller, large ClaI fragment of either 5.7 or 6.0 kb. Thus the size of the intergenic region tends to group our isolates of C. ips on the basis of geographic origin.

Unfortunately, the ClaI profiles did not provide enough information to estimate the degree of relatedness amongst C. ips, C. montia, C. pilifera, Sph. fimicola, or C. penicillata (Table 2). A 1.2 kb fragment is shared amongst the two C. montia isolates and the C. pilifera isolate,

otherwise no additiional shared ClaI fragments were observed amongst the non ips isolates. Surprisingly, in light of the results obtained with the C. ips isolates, the two C. montia isolates shared only the 1.2 kb fragment. It is therefore quite possible that six-cutter profiles will not generate enough fragments to serve as a basis for relatedness appraisal.

While six-cutter endonucleases usually generate only two or three fragments per restriction, four-cutters (e.g. RsaI, MspI) have a much higher restriction frequency, and can yield up to 10 or more fragments per restriction. Therefore four-cutter profiles represent a more thorough sampling of sequences, and yield more information which can be used to support taxonomic decisions made on more traditional grounds.

The MspI and RsaI profiles (Figs. 3,4) of the C. ips isolates were identical. Each comprised eight recognizable fragments, suggesting that a total of 16 sites had been restricted in each isolate by the two restriction endonucleases. And as the sizes, and therefore the positioning of the fragments on the gels, depend upon the distance between two adjacent restriction sites, the identity of the profiles also suggest insertions and deletions are lacking in the various isolates. These results further suggest that: (i) the isolates employed belong to one species despite the morphological variation encountered amongst them; and (ii) that sampling sequences by means of

four-cutter profiles can be useful in evaluating sequence divergence between closely related species.

The restriction fragments were visualized by Southern blotting and hybridization with pMF2, a plasmid which contains the entire coding region, plus 300 bases of the IGR flanking either side of the coding region of the rDNA repeat unit of Neurospora crassa (Free et al. 1979). Thus the obtained four-cutter profiles probably represent mainly fragments within the coding regions of the rDNA repeat unit of C. ips, as it is unlikely pMF2 would hybridize to IGR fragments unless they included parts of the adjacent coding regions.

To evaluate the relationship of species such as C. montia, C. adjuncta, C. ossiformis, O. flexuosum, C. hyalothecium, and the isolate designated as Ceratocystis sp. to C. ips, four-cutter profiles were generated with MspI and MboI and compared (Fig. 5). The data were analyzed by determining the fraction of common bands or F-value [F=2(number of fragments common to two isolates) divided by the total number of restriction fragments in the two isolates] between isolates of all species tested (Kozlowski and Stepien 1982). And although F-values were calculated for each pair of isolates with both MspI and MboI, only the averaged F-values for each pair of isolates are reported. The two data sets were consistent with each other for all of the comparisons.

F-values are a reflection of sequence divergence due to

either a mutation at a particular restriction site which results in the loss of a fragment due to restriction failure, or to a change in the position of a particular fragment on a gel due to a size change resulting from insertions or deletions. Therefore F-values, when they are high and when they are confirmed by independent data sets, are useful measures of relatedness as they are indicators of evolutionary change at the DNA sequence level.

The four-cutter profiles (Fig. 5, Table 3) suggest that C. montia, C. adjuncta, O. flexuosum, C. ossiformis, C. hyalothecium, and the Ceratocystis sp. all have distinct RFLP phenotypes, and therefore are very likely to be distinct species. Four species were included here as outgroups to establish F-values for clearly morphologically distinct species which one would not expect to be too closely related to C. ips. Of these, C. pilifera does share a high degree of similarity with C. ips and the other ips-like species; this could indicate a potentially close evolutionary kinship. On the other hand, the F-values for C. fimbriata, C. penicillata, and Sph. fimicola with the other species tested are very low, suggesting these three have a more distant evolutionary relationship with C. ips, C. pilifera etc., with evolutionary distance appearing most pronounced with C. fimbriata and Sph. fimicola.

While the ClaI profiles had earlier suggested the two isolates of C. montia tested were distinct, the four-cutter profiles confirmed that the two were nevertheless closely

related. A Ceratocystis sp. was included in this study which had been received identified as a C. ips isolate, but it did not produce perithecia in culture. Based on four-cutter profiles and F-values, it was found to be closely related to the Ips complex. What this isolate actually represents will not be determined unless it can be induced to fruit in culture.

The study was expanded to investigate the position of C. ips within Ceratocystis s.l., by comparing four-cutter profiles of isolates of 14 species chosen to represent various aspects of the genus, with the profiles of C. ips; an isolate of Neosartorya fischeri and of a Chalara species were included as outgroups. The endonucleases employed were MboI, RsaI, MspI, HhaI, and AluI. Figure 6 illustrates the four-cutter profiles obtained with MboI and HhaI, while Table 4 records the average F-values obtained with all five four-cutters for each isolate pair compared.

These F-values indicate that the isolates identified as C. autographa and Cop. falcata, both species with Chalara anamorphs, have little affinity with any of the other Ceratocystis or Europhium species tested. Nor does Cop. falcata have much affinity with Cop. minuta, the type species of the genus Ceratocystiopsis to which both have been assigned by some authors. Based on the isolates tested, the results suggest that C. autographa and Cop. falcata should be excluded from the genus Ceratocystis s.l., that S. fimicola has virtually no affinity towards any of the

species tested, and that the latter's true placement must be sought elsewhere. The only species with which C. ips shares a relatively high F-value (F=0.64) is C. pilifera, and this was to be expected from the results reported above.

In general, the obtained four-cutter profiles suggested that collectively, the genus Ceratocystis s.l. is a very heterogeneous assemblage of species within which groups of closely related species are to be found. The latter was exemplified by the results obtained with C. fimbriata, C. radicicola, C. adiposa, and C. paradoxa, all species with Chalara anamorphs and the fimbriata-type of ascospore (Olchowecki and Reid 1974). These shared high F-values with each other, but low values (<0.30) with the other species tested. C. penicillata and C. piceaperda, two species with fimbriata-type ascospores but which lack Chalara anamorphs, failed to group with C. fimbriata and related species. This supports the belief of de Hoog and Scheffer (1984) that Ceratocystis species with Chalara anamorphs form a discrete group (note the exception of C. autographa).

Recently it has been proposed that yeasts with galeate ascospores could have a kinship with the Ophiostomatales, perhaps belonging to the Ophiostomataceae. To test this hypothesis, two isolates of Cephaloascus fragrans and one of Endomyces decipiens were analyzed by comparing their HaeIII and MspI profiles with those of the Ceratocystis species which have Fimbriata-type ascospores (Fig. 7). The RFLP phenotypes of the two yeast species had little in common

with those of the Ceratocystis species tested. Indeed the profiles even suggest the two yeast species have little in common with each other. However, since without restriction mapping the contribution of length mutations towards the F-values cannot be assessed, therefore common descent of galeate-spored fungi cannot be definitively precluded. In addition it has been noted that the level of taxonomic resolution of rDNA RFLPs is probably limited at or below the genus level (Kohn 1992). C. coerulescens, which along with C. fimbriata possesses an endoconidial anamorph was included here, and the restriction profiles obtained indicate that it, too, was closely related to C. fimbriata, and would fit in the group of species with Chalara anamorphs noted above.

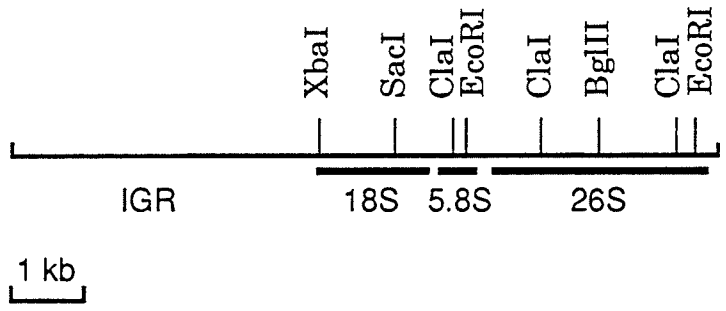
The possible use of mitochondrial profiles as molecular characters was also investigated by analyzing intraspecific variability at the mtDNA level in the isolates of C. ips. Two four-cutter profiles were generated by restricting total DNA samples with HaeIII and MspI, and hybridizing the derived Southern blots with a probe consisting of radio-labelled pure mtDNA prepared from isolate 57b (Figs 8-10). The average F-values for these restriction profiles are listed in Table 5. The mitochondrial profiles clearly distinguished between isolates from New Zealand and Scandinavia, except for isolate 88-100B which clustered with Scandinavian isolates. The F-values that resulted from comparing the New Zealand isolates with the Scandinavian isolates range from 0.50 to 0.62, whereas within the

Scandinavian set of isolates the F-values ranged from 0.7 to 0.93. And within the New Zealand isolates, F-values ranged from 0.82 to 1.0. There was also an indication that the New Zealand isolates could represent at least two clusters [(88-105, 88-131, 88-138, 88-141, 88-134, 82-185) and (82-83d, 82-70a, 82-83a, 82-87a, 82-70b, 82-57b, 82-85b)] corresponding to the two separate geographical regions of New Zealand from which the samples yielding these isolates were obtained.

The F-values for the outgroups included within this survey ranged from 0.09 to 0.24. And when the restriction profiles of C. adjuncta, C. montia, and C. pilifera were compared to those of the C. ips isolates, the F-values never exceeded 0.2. The two C. montia isolates shared an F-value of 0.58, which is within the range observed within the C. ips isolates. The mtDNA profiles confirmed the decision to exclude the isolate labelled Ceratocystis sp. from C. ips, as the F-values for this isolate never exceeded 0.23 when compared to the other isolates of C. ips tested.

Overall the mtDNA profiles were found to be quite diverse, suggesting that mtDNA might be useful for DNA typing of isolates, but not for studying relationships between more distantly related species.

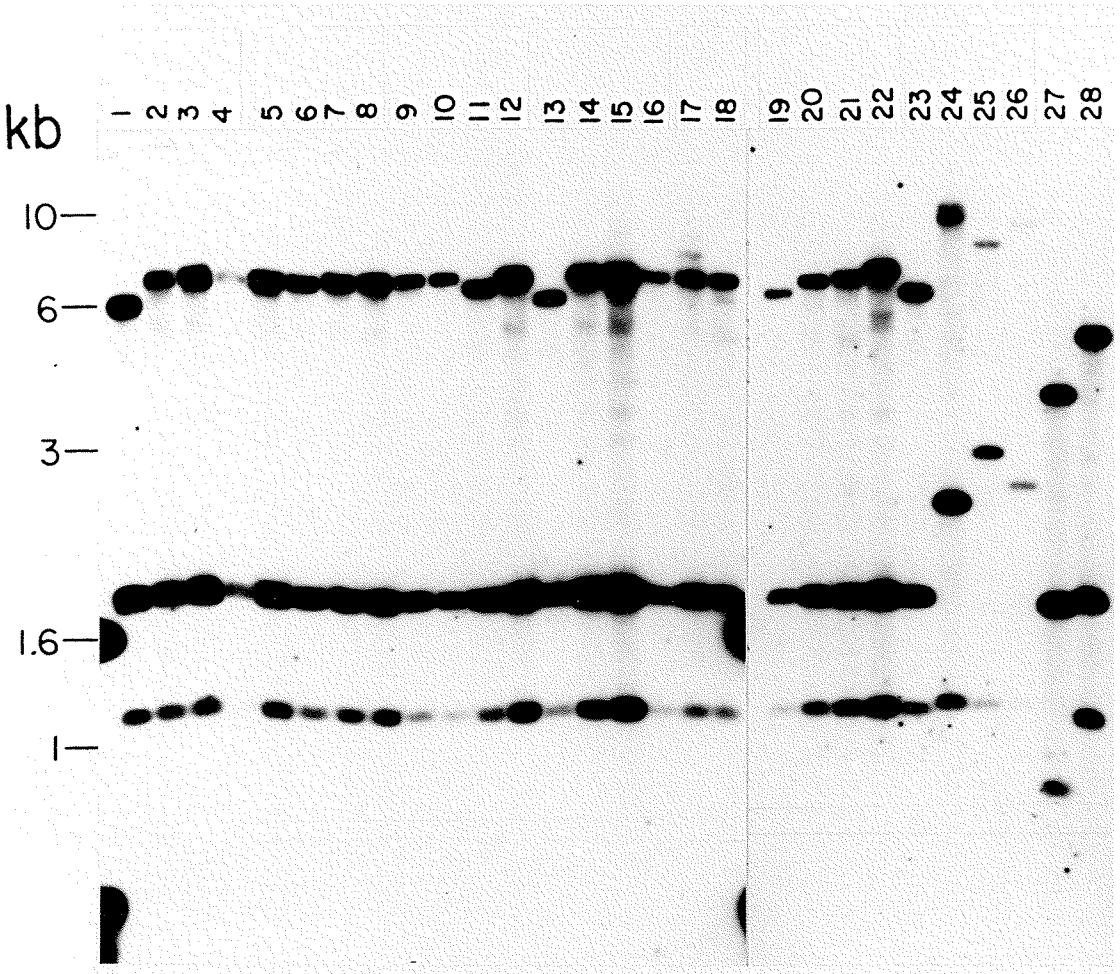
Fig. 1. Restriction enzyme site map of the 9.4 Kb rDNA repeat unit of C. ips (WIN(M) 82-57b).


XbaI
SacI
ClaI
EcoRI
ClaI
BglII
ClaI
EcoRI
IGR
18S
5.8S
26S
1 kb

C. ips

9.4 kb

Fig. 2. Southern hybridization of ClaI digested whole-cell DNAs with radiolabelled, rDNA-containing plasmid pMF2. Twenty-seven isolates representing five fungus species were tested. C. ips isolates, all WIN(M), lanes 1 to 23. Lanes 1 & 19, 88-100b; 2, 88-105; 3, 88-131; 4, 88-134; 5, 88-138; 6, 88-508; 7, 88-141; 8, 88-135; 9, 82-185; 10, 82-83d; 11, 92; 12, 82-77b; 13, 96; 14, 82-83a; 15, 82-70b; 16, 82-85b; 17, 82-87a; 18, 82-57b; 20, 114; 21, 182; 22, 82-70a; 23, 391; 24, C. montia, CBS 151.78; 25, C. montia,, C. 450; 26, C. pilifera, B. 94; 27, Sph. fimicola; 28, C. penicillata.


kb
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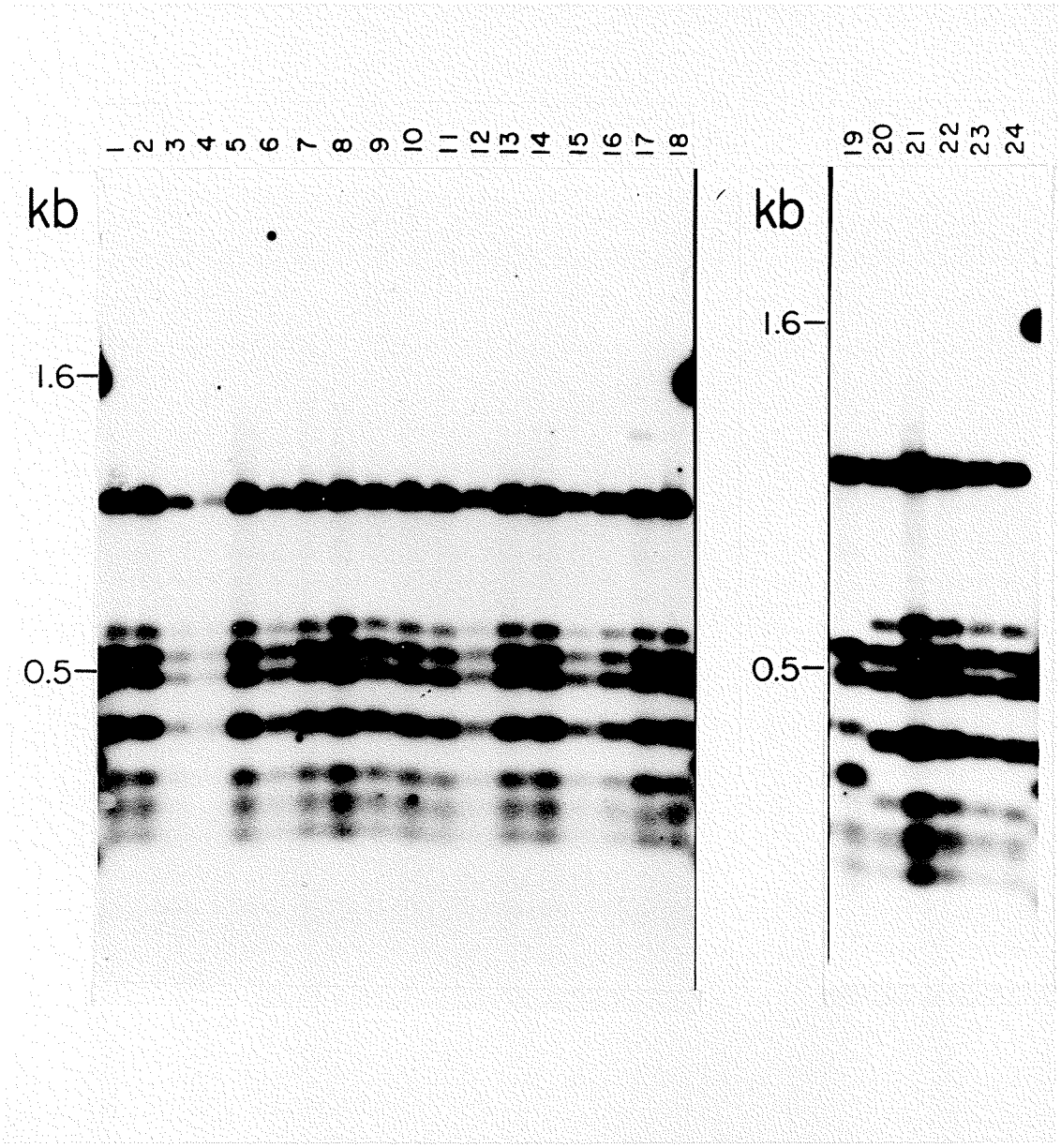
10
6
3
1.6
1

Table 2. Sizes of the rDNA fragments that hybridized to the pMF_2 probe following restriction with ClaI.

	kb
WIN(M) 92	6.0, 1.8, 1.2
WIN(M) 96	5.7, 1.8, 1.2
WIN(M) 114	6.0, 1.8, 1.2
WIN(M) 182	6.0, 1.8, 1.2
WIN(M) 391	5.7, 1.8, 1.2
WIN(M) 88-100B	5.7, 1.8, 1.2
WIN(M) New Zealand* Isolates of *C. ips*	6.4, 1.8, 1.2
C. montia CBS 151.78	8.7, 2.4, 1.2
C. montia C 450	6.6, 2.9, 1.2
C. pilifera WIN(M) 82-129d	8.2, 2.5, 1.2
Sph. fimicola	3.7, 1.6, 0.5
C. penicillata	4.6, 1.6, 1.0

*The following New Zealand isolates identified as *C. ips* all yielded identical fragment sizes: 82-57b, 82-70a, 82-70b, 82-77b, 82-83a, 82-83d, 82-85b, 82-87a, 88-105, 88-131, 88-134, 88-135, 88-138, 88-141, 88-185, 88-508.

Fig. 3. Southern hybridization of MspI digested whole-cell DNAs with radiolabelled, rDNA-containing plasmid pMF2. C. ips isolates, all WIN(M): Lanes 1 & 24, 88-100b; 2, 88-105; 3, 88-131; 4, 88-134; 5, 88-138; 6, 88-508; 7, 88-141; 8, 88-135; 9, 82-185; 10, 82-83d; 11, 92; 12, 82-77b; 13, 96; 14, 82-83a; 15, 82-70b; 16, 82-85b; 17, 82-87a; 18, 82-57b; 20, 391; 21, 82-70a; 22, 182; 23, 114; 19, C. montia, CBS 151.78.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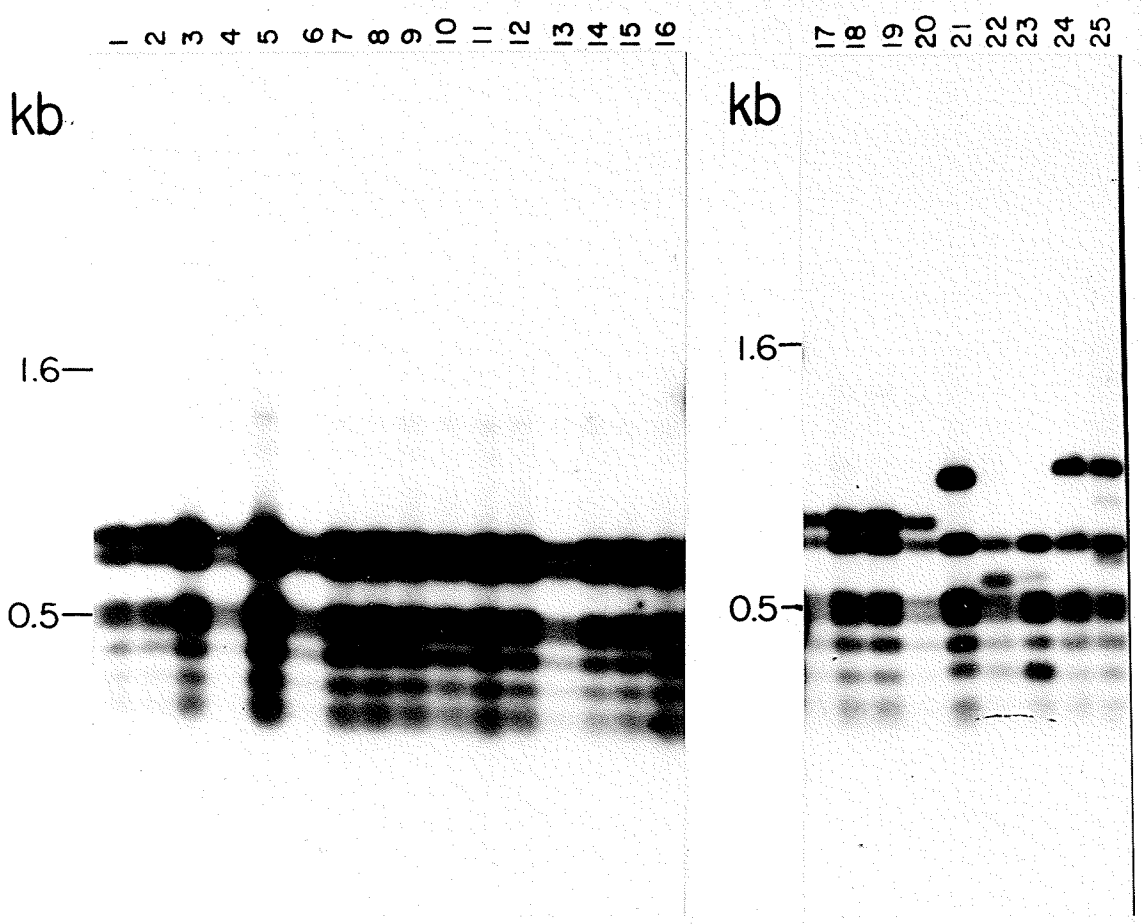
kb
1.6
0.5
19 20 21 22 23 24
kb
1.6
0.5

Fig. 4. Southern hybridization of RsaI digested whole cells DNAs with radiolabelled, rDNA-containing plasmid pMF2. C. ips isolates, all WIN(M): Lane 1, 88-141; 2, 88-135; 3, 82-185; 4, 82-83d; 5, 88-138; 6, 88-134; 7, 88-508; 8, 92; 9, 82-77b; 10, 96; 11, 82-83a; 12, 82-100B; 13, 82-70b; 14, 82-85b; 15, 82-87a; 16, 82-57b; 17, 88-131; 18, 182; 19, 82-70a; 20, 391; 21, C. montia, CBS 151.78; 22, C. montia, C-450; 23, C. adjuncta; 24, Ceratocystis sp.; 25, C. pilifera, B-94.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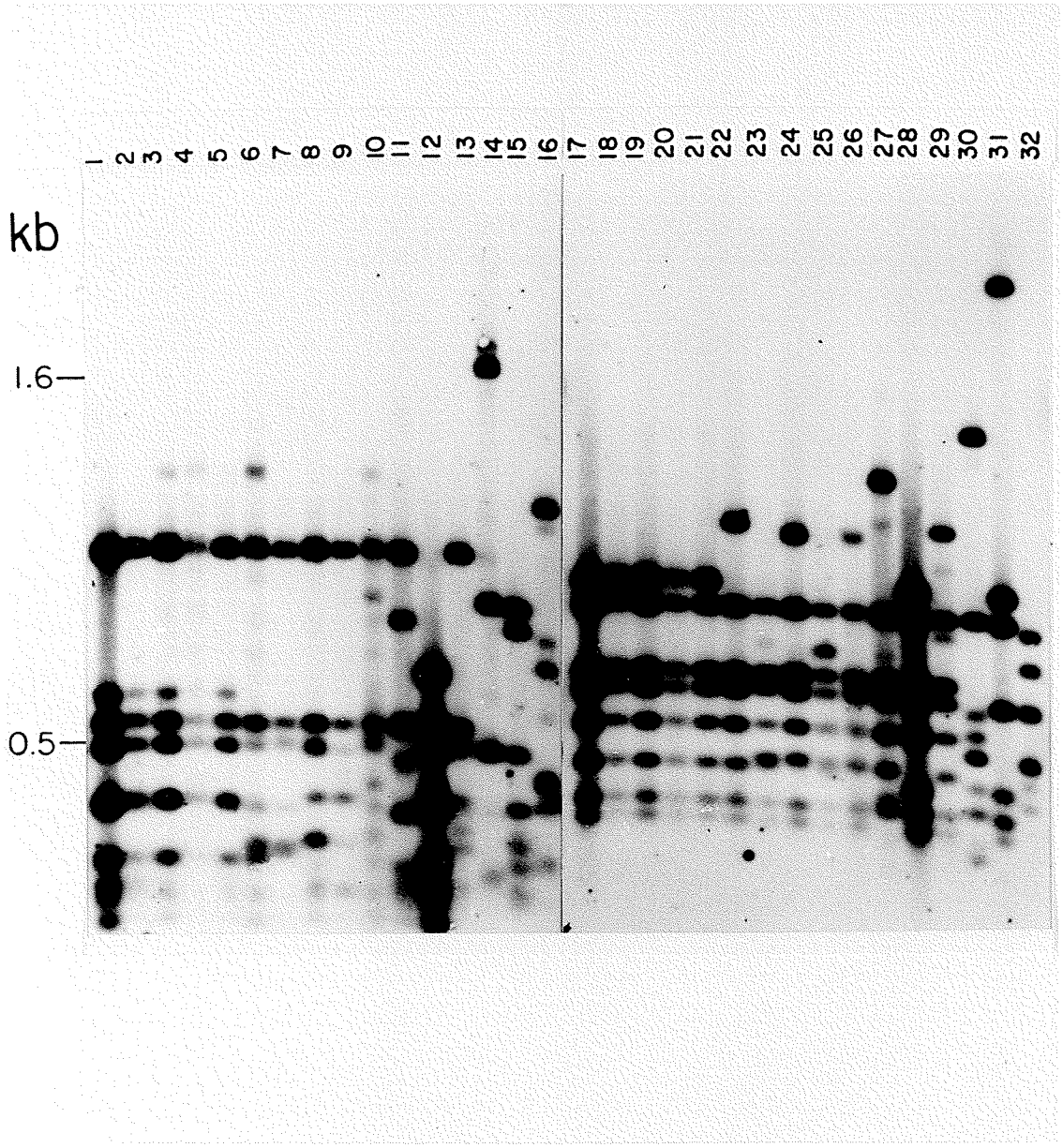
1.6
0.5
17 18 19 20 21 22 23 24 25
kb
1.6
0.5

Fig. 5. Southern hybridizations of MspI (lanes 1-16) and MboI (lanes 17-32) digested whole cell DNAs with radiolabelled, rDNA-containing plasmid pMF2. Sixteen different isolates representing ten species were tested. C. ips isolates, all WIN(M): Lanes 1 & 17, 82-58; 2 & 18, 114; 3 & 19, 82-70a; 4 & 20, 88-105; 5 & 21, 88-100B; 6 & 22, Ceratocystis sp.; 7 & 23, C. adjuncta; 8 & 24, C. montia, CBS 151.78; 9 & 25, C. montia, C-450; 10 & 26, C. ossiformis; 11 & 27, O. flexuosum; 12 & 28, C. hyalothecium; 13 & 29, C. pilifera, B-94; 14 & 30, C. fimbriata; 15 & 31, C. penicillata; 16 & 32, Sph. fimicola.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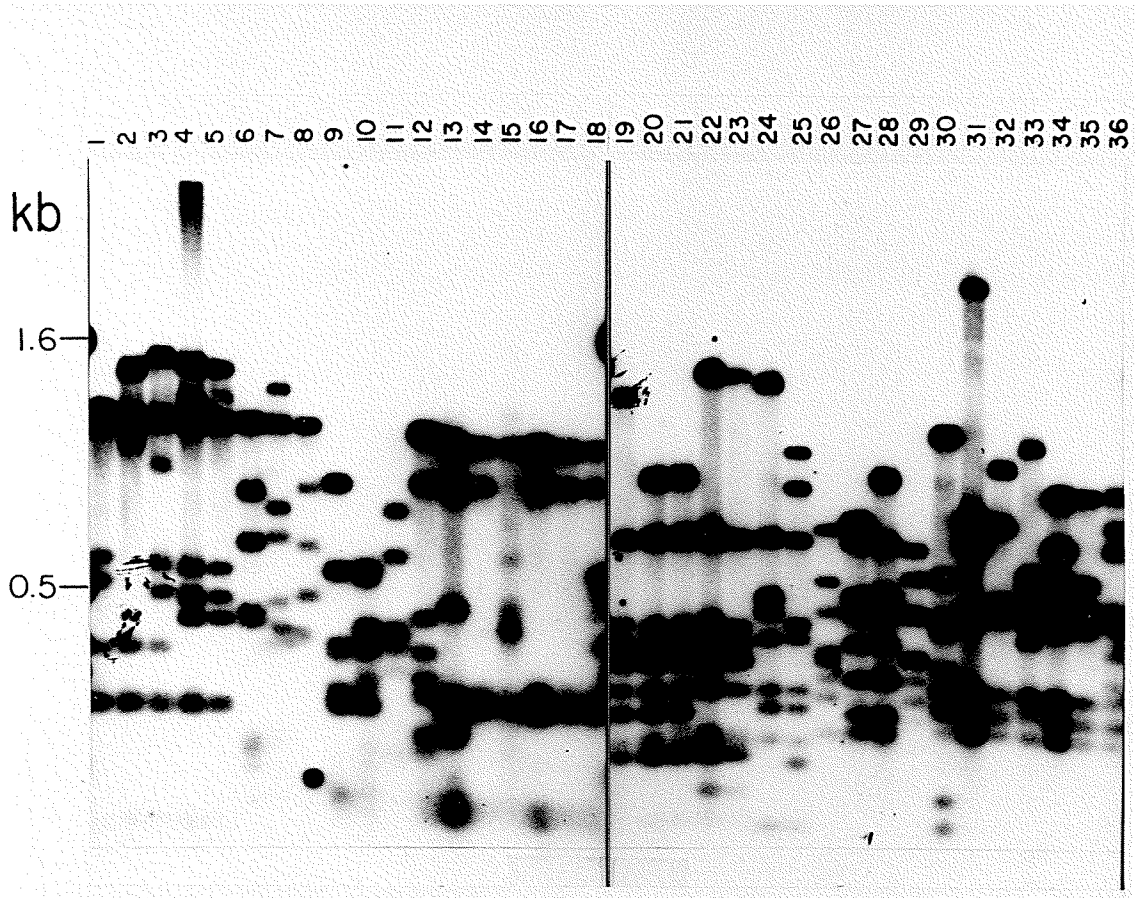
kb
1.6
0.5

Table 3. Combined F-values (fraction of common bands between two tested species) following restriction of rDNA with MboI and MspI for twelve presumed species of the Ophiostomatales.

	C.i. 82-57b	*C. sp.*	*C.a.*	*C.m.* 151.78	*C.m.* 450	*C.o.*	*O.f.*	*C.h.*	*C.f.* 82-129d	*C.f.*	*C.p.*
C. ips WIN(M)82-57b											
Ceratocystis sp.	0.85										
C. adjuncta	0.73	0.77									
C. montia CBS 151.78	0.71	0.69	0.76								
C. montia C450	0.71	0.69	0.76	0.93							
C. ossiformis	0.76	0.74	0.81	0.85	0.73						
O. flexuosum	0.71	0.63	0.76	0.67	0.67	0.61					
C.hyalothecium	0.81	0.67	0.83	0.73	0.73	0.73	0.58				
C. pilifera WIN(M) 82-129d	0.71	0.75	0.83	0.93	0.80	0.79	0.65	0.65			
C. fimbriata	0.13	0.13	0.14	0.14	0.21	0.13	0.07	0.07	0.07		
C. penicillata	0.27	0.32	0.36	0.41	0.34	0.31	0.27	0.27	0.28	0.21	
Sph. fimicola	0.07	0.07	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0	0.08

Fig. 6. Southern hybridizations of HhaI (lanes 1-18) and MboI (lanes 19-36) digested whole cell DNAs with radiolabelled, rDNA-containing plasmid pMF2. Eighteen species were tested. Lanes 1 & 19, C. fimbriata; 2 & 20, C. paradoxa; 3 & 21, C. radicicola; 4 & 22, C. adiposa; 5 & 23, C. major; 6 & 24, Chalara sp.; 7 & 25, C. autographa; 8 & 26, Cop. falcata; 9 & 27, C. ips, WIN(M) 82-57b; 10 & 28, C. pilifera, WIN(M) 82-129d;, 11 & 29, Sph. fimicola; 12 & 30, N. fischeri; 13 & 31, C. penicillata; 14 & 32, C. piceaperda; 15 & 33, Cop. minuta; 16 & 34, E. aureum; 17 & 35, E. clavigerum; 18 & 36, E. triancriforme.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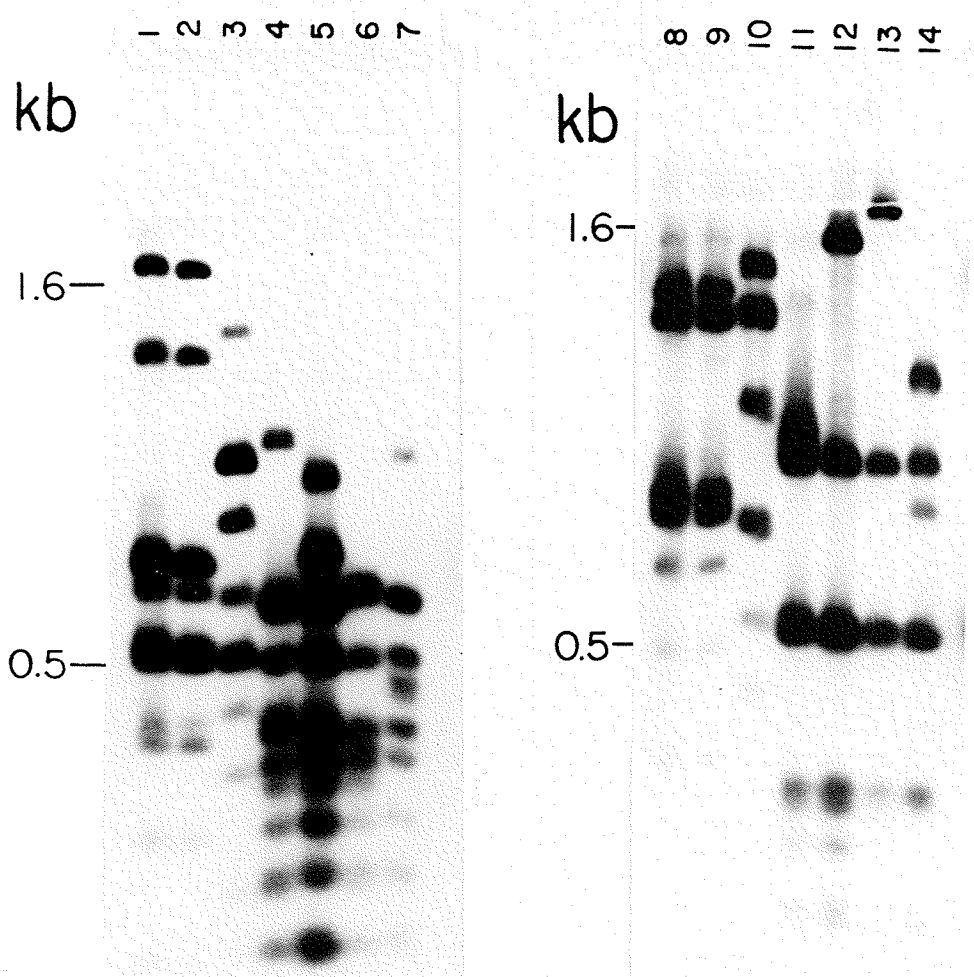
kb
1.6
0.5

Table 4. Combined F-values (fraction of common bands between two tested species) following restriction of rDNA with MboI and HhaI for fifteen presumed species of the Ophiostomatales and two unrelated species.

	C. fi.	*C. pa.*	*C.r.*	*C.a.*	*Ch.* sp.	*C. au.*	*C. fa.*	*C.i.* 82-57b	*C.p.* B-94	*S.f.*	*N.f.*	*C. pe.*	*C. pi.*	*C. mi.*	*E.a.*	*E.c.*
C. fimbriata																
C. paradoxa	0.78															
C. radicicola	0.82	0.86														
C. adiposa (= C. major)	0.76	0.75	0.76													
Chalara sp.	0.25	0.34	0.35	0.26												
C. autographa	0.25	0.25	0.25	0.27	0.38											
Cop. falcata	0.10	0.10	0.10	0.09	0.13	0.07										
C. ips WIN(M)82-57b	0.32	0.33	0.32	0.31	0.15	0.13	0.06									
C. pilifera B-94	0.29	0.27	0.22	0.26	0.01	0.10	0.03	0.64								
Sph. fimicola	0	0	0	0	0	0	0	0.03	0.03							
N. fischeri	0.12	0.12	0.12	0.15	0.06	0.03	0.06	0.20	0.15	0.02						
C. penicillata	0.29	0.30	0.29	0.31	0.12	0.06	0.06	0.41	0.36	0	0.25					
C. piceaperda	0.30	0.30	0.30	0.30	0.11	0.11	0.06	0.42	0.36	0	0.22	0.70				
Cop. minuta	0.21	0.21	0.21	0.23	0.05	0.08	0.03	0.32	0.27	0	0.12	0.45	0.49			
E. aureum	0.21	0.21	0.21	0.16	0.29	0.09	0.03	0.32	0.25	0	0.16	0.55	0.55	0.50		
E. clavigerum	0.22	0.22	0.22	0.22	0.05	0.08	0.03	0.30	0.26	0	0.15	0.54	0.55	0.51	0.89	
E. triancriforme	0.21	0.22	0.22	0.21	0.05	0.08	0.03	0.28	0.25	0	0.15	0.50	0.50	0.43	0.79	0.74

Fig. 7. Southern hybridizations of HaeIII (lanes 1-7) and MspI (lanes 8-14) digested whole cell DNAs with radiolabelled, rDNA-containing plasmid pMF2. Lanes 1 & 8, C. fragrans, ATCC 60760; 2 & 9, C. fragrans, ATCC 36174; 3 & 10, E. decipiens; 4 & 11, C. paradoxa; 5 & 12, C. adiposa; 6 & 13, C. fimbriata; 7 & 14, C. coerulescens.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8 9 10 11 12 13 14
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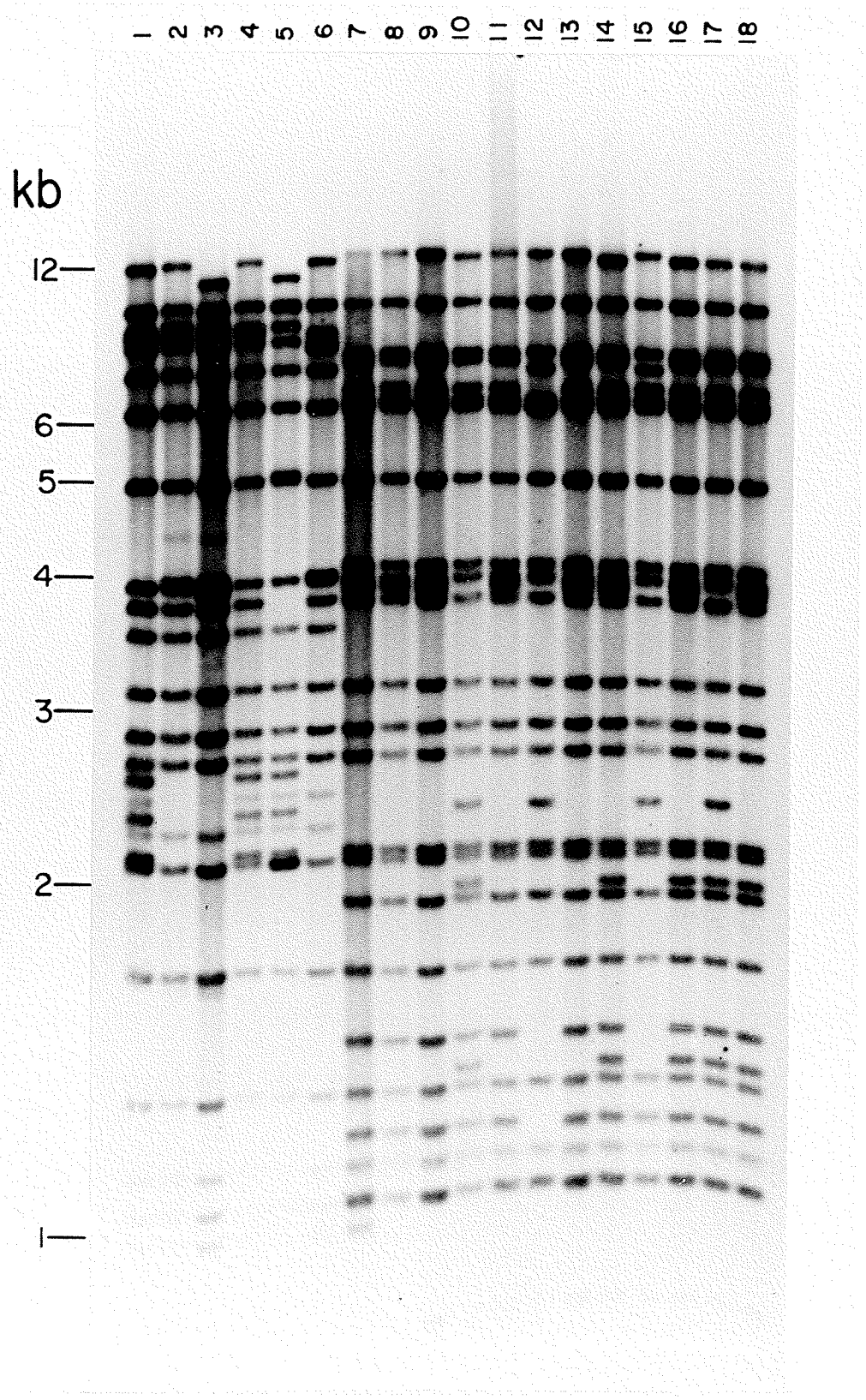
Fig. 8. Southern hybridizations of HaeIII digested whole cell DNAs of eighteen C. ips isolates, all WIN(M), with radiolabelled, mtDNA from C. ips isolate 82-57b. Lane 1, 82-100b; 2, 114; 3, 182; 4, 391; 5, 96; 6, 92; 7, 88-105; 8, 88-131; 9, 88-138; 10, 88-508; 11, 88-141; 12, 88-135; 13, 82-185; 14, 82-83d; 15, 82-77b; 16, 82-83a; 17, 82-70b; 18, 82-85b.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
kb
12
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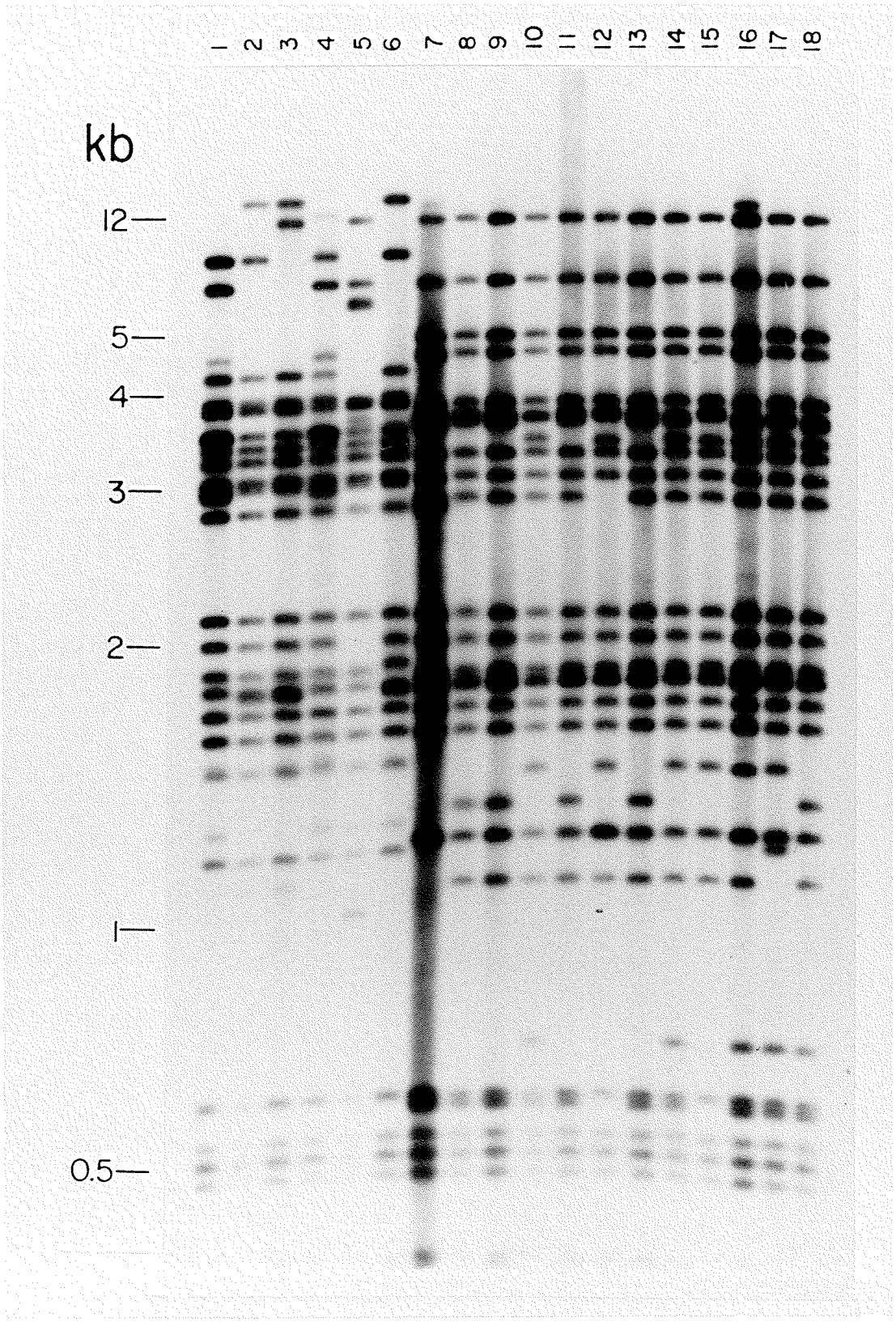
5
4
3
2
1

Fig. 9. Southern hybridizations of MspI digested whole cell DNAs of eighteen C. ips isolates, all WIN(M), with radiolabelled, mtDNA from C. ips isolate 82-57b. The arrangement of the isolates is the same as in figure 8.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12
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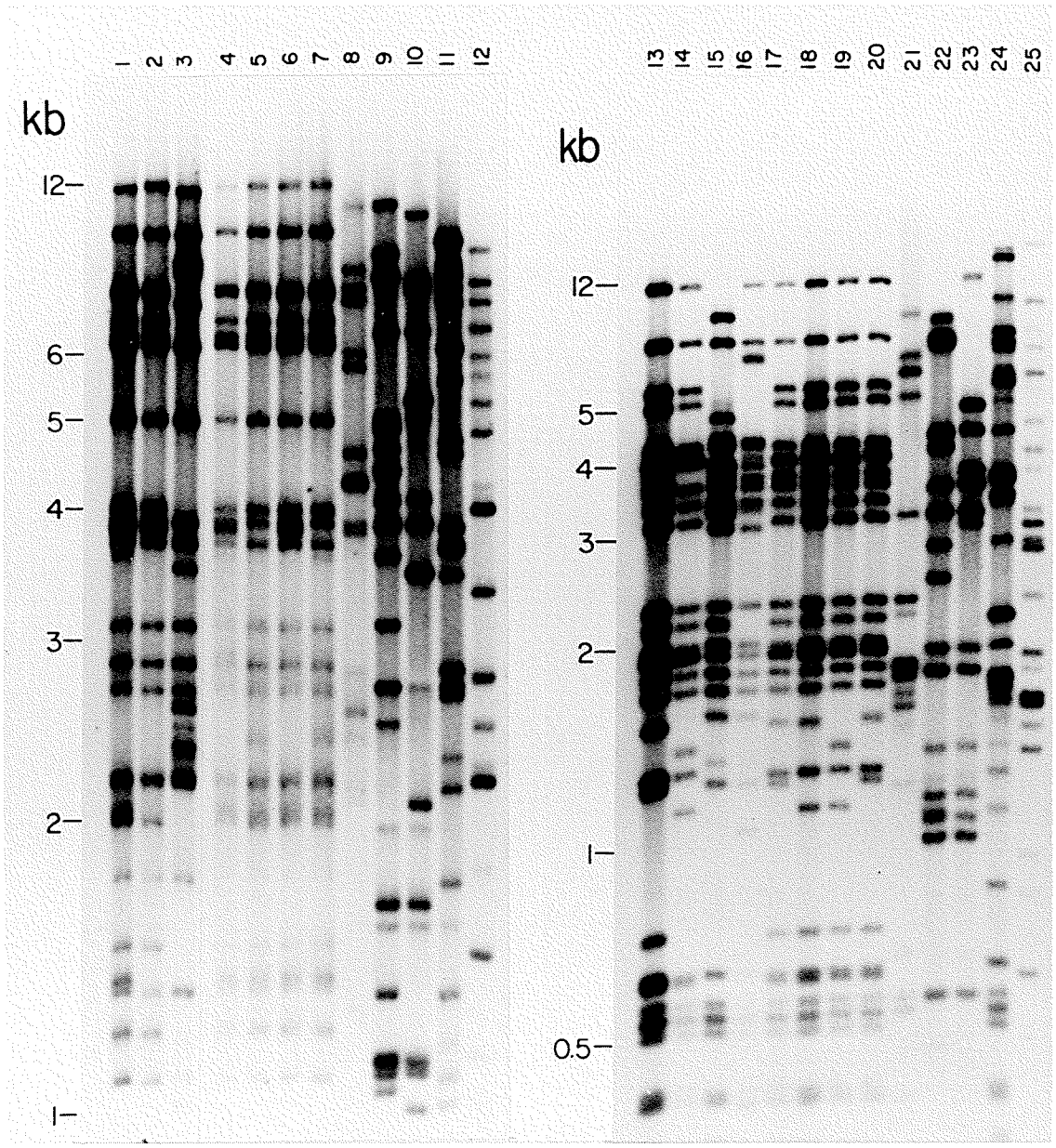
Fig. 10. Southern hybridizaitons of HaeIII (lanes 1-12) and MspI (lanes 13-25) digested whole cell DNAs with radiolabelled, mtDNA from C. ips isolate 82-57b. Twelve isolates representing five species were tested. C. ips isolates, all WIN(M): Lanes 1 & 16, 82-87a; 2 & 13, 88-134; 3 & 14, 88-100b; 4 & 17, 82-57b; 5 & 18, 82-70a; 6 & 19, 82-85b; 7 & 20, 82-70b; 15, 96; 8 & 12, C. adjuncta; 9 & 22, C. montia, CBS 151.78; 10 & 23, C. montia, C-450; 11 & 24, Ceratocystis sp.; 12 & 25, C. pilifera, B-94. NOTE: There is no HaeIII digestion for WIN(M) 96.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13 14 15 16 17 18 19 20 21 22 23 24 25
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Table 5. Combined F-values (fraction of common bands between two tested isolates) following restriction of mtDNA with HaeIII and MspI of twenty-seven isolates of six species of the Ophiostomatales.

	88[1]-100b	182	92	114	96	88-105	88[2]-131	88-135	82-77b	88[3]-508	82-83a	82[4]-87a	82[5]-57b	*C.a.*	*C.m.* 151.78	*C.m.* 450	*C.* sp.
WIN(M) 88-100b																	
182	0.84																
92	0.84	0.88															
114	0.84	0.93	0.92														
96	0.80	0.75	0.70	0.73													
88-105	0.56	0.57	0.52	0.52	0.60												
88-131	0.56	0.54	0.52	0.52	0.54	0.98											
88-135	0.55	0.58	0.53	0.56	0.62	0.81	0.83										
82-77b	0.55	0.55	0.54	0.55	0.61	0.86	0.90	0.95									
88-508	0.55	0.52	0.52	0.50	0.54	0.88	0.88	0.86	0.91								
82-83a	0.55	0.54	0.53	0.52	0.55	0.87	0.87	0.85	0.90	0.99							
82-87a	0.55	0.54	0.54	0.52	0.56	0.88	0.88	0.86	0.88	0.98	0.97						
82-57b	0.53	0.54	0.54	0.52	0.56	0.92	0.92	0.82	0.92	0.94	0.93	0.96					
C. adjuncta	0.14	0.15	0.15	0.15	0.15	0.18	0.18	0.19	0.18	0.18	0.17	0.11	0.17				
C. montium CBS 151.78	0.16	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.16	0.16	0.16	0.12			
C.montium C450	0.19	0.18	0.18	0.18	0.18	0.18	0.18	0.19	0.18	0.18	0.17	0.17	0.17	0.13	0.58		
Ceratocystis sp.	0.24	0.23	0.23	0.23	0.23	0.23	0.18	0.24	0.23	0.23	0.16	0.16	0.16	0.12	0.16	0.12	
C. pilifera	0.09	0.09	0.09	0.09	0.09	0.09	0.18	0.11	0.09	0.09	0.09	0.09	0.09	0.14	0.11	0.09	0.09

1. Isolate 391 had an F-value identical to isolate 88-100b.
2. Isolates 88-134, 88-138, and 88-141 had F-values identical to isolate 88-131.
3. Isolates 82-70a and 82-83d had F-values identical to isolate 88-508.
4. Isolate 82-70b had an F-value identical to isolate 82-87a.
5. Isolate 82-85b had an F-value identical to isolate 82-57b.

DISCUSSION

No classification system for fungi assigned to Ceratocystis s.l. has yet received full acceptance, although in several publications C. ips is considered to be the central species of the Ips spore-group (Olchowecki and Reid 1974; Benny and Kimbrough 1980; Upadhyay 1981). This group is characterized by species whose ascospores appear rectangular, ossiform, or pillow-shaped in side or plan view, spherical in end view, and are never curved (Olchowecki and Reid 1974). These spore characters still seem to set this group of species apart regardless of which of the current taxonomic treatments of Ceratocystis s.l. one follows e.g. Upadhyay (1981), de Hoog and Scheffer (1984), or von Arx and van der Walt (1987).

The results suggest that in C. ips, ascospore size and shape are the only reliable morphological characters that can be used to identify this species. Isolates that differed in the secondary characters noted earlier (e.g. presence or absence of perithecial appendages, colour of the perithecial bases, perithecial size etc.), but were identical in ascospore shape and size range, were confirmed as C. ips by the molecular techniques employed. Further, based on the limited number of C. montia isolates tested to date, molecular characters suggest that this species is distinct from C. ips. Upadhyay's (1981) decision to synonymyze C. montia with C. ips was based on the overlap in size of the

respective ascocarp bases, which does occur, but Rumboldt (1941) distinguished the two species on a number of characters e.g. ascospore size, temperature range for growth, anamorph complexity, etc. The latter differences do exist, and they accord with the molecular separation of C. montia from C. ips.

Unfortunately, the two isolates of C. montia do pose a problem if they are correctly identified. For all C. ips isolates tested, the rDNA four-cutter restriction profiles were identical, but those of the C. montia isolates differed not only from the C. ips isolates, but from each other. However, the mitochondrial restriction patterns for the C. montia isolates indicated that the observed divergence is well within the mtDNA divergence range observed for the C. ips isolates. Thus if both isolates are C. montia, the ribosomal sequences would appear to be evolving at a faster rate relative to the mtDNA sequences than is the case in C. ips, and this would be most unusual! Unfortunately, neither isolate of C. montia has produced perithecia in culture so their actual identities are uncertain. Clearly additional authentic isolates must be studied to clarify the nature of the apparent intraspecific divergence in C. montia.

These results also illustrate the importance of examining both rDNA and mtDNA when morphologically similar isolates or species are being examined, although mtDNA is not very useful when more distantly related species are being studied (Weber et al. 1986; Smith and Anderson 1989;

Taylor and Natvig 1989).

Thus amongst strains of C. ips examined during this study, the restriction enzyme data suggest that the morphological variation encountered is probably a manifestation of intraspecific morphological variation, and not an indicator of speciation. Also the mtDNA analysis based on the C. ips strains suggests that C. ips was introduced into New Zealand. The range of F-values for the Scandinavian isolates is greater than that for the New Zealand isolates. This is probably indicative of the Scandinavian isolates being members of an older population. In addition, the New Zealand strains were isolated from introduced conifer species. It would be interesting to examine the source of the introduced conifer species, as this might provide a clue as to the origin of the New Zealand C. ips isolates. The mtDNA RFLP differences observed within the New Zealand strains could represent divergence since introduction rather than separate introductions as the RFLP phenotypes of all New Zealand isolates, except for isolate 88-100B, are very similar.

Although ascospore shape proved to be very important in defining C. ips, the results do not fully support the division of Ceratocystis s.l. into four spore groups (Olchowecki and Reid 1974; Benny and Kimbrough 1980). C. pilifera, the central species of the Pilifera spore-group appears to be closely related to C. ips, indeed just as close as C. ossiformis and O. flexuosum which have typical

ips-type ascospores; this runs counter to a clear separation between the Ips and Pilifera spore-groups. Conversely, the Fimbriata spore-group appears partially justified based on the results obtained with C. fimbriata, C. adiposa, C. paradoxa, and C. radicicola; the results show that these species have a high degree of relatedness. Further, C. coerulescens, which Olchowecki and Reid (1974) placed in their Pilifera group "Although there is a thin gelatinous sheath associated with the ascospores, it is frequently not seen, and for this reason C. coerulescens is included in this group", appears to be closely related to C. fimbriata and to C. adiposa, C. paradoxa, and C. radicicola.

The above grouping of the five species which have Chalara anamorphs partially supports von Arx (1987), who suggested that only species with endoconidial anamorphs should be retained within the genus Ceratocystis. This suggestion is also supported by the fact that many members of the species which would then be assigned to Ophiostoma have both cellulose and rhamnose as cell wall components; both are lacking in species of Ceratocystis s.s. (de Hoog and Scheffer 1984). Further, Ophiostoma species are also reported to have an unusually high tolerance of cycloheximide (Harrington 1987; de Hoog & Scheffer 1984), a feature also absent in species of Ceratocystis s.s. However, the data reported herein suggest that C. autographa, a species with a Chalara anamorph, is not related to C. fimbriata and associated species; indeed the

data indicate that it should be excluded from Ceratocystis s.l. This species was noted as being anomalous by de Hoog and Scheffer (1984); perhaps the cultures currently available do not actually represent Bakshi's C. autographa.

Upadhyay and Kendrick (1975) erected the genus Ceratocystiopsis for the members of the Minuta spore group. In this study Cop. falcata, a species with two-celled Minuta-type ascospores and two discrete Chalara synanamorphs (Hutchison and Reid 1988), showed little relatedness to Cop. minuta, the type species of Ceratocystiopsis, nor did it to any other species of Ceratocystis s.l. reported on herein. It is very unlikely that it is related to either Ophiostoma or Ceratocystis s.s., and it probably should be placed elsewhere. However, if it is proven to be related to Pyxidiophora (de Hoog and Scheffer 1984), then the suggestion that Ceratocystis s.s. also belongs in the Pyxidiophoraceae (von Arx and van der Walt, 1987) would seem to be in error.

Four-cutter analysis of rDNA and mtDNA is a very rapid method of screening large collections of isolates and, in addition, calculating F-values allows for an estimation to be made of evolutionary change due to point mutations at restriction sites, or change due to insertions or deletions in DNA sequences. Morphological change and molecular divergence are thought to be quite independent, and to respond to different evolutionary pressures (Wilson et al.

1974; Wilson et al. 1977), but molecular taxonomy can be important in assessing the significance of apparent morphological divergence.

Although restriction patterns are useful in studying closely related species, problems can arise when more distant species are examined. Kessler and Avise (1985) have noted that simple fragment comparisons should not be used where less than 25% of the fragments are shared (i.e. F=0.25), as there is a possibility that two unrelated DNA samples could contain fragments of the same size, generated by different cleavage sites. This problem could be avoided by restriction mapping, but restriction mapping is very time consuming. However, Dowlin et al. (1990) pointed out that cleavage sites may be convergent and, in addition, the loss of a particular restriction site requires only a single point mutation to occur in the cleavage site, whereas the gain of a site requires a very specific base substitution (Templeton 1983).

Ultimately the establishment of more distant relationships within the genus Ceratocystis requires a more intensive study, such as DNA sequence analysis of the ribosomal genes. But the genus Ceratocystis s.l. is very large, and thus sequencing all of the members of this genus would be very time consuming and expensive. Therefore the approach presented in this chapter could be useful in screening and grouping isolates and species for the purpose of determining which species are of great enough

phylogenetic interest to justify full DNA sequence analysis.

CHAPTER 2

DO GALEATE-ASCOSPORE MEMBERS OF THE CEPHALOASCACEAE, ENDOMYCETACEAE AND OPHIOSTOMATACEAE SHARE A COMMON PHYLOGENY?

INTRODUCTION

Relationships proposed for the ophiostomataceous fungi remain controversal. Since Nannfeldt (1932) erected the family Ophiostomataceae based on Ophiostoma H. & P. Sydow (1919) but to include Microascus Zukal (1885), it has been variously disposed. Assigned to the Microascales Luttrell (Luttrell 1951), a nomen nudum validated by Benny and Kimbrough (1980), along with the Microascaceae Luttrell, which was also a nomen nudum but which was validated by Malloch (1970), the family was later submerged in an expanded and redefined concept of the Endomycetaceae Schroet. of the Endomycetales, by Redhead and Malloch (1977); the latter was based on earlier suggestions by Cain (1972) and von Arx (1977). However Benny and Kimbrough (1980) returned to a more restricted concept, and made the Ophiostomataceae, consisting of four genera, the sole family of the new order Ophiostomatales. Next, Upadhyay (1981) formally emended the Ophiostomataceae Nannf., and placed it once again within the Microascales along with the family Microascaceae.

In 1987, von Arx and van der Walt expanded the concept of the Ophiostomatales to include the Cephaloascaceae Batra, Pyxidiophoraceae Arnold, and Pseudoeurotiaceae Malloch & Cain, in addition to the Ophiostomataceae. They recognized that such an order would be polyphyletic, but their circumscription of the Ophiostomataceae was narrower than

that of any other author since Nannfeldt. Most recently, Barr (1990) has followed that circumscription of von Arx and van der Walt, but she included the Ophiostomataceae, along with the Microascaceae, in the Microascales.

Not unexpectedly, the foregoing has contributed to an energetic debate concerning generic limits and relationships between the fungi deemed assignable or related to the Ophiostomatales.

One controversy is whether an evolutionary connection exists between the yeasts and the Ophiostomataceae (Cain 1972; von Arx 1974; Redhead and Malloch 1977; von Arx and van der Walt 1986, 1987). This possibility has been predicated primarily on the belief that galeate (hat-shaped) ascospores are not likely to have had multiple originations during evolution. But has been disputed on cell-wall composition data (Weijman 1976), differences in septal ultrastructure between the filamentous ascomycetes and the yeasts (Benny and Kimbrought 1980), and by studies which have shown that there are different developmental patterns of galeate ascospores when these are studied at the ultrastructural level (Bandoni et al. 1967; Stiers 1976; Samuels and Müller 1979; van Wyk and Wingfield 1991a, 1991b; van Wyk et al. 1991). However, as Kendrick et al. (1990) have stated, additional studies are needed before unequivocal relationships amongst these organisms can be established.

In this study rDNA comparisons were used to assist in

determining whether a phylogenetic relationship does exist between galeate-spored yeast-like species of the genera Endomyces and Cephaloascus, and similar-spored species of the Ophiostomatales.

MATERIALS AND METHODS

The strains used in this study, and their origins, are listed in Table 1.

The V3, V4, and V9 regions of the SSrDNA (Neefs et al. 1990) were obtained and sequenced as described in the Materials and Methods. Similarly, the PCR amplification protocol used to generate sequencing templates, the preparation of DNA sequencing templates, and the sequencing of double-stranded PCR products were as described previously (Materials and Methods). Ribosomal DNA sequences of Neurospora crassa Shear & Dodge (Sogin et al. 1986b), Saccharomyces cerevisiae Meyen ex Hansen (Rubstov et al. 1980), Aspergillus fumigatus Fresen. (Barns et al. 1991), Kluyveromyces lactis (Domb.) van der Walt (EMBL: X51830), Torulaspora delbrueckii Lindner (GENBANK: TOUSRSR), Penicillium notatum Westling (GENBANK: PNNDA), and Podospora anserina (Ces.) Niessl (GENBANK: PANRRNASS), were included in the analysis for comparative purposes. Schizophyllum commune and Sclerotium hydrophilum were also included in the analysis to serve as outgroups; the latter is a filamentous fungus which has been characterized as having dolipore septa, and therefore it also belongs to the Basidiomycotina (Punter et al. 1984).

The nucleotide sequences were initially aligned with CLUSTAL V (PC/Gene), and with MASE. The combined SSrDNA

sequence data (about 700 positions) were analyzed using the programs contained within PHYLIP (Version 3.4; Felsenstein 1991).

The estimate of phylogeny is based on parsimony and distance methods. Divergence (or distance) between two sequences was calculated by DNADIST using Kimura's two parameter model (Kimura 1980). The FITCH program was used to carry out Fitch and Margoliash's least-square method for estimating phylogenies from distance matrices. The DNAPARS program, which was used to carry out unrooted parsimony analysis on DNA sequences to estimate phylogenies, minimizes the number of mutational events necessary to account for the sequence differences.

For both distance and parsimony methods, bootstrap analysis was performed on the complete aligned dataset, as well as on a dataset from which potentially ambiguously aligned positions were omitted. SEQBOOT was used to generate 1000 boostrap replicates which were analyzed with both the DNAPARS program and the FITCH program. A majority-rule consensus tree was produced by CONSENSE. According to Felsenstein (1985), a consensus tree from a bootstrap analysis can be considered an overall estimate of the phylogeny.

Table 1: List of fungal strains studied.

Species and Isolate No.	Source	Geographic origin
Cepaloascus fragrans Hanawa ATCC 36174	Unknown	Japan
Ceratocystis fimbriata Ell. & Bakshi DAOM 195303	Kerria japonica (L.) DC	France
Ceratocystis moniliformis (Hedgc.) C. Moreau CBS 773.77	Unknown	Unknown
Ceratocystis vesca R.W. Davidson CBS 800.73	Picea engelmannii Parry ex Engelman.	U.S.A
Endomyces decipiens (Tulasne) Reess ATCC 11647	Unknown	Unknown
Europhium aureum Robinson-Jeffrey & R.W. Davidson CBS 438.69	Pinus contorta Dougl. & Loud. var. latifolia Engelm. ex. S. Wats.	Canada
Europhium clavigerum Robinson-Jeffrey & R.W. Davidson CBS 493.77	Pinus ponderosa Douglas ex P. Laws. & C. Laws.	Unknown
Europhium triancriforme Parker CFB 527	Pinus monticola Douglas ex D.Don	Unknown
Gelasinospora tetrasperma Dowding ATCC 11345	Parsnip seed	Canada
Neosartorya fischeri (Wehmer) Malloch & Cain var. fischeri CBS 525.65	Unknown	Unknown
Ophiostoma cucullatum H. Solheim NFRI 81-83/16	Picea abies (L.) H. Karst	Norway
Schizophyllum commune Fr. WIN(M) 916	Tilia americana L.	Canada
Sclerotium hydrophilum Sacc. WIN(M) 917	Zizannia aquatica L.	Canada
Sordaria fimicola (Roberge) Ces. & DeNot. ATCC 6739	Unknown	Unknown

ATCC, American Type Culture Collection, Rockville, Maryland, USA;
CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands;
CFB, Northern Forest Research Centre, Edmonton, Canada;
DAOM, Biosystematics Research Institute, Ottawa, Canada;
NFRI, Norwegian Forest Research Institute, As, Norway;
WIN(M), Culture collection of J. Reid, University of Manitoba, Department of Botany, Winnipeg, Manitoba, Canada.

RESULTS

From the 21 partial sequences analyzed (Fig. 1), distance and parsimony methods generated phylogenetic trees with identical topologies, although the bootstrap support for particular branches varied somewhat depending upon the method of analysis (Fig. 2). Overall levels of >95% indicate strong statistical support for branches (Felsenstein 1985), and the estimate of phylogeny, based on an unrooted tree generated by FITCH (Fig. 2), indicates significant bootstrap support (>95% confidence) for the potential monophyly of the following groupings: (1) the members of the class Hemiascomycetes, order Endomycetales (C. fragrans, E. decipiens, K. lactis, T. delbrueckii, and S. cerevisiae); (2) the Eurotiales (P. notatum, A. fumigatus, and N. fischeri); (3) the Sordariales (S. fimicola, P. anserina, G. tetrasperma, and N. crassa); (4) the Ophiostoma-complex, including three galeate-spored species of Europhium (= Ophiostoma fide Harrington 1987), E. aureum, E. clavigera and E. triancriforme, plus O. cucullatum and C. vesca which also have hat-shaped ascospores (although never formally transferred, C. vesca lacks a Chalara anamorph and belongs in Ophiostoma); and (5) species of Ceratocystis s.s. (C. fimbriata and C. moniliformis).

Fig. 1. Aligned sequences of the V3, V4 (positions 402-886), and V9 (positions 1546-1748) containing segments of the SSrDNA gene from 21 different species: 1 = C. vesca, 2 = E. aureum, 3 = E. clavigerum, 4 = E. triancriforme, 5 = O. cucullatum, 6 = C. fimbriata, 7 = C. moniliformis, 8 = N. crassa, 9 = P. anserina, 10 = S. fimicola, 11 = G. tetrasperma, 12 = P. notatum, 13 = A. fumigatus, 14 = N. fischeri, 15 = C. fragrans, 16 = E. decipiens, 17 = S. cerevisiae, 18 = K. lactis, 19 = T. brueckii, 20 = S. commune, 21 = S. hydrophilum. Nucleotides 402-886 and 1546-1746 correspond to positions in the SSrRNA of S. cerevisiae (Rubstov et al., 1980). Positions that could not be unambigiously aligned are underlined.

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                 402
 1 (C.ves.)  GGCTACTACA TCCAAGGAAG GCAGCAGGCG CGCAAATTAC CCAATCCCGA TTCGGGGAGG
 2 (E.aur.)  .......... .......... .......... .......... .......... C.........
 3 (E.cla.)  .......... .......... .......... .......... .......... CA........
 4 (E.tri.)  .......... .......... .......... .......... .......... C.........
 5 (O.cuc.)  .......... .......... .......... .......... .......... ..........
 6 (C.fim.)  ......C..T .TT..... .......... .......... .......... CA........
 7 (C.mon.)  ......C..T .TT..... .......... .......... .......... CA........
 8 (N.cra.)  .......... .......... .......... .......... .......... CA........
 9 (P.ans.)  .......... .......... .......... .......... .......... CA........
10 (S.fim.)  .......... .......... .......... .......... .......... CA........
11 (G.tet.)  .......... .......... .......... .......... .......... CA........
12 (P.not.)  ......C... .......... .......... .......... .......... .A........
13 (A.fum.)  ......C... .......... .......... .......... .......... CA........
14 (N.fis.)  ......C... .......... .......... .......... .......... CA........
15 (C.fra.)  ......C... .......... .......... .......... .......... CA........
16 (E.dec.)  ......C... .......... .......... .......... .......... CA........
17 (S.cer.)  ......C... .......... .......... .......... .......TA. ...A......
18 (K.lac.)  ......C... .......... .......... .......... .......TA. ...A......
19 (T.del.)  ......C... .......... .......... .......... .......TA. .A.A......
20 (S.com.)  ......C... .......... .......... .......... .......... CA........
21 (S.hyd.)  ......C... .......... .......... .......... .......... CA........

                 462
 1 (C.ves.)  TAGTGACAAT AAATACTGAT ACAGGGCTCT TTTGGGTCTT GTAATTGGAA TGAGTACAAT
 2 (E.aur.)  .......... .......... .......... .......... .......... ..........
 3 (E.cla.)  .......... .......... .......C.. .A........ .......... ....A.....
 4 (E.tri.)  .......... .......... .......... .......... .......... ..........
 5 (O.cuc.)  .......... .......... .......... .......... .......... ..........
 6 (C.fim.)  .......... .......... .......... .A........ .....C.... ..........
 7 (C.mon.)  .......... .......... .......... .A........ .....C.... ..........
 8 (N.cra.)  .......... .......... .......... .......... .......... ..........
 9 (P.ans.)  .......... .......... .......... .......... .......... ..........
10 (S.fim.)  .......... .......... .......... .......... .......... ..........
11 (G.tet.)  .......... .......... .......... .......... .......... ..........
12 (P.not.)  .......... .......... ..G....... .........C .......... ....A.....
13 (A.fum.)  .......... .......... ..G....... .........C .......... ..........
14 (N.fis.)  .......... .......... ..G....... .........C .......... ..........
15 (C.fra.)  .......... .....AC... .......C.A .......... .......... ..........
16 (E.dec.)  .......... .....AC... ..G...-..C A....A...C .-........ ....A.....
17 (S.cer.)  .......... .....AC... .......C.A ..C..... .......... ..........
18 (K.lac.)  .......... .....AC... .......C.A ..C-.... .......... ..........
19 (T.del.)  .......... .....AC... .......C.A ..C..... .......... ..........
20 (S.com.)  .......... .....ACA.. .T........ ..C.....C. A........ ..........
21 (S.hyd.)  .......... .....ACA.. .T.....G.C .......... .......... ..........

                 522
 1 (C.ves.)  TTAATTCCCT TAACGAGGAA CAATTGGAGG GCAAGTCTGG TGCCAGCAGC CGCGGTAATT
 2 (E.aur.)  .......... .......... .......... .......... .......... ..........
 3 (E.cla.)  ....A..... .......... .......... .......... .......... ..........
 4 (E.tri.)  .......... .......... .......... .......... .......... ..........
 5 (O.cuc.)  .......... .......... .......... .......... .......... ..........
 6 (C.fim.)  ....A..... .......... .......... .......... .......... ..........
 7 (C.mon.)  ....A..... .......... .......... .......... .......... ..........
 8 (N.cra.)  ....A..... .......... .......... .......... .......... ..........
 9 (P.ans.)  ....A..... .......... .......... .......... .......... ..........
10 (S.fim.)  ....A..... .......... .......... .......... .......... ..........
11 (G.tet.)  ....A..... .......... .......... .......... .......... ..........
12 (P.not.)  ....A..... .......... .......... .......... .......... ..........
13 (A.fum.)  C...A..... .......... .......... .......... .......... ..........
14 (N.fis.)  C...A..... .......... .......... .......... .......... ..........
15 (C.fra.)  ....A.A... .......... .......... .......... .......... ..........
16 (E.dec.)  ....A-A... .......... ...G.A.... .......... .......... ..........
17 (S.cer.)  G...A.A... .......... .......... .......... .......... ..........
18 (K.lac.)  G...A.A... .......... ...C...... .......... .......... ....-.....
19 (T.del.)  G...A.A... .......... .......... .......... .......... ....-.....
20 (S.com.)  ....A..... .........T .......... .......... .......... ..........
21 (S.hyd.)  ....A..... .........T .......... .......... .......... ..........
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                582
 1 (C.ves.)  CCAGCTCCAA TAGCGTATAT TAAAGTTGTT GCAGTTAAAA AGCTCGTAGT TGAACCTT-G
 2 (E.aur.)  .......... .......... .......... .......... .......... ..........
 3 (E.cla.)  .......... .......... .......... .......... .......... ..........
 4 (E.tri.)  .......... .......... .......... .......... .......... ..........
 5 (O.cuc.)  .......... .......... .......... .......... .......... ..........
 6 (C.fim.)  .......... .......... .......... .TG....... .......... ..........
 7 (C.mon.)  .......... .......... .......... .TG....... .......... ..........
 8 (N.cra.)  .......... .......... .......... .AG....... .......... ..........
 9 (P.ans.)  .......... .......... .......... .AG....... .......... ..........
10 (S.fim.)  .......... .......... .......... .......... .......... ..........
11 (G.tet.)  .......... .......... .......... .AG....... .......... ..........
12 (P.not.)  .......... .......... .......... .......... .......... ..........
13 (A.fum.)  .......... .......... .......... .......... .......... ..........
14 (N.fis.)  .......... .......... .......... .......... .......... ..........
15 (C.fra.)  .......... A......... .......... .......... .......... ..........
16 (E.dec.)  .......G.C G......... .......... .......... .......... ....A.....
17 (S.cer.)  .......... .......... .......... .......... .......... .....T....
18 (K.lac.)  .........G .......... .......... .......... .......... .....T....
19 (T.del.)  .......... .......... .......... .......... .......... .....T....
20 (S.com.)  .......... .......... .......... .......... .......... ........CA
21 (S.hyd.)  .......... .......... .......... .......... .......... ........CA

                641
 1 (C.ves.)  GGCCTGGCTG G-CCGGTCCG CCTCACCGCG TGCACT--GG -TCCGG--CC GGGCCTTTC-
 2 (E.aur.)  .......... .......... .......... .......... .......... ..........
 3 (E.cla.)  .......... ..T....... .......... .........A .......... ...T......
 4 (E.tri.)  .......... .......... .......... .......... .......... ..........
 5 (O.cuc.)  .......... .......... .......... .......... .......... ..........
 6 (C.fim.)  .......... .........C ........G. .......... T......... ...T......
 7 (C.mon.)  .......... .........C ........G. .......... ..T.C.G.... ...T......
 8 (N.cra.)  ...TC...C. -.T....... .......... .........A .CTG....T. ........T.
 9 (P.ans.)  ....C...CC T........C ........G. .......... .CT....... ..........
10 (S.fim.)  ....CA..C. .......... .......... .......... .ATA....TT ..........
11 (G.tet.)  ....C...CC ..T....... .......... .........A .CTG....T. ..........
12 (P.not.)  ..T....... .......... .......... A.T....... .........T ..A.......
13 (A.fum.)  ..T....... .......... .......... A.T....... .........T ..A.......
14 (N.fis.)  ..T....... .......... .......... A.T....... .........T ..A.......
15 (C.fra.)  ...T...T.. A..T...... .T.TTTG..- A.T......- .-A.TCAA.. .A........
16 (E.dec.)  .--...TT.. ..TG...... .T.AGT-... A.-.....C- .-AG.CGAA. .A......A.
17 (S.cer.)  ....C..T.. .......... AT.TTTT-.. ..T....... .ATTTCCAA. ...G.C..TC
18 (K.lac.)  ..T....T.. T......... A..TTAT.-T C..G.ACT.. ..TTTCAA.. ..AT......
19 (T.del.)  .......T.. .......... AT.TTTT-.. ..T....... ..TTCCAA.. ..........
20 (S.com.)  ........C. ..G.....T. ...A..G.TA ..T.......- ...T...... ...T...A..
21 (S.hyd.)  .......T.. ..G.....T. .....G.TA ..T.......- .....A.... ...T...A..

                696
 1 (C.ves.)  CCTCTGGGGA GCCG-CATGC CCTT-----C GCTGGGCGTG TCGGGGAACC AGGACTTTTA
 2 (E.aur.)  .......... .......... .......... A......... .......... ..........
 3 (E.cla.)  .......... A..T.....T .........T AT........ .T........ ..........
 4 (E.tri.)  .......... .......... .......... A......... .......... .....A....
 5 (O.cuc.)  .......... .......... .......... .......... .......... ..........
 6 (C.fim.)  ......T... A......... .......... A.....T... ........A. ..........
 7 (C.mon.)  ........-- A.......C. .......... A.....T... ........A. ..........
 8 (N.cra.)  TTC....A.. A......... .......... A.....T... .......... ..........
 9 (P.ans.)  .T.....A.. A......... .......... A......... C......... ..........
10 (S.fim.)  .T.....A.. A......... .......... A.....T... .......... ..........
11 (G.tet.)  TT.....A.. A......... .......... A.....T... .......... ..........
12 (P.not.)  .T........ A..T.....G .......... A....CT... GG...-.... ..........
13 (A.fum.)  .T........ A..T.....G .......... A....CT... GG...-.... ..........
14 (N.fis.)  .T........ A..T.....G .......... A....CT... GG........ ..........
15 (C.fra.)  .T.....CTT C-T..GC.A. T...C..TCT TTC...A..T ---.A..... ..........
16 (E.dec.)  AT.TG..... -----.GTGTG TT..ATTTAT TGA.A.-.CA .--AC...G. CAA..A....
17 (S.cer.)  .T.....CT. A..T.TGA.T .........- -G...CTC.T G--.C..... ..........
18 (K.lac.)  .T.....CT. A..T.GTACT .........- -G....T.CA G--.C..... ..........
19 (T.del.)  .T.....CT. A..T.TGG.T .........- -G...C.C.T G--.C..... ..........
20 (S.com.)  .TCT...T.. A...G.G... T........T A.......C. ....C..... ..........
21 (S.hyd.)  .TCT...T.. ....G..... T........A A.....T... ....C..... ..........
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                748
 1 (C.ves.)  CTTTGAAAAA ATTAGAGTGT TCAAAGCAGG C-TTATGCTC GGATA-CATT AGCATGGAAT
 2 (E.aur.)  .......... .......... .......... .......... .......... ..........
 3 (E.cla.)  .......... .......... .......... ..C....... .A........ ..........
 4 (E.tri.)  .......... .......... .......... .......... .A........ ..........
 5 (O.cuc.)  .......... .......... .......... .......... .......... ..........
 6 (C.fim.)  .......... .........C ..C.G..... ..C....... .A..CGT.C- ..........
 7 (C.mon.)  .......... .........C ..C.G..... ..C....... .A...GT.C- ..........
 8 (N.cra.)  .CG....C.. ..C...TC.C ......A... ..C....... .A..G.T.C. ..........
 9 (P.ans.)  ..C....C.. ......TC.C .T....A... ..C....... .A....GTC. ..........
10 (S.fim.)  ..C....C.. ......TC.C .T....A... ..C....... .A........ ..........
11 (G.tet.)  .CG....C.. ..C...TC.C .T....A... ..C....... .A..-GT.C. ..........
12 (P.not.)  ..G....... .......... .......... ..C.T..... .A........ ..........
13 (A.fum.)  ..G....... .......... .......... ..C.T..... .A........ ..........
14 (N.fis.)  ..G....... .......... .......... ..C.T..... .A........ ..........
15 (C.fra.)  .......... .......... .......... ..C.T..... .A....T... ..........
16 (E.dec.)  .......... .......... .......... ..C.T..... .A....T... ..........
17 (S.cer.)  .......... .......... .......... .G.AT..... .A....T... ..........
18 (K.lac.)  .......... .......... .......... ..GA.A.... .A....T... ..........
19 (T.del.)  .......... .......... .......... .G.AT..... .A....T... ..........
20 (S.com.)  .C....G... .......... .......... .....C..C. .A........ ..........
21 (S.hyd.)  ......G... .......... .......... ..C.....C. .A..______ ..........

                807
 1 (C.ves.)  AATAGAATAG GACGT-GCGG TTCTATTTTG TTGGTTTCTA GGACCGCCGT AATGATTAAT
 2 (E.aur.)  .......... .......T.. .......... .......... .......... ..........
 3 (E.cla.)  .......... .......T.. .......... .......... .......... ..........
 4 (E.tri.)  .......... ....C..... .......... .......... .......... ..........
 5 (O.cuc.)  .......... .......... .......... .......... .......... ..........
 6 (C.fim.)  .......... .......T.. .......... .......... .......... ..........
 7 (C.mon.)  .......... .......T.. .......... .......... .......... ..........
 8 (N.cra.)  .......... .......T.. .......... .......... .......... ..........
 9 (P.ans.)  ...G...... .......T.. .......... .......... .......... ..........
10 (S.fim.)  .......... .......T.. .......... .......... .......... ..........
11 (G.tet.)  .......... .......T.. .......... .......... .......... ..........
12 (P.not.)  .......... .......T.. .......... .......... .......... ..........
13 (A.fum.)  .......... .......... .......... .......... .......... ..........
14 (N.fis.)  .......... .......... .......... .......... .......... ..........
15 (C.fra.)  .......... .....TAT.. .......... .......... .....AT... ..........
16 (E.dec.)  .......... ......AT.. .......... .......... ......T... ..........
17 (S.cer.)  .......... ......TT.. .......... .......... .....AT... ..........
18 (K.lac.)  ...G...... ......TT.. .......... .......... .....AT... ..........
19 (T.del.)  .......... ......TT.. .......... .......... .....AT... ..........
20 (S.com.)  ....A..... .......... .C........ .......... ...T...... ..........
21 (S.hyd.)  ....A..... .......... .......... .......... .AGT...... ..........

                867                                  1546
 1 (C.ves.)  AGGGACAGTC GGGGGCATCA GT-----TAT TGCTCTTCAA CGAGGAATCC CTAGTAAGCG
 2 (E.aur.)  .......... .......... ..      ... .......... .......... ..........
 3 (E.cla.)  .......... .....TG... ..      ... .......... .......... ..........
 4 (E.tri.)  .......... .......... ..      ... .......... .......... ..........
 5 (O.cuc.)  .......... .......... ..      ... .......... .......... ..........
 6 (C.fim.)  .......... .......... ..      ... .......... .......... ..........
 7 (C.mon.)  .......... .......... ..      ... .......... .......... ..........
 8 (N.cra.)  .......... .......... ..      ... .......... .......... ..........
 9 (P.ans.)  .......... .......... ..      ... .......... ........T. ..........
10 (S.fim.)  .......... .......... ..      ... .......... .......... ..........
11 (G.tet.)  .......... .......... ..      ... .......... .......... ..........
12 (P.not.)  .....T.... ......G... ..      ... .......... ........G. ......G..A
13 (A.fum.)  .....T.... ......G... ..      ... .......... ........G. ......G..A
14 (N.fis.)  .......... ......G... ..      ... .......... ........G. ......G..A
15 (C.fra.)  ......G... .......... ..      ... .......... ........T. ..........
16 (E.dec.)  .......... .......... ..      ... .......... .......... ..........
17 (S.cer.)  ......G... .........G ..      ... .......... ........T. ..........
18 (K.lac.)  ......G... .......... ..      ... .......... ........T. ..........
19 (T.del.)  ......G... .......... ..      ... .......... ........T. ..........
20 (S.com.)  .....T...T ........TG ..      ... .......... ........A. ..........
21 (S.hyd.)  .....T...T ........T. ..      ... .......... ........T. ..........
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                1579
 1 (C.ves.)     CAAGTCATCA ACTTGCGTTG ATTACGTCCC TGCCCTTTGT ACACACCGCC CGTCGCTACT
 2 (E.aur.)     .......... .......... .......... .......... .......... ..........
 3 (E.cla.)     .......... .......... .......... .......... .......... ..........
 4 (E.tri.)     .......... .......... .......... .......... .......... ..........
 5 (O.cuc.)     .......... .......... .......... .......... .......... ..........
 6 (C.fim.)     .......... G......... .......... .......... .......... ..........
 7 (C.mon.)     .......... G......... .......... .......... .......... ..........
 8 (N.cra.)     .......... G......... .......... .......... .......... ..........
 9 (P.ans.)     .......... G......C.. .......... .......... .......... ..........
10 (S.fim.)     .......... G......... .......... .......... .......... ..........
11 (G.tet.)     .......... G......... .......... .......... .......... ..........
12 (P.not.)     .G........ G..C.T.CC. .......... .......... .......... ..........
13 (A.fum.)     .G........ G..C.T.CC. .......... .......... .......... ..........
14 (N.fis.)     .G........ G..C.T.CC. .......... .......... .......... ..........
15 (C.fra.)     .......... G......... .......... .......... .......... ..........
16 (E.dec.)     .......... G......... .......... .......... .......... ..........
17 (S.cer.)     .......... G......... .......... .......... .......... ..........
18 (K.lac.)     .......... G......... .......... .......... .......... ........G.
19 (T.del.)     .......... G......... .......... .......... .......... ........G.
20 (S.com.)     TG........ G..C...... .......... .......... .......... ..........
21 (S.hyd.)     TG.......- G..C...... .......... .......... .......... ..........

                1639
 1 (C.ves.)     ACCGATTGAA TGGCTCAGTG AGGCTCTCGG ACTGGCCCAG GGGGGCGGG- CAACCCCCCC
 2 (E.aur.)     .......... .......... .....T.... .......... .......... ..........
 3 (E.cla.)     .......... .......... .....T.... .......... .......... ..........
 4 (E.tri.)     .......... .......... .....T.... .......... .......... ..........
 5 (O.cuc.)     .......... .......... .......... .......... .......... .....A....
 6 (C.fim.)     ........G. .......... ..A.CTC... .......G.. A.A..T.... A...TA....
 7 (C.mon.)     ........G. .......... ..A.CTC... .......G.. A.A..T.... A...TA....
 8 (N.cra.)     .......... .......... .....TC... .......... ..A..TC... ....GA..A.
 9 (P.ans.)     .......... .......... .....TC... .......... ..A..TC... ....G..AA.
10 (S.fim.)     .......... .......... .....TC... .......... ..A..TC... ....GA.A..
11 (G.tet.)     .......... .......... .....TC... .......... ..A..TC... ....GA.A..
12 (P.not.)     .......... .......... ....CT.G.. .T....TT.. .A...TT... ....GA....
13 (A.fum.)     .......... ......G... ....CT.... ......T... ...A.TT... ....GA.T..
14 (N.fis.)     .......... ......G... ....CT.... ......T... ...A.TT... ....GA.T..
15 (C.fra.)     .......... .....T.... ...ACTC... .T...TT... .A.TAGA..G ....TT.AA.
16 (E.dec.)     ......C... .....T.... .....TC... .T..A.TGTT TA-..A.... AG..TTTTTA
17 (S.cer.)     .......... .....T.... ....CTCA.. .TCT..TT.. A.AA.G.... ....T..AT.
18 (K.lac.)     .......... .....T.... ....CTCA.. .T.T..TT.. A.AA.G.... ....T..AT.
19 (T.del.)     .......... .....T.... ....CTCA.. .TCT..TT.. A.AA.G.... ....T..AT.
20 (S.com.)     .......... .....T.... ...TCT.... .TC...TTT. ...A.AA... ....GG.A..
21 (S.hyd.)     .......... .....T.... ...TCT.... .T....TTTT ...A..C... ....GG.A.T

                1699
 1 (C.ves.)     CCCGGGCGG- -GGAAGTTAT CCAAACTCGG TCATTTAGAG GAAGTAAAAG T
 2 (E.aur.)     .......... ..A....... .......... .......... .......... .
 3 (E.cla.)     .......... ..A....... .......... .......... .......... .
 4 (E.tri.)     .......... ..A....... .......... .......... .......... .
 5 (O.cuc.)     .......... .......... .......... .......... .......... .
 6 (C.fim.)     T.AT...C.. ..A...C.G. T......... .....C.... .......... .
 7 (C.mon.)     T.AT...C.. ..A...C... T......... ....C..... .......... .
 8 (N.cra.)     ..A....C.. ..A...C... .......... .......... .......... .
 9 (P.ans.)     ..A....C.. ..A...C... .......... .......... .......... .
10 (S.fim.)     .AG..AG..C G.A...C... .......... .......... .......... .
11 (G.tet.)     .-A....C.. ..A...C... .......... .......... .......... .
12 (P.not.)     ..A.A..C.. .AA..C..GG T......... .......... .......... .
13 (A.fum.)     ..A.A..C.. ..A.....GG T.....C... .......... .......... .
14 (N.fis.)     ..A.A..C.. ..A.....GG T.....C... .......... .......... .
15 (C.fra.)     TTG.AA.C.. .A-...C.G. T......T.. .......... .......... .
16 (E.dec.)     GA..A.AT.. .A....C..G T......T.. .......... .......... .
17 (S.cer.)     T.A.A..... .A...T..GG A......T.. .......... ...C...... .
18 (K.lac.)     T.A.A...A. .A...TC.GG T......T.. .......... ...C...... .
19 (T.del.)     T.A.A..... .A...TC.GG T......T.. .......... ...C...... .
20 (S.com.)     T.ATT..T.. .A......GA T......T.. .......... .......... .
21 (S.hyd.)     .GATT..T.. .A......GA T......T.. .......... .......... .
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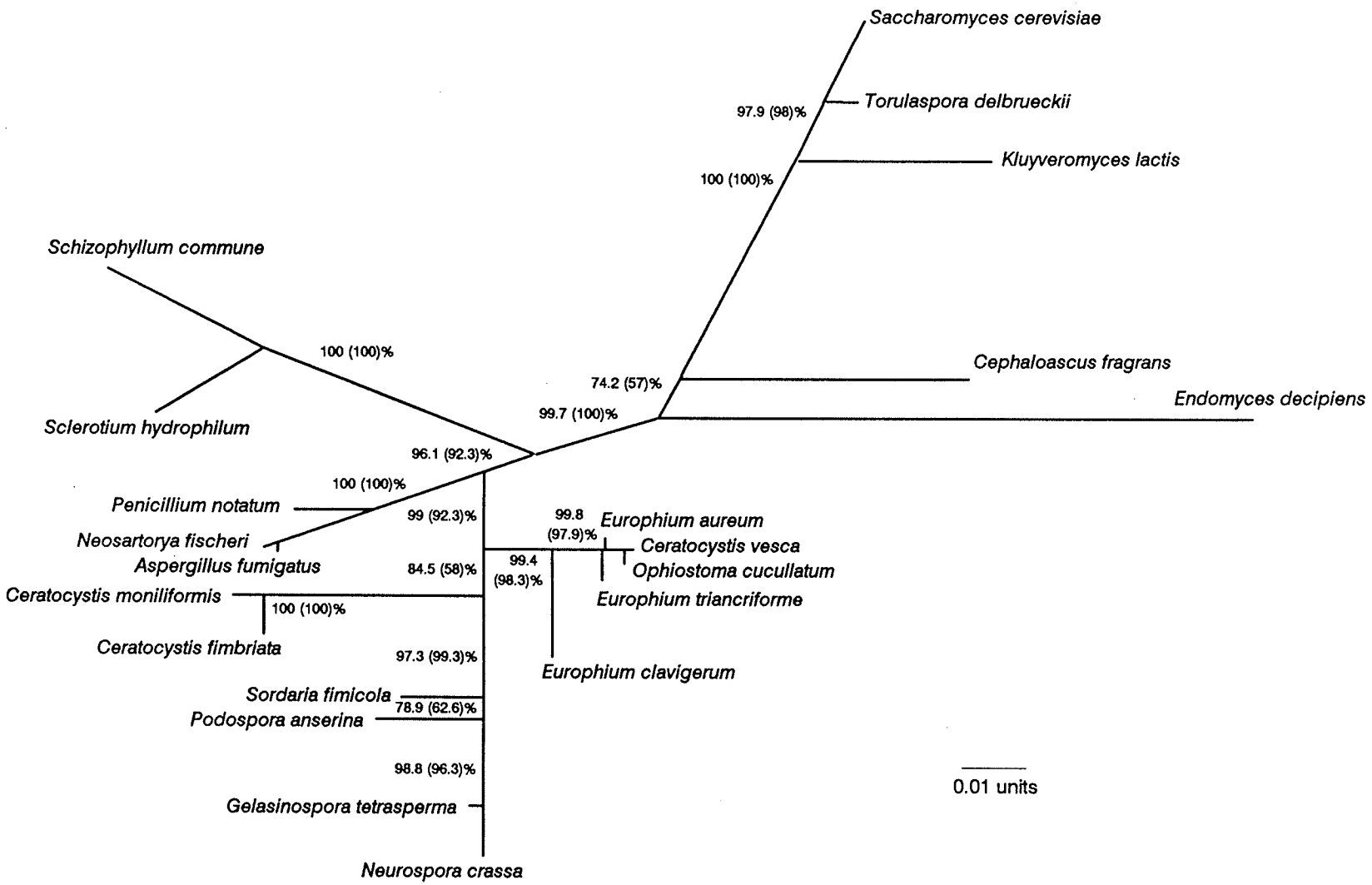
Fig. 2. Unrooted Fitch-Margoliash network showing the phyletic relationships between yeast-like genera that produce galeate ascospores and similar-spored members of the Ophiostomatales, based on the analysis of partial SSrDNA sequences (about 700 positions) with the FITCH program. The branch lengths are those as determined by the FITCH program and the sum of squares for this tree was 0.502 with a average percent standard deviation of 3.465. The percentages along the branches of the network are an indication of the level of confidence for major branches as determined by bootstrap analysis. The percentages given in brackets are the bootstrap support for major branches when the data was analyzed by DNAPARS.


Saccharomyces cerevisiae
Torulaspora delbrueckii
97.9 (98)%
Kluyveromyces lactis
100 (100)%
Schizophyllum commune
100 (100)%
Cephaloascus fragrans
74.2 (57)%
Endomyces decipiens
99.7 (100)%
Sclerotium hydrophilum
96.1 (92.3)%
100 (100)%
Penicillium notatum
99 (92.3)%
99.8
(97.9)%
Europhium aureum
Ceratocystis vesca
Neosartorya fischeri
Aspergillus fumigatus
84.5 (58)%
99.4
(98.3)%
Ophiostoma cucullatum
Ceratocystis moniliformis
Europhium triancriforme
100 (100)%
Ceratocystis fimbriata
97.3 (99.3)%
Europhium clavigerum
Sordaria fimicola
78.9 (62.6)%
Podospora anserina
98.8 (96.3)%
Gelasinospora tetrasperma
Neurospora crassa
0.01 units

DISCUSSION

Ascospores differ in shape, colour, septation, ornamentation and size, and all of these features have been used extensively in the delimitation of species and genera. Ascospore shape has also been used as an important character to aid in delimiting species and genera in both the Ophiostomataceae and the Endomycetales (e.g., Olchowecki and Reid 1974; Redhead and Malloch 1977; Upadhyay 1981; von Arx and van der Walt 1987). The production of ascospores that are hat- or helmet-shaped (galeate), with an apparent basal circumfluent brim in species such as C. fimbriata, E. triancriforme, C. fragrans, and E. decipiens, have led some authors to speculate that a close relationship exists between certain yeast-like genera and Ceratocystis s.l. (Cain 1972; von Arx 1974; Redhead and Malloch 1977). And Weijman (1976) ruled out a possible relationship of C. fragrans with galeate-spored E. aureum and O. ulmi, because he reported the latter two species had cellulose and rhamnose in their cell walls, while C. fragrans did not. However, although his results did not preclude a relationship between C. fragrans and C. fimbriata, the latter also lacks these sugars in its walls, he felt morphological characters lessened the likelihood of such a relationship. Nonetheless, although recognizing that the Ophiostomatales in their circumscription was polyphyletic,

von Arx and van der Walt (1987) continued to suggest that C. fragrans "had a kinship with the Ophiostomatales rather than the Endomycetales" because its ascophores resemble Leptographium conidiophores, it has poroid septa (E. decipiens does not), and its galeate ascospores are similar to those of E. triancriforme. However, both E. aureum and E. triancriforme have anamorphs solely assignable to Leptographium (Harrington 1988), while E. clavigera produces a Leptographium state as one of its several synanamorphs (Tsuneda and Hiratsuka 1984), and yet the results show C. fragrans has no affinity with the Europhium species tested. This counters von Arx and van der Walt's (1987) contention that the resemblance of C. fragrans ascophores to Leptographium conidiophores might, in some way, reflect a kinship of C. fragrans with the Ophiostomatales.

The sequence data clearly shows that C. fragrans and E. decipiens are not related to either the Europhium species or the species of Ceratocystis s.s. tested. And as parsimony and distance analysis placed C. fragrans and E. decipiens, along with three members of the Endomycetales, within a probably discrete monophyletic group outside of the clusters of the plectomycetous and pyrenomycetous species examined, the sequence data supports returning the Cephaloascaceae to the Endomycetales.

Both parsimony and distance analyses indicate that the Ophiostomataceae is polyphyletic. The two species representing Ceratocystis s.s. (de Hoog and Scheffer 1984)

and the five representing the Ophiostoma-complex did not cluster together. This supports de Hoog (1974) and de Hoog and Scheffer's (1984) separation of Ceratocystis Ell. & Halst. sensu lato into Ceratocystis s.s., which is restricted to species with Chalara anamorphs, and Ophiostoma in which all other species are placed regardless of their anamorphs. However, the ultimate dispositon of Ceratocystis s.s. and Ophiostoma remains unresolved, but will be explored further in the following chapters.

Recently, in a search for new criteria which might be applied in the taxonomy of Ceratocystis s.l., ascospore sheath ontogeny, spore wall complexity, and centrum development at the ultrastructural level were investigated (van Wyk and Wingfield 1991a, 1991b; van Wyk et al. 1991). Two species with galeate-ascospores, C. fimbriata (Stiers 1976) and C. moniliformis (van Wyk et al. 1991), are considered by van Wyk et al. to differ at the ultrastructural level in ascospore walls and wall delimiting structures, and by the presence of auxiliary cells. However, the analysis of the data by parsimony and distance methods supported the grouping of these two species at the 100% confidence level.

Van Wyk and Wingfield (1991a, 1991b) further noted that the ascospore walls in O. cucullatum and O. davidsonii were also hat-shaped, and while their ascospore developmental patterns were similar, those patterns appeared to differ significantly from what occurs in either C. moniliformis or

C. fimbriata. Interestingly, the rDNA sequence analysis shows that O. cucullatum and C. vesca do not cluster with C. fimbriata or C. moniliformis.

It would appear, then, that ultrastructural studies of ascosporogenesis in species of Ceratocystis s.l. support the view that this genus represents a polyphyletic grouping. However, caution will be required in interpreting the significance of ascospore development data as taxonomic criteria; C. fimbriata and C. moniliformis differ in their ascospore developmental patterns, and yet they are united by molecular data.

Overall the results strongly support the proposition that the evolution of galeate ascospores is an example of convergent evolution in fungi. Genera such as Cephaloascus, Endomyces, Ceratocystis, and Ophiostoma all include species which produce galeate ascospores, but they also have additional characters in common i.e., insect dispersal, growth on exudates of higher plants, blastic conidial states (including yeast-like daughter cells), and subglobose to short clavate asci which frequently lack croziers (Redhead and Malloch 1977). Therefore it is very likely that galeate ascospores, along with the other morphological features noted above, have evolved independently in different fungal groups due to adaptation to a similar habitat or insect association. The production of hat-shaped ascospores by itself should therefore not be construed as an indicator of evolutionary relatedness.

CHAPTER 3

ON THE SUBDIVISION OF *Ceratocystis* s.l., BASED ON PARTIAL RIBOSOMAL DNA SEQUENCES

INTRODUCTION

The disposition of the genus Ceratocystis Ell. and Halst. s.l. within the Ascomycotina is unresolved; additionally its subdivision into smaller groups has long been a source of controversy. De Hoog and Scheffer (1984) placed species of Ceratocystis s.l. that lack Chalara anamorphs, are resistant to cycloheximide, and have rhamnose in their cell walls in Ophiostoma H. & P. Sydow., while they assigned those with Chalara anamorphs to Ceratocystis s.s. Moreover, de Hoog and Scheffer also recognized Ceratocystiopsis, a genus erected by Upadhyay and Kendrick (1975) to accommodate species of Ceratocystis s.l. that produce elongate or falcate-type ascospores. Von Arx and van der Walt (1987) also accepted three genera in the Ophiostomataceae, Ophiostoma, Ceratocystiopsis, and Europhium Parker, but placed Ceratocystis s.s. in the Pyxidiophoraceae Arnold in their polyphyletic concept of the Ophiostomatales. Upadhyay considered Europhium to be a synonym of Ceratocystis s.l., whereas Benny and Kimbrough (1980) and Harrington (1987) placed Europhium in synomyny with Ophiostoma. Recently Barr (1990) followed von Arx and van der Walt's narrow circumscription of the Ophiostomataceae, but she assigned Ceratocystis s.s. to the Lasiosphaeriaceae (Sordariales), and aligned the Ophiostomataceae with the Microascaceae in the Microascales.

In addition to the foregoing, the previous chapter showed that Europhium species are closely related to Ophiostoma species.

This strongly supports those who believe Europhium should be reduced to synonymy with Ophiostoma.

De Hoog (1974) recognized only 11 species of Ceratocystis s.s.; these have Chalara, Chalaropsis or Thielaviopsis anamorphs, and are biologically heterogenous when compared to those of the Ophiostoma complex (Harrington 1987). Ceratocystis s.s. includes temperate and tropical species that grow on a wide variety of herbaceous and woody plants, whereas most Ophiostoma species are found in the woody xylem or phloem of temperate forest trees. Ophiostoma species are primarily vectored by subcortical insects such as bark beetles; in contrast, species of Ceratocystis s.s. also have flies and sapfeeding insects as important agents of spore dispersal (Harrington 1987; Upadhyay 1981). As pathogens, species of Ceratocystis s.s. may be of great economic importance: Ceratocystis fimbriata causes rot in sweet potatoes and wilt of coffee and rubber trees; Ceratocystis paradoxa, diseases in palms and sugar cane; Ceratocystis adiposa, black rot of sugar cane; Ceratocystis fagacearum, oak wilt; and Ceratocystis coerulescens, maple sapstreak.

To obtain additional evidence that species assigned to Ceratocystis s.s. do comprise a valid genus, partial ribosomal DNA (rDNA) sequences were determined from the large subunit gene (LSrRNA). Also, partial rDNA sequences were collected from the small ribosomal subunit gene (SSrRNA) of two members of Ceratocystis s.s. (C. fimbriata and C. moniliformis) and other species included within the Ophiostomataceae in order to gain a better understanding of the taxonomic disposition of Ceratocystis

within the Ascomycotina.

MATERIALS AND METHODS

The fungal strains that were sequenced, and their sources are listed in Table 1.

For this investigation, partial SSrDNA sequences which comprised the V3, V4 and V9 regions were employed, along with partial rDNA sequences from the 5' end of the LSrDNA gene that included Domain I. These were obtained by the procedures described in the Materials and Methods.

The rDNA sequences of Saccharomyces cerevisiae Meyen ex Hansen (Rubstov et al. 1980; Georgiev et al. 1981) Neurospora crassa Shear & B.O. Dodge (Sogin et al. 1986b; Guadet et al. 1989), Kluyveromyces lactis (Domb.) van der Walt (EMBL: X51830), Torulaspora delbrueckii (Lindner) Lindner (GENBANK: TOUSRSR), Penicillium notatum Westling (GENBANK: PNNDA), Aspergillus fumigatus Fresen. (GENBANK: ASNDA), and Podospora anserina (Ces.) Niessl (GENBANK: PANRRNASS), were included in the analysis for comparison. Schizophyllum commune and Sclerotium hydrophilum were included in the analysis to serve as outgroups.

The nucleotide sequences were aligned using CLUSTAL V and the MASE programs. The partial rDNA sequence data were analyzed using the programs contained within PHYLIP (Version 3.4; Felsenstein 1991).

Phylogenetic trees were obtained by parsimony (DNAPARS) and distance methods (KITSCH and FITCH). Divergence (or distance) between two sequences was calculated by DNADIST using Kimura's two parameter model (Kimura 1980). The FITCH and KITSCH programs

were used to carry out Fitch and Margoliash's least-square method of estimating phylogenies from distance matrices. The FITCH program will fit a tree that has the branch lengths unconstrained, whereas the KITSCH program assumes the validity of an evolutionary clock, and thus creates a tree in which the branch lengths from the root of the tree to each tip are equal. The DNAPARS program was used to carry out unrooted parsimony on DNA sequences for estimate phylogenies.

In order to estimate the confidence in all putatively monophyletic groups based on parsimony or distance criteria, bootstrap analysis was carried out by generating 1000 (500 if more than 20 DNA sequences were involved) bootstrap replicates with SEQBOOT. For bootstrap analysis, both the complete aligned dataset, and a dataset from which potentially ambiguously aligned positions were omitted, were utilized. Each bootstrap replicate was analyzed by DNAPARS, KITSCH, and FITCH and a majority-rule consensus tree was generated by CONSENSE.

Table 1. List of fungal strains studied.

Species and Isolate No.	Source	Geographic origins
Ceratocystis adiposa (Butler) C. Moreau		
CBS 127.27	Not given	Not given
Ceratocystis autographa Bakshi		
CBS 650.75	Juniperus communis L.	Not given
Ceratocystis coerulscens (Münch) Bakshi		
WIN(M) 98	Picea abies (L.) H. Karst.	Norway
Ceratocystis fagacearum (Bretz) Hunt		
ATCC 24789	Quercus nigra L.	U.S.A
ATCC 24790	Quercus rubra L.	U.S.A
ATCC 11090	Not given	Not given
Ceratocystiopsis falcata (Wright & Cain) Upadh.		
WIN(M) 792	Pinus radiata D. Don	New Zealand
WIN(M) 793	P. radiata	New Zealand
Ceratocystis fimbriata Ell. & Halst.		
DAOM 195303	Kerria japonica (L.) DC.	France
Ceratocystis ips (Rumb.) Nannf.		
WIN(M) 88-58	P. radiata	New Zealand
WIN(M) 88-141	P. radiata	New Zealand
WIN(M) 88-508	Pinus contorta Douglas ex Loud.	New Zealand
WIN(M) 92	Pinus sylvestris L.	Norway
WIN(M) 114	P. sylvestris	Norway
Ceratocystiopsis minuta (Siem.) Upadh. & Kendrick		
CBS 463.77	Picea engelmannii Parry ex Engelm.	U.S.A
Ceratocystis major (von Beyma) C. Moreau		
CBS 138.34	Isolated from air	Not given
Ceratocystis moniliformis (Hedgc.) C. Moreau		
CBS 773.77	Not given	Not given
Ceratocystis paradoxa (Dade) C. Moreau		
CBS 107.22	Cocos nucifera L.	Not given
Ceratocystis piceae (Münch) H. & P. Sydow		
DAOM 145433	Abies balsamea (L.) Mill.	Canada
Ceratocystis pilifera (Fr.) C. Moreau		
WIN(M) 932	P. radiata	New Zealand
Ceratocystiopsis proteae Wingfield, Van Wyk & Marasas		
CBS 486.88	Proteae repens (L.) L.	South Africa
Ceratocystis radicicola (Bliss.) C. Moreau		
CBS 114.47	Phoenix dactylifera L.	Not given
Ceratocystiosis ranaculosus Bridges & Perry		
WIN(M) 919	Pinus taeda L.	U.S.A
Chalara sp.		
WIN(M) 632	Podocarpus sp.	New Zealand

Gelasinospora tetrasperma Dowding		
ATCC 11345	Parsnip seed	Canada
Kernia pachypleura Malloch & Cain		
WIN(M) 253	Soil	Canada
Neosartorya fischeri (Wehmer) Malloch & Cain var. fischeri		
CBS 525.65	Not given	Not given
Schizophyllum commune Fr.		
WIN(M) 916	Tilia americana L.	Canada
Sclerotium hydrophilum Sacc.		
WIN(M) 917	Zizania aquatica L.	Canada
Sphaeronaemella fimicola Marchal		
WIN(M) 818	P. radiata	New Zealand
WIN(M) 827	P. radiata	New Zealand
Sordaria fimicola (Roberge) Ces. & DeNot.		
ATCC 6739	Not given	Canada

ATCC, American Type Culture Collection, Rockwille, Maryland, USA;
CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands;
DAOM, Biosystematics Research Institute, Ottawa, Canada;
WIN(M), The culture collection of J. Reid, University of Manitoba, Department of Botany, Winnipeg, Manitoba, Canada.

RESULTS

The 5'-terminal region (250 nucleotides) of the LSrDNA gene was determined for 13 different species (Fig. 1a); nine of these have Chalara anamorphs, and therefore can be assigned to Ceratocystis s.s.. Phylogenetic trees generated by both distance and parsimony methods had essentially the same topologies, and grouped all species of Ceratocystis s.s. together (Fig. 2, node 7), except C. autographa. This occurred in 93.5% and 97.8% of the bootstrap replicates in the phylogenetic estimates based on distance methods (FITCH and KITCH respectively), and in 94.4% of the bootstrap replicates in parsimony analysis. According to Felsenstein (1985), levels of >95% indicate strong statistical support for nodes, and therefore suggest a very close phylogenetic relationship for species clustered by such nodes.

To obtain an estimate of the relationship of the species of Ceratocystis s.s. to those of the genera Ophiostoma and Sphaeronaemella, the latter having also been assigned previously to the Ophiostomataceae, partial SSrDNA sequences from regions V3, V4, and V9 were employed. Sequences of members of the Basidomycotina, Endomycetales, Eurotiales and Sordariales were included for comparison. The aligned data set (Fig. 1b), which includes both published sequences (GenBank, EMBL) and partial rDNA sequences obtained for this study, was analyzed by the FITCH, KITSCH, and DNAPARS programs.

The estimate of phylogeny based on an unrooted tree generated by FITCH (Fig. 3) indicates significant bootstrap

support (>95% confidence) for the following groupings: (1) the members of the class Hemiascomycetes, order Endomycetales (node 2; K. lactis, T. delbrueckii, and S. cerevisiae); (2) the Eurotiales (node 5; P. notatum, A. fumigatus, and Neosartorya fischeri); (3) the Ophiostoma-complex (node 8; C. ips, C. pilifera, C. piceae, Cop. minuta, and Cop. ranaculosus); (4) the Sordariales (node 7; S. fimicola, P. anserina, N. crassa, and G. tetrasperma); and (5) species of Ceratocystis s.s. (node 11; C. fimbriata and C. moniliformis). The potential monophyly (node 6) of a grouping consisting of the Ophiostoma-complex, Sordariales, Sph. fimicola, Kernia pachypleura, Cop. falcata and Ceratocystis s.s. is supported at the 97.2% confidence level.

Distance methods and parsimony analysis generated phylogenetic trees with essentially the same topologies, except that the phylogenetic trees generated by KITSCH placed both Cop. falcata and Sph. fimicola outside of node 4 (Fig. 3). As it is this node that defined the ascocarpic ascomycetes we tested, it is unlikely that the KITSCH program was placing these two species accurately. Consequently the sequences for these two species were excluded from data sets when phylogenies were generated by KITSCH, but FITCH and DNAPARS were run both with and without these sequences. KITSCH is a program that assumes the validity of an evolutionary clock. As both the Sph. fimicola and the Cop. falcata partial sequences appear to have diverged significantly from those of the other ascomycete members in this analysis, the assumption of an evolutionary clock might not hold. The effect of accelerated evolution on phylogenetic analysis has been discussed

by Woese (1991).

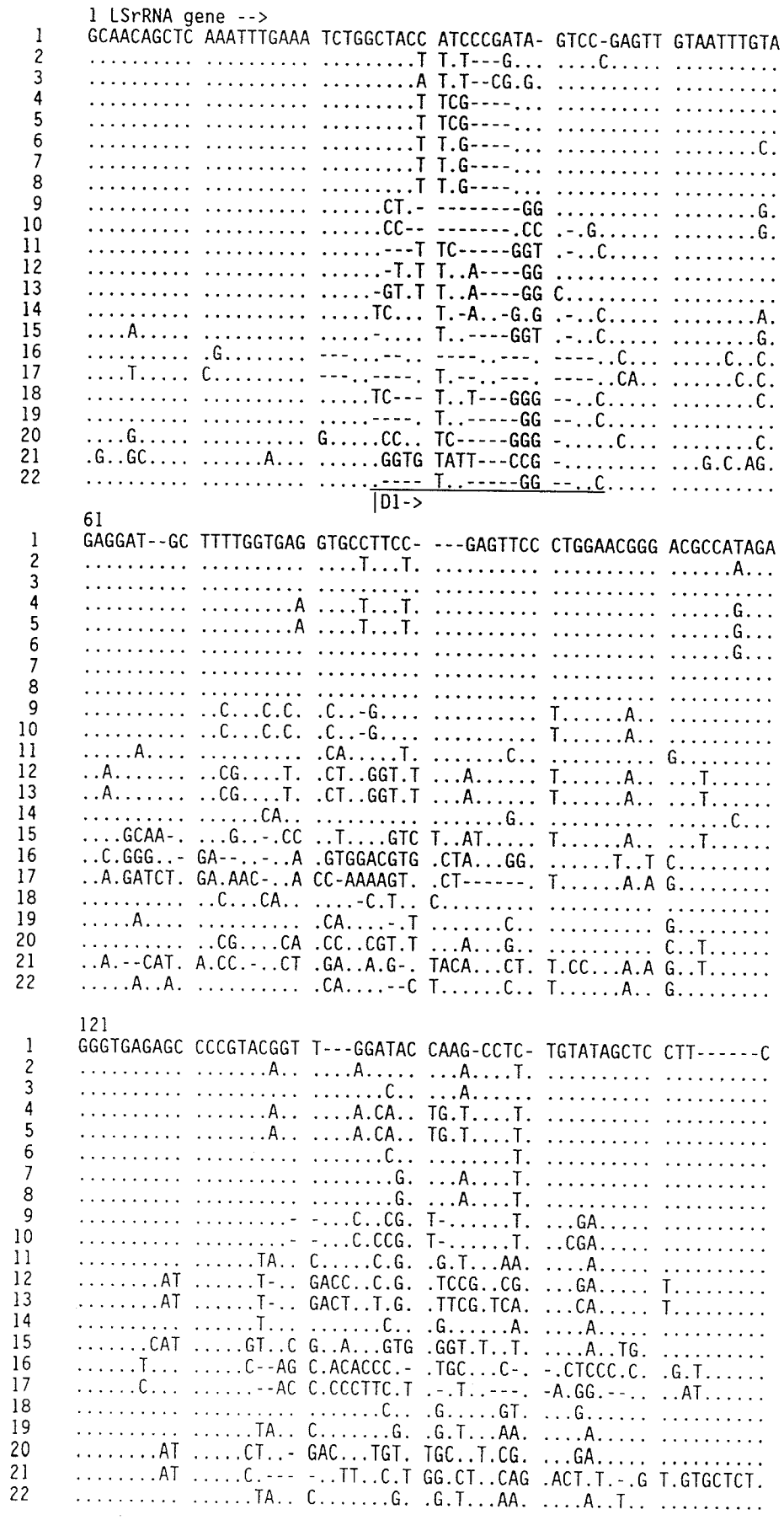
The exclusion of Sph. fimicola and Cop. falcata sequences increased the confidence in the potential monophyly of the cluster consisting of K. pachypleura, Cop. proteae, and Ceratocystis s.s. (node 10); this was also observed when the data was analyzed by DNAPARS (Table 2). Overall the bootstrap support remained the same, or increased slightly, for all major nodes in trees generated by FITCH or DNAPARS when Sph. fimicola and Cop. falcata were omitted from the analysis (Table 2). However, bootstrap support for node 4 in the DNAPARS tree dropped from 83.8% to 55%. This node links the Eurotiales with the Parenchymatomycetidae (Barr 1990). This surprising result points to a weakness in the data, so that no statement can be made at this time regarding this relationship. Node 9, which grouped Sph. fimicola and Cop. falcata together with K. pachypleura, Cop. proteae, and Ceratocystis s.s., also has low confidence levels (FITCH 62.9%, DNAPARS 78.4%). When Sph. fimicola and Cop. falcata were excluded from the analysis node 9 disappeared, and the grouping of Ceratocystis s.s., K. pachypleura, and Cop. proteae was significantly supported by the FITCH (98.9% confidence) and DNAPARS (99.1% confidence) analyses; however KITSCH analysis gave only 93.3% confidence for this group, and thus failed to support strict monophyly.

Following Kurtzman's example of combining partial rDNA sequence data from the SSrDNA and LSrDNA for the purpose of estimating relationships amongst fungi (Kurtzman and Robnett 1991; Guého et al. 1990), an additional data set was generated

which combined the sequences from the V3, V4, and V9 regions of the SSrDNA gene and those of Domain I of the LSrDNA gene (Fig. 1a and 1b), from strains of 14 species. The data set was analyzed hoping that the results would support the groupings obtained by the analysis of the SSrDNA sequences. Phylogenetic trees constructed by both distance (Fig. 4) and parsimony methods had similar topologies, although bootstrap support for nodes varied somewhat depending upon the method of analysis (Table 2). This data set largely confirmed the groupings derived by analysis of the SSrDNA data (Fig. 3); but with one significant exception. Here Sph. fimicola and Cop. falcata do not cluster near Ceratocystis s.s., as they did previously (Fig. 3), instead they are grouped separately at the 92.5% confidence level with FITCH and 99.2% confidence with parsimony analysis.

Fig. 1. (a) Alignments of the 5' terminal end of the LSrDNA. Nucleotide postions 1-271 within the alignment correspond to positions 102-360 in the S. cerevisiae LSrDNA gene (Guadet et al. 1989). Positions which are underlined were excluded for generating phylogenetic estimates as it was difficult to obtain reliable alignments.

Fig. 1. (b) Alignments for the V3, V4 (positions 402-886) and V9 (positions 1546-1748) containing segments of the SSrDNA gene. Nucleotide positions correspond to the nucleotide numbering of the SSrDNA of S. cerevisiae (Rubstov et al. 1980).


1 LSrRNA gene -->
D1->
61
121

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      181
 1    AACGAGTCGA GTAGTTTGGG AATGCTGCTC TAAATGGGAG GTATATCTCT TCTAAAGCTA
 2    G......... .......... .......... .......... .......... ..........
 3    .......... .......... .......... .........C C......... ..........
 4    G......... .......... .......... .......... .......... ..........
 5    G......... .......... .......... .......... .......... ..........
 6    .......... .......... .......... .......... .......... ..........
 7    .......... .......... .......... .......... .......... ..........
 8    .......... .......... .......... .......... .......... ..........
 9    G......... .......... .......... A......... ...A..T... ..........
10    G......... .......... .......... A......... ...A..T... ..........
11    G......... .......... .......... A......... ...A..G... ..........
12    G......... ..T....... .....A.... A.......T. ...A..T..A ..........
13    G......... ..T....... .....A.... A.......T. ...A..T..A ..........
14    G......... .......... .......... A......... ...A.C.C.. ..........
15    G.A....... ..T....... .....A.... ...G....T. ...A..TC.A ..........
16    G......... .......... .......... ....C..... .....C.C.. ..........
17    G.A....... .......... .......... ...-...... ...A...C.. C.........
18    .......... .......... .......... .......... .......... ..........
19    G......... .......... .......... A......... ...A..T... ..........
20    G......... ..T....... .....A.... ........T. ...A..T..A ..........
21    .......... ..T....... .....A.... A.......T. ...A..TC.A ..........
22    G......... .......... .......... A......... ...A..T... ..........
     <-|D1

      241
 1    AAT-ATAGGC TAGAGACCGA TAGCGCACAA  C. fimbriata
 2    .......... .......... ..........  C. fagacearum
 3    .......... .......... ..........  C. moniliformis
 4    .......... .......... ..........  C. major
 5    .......... .......... ..........  C. adiposa
 6    .....CC... .......... ..........  C. coerulescens
 7    .....CC... .......... ..........  C. radicicola
 8    .......... .......... ..........  C. paradoxa
 9    .....CC... C......... ..........  C. ips
10    .....CC... C......... ..........  C. pilifera
11    ......T... C......... ..........  N. crassa
12    ......T... C......... ..........  Chalara sp.
13    ......T... C......... ..........  C. autographa
14    ......T... C......... ..........  K. pachypleura
15    ......T... G......... .....A....  S. cerevisiae
16    .....CCC.. CT........ ..........  Sph. fimicola
17    .....CTT.. C....-.... ..........  Cop. falcata
18    .....CC... C......... ..........  Cop. proteae
19    .....CC... C......... ..........  G. tetrasperma
20    .....CT... CG........ ..........  N. fischeri
21    ..AT..T... G......... .....A....  S. hydrophilum
22    .......... C......... ..........  S. fimicola
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      402 SSrRNA gene -->
 1    GGCTACCACA TCCAAGGAAG GCAGCAGGCG CGCAAATTAC CCAATCCCGA CACGGGGAGG
 2    .......... .......... .......... .......... .......... ..........
 3    .......... .......... .......... .......... .......TA. TT.A......
 4    .......... .......... .......... .......... .......TA. TT.A......
 5    .......... .......... .......... .......... .......TA. T..A......
 6    .......... .......... .......... .......... .......... T.........
 7    .......... .......... .......... .......... .......... ..........
 8    .......... .......... .......... .......... .......... ..........
 9    ......T... .......... .......... .......... .......... ..........
10    ......T... .......... .......... .......... .......... ..........
11    ......T... .......... .......... .......... .......... ..........
12    ......T... .......... .......... .......... .......... ..........
13    ......T... .......... .......... .......... .......... .G........
14    ......T... .......... .......... .......... .......... .G........
15    ......T... .......... .......... .......... .......... .T........
16    ......T... .......... .......... .......... .......... .T........
17    ......T... .......... .......... .......... .......... .T........
18    ......T... ..T....... .......... .......... ........C. --........
19    ......T... .......... .......... .........T .....A.... TT...T...A
20    ......T... ..T....... .......... .......... .......... .G........
21    .........T .TT....... .......... .......... .......... ..........
22    .........T .TT....... .......... .......... .......... ..........
23    ......T... .......... .......... .......... .......... ..........

      462
 1    TAGTGACAAT AAATAACAAT ATAGGGCTCT TTCGGGTCCT ATAATTGGAA TGAGTACAAT
 2    .......... .......... .......G.C ..T.....T. G......... ..........
 3    .......... .......G.. .C.....C.A ........T. G......... ..........
 4    .......... .......G.. .C.....C.A ...-....T. G......... ..........
 5    .......... .......G.. .C.....C.A ........T. G......... ..........
 6    .......... .....CTG.. .CG....... ..T.....TC G......... ....A.....
 7    .......... .....CTG.. .CG....... ..T.....TC G......... ..........
 8    .......... .....CTG.. .CG....... ..T.....TC G......... ..........
 9    .......... .....CTG.. .C........ ..T.....T. G......... ..........
10    .......... .....CTG.. .C........ ..T.....T. G......... ..........
11    .......... .....CTG.. .C........ ..T.....T. G......... ..........
12    .......... .....CTG.. .C........ ..T.....T. G......... ..........
13    .......... .....CTG.. .C........ ..T.....T. G......... ..........
14    .......... .....CTG.. .C........ ..T.....T. G......... ..........
15    .......... .....CTG.. .C........ ..T.....T. G......... ..........
16    .......... .....CTG.. .C........ ..T.....T. G......... ..........
17    .......... .....CTG.. .C........ ..T.....T. G......... ..........
18    .......... .....CTG.. .C........ ..T.....T. G....C.... ..........
19    .......... .....CTG.. .CG....... ..T....... G....C.... ..........
20    .......... .....CTG.. .C........ ..T.....T. G....C.... ..........
21    .......... .....CTG.. .C........ .AT.....T. G....C.... ..........
22    .......... .....CTG.. .C........ .AT.....T. G....C.... ..........
23    .......... .....CTG.. .C........ ..T.....T. G....C.... ..........
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      522
 1    TTAAATCCCT TAACGAGGAT CAATTGGAGG GCAAGTCTGG TGCCAGCAGC CGCGGTAATT
 2    .......... .......... .......... .......... .......... ..........
 3    G.....A... .........A .......... .......... .......... ..........
 4    G.....A... .........A ...C...... .......... .......... ....-.....
 5    G.....A... .........A .......... .......... .......... ....-.....
 6    .......... .........A .......... .......... .......... ..........
 7    C......... .........A .......... .......... .......... ..........
 8    C......... .........A .......... .......... .......... ..........
 9    .......... .........A .......... .......... .......... ..........
10    .......... .........A .......... .......... .......... ..........
11    .......... .........A .......... .......... .......... ..........
12    .......... .........A .......... .......... .......... ..........
13    ....T..... .........A .......... .......... .......... ..........
14    ....T..... .........A .......... .......... .......... ..........
15    ....T..... .........A .......... .......... .......... ..........
16    ....T..... .........A .......... .......... .......... ..........
17    ....T..... .........A .......... .......... .......... ..........
18    .......... .........A .C.G...... .......... .......... .........A
19    .......T.. .........A ...GAA.... .......... .......... ..........
20    .......... .........A .C........ .......... .......... ..........
21    .......... .........A .......... .......... .......... ..........
22    .......... .........A .......... .......... .......... ..........
23    .......T.. .........A .......... .......... .......... ..........

      582
 1    CCAGCTCCAA TAGCGTATAT TAAAGTTGTT GCAGTTAAAA AGCTCGTAGT TGAACCTTCA
 2    .......... .......... .......... .......... .......... ..........
 3    .......... .......... .......... .......... .......... .....T..-G
 4    .........G .......... .......... .......... .......... .....T..-G
 5    .......... .......... .......... .......... .......... .....T..-G
 6    .......... .......... .......... .......... .......... .........-G
 7    .......... .......... .......... .......... .......... .........-G
 8    .......... .......... .......... .......... .......... .........-G
 9    .......... .......... .......... .AG....... .......... .........-G
10    .......... .......... .......... .AG....... .......... .........-G
11    .......... .......... .......... .......... .......... .........-G
12    .......... .......... .......... .AG....... .......... .........-G
13    .......... .......... .......... .......... .......... .........-G
14    .......... .......... .......... .......... .......... .........-G
15    .......... .......... .......... .......... .......... .........-G
16    .......... .......... .......... .......... .......... .........-G
17    .......... .......... .......... .......... .......... .........-G
18    .........C .......... .......... .TG....... .......... ........A-G
19    .......TTC G........C .......... ..G....... .......... ....GG..-G
20    .......... .......... .......... .TG....... .......... C........-G
21    .......... .......... .......... .TG....... .......... .........-G
22    .......... .......... .......... .TG....... .......... .........-G
23    .......... .......... .......... .TG....... .......... C........-G
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     641
 1   GGCCTGGCCG G-GCGGTCTG CCTAACGGTA TGTACT--G- -TCTGG--CC GGGTCTTAC-
 2   .......TT. .......... ...C...... .......... ...C.A.... ..........
 3   ....C..TT. ..C.....C. AT.TTTT-CG .........G .AT.TCCAA. ...G.C.TTC
 4   ..T....TT. T.C.....C. A..TTAT.-T C.CG.ACT.G ..T.TCAA.. ..A....T..
 5   .......TT. ..C.....C. AT.TTTT-CG .........G ..T.CCAA.. ...C...T..
 6   ..T.....T. ..C.....C. ...C..C.CG A........G ...C..CT-- ..AC...T..
 7   ..T.....T. ..C.....C. ...C..C.CG A........G ...C.....T ..AC...T..
 8   ..T.....T. ..C.....C. ...C..C.CG A........G ...C.....T ..AC...T..
 9   ...TC..... -.T.....C. ...C..C.CG ..C......A .CTG..TC-- ...C...TT.
10   ....C....C T.C.....CC ...C..C.GG ..C......G .CTC..CC-- ...C...T..
11   ....CA.... ..C.....C. ...C..C.CG ..C......G .ATA..T.-T ...C...T..
12   ....C....C ..T.....C. ...C..C.CG ..C......A .CTG..T.-. ...C...T..
13   ........T. ..C.....C. ...C..C.CG ..C......G ...C...... ...C...T..
14   ........T. ..C.....C. ...C..C.CG ..C......G ...C...... ...C...T..
15   ........T. ..C.....C. ...C..C.CG ..C......G ...C...... .......T..
16   ........T. ..C.....C. ...C..C.CG ..C......G ...C...... .......T..
17   ........T. ..C.....C. ...C..C.CG ..C......G ...C...... .......T..
18   T.GTCTA.T. T.CTTC.-GT ...C..C.-. ..G.A-...A T.--.....-T ...C.--...
19   ...G..--T. .T..T...C- --.CCGC.AG GAC-G...-A .CGGAAACA. AC.C...T-.
20   ........T. ..C.....CC ...C..C.GG ..C......G ...C...... ...C...T..
21   ........T. ..C.....CC ...C..C.GG ..C......G T..C...... .......T..
22   ........T. ..C.....CC ...C..C.GG ..C......G ..TCC.G... .......T..
23   ........T. ..C.....CC ...C..C.GG ..C......A ...CA..... ...C...T..

     696
 1   CTCTTGGTGA ACCGGCGTGC TCTTTACTGG GCGCGTCGGC GAACCAGGAC TTTTACCTTG
 2   .......... G.....A... ....A..... .T.T.....G .......... ......T...
 3   ..TC...CT. ...T-T.A.T C...--G... CTCTTG--.. .......... ......T...
 4   ..TC...CT. ...T-GTACT C...--G... .T..AG--.. .......... ......T...
 5   ..TC...CT. ...T-T.G.T C...--G... C.CTTG--.. .......... ......T...
 6   ..TC...G.. ...T-.A..G C...C..... CT.T.GG..G -......... ......TG..
 7   ..TC...G.. ...T-.A..G C...C..... CT.T.GG..G -......... ......TG..
 8   ..TC...G.. ...T-.A..G C...C..... CT.T.GG..G .......... ......TG..
 9   T..C...A.. ....-.A... C...C..... .T.T.....G .......... .......G..
10   ..TC...A.. ....-.A... C...C..... ...T.C...G .......... ......TC..
11   ..TC...A.. ....-.A... C...C..... .T.T.....G .......... ......TC..
12   T.TC...A.. ....-.A... C...C..... .T.T.....G .......... .......G..
13   .CTC...G.. G...-.A... C...C..... ...T.....G .......... ......T...
14   .CTC...G.. G...-.A... C......... ...T.....G .......... ......T...
15   .CTC...G.. G...-.A... C...C..... ...T.....G .......... A.....T...
16   .CTC...G.. G...-.A... C...CG.... ...T.....G .......... A.....T...
17   .CTC...G.. G...-.A... C...C..... ...T.....G .......... A.....T...
18   .A.CG.TC.- --T.--A..G .AACGT.AC. ..C------- ---------. GA....T...
19   TGTC..TG.. G.G.-.A..T C...C..... ...T.....G AC..AG.-.. GG....T...
20   .CTC..TG.. ..T.-.A... C......... ...T..-A.G ...A...... ......T...
21   .CTC..TG.. ....-.A... C...C..... .T.T.....G ...A...... ......T...
22   .CTC...--. ....-.A.C. C...C..... .T.T.....G ...A...... ......T...
23   .CTC..TG.. ...C-.A..G C...C..... CT.T.CG..G ...A...... ......TG..
```

1b

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      751
 1    AGAAAATTAG AGTGTTCAAA GCAGGC-TTA CGCCCGAATA -CATTAGCAT GGAATAATAA
 2    .......... .......... .......C.. T......... .......... ..........
 3    .A........ .......... ......G.AT T..T...... .T........ .........G
 4    .A........ .......... .......GA. A..T...... .T........ ........GG
 5    .A........ .......... ......G.AT T..T...... .T........ .........G
 6    .A........ .......... .......C.T T..T...... .......... .........G
 7    .A........ .......... .......C.T T..T...... .......... .........G
 8    .A........ .......... .......C.T T..T...... .......... .........G
 9    .AC....C.. .TC.C..... .A.....C.. T..T.....G .T.C...... .........G
10    .AC....... .TC.C.T... .A.....C.. T..T...... .GTC...... ........GG
11    .AC....... .TC.C.T... .A.....C.. T..T...... .......... .........G
12    .AC....C.. .TC.C.T... .A.....C.. T..T.....- GT.C...... .........G
13    .A........ .......... .......... T..T...... .......... .........G
14    .A........ .......... .......... T..T...... .......... .........G
15    .A........ .......... .......... T..T...... .......... .........G
16    .A........ .......... .......... T..T...... .......... .........G
17    .A........ .......... .......... T..T...... .......... .........G
18    .A........ ....C..C.G .......... T..T...... GTTC-..... ..........
19    .AG.....G. ....C..... .......GC. T..T....C. GTTC-..... .......C-G
20    .A........ ....C..C.G .......A.. T..T...... CTTC-..... .........G
21    .A........ ....C..C.G .......C.. T..T.....C GT.C-..... .........G
22    .A........ ....C..C.G .......C.. T..T...... GT.C-..... .........G
23    .A........ ....C..C.G .......C.. T..T...... .......... .........G

      810
 1    AATAGGACGT GCGGTCCTAT TTTGTTGGTT TCTAGGATCG CCGTAATGAT TAATAGGGAT
 2    .......... .....T.... .......... .....AG... .......... ..........
 3    .......... TT...T.... .......... .......C.A T......... .........C
 4    .......... TT...T.... .......... .......C.A T......... .........C
 5    .......... TT...T.... .......... .......C.A T......... .........C
 6    .......... .T...T.... .......... .......C.. .......... ..........
 7    .......... .....T.... .......... .......C.. .......... ..........
 8    .......... .....T.... .......... .......C.. .......... .........C
 9    .......... .T...T.... .......... .......C.. .......... .........C
10    .......... .T...T.... .......... .......C.. .......... .........C
11    .......... .T...T.... .......... .......C.. .......... .........C
12    .......... .T...T.... .......... .......C.. .......... .........C
13    .......... .T...T.... .......... .......C.. .......... .........C
14    .......... .T...T.... .......... .......C.. .......... .........C
15    .......... .T...T.... .......... .......C.. .......... .........C
16    .......... .T...T.... .......... .......C.. .......... .........C
17    .........C .....T.... .......... .......C.. .......... .........C
18    .......... .G...T.... .......... .......C.A G......... G........C
19    ..ATA.GACG CT.A.T.... .-C....... ..-..A...T G......... .........C
20    .......... .T...T.... .......... .......C.. .......... .........C
21    .......... .T...T.... .......... .......C.. .......... .........C
22    .......... .T...T.... .......... .......C.. .......... .........C
23    .......... .T...T.... .......... .......C.. .......... .........C
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      870                          1546 SSrRNA gene -->
 1    AGTTGGGGGC ATTGGT---- -TATTGCTCT TCAACGAGGA ATACCTAGTA AGCGTGAGTC
 2    .......... ...A..     ......... .......... ..T....... ..........
 3    G..C...... ..C...     ......... .......... ..T....... ....CA....
 4    G..C...... ..CA..     ......... .......... ..T....... ....CA....
 5    G..C...... ..CA..     ......... .......... ..T....... ....CA....
 6    ...C...... G.CA..     ......... .......... ..G....... G..AC.....
 7    ...C...... G.CA..     ......... .......... ..G....... G..AC.....
 8    ...C...... G.CA..     ......... .......... ..G....... G..AC.....
 9    ...C...... ..CA..     ......... .......... ..C....... ....CA....
10    ...C...... ..CA..     ......... .......... ..T....... ....CA....
11    ...C...... ..CA..     ......... .......... ..C....... ....CA....
12    ...C...... ..CA..     ......... .......... ..C....... ....CA....
13    ...C...... ..CA..     ......... .......... ..C....... ....CA....
14    ...C...... ..CA..     ......... .......... ..C....... ....CA....
15    ...C...... ..CA..     ......... .......... ..C....... ....CA....
16    ...C...... ..CA..     ......... .......... ..C....... ....CA....
17    ...C...... ..CA..     ......... .......... ..C....... ....CA....
18    ...C...... ..CC..     .G....... .......... ..T....... ....CA....
19    ...C...... ..CA..     ......... .......... ..C....... GT..CAT...
20    ...C...... ..CA..     ......... .......... ..C....... ....CA....
21    ...C...... ..CA..     ......... .......... ..C....... ....CA....
22    ...C...... ..CA..     ......... .......... ..C....... ....CA....
23    ...C...... ..CA..     ......... .......... ..C....... ....CA....

      1585
 1    ATCAGCTCGC GTTGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TACTACCGAT
 2    ...-...... .......... .......... .......... .......... ..........
 3    .......T.. .......... .......... .......... .......... ..G.......
 4    .......T.. .......... .......... .......... .......... ..G.......
 5    .......T.. .......... .......... .......... .......... ..G.......
 6    .........T .CC....... .......... .......... .......... ..........
 7    .........T .CC....... .......... .......... .......... ..........
 8    .........T .CC....... .......... .......... .......... ..........
 9    .......T.. .......... .......... .......... .......... ..........
10    .......T.. .C........ .......... .......... .......... ..........
11    .......T.. .......... .......... .......... .......... ..........
12    .......T.. .......... .......... .......... .......... ..........
13    ....A..T.. .......... .......... .......... .......... ..........
14    ....A..T.. .......... .......... .......... .......... ..........
15    ....A..T.. .C........ .......... .......... .......... ..........
16    ....A..T.. .C........ .......... .......... .......... ..........
17    ....A..T.. .C........ .......... .......... .......... ..........
18    .......T.. .......... .......... .......... .......... ..........
19    ......AT.. .AC....... .......... .......... .......... ..........
20    .......T.. .......... .......... .......... .......... ..........
21    .......T.. .......... .......... .......... .......... ..........
22    .......T.. .......... .......... .......... .......... ..........
23    .......T.. .......... .......... .......... .......... ..........
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      1645
 1    TGAATGGCTT AGTGAGGTCT TCGGATCGGC TTTGGGGAGA AGGCAACGGC ACCTCATTGC
 2    .......... .......... ......T... ...T.....C C......... ..TCG.....
 3    .......... .......C.. CA.....T.. ..A.A.A..G G......TC. .T....GA..
 4    .......... .......C.. CA....TT.. ..A.A.A..G G......TC. .T....GA..
 5    .......... .......C.. CA.....T.. ..A.A.A..G G......TC. .T....GA..
 6    .........C .......C.. .G....T... ..A..A.G.T T.......A. C..C..GA..
 7    .........C G......C.. .....CT... .CA......T T.......A. T..C..GA..
 8    .........C G......C.. .....CT... .CA......T T.......A. T..C..GA..
 9    .........C .......CT. C....CT... CCA...AG.T C.......A. CA.C..GG..
10    .........C .......CT. C....CT... CCA...AG.T C.......C. .A.C..GG..
11    .........C .......CT. C....CT... CCA...AG.T C.......A. ...CAGGGAG
12    .........C .......CT. C....CT... CCA...AG.T C.......A. ...C-.GG..
13    .........C .......CT. C....CT... CCA..A.G.T G......CA. C..C.CGG..
14    .........C .......CT. C....CT... CCA..A.G.T G......CA. C..C.CGG..
15    .........C .......CT. C....CT... CCA.A..G.T G......TA. C.T..CGG..
16    .........C .......CT. C....CT... CCA.A..G.T G......TA. C.TC.CGG..
17    .........C .......CT. C....CT... CCA.A..G.T G......TA. C.T..CGG..
18    .........C .......C.A .....CT... C..TTTAG-C G......T.. TGAGA.-G..
19    ..G......C .......CT. CT....T... GC..-----T G......T.. .G..G----.
20    ..G......C ......AC.. C....CT... C.G.A.AG.C G..A...T.. CA...CGG..
21    ..G......C ......AC.. C....CT... CGA.A.AG.T G..A...TA. C......G..
22    ..G......C ......AC.. C....CT... CGA.A.AG.T G..A...TA. C......G..
23    .........C ......AC.. C....CT... CCA.A.AG.T G......TAG GA....GG..

      1705
 1    TG--AGAAGT TGATCAAACT TGGTCATTTA GAGGAAGTAA AAGT S. commune
 2    .......... .......... .......... .......... .... S. hydrophilum
 3    G.......T. ..GA...... .......... ......C... .... S. cerevisiae
 4    GA......TC ..G....... .......... ......C... .... K. lactis
 5    G.......TC ..G....... .......... ......C... .... T. delbrueckii
 6    C....A..C. ..G....... C......... .......... .... P. notatum
 7    C...GA.... ..G......C C......... .......... .... A. fumigatus
 8    C...GA.... ..G......C C......... .......... .... N. fischeri
 9    C...GA...C .ATC...... C......... .......... .... N. crassa
10    C...GA...C .ATC...... C......... .......... .... P. anserina
11    G.CGGA...C .ATC...... C......... .......... .... S. fimicola
12    C...GA...C .ATC...... C......... .......... .... G. tetrasperma
13    C...GA...C .ATC...... C......... .......... .... Cop. minuta
14    C...GA.... .ATC...... C......... .......... .... Cop. ranaculosus
15    C...GA...C .ATC...... C......... .......... .... C. ips
16    C...GA...C .ATC...... C......... .......... .... C. pilifera
17    C...GA...C .ATC...... C......... .......... .... C. piceae
18    C...GA.... ..TC...... C......... .......... .... Cop. falcata
19    C.......AC .AT....... C.......C. .......... .... Sph. fimicola
20    C...GA...C ..T....... C.......C. .......... .... Cop. proteae
21    C...GA...C ..T....... C.......C. .......... .... C. fimbriata
22    C...GA...C .AT....... C......C.. .......... .... C. moniliformis
23    C...GA.... ..T....... C......... .......... .... K. pachypleura
```

Fig. 2. Unrooted Fitch-Margoliash network showing the phyletic relationship between species of Ceratocystis s.s. and Ophiostoma, based on the analysis of partial LSrDNA sequences (250 positions) with the FITCH program. The percentages are an indication of the level of confidence for major nodes (1-7) as determined by bootstrap analysis. The data was analyzed by three programs and the bootstrap support as determined for each method is listed in the following order: (1) FITCH, (2) KITSCH, (3) DNAPARS.

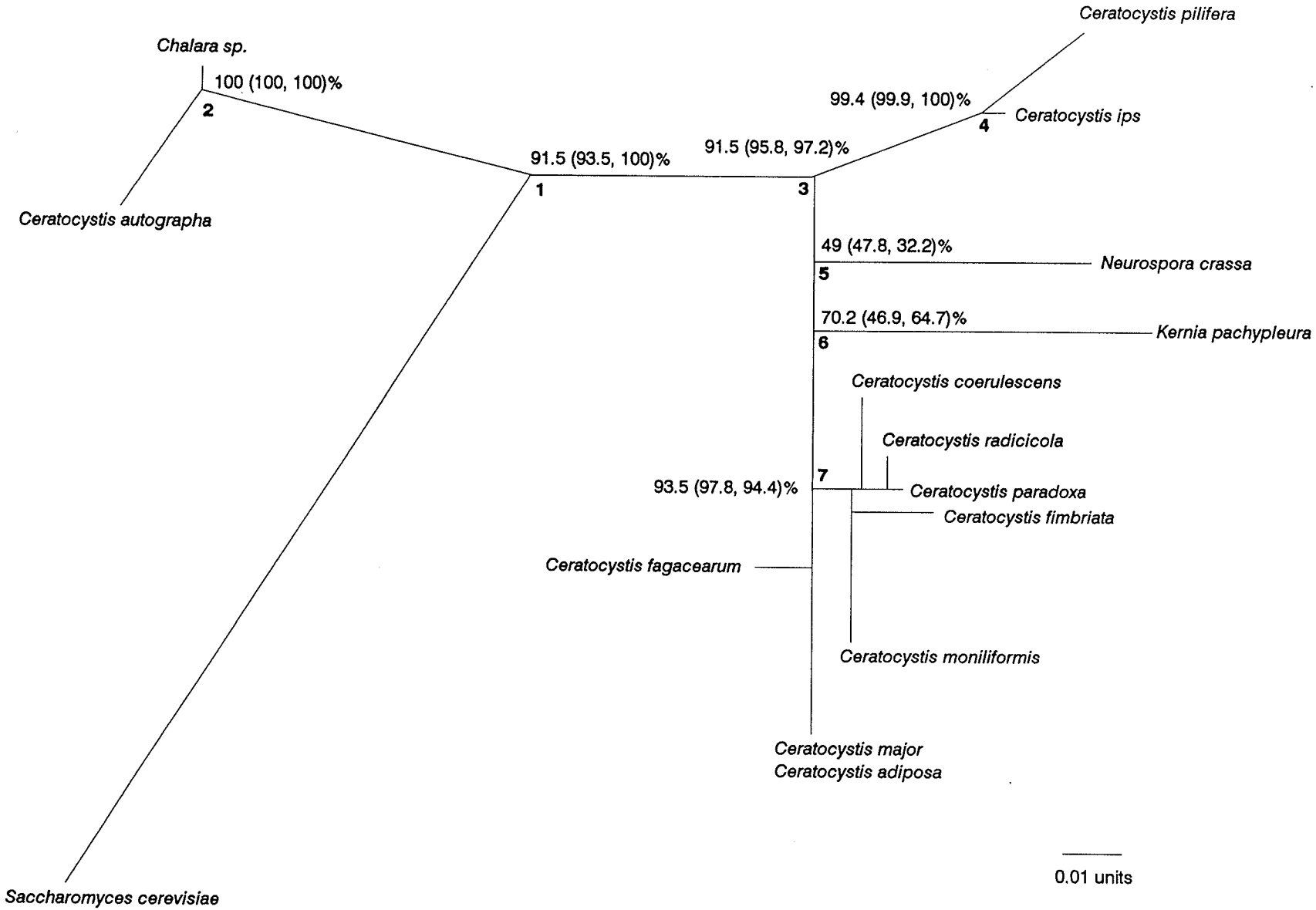

Ceratocystis pilifera
Chalara sp.
100 (100, 100)%
2
99.4 (99.9, 100)%
Ceratocystis ips
4
91.5 (95.8, 97.2)%
91.5 (93.5, 100)%
1
3
Ceratocystis autographa
49 (47.8, 32.2)%
Neurospora crassa
5
70.2 (46.9, 64.7)%
Kernia pachypleura
6
Ceratocystis coerulescens
Ceratocystis radicicola
7
93.5 (97.8, 94.4)%
Ceratocystis paradoxa
Ceratocystis fimbriata
Ceratocystis fagacearum
Ceratocystis moniliformis
Ceratocystis major
Ceratocystis adiposa
0.01 units
Saccharomyces cerevisiae

Fig. 3. Unrooted Fitch-Margoliash network illustrating the phyletic relations of ophiostomataceous fungi within the Ascomycotina. The network was generated by the FITCH program based on partial SSrDNA sequences (about 700 positions). The bootstrap support is indicated for the major nodes with levels of confidence >60%. For the results of the bootstrap analysis obtained when the data was analyzed by KITSCH and DNAPARS see Table 2. Percentages noted in brackets (e.g. node 10) are the level of confidence when Sph. fimicola and Cop. falcata were excluded from the analysis.

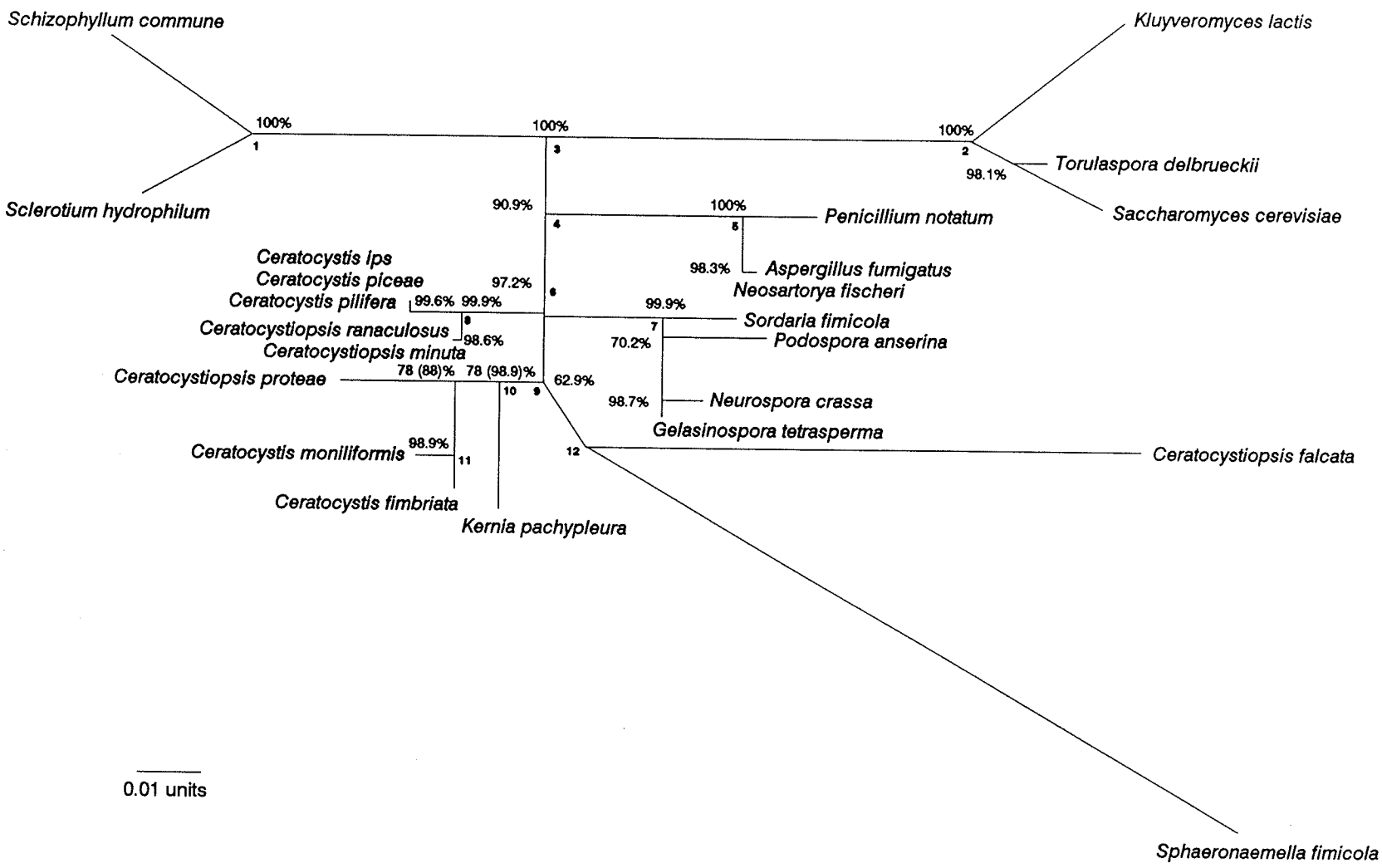

Schizophyllum commune
Kluyveromyces lactis
100%
1
100%
3
100%
2
Torulaspora delbrueckii
98.1%
Saccharomyces cerevisiae
Sclerotium hydrophilum
90.9%
100%
Penicillium notatum
4
5
Ceratocystis ips
Ceratocystis piceae
97.2%
Ceratocystis pilifera
99.6% 99.9%
98.3%
Aspergillus fumigatus
Neosartorya fischeri
6
99.9%
Sordaria fimicola
Ceratocystiopsis ranaculosus
8
98.6%
7
70.2%
Podospora anserina
Ceratocystiopsis minuta
78 (88)% 78 (98.9)%
62.9%
Ceratocystiopsis proteae
10
9
98.7%
Neurospora crassa
Gelasinospora tetrasperma
98.9%
Ceratocystis moniliformis
11
12
Ceratocystiopsis falcata
Ceratocystis fimbriata
Kernia pachypleura
0.01 units
Sphaeronaemella fimicola

Fig. 4. Unrooted Fitch-Margoliash network showing the phyletic relationships between ophiostomataceous fungi based on the FITCH analysis of partial SSrDNA and LSrDNA sequences (about 950 positons). Bootstrap support is given for all nodes with a level of confidence >60%. See Table 2 for the level of confidence of major nodes obtained when the data was analyzed by the KITSCH and DNAPARS program.

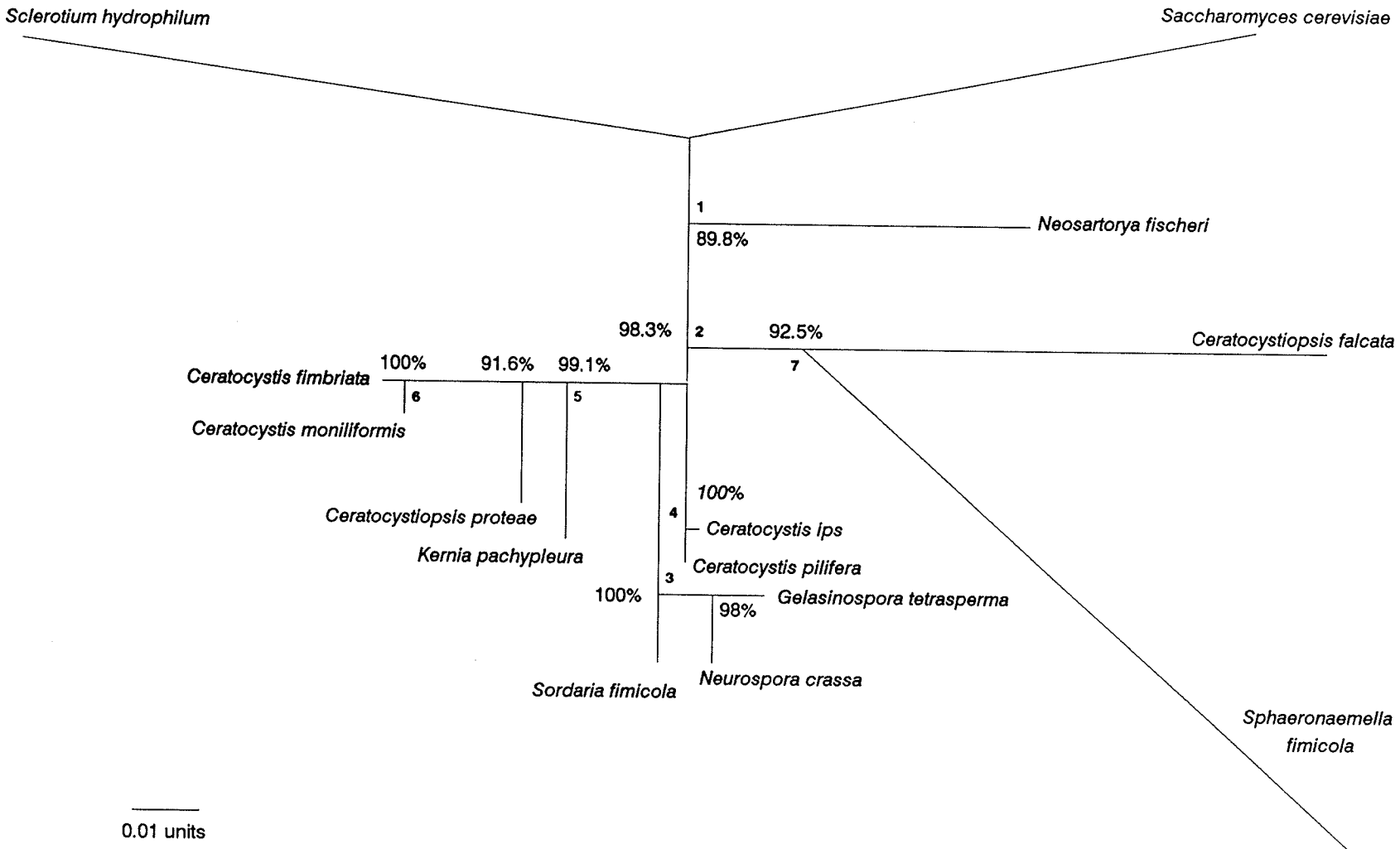

Sclerotium hydrophilum
Saccharomyces cerevisiae
1
89.8%
Neosartorya fischeri
98.3%
2
92.5%
Ceratocystiopsis falcata
7
100%
91.6%
99.1%
Ceratocystis fimbriata
6
5
Ceratocystis moniliformis
Ceratocystiopsis proteae
Kernia pachypleura
100%
4
Ceratocystis ips
Ceratocystis pilifera
3
100%
Gelasinospora tetrasperma
98%
Sordaria fimicola
Neurospora crassa
Sphaeronaemella fimicola
0.01 units

Table 3. Summary of the level of confidence for the major nodes, as determined by bootstrap analysis, for phyletic estimates obtained by distance and parsimony methods. Note, for FITCH and DNAPARS, datasets were analysed that excluded (1) or included (2) the partial rDNA sequences of *Sph. fimcola* and *Cer. falcata*.

Data set	Node	Taxonomic unit clustered by nodes	Bootstrap support for major nodes: FITCH 1	FITCH 2	KITSCH	DNAPARS 1	DNAPARS 2
partial SSrDNA (Fig. 3)	1	Basidiomycotina	100%	100%	100%	-	-
	2	Endomycetales	100%	100%	100%	100%	100%
	3	Hymenoascomycetes	100%	100%	100%	100%	100%
	4	Eurotiales and Parenchymatomycetidae	91.6%	90.9%	100%	55%	83.8%
	5	Eurotiales	98.3%	100%	100%	100%	100%
	6	Parenchymatomycetidae	99.8%	97.2%	94.7%	94%	84.8%
	7	Sordariales	100%	99.9%	98.9%	99.9%	99%
	8	*Ophiostoma*-complex	99.8%	99.9%	100%	98.2%	94.4%
	9	*Ceratocystis*, *Kernia*, *Sphaeronaemella*, *C. proteae* *Cer. falcata*	-	62.9%	-	-	78.4%
	10	*Ceratocystis*, *Kernia*, *C. proteae*	98.9%	78%	93.3%	99.1%	-
	11	*Ceratocystis*	98.6%	98.9%	99.8%	96.9%	97.6%
	12	*Sphaeronaemella*, *C. falcata*	-	51%	-	-	81.8%
partial SSrDNA & LSrDNA (Fig. 4)	1	Hymenoascomycetes		89.8%	100%	92.2%	89.1%
	2	Parenchymatomycetidae		98.3%	100%	99.6%	98.6%
	3	Sordariales		100%	100%	100%	100%
	4	*Ophiostoma*		100%	100%	100%	99.9%
	5	*Ceratocystis*, *Kernia* *C. proteae*		99.1%	95.2%	98.6%	61.3%
	6	*Ceratocystis*		100%	100%	100%	100%
	7	*Sphaeronaemella*, *C. falcata*		92.5%	-	-	99.2%

DISCUSSION

The genus Ceratocystis s.l. has been subdivided based on ascospore morphology (Griffin 1968; Olchowecki and Reid 1974; Upadhyay 1981). Olchowecki and Reid (1974) recognized four spore groups within the genus: Ceratocystis s.s. comprises the members of their Fimbriata-group with Chalara anamorphs; Ceratocystiopsis equals their Minuta-group; and Ophiostoma includes their Ips and Pilifera-groups, plus their Fimbriata-group species that lack Chalara anamorphs. Although in many instances ascospore shape can be important in defining a species or even a genus, the results fail to support a subdivision of Ceratocystis s.l. based on ascospore morphology. For example Olchowecki and Reid (1974) placed C. coerulescens in their Pilifera-spore group, but the rDNA data show that it is actually closely related to species of Ceratocystis s.s. Nor do the the results support Upadhyay (1981) who placed C. fimbriata, C. adiposa, and C. moniliformis in section Ceratocystis of Ceratocystis sensu Upadhyay, but C. radicicola, C. paradoxa, C. coerulescens, and C. fagacearum into section Endoconidiophora.

Ceratocystis ips and C. pilifera fall outside of the Ceratocystis s.s. cluster at the >99% confidence level in all phylogenetic estimates which were generated (Fig. 2, node 4), thus supporting de Hoog and Scheffer's (1984) contention that only species with endoconidial anamorphs belong in Ceratocystis s.s. Their contention is also supported by the cell wall chemistry of species of Ceratocystis s.s., and the sensitivity of

these species to cycloheximide (Weijman and de Hoog 1975; Jewell 1974; Harrington 1981). However, based on the present rDNA analysis, C. autographa must be excluded from Ceratocystis s.s. De Hoog and Scheffer (1984) noted that Chalara strains that fit the description of the anamorph of C. autographa are insensitive to cycloheximide, as are the majority of strains of species of the anamorphic genus Chalara that have been tested, and this is in contrast to the Chalara anamorphs of Ceratocystis s.s. It is likely that strains currently named C. autographa are misidentified, and the disposition of this species must await the availability of strains from fresh teleomorph material. The C. autographa strain showed no affinity with either the species of Ceratocystis s.s. or C. ips and C. pilifera; the latter are Ophiostoma species (fide Harrington 1987). Instead, it clustered with an anamorphic Chalara sp. at the 100% confidence level (Fig.2, node 2).

The analysis of the partial SSrDNA data also supported the results obtained from the partial LSrDNA data, confirming that Ceratocystis is a distinct genus and should be maintained for species of Ceratocystis s.l. with Chalara anamorphs.

Cop. proteae and K. pachypleura are two species that appear to show an affinity to Ceratocystis s.s., although, based on their ecology, anamorphs, and partial rDNA sequences (Fig. 3), most Ceratocystiopsis species should be regarded as belonging to the complex of "Ophiostoma"-like, blue staining fungi (de Hoog and Scheffer 1984; Wingfield et al. 1988a). However Wingfield et al. (1988a, 1988b) have already noted that Cop. proteae is a

rather unusual Ceratocystiopsis species because of its long-necked perithecia that possess divergent ostiolar hyphae and its sensitivity to cycloheximide; cycloheximide sensitivity is found in species of Ceratocystis s.s.

Ceratocystiopsis Upad. & Kendrick (1975) was erected to accommodate those species of Ceratocystis s.l. whose ascospores appear elongate or falcate, and are surrounded by a gelatinous sheath; essentially the Minuta-spore group of Olchowecki and Reid (1974). And although for this study only four species assignable to Ceratocystiopsis were included (Fig. 3), two of them failed to cluster with Cop. minuta, or even within the Ophiostoma-complex; this clearly suggests that a reappraisal of Ceratocystiopsis is required (see Chapter 4).

Sphaeronaemella fimicola failed to cluster with either Ceratocystis s.s. or Ophiostoma; instead it grouped with Cop. falcata. Upadhyay (1981) synonymized Sphaeronaemella Karst. with Ceratocystis and assigned Sph. fimicola to the section Ophiostoma of Ceratocystis sensu Upadhyay. De Hoog and Scheffer (1984) noted that this was an unfortunate decision; they felt that Sphaeronaemella with its oblate ascospores with narrow germ slits, fleshy, pink to reddish white ascomata, and a Gabarnaudia-like anamorph (Samson 1974), should be assigned to the Hypocreaceae (von Arx 1974; Barr 1990). If it is proven that Cop. falcata is related to Pyxidiophora (de Hoog and Scheffer 1984) and Sph. fimicola related to Hypocrea Fr. then the suggestion that the Pyxidiophoraceae could be accommodated within the Hypocreales (Barr 1990), would be supported by the parsimony

analysis of the rDNA sequence data (Table 3). This data appears to show that Sph. fimicola and Cop. falcata are related despite great morphological differences. Obviously more rDNA sequences have to be collected from appropriate groups of fungi in order to clarify the position of Sph. fimicola and Pyxidiophora-like fungi.

Nannfeldt (1932), Luttrell (1951), and Benny and Kimbrough (1980) all placed the Ophiostomataceae in the class Plectomycetes (Plectoascomycetidae sensu Barr (1990)), because in members of this family the asci are deliquescent and are not organized into a single layer in the perithecial centrum. However the results reported herein show that the species of this family that were studied are more closely related to members of the Sordariales than they are to representatives of the Order Eurotiales (Plectoascomycetideae). Thus the family belongs in the Parenchymatomycetideae (Barr 1990). This would be in agreement with Berbee and Taylor (1992b) who demonstrated that, based on SSrDNA sequences, Ophiostoma ulmi (Buisman) Nannf., has its affinities within the Pyrenomycetes; the four genera they classified as Pyrenomycetes are all referrable to the Parenchymatomycetideae.

The genus Kernia Nieuwl., which includes species with thick-walled, nonostiolate ascocarps, has been accommodated within the family Microascaeae, order Microascales (Benny and Kimbrough 1980; Barr 1990). The clustering of K. pachypleura towards Ceratocystis s.s. would suggest that the inclusion of Ceratocystis within the Lasiosphaeriaceae (Sordariales) (Barr

1990) is inappropriate. Conversely the position of the Ophiostomataceae as defined by Barr (1990), Luttrell (1951), or Upadhyay (1981), which places Ophiostoma and its species within the Microascales, has to be reevaluated. Overall the sequence data indicates that Ceratocystis s.s. is best disposed in the Microascales, whereas the Ophiostomataceae with its included species should remain the sole family within the Ophiostomatales. Ordinal relationships for the ophiostomataceous fungi will be treated in more detail in Chapter 5.

CHAPTER 4

THE GENUS *Ceratocystiopsis*: A REAPPRAISAL BASED ON MOLECULAR CRITERIA

INTRODUCTION

Upadhyay and Kendrick (1975) erected Ceratocystiopsis based on the Minuta-spore group of Olchowecki and Reid (1974). This group was to be characterized by one-celled, elongate, falcate-appearing ascospores, whose sheaths are attenuated at their ends. Additionally, most species in this proposed group produce perithecia with short to conical beaks, usually with convergent, but sometimes parallel, ostiolar hyphae. Based on their ecology and anamorphs (holoblastic, sympodial, or annellidic-appearing condiogenous cells), the majority of these species appear to be related to Ophiostoma H. & P. Sydow (1919). Finally, species of both Ceratocystiopsis and Ophiostoma which had been tested were insensitive to cycloheximide (Harrington 1981).

From the beginning there were problems with the circumscription of Ceratocystiopsis. Ceratocystiopsis falcata (1981) has two-celled ascospores and two enteroblastic Chalara synanamorphs; these features render it incompatable with the Ophiostoma-complex. Also the ascospores of Cop. ochracea (Griffin) Upadh. (1981) are not falcate, and the perithecia of Cop. retusi (1981) are quite atypical. Cop. proteae Wingfield et al. (1988a) is cycloheximide sensitive, and its perithecia have relatively long necks with divergent ostiolar hyphae; it also has a

very distinctive anamorph. Other related problems led Wingfield *et al*. (1988b) and Wingfield (1990) to suggest a reduction of *Ceratocystiopsis* to synonymy with *Ophiostoma*. However, Zambino and Harrington (1992) studied isozyme variation in species of the anamorphic genus *Leptographium*, and in *Ophiostoma* species with *Leptographium* anamorphs, and found some justification for subdividing *Ophiostoma* into sections or groups based on ascospore morphology as proposed by Olchowecki and Reid (1974). This could be construed as supporting generic status for *Ceratocystiopsis* and, possibly, the segregating out of additional genera in the future.

It is questionable whether *Ceratocystiopsis* as presently circumscribed represents a monophyletic group. Therefore partial ribosomal DNA (rDNA) sequences were obtained from both the small subunit (SSrDNA) and large ribosomal subunit (LSrDNA) genes from presumed authentic strains of selected species of *Ceratocystis s.l.* species. These were analyzed in an attempt to determine the true relationships of the examined species.

MATERIALS AND METHODS

The strains employed are listed in Table 1. Of the 18 species of Ceratocystis s.l. included, ten were assigned to Ceratocystiopsis by Upadhyay (1981), and three were added by later authors (Bridges and Perry 1987; Wingfield et al. 1988a; Marmolejo and Butin 1990). Three as yet undescribed species are also included, since they are similar to species currently assigned to Ceratocystiopsis.

The nucleotide sequences were aligned with the CLUSTAL V and the MASE programs. The combined large and small subunit rDNA sequence data (approximately 950 nucleotides) were analyzed using the programs contained within PHYLIP (Felsenstein 1991).

The divergence (or distance) between two sequences was calculated by DNADIST using Kimura's two parameter model (Kimura 1980). SEQBOOT was used to generate 1000 bootstrap replicates, and the distance matrices generated by DNADIST from these bootstrap replicates were analyzed by the KITSCH program (based on the Fitch and Margoliash least-square clustering procedure, assuming a constant evolutionary clock) and a majority-rule consensus tree was produced by CONSENSE. Bootstrap analysis was performed on the data set once positions were removed which could only be ambiguously aligned. Phylogenentic trees were also generated by the FITCH program (this uses the same algorithm as KITSCH, except that an evolutionary clock is not assumed), by the

neighbor-joining method of the NEIGHBOR program, and by parsimony criteria (DNAPARS program); however the latter tree topologies were identical to those generated by KITSCH. Ribosomal DNA sequences of Saccharomyces cerevisiae Meyen ex Hansen (Rubstov et al. 1980; Georgiev, Nikolaev & Hadjiolov, 1981) and Neurospora crassa Shear & Dodge (Sogin et al. 1986b; Guadet et al. 1989) were included for comparison, with the partial rDNA sequences of Sclerotium hydrophilum serving as the outgroup.

The sensitivity of selected species towards cycloheximide was tested as previously described (Materials and Methods).

Table 1. List of fungal strains studied.

Species and Isolate No.	Source	Georgraphic oringin
Ceratocystiopsis alba (DeVay et al.) Upadh.		
CBS 797.73	Prunus amygdalus Batsch	U.S.A
Ceratocystiopsis colifera Marmolejo & Butin		
CBS 126.89	Pinus teocote Schlecht. & Cham.	Mexico
Ceratocystiopsis crassivaginata (Griffin) Upadh.		
UAMH 700H	Populus tremuloides Michx.	Canada
CBS 402.77	Pinus sp.	U.S.A.
Ceratocystiopsis falcata (Wright & Cain) Upadh.		
WIN(M) 792	Pinus radiata D. Don	New Zealand
WIN(M) 793	P. radiata	New Zealand
Ceratocystiopsis fasciata (Olchow. & Reid) Upadh.		
WIN(M) 56	Pseudotsuga menziesii (Mirb.) Franco	Canada
Ceratocystis fimbriata Ell. & Halst.		
DAOM 195303	Kerria japonica (L.) DC	Canada
Ceratocystis ips (Rumb.) C. Moreau		
WIN(M) 82-58	P. radiata	New Zealand
WIN(M) 88-141	P. radiata	New Zealand
WIN(M) 88-508	Pinus contorta Douglas ex Loud.	New Zealand
WIN(M) 92	Pinus sylvestris L.	Norway
WIN(M) 114	P. sylvestris	Norway
Ceratocystiopsis longispora (Olchow. & Reid) Upadh.		
WIN(M) 48	Pinus banksiana Lamb.	Canada
Ceratocystiopsis minima (Olchow. & Reid) Upadh.		
WIN(M) 61	P. banksiana	Canada
Ceratocystiopsis minuta (Siem.) Upadh. & Kendrick		
CBS 145.59	Unknown	U.S.A. (?)
CBS 463.77	Picea engelmannii Parry ex Engelm.	U.S.A.
Ceratocystiopsis minuta-bicolor (Davidson) Upadh. & Kendrick		
WIN(M) 479	P. contorta	Canada
WIN(M) 480	P. contorta	Canada
Ceratocystiopsis parva (Olchow, & Reid) Upadh.		

WIN(M) 59	Abies balsamea (L.) Mill.	Canada
Ceratocystiopsis proteae Wingfield, Van Wyk & Marasas		
CBS 486.88	Protea repens (L.)L.	South Africa
Ceratocystiopsis ranaculosus Bridges & Perry		
WIN(M) 919	Pinus taeda L.	U.S.A.
Ceratocystiopsis retusi (Davids. & Hinds) Upadh.		
ATCC 22324	Populus sp.	U.S.A.
C.tax.sp.1		
WIN(M) 855	Trypodendron lineatum (Olivier)	U.S.A.
C.tax.sp.2		
WIN(M) 110	P. sylvestris	Norway
WIN(M) 113	P. sylvestris	Norway
C.tax.sp.3		
WIN(M) 237	Pinus resinosa Ait.	Canada
Sclerotium hydrophilum Sacc.		
WIN(M) 917	Zizania aquatica L.	Canada

ATCC, American Type Culture Collection, Rockville, Maryland, USA;
CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands;
DAOM, Biosystematics Research Institute, Ottawa, Canada;
UAMH, University of Alberta Microfungus Collection, Edmonton, Canada;
WIN(M), The culture collection of J. Reid, University of Manitoba, Department of Botany, Winnipeg, Manitoba, Canada.

RESULTS AND DISCUSSION

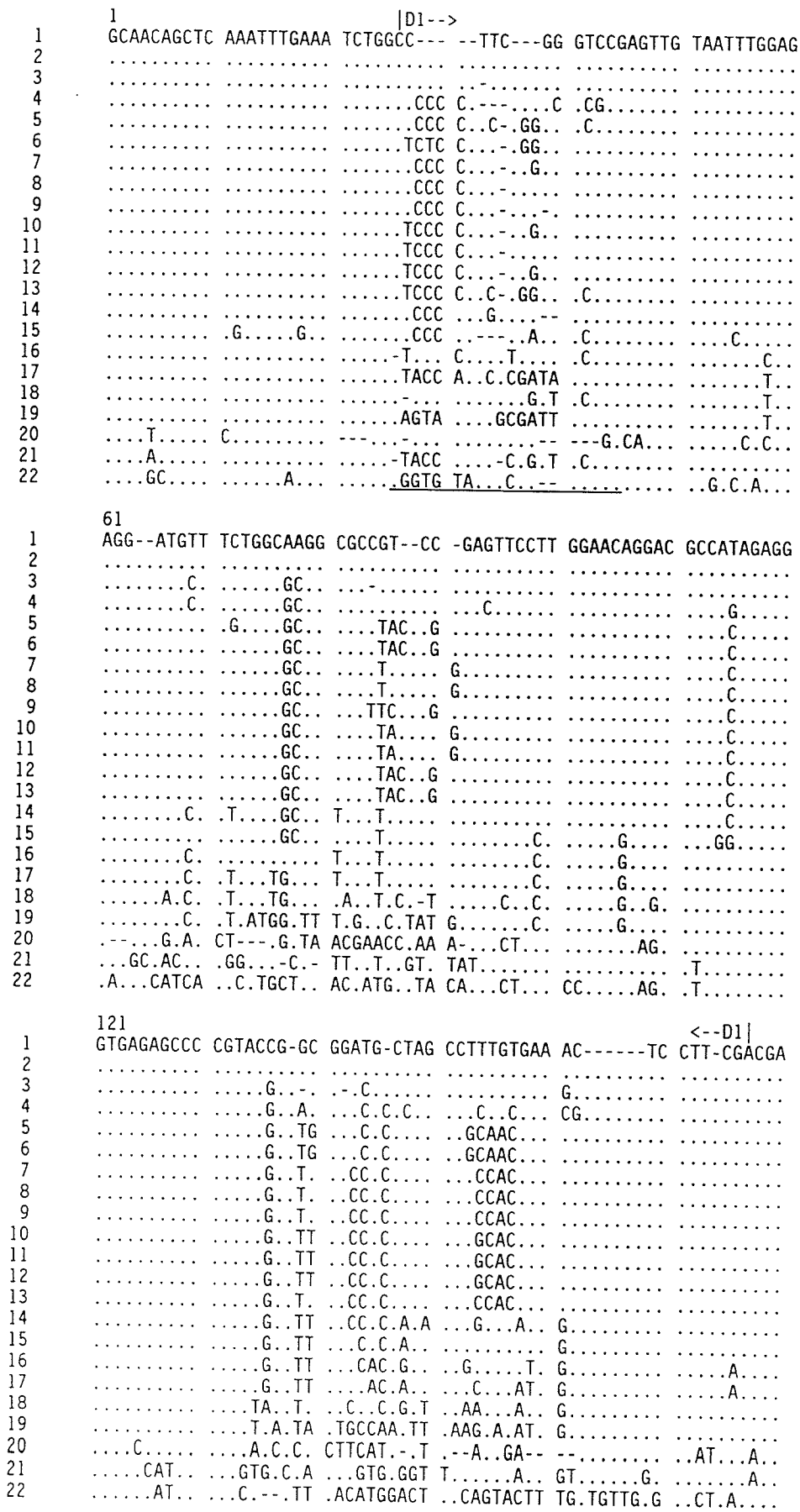
Sequencing the four specified regions of the rDNA allowed the determination of 685 nucleotides from the SSrDNA gene, and 250 from the LSrDNA gene for all species. Following Kurtzman's example, partial rDNA sequence data from both the SSrDNA and LSrDNA genes were combined to estimate the phylogenies (Gueho *et al*. 1990; Kurtzman 1992). Although the SSrDNA and LSrDNA data were also analyzed individually, the topologies of the resulting phylogenetic trees were in agreement with the phyletic estimates obtained from analyzing the combined data set.

Gaps were introduced into the sequences to facilitate alignment (Fig. 1a and 1b), and from these a majority-rule consensus tree (Fig. 2) was created based on 1000 bootstrap replicates; in this tree three major nodes are supported by >99% confidence in bootstrap analysis. As levels >95% indicate strong statistical support for branches, they suggest a high degree of confidence for putatively monophyletic groups (Felsenstein 1985).

Of the 19 *Ceratocystis s.l.* strains studied, 15 were clustered in a potentially monophyletic group (Fig. 2, node 2) by 100% of the bootstrap replicates. These 15, one of which is a strain of *C. ips* (=*Ophiostoma ips* (Rumb.)Nannf., in Melin and Nannfeldt 1934), could be considered part of an *Ophiostoma*-complex; Wingfield *et al*. (1988b) and Wingfield

Fig. 1. (a) Alignments of the 5' terminal end of the LSrDNA. Nucleotide postions 1-267 within the alignment correspond to postions 102-360 in the S. cerevisiae LSrDNA gene (Guadet et al. 1989). Positions which are underlined were excluded for generating phylogenetic estimates as it was difficult to obtain reliable alignments.

Fig. 1. (b) Alignments for the V3, V4 (positions 402-886) and V9 (positions 1546-1748) containing segments of the SSrDNA gene. Nucleotide positions correspond to the nucleotide numbering of the SSrDNA of S. cerevisiae (Rubstov et al. 1980). 1 = Cop. retusi; 2 = C.tax.sp.1; 3 = C. ips; 4 = Cop. fasciata; 5 = Cop. minuta-bicolor; 6 = Cop. = minuta CBS 463. 77; 7 = Cop. parva; 8 = Cop. minuta CBS 145.59; 9 = Cop. minima; 10 = Cop. colifera; 11 = Cop. ranaculosus; 12 = C.tax.sp.3; 13 = C.tax.sp.3; 14 = Cop. longispora; 15 = Cop. crassivaginata; 16 = Cop. proteae; 17 = C. fimbriata; 18 = N. crassa; 19 = Cop. alba; 20 = Cop. falcata; 21 = S. cerevisiae; 22 = S. hydrophilum.



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          181
 1        GTCGAGTAGT TTGGGAATGC TGCTCAAAAT GGGAGGTAAA TTTCTTCTAA AGCTAAA-TA
 2        .......... .......... .......... .......... .......... .........
 3        .......... .......... .......... .......... .......... .........
 4        .......... .......... .......... .......... .......... .........
 5        .......... .......... .......... .......... .......... .........
 6        .......... .......... .......... .......... .......... .........
 7        .......... .......... .......... .......... .......... .........
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 9        .......... .......... .......... .......... .......... .........
10        .......... .......... .......... .......... .......... .........
11        .......... .......... .......... .......... .......... .........
12        .......... .......... .......... .......... .......... .........
13        .......... .......... .......... .......... .......... .........
14        .......... .......... .......... .......... .......... .........
15        .......... .......... .......... .......... .......... .........
16        .......... .......... .....T.... ........T. .C........ .........
17        .......... .......... .....T.... ........T. .C........ .........
18        .......... .......... .......... .......... .G........ .........
19        .......... .......... .......... .......... .......... .........
20        .......... .......... .....T..-. .......... .CC..C.... .........
21        .......T.. .......... A....T..G. ...T...... ..C.A..... .........
22        .......T.. .......... A......... ...T...... ..C.A..... .......A..

          241
 1        TTGGCCAGAG ACCGATAGCG CACAA (Ceratocystiopsis retusi)
 2        .......... .......... ..... (C.tax.sp.1)
 3        CC........ .......... ..... (Ceratocystis ips)
 4        CC........ .......... ..... (Ceratocystiopsis fasciata)
 5        CC........ .......... ..... (Ceratocystiopsis minuta-bicolor)
 6        CC........ .......... ..... (Ceratocystiopsis minuta CBS 463.77)
 7        C......... .......... ..... (Ceratocystiopsis parva)
 8        C......... .......... ..... (Ceratocystiopsis minuta CBS 145.59)
 9        C......... .......... ..... (Ceratocystiopsis minima)
10        CC........ .......... ..... (Ceratocystiopsis colifera)
11        CC........ .......... ..... (Ceratocystiopsis ranaculosus)
12        CC........ .......... ..... (C.tax.sp.3)
13        CC........ .......... ..... (C.tax.sp.2)
14        .......... .......... ..... (Ceratocystiopsis longispora)
15        CC........ .......... ..... (Ceratocystiopsis crassivaginata)
16        CC........ .......... ..... (Ceratocystiopsis proteae)
17        .A...T.... .......... ..... (Ceratocystis fimbriata)
18        .......... .......... ..... (Neurospora crassa)
19        .......... .......... ..... (Ceratocystiopsis alba)
20        C.T....... -......... ..... (Ceratocystiopsis falcata)
21        .....G.... .......... A.... (Saccharomyces cerevisiae)
22        .....G.... .......... A.... (Sclerotium hydrophilum)
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          402
 1        GGCTACTACA TCCAAGGAAG GCAGCAGGCG CGCAAATTAC CCAATCCCGA CGCGGGGAGG
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... .T........
 4        .......... .......... .......... .......... .......... .A........
 5        .......... .......... .......... .......... .......... ..........
 6        .......... .......... .......... .......... .......... ..........
 7        .......... .......... .......... .......... .......... ..........
 8        .......... .......... .......... .......... .......... ..........
 9        .......... .......... .......... .......... .......... ..........
10        .......... .......... .......... .......... .......... ..........
11        .......... .......... .......... .......... .......... ..........
12        .......... .......... .......... .......... .......... ..........
13        .......... .......... .......... .......... .......... ..........
14        .......... .......... .......... .......... .......... .A........
15        .......... .......... .......... .......... .......... .A........
16        .......... ..T....... .......... .......... .......... ..........
17        ......C..T .TT....... .......... .......... .......... .A........
18        .......... .......... .......... .......... .......... .A........
19        .......... .......... .......... .......... .......... .A........
20        .......... ..T....... .......... .......... .......C. ..-...-...
21        ......C... .......... .......... .......... .......TA. TT.A......
22        ......C... .......... .......... .......... .......... .A........

          462
 1        TAGTGACAAT AAATACTGAT ACAGGGCTCT TTTGGGTCTT GTAATTGGAA TGAGTACAAT
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... .......... ..........
 5        .......... .......... .......... .......... .......... ..........
 6        .......... .......... .......... .......... .......... ..........
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 9        .......... .......... .......... .......... .......... ..........
10        .......... .......... .......... .......... .......... ..........
11        .......... .......... .......... .......... .......... ..........
12        .......... .......... .......... .......... .......... ..........
13        .......... .......... .......... .......... .......... ..........
14        .......... .......... .......C.. .......... .......... .........A
15        .......... .......... .......... .......... .......... ..........
16        .......... .......... .......... .......... .....C.... ..........
17        .......... .......... .......... .A........ .....C.... ..........
18        .......... .......... .......... .......... .......... ..........
19        .......... .......... .......C.. .A........ .......... ....A.....
20        .......... .......... .......... .......... .....C.... ..........
21        .......... .....AC... .......C.A ..C....... .......... ..........
22        .......... .....ACA.. .T.....G.C .......... .......... ..........

          522
 1        TTAATT-CCC TTAACGAGGA ACAATTGGAG GGCAAGTCTG GTGCCAGCAG CCGCGGTAAT
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... .......... ..........
 5        .......... .......... .......... .......... .......... ..........
 6        .......... .......... .......... .......... .......... ..........
 7        .......... .......... .......... .......... .......... ..........
 8        .......... .......... .......... .......... .......... ..........
 9        .......... .......... .......... .......... .......... ..........
10        .......... .......... .......... .......... .......... ..........
11        .......... .......... .......... .......... .......... ..........
12        .......... .......... .......... .......... .......... ..........
13        .......... .......... .......... .......... .......... ..........
14        ......T... .......... .......... .......... .......... ..........
15        .......... .......... .......... .......... .......... ..........
16        ....A..... .......... ..C....... .......... .......... ..........
17        ....A..... .......... .......... .......... .......... ..........
18        ....A..... .......... .......... .......... .......... ..........
19        ....A..... .......... .......... .......... .......... ..........
20        ....A..... .......... ..C.G..... .......... .......... ..........
21        G...A..A.. .......... .......... .......... .......... ..........
22        ....A..... .......... T......... .......... .......... ..........
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          581
 1        TCCAGCTCCA ATAGCGTATA TTAAAGTTGT TGCAGTTAAA AAGCTCGTAG TTGAACCTTG
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... .......... ..........
 5        .......... .......... .......... .......... .......... ..........
 6        .......... .......... .......... .......... .......... ..........
 7        .......... .......... .......... .......... .......... ..........
 8        .......... .......... .......... .......... .......... ..........
 9        .......... .......... .......... .......... .......... ..........
10        .......... .......... .......... .......... .......... ..........
11        .......... .......... .......... .......... .......... ..........
12        .......... .......... .......... .......... .......... ..........
13        .......... .......... .......... .......... .......... ..........
14        .......... .......... .......... .......... .......... ..........
15        .......... .......... .......... .......... .......... ..........
16        .......... .......... .......... ..TG...... .......... .C........
17        .......... .......... .......... ..TG...... .......... ..........
18        .......... .......... .......... ..AG...... .......... ..........
19        .......... .......... .......... .......... .......... ..........
20        A......... C......... .......... ..TG...... .......... ........A.
21        .......... .......... .......... .......... .......... ......T...
22        .......... .......... .......... .......... .......... .........C

          641
 1        -GGCCTGGCT GGC--CGGTC CGCCTCACCG CGTGCACTGG TCCGGCC--- GGGTCTTTCC
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... .......... ...C......
 5        .......... .......... .......... .......... .......... ...C......
 6        .......... .......... .......... .......... .......... ...C......
 7        .......... .......... .......... .......... .......... ...C......
 8        .......... .......... .......... .......... .......... ...C......
 9        .......... .......... .......... .......... .......... ...C......
10        .......... .....-.... .......... .......... .......... ...C......
11        .......... .......... .......... .......... .......... ...C......
12        .......... .....-.... .......... .......... .......... ...C......
13        .......... .......... .......... .......... .......... ...C......
14        .......... .......... .......... .......... .......... ...C......
15        .......... .......... .......... .......... .......... ...C......
16        .......... .......... .C........ G......... .......... ...C......
17        .......... .......... .C........ G......... .T.C.G.C.. ..........
18        ....TC...- C.T....... .......... .........A CTG..T.... ...C....TT
19        .......... ..T....... .......... .........A .......... ..........
20        .T.GTCTA.. .T.TT.T..- --........ -A..G.AG-A .-T..T-... ...C.-----
21        .....C..T. .......... ..AT.TTTT- ....T..... ATTTC.AACG ...C......
22        A.......T. ..-.G..... T.......G. TA..T...-. ....A..... .......A..

          697
 1        CTCTGGGGAG CCGC-ATGCC CTTCACT-GG GCGTGTCGGG GAACCAGGAC ATTTACTTTG
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .....C.... .......... G.........
 5        .......... .......... .......... .......... .......... T.........
 6        .......... .......... .......... .......... .......... T.........
 7        .......... .......... .......... .......... .......... G.........
 8        .......... .......... .......... .......... .......... G.........
 9        .......... .......... .......... .......... .......... T.........
10        .......... .......... ...TG..... .A........ .......... T.........
11        .......... .......... ...T...... .......... .......... T.........
12        .......... ........T. .......... .......... .......... T.........
13        .......... .......... .......... .......... .......... T.........
14        .......... .......... .......... .T........ .......... G.........
15        .......... .......... ...T...... .......... .......... T.........
16        .....T...A .T........ ...T...... .......-A.. ...A...... T.........
17        .....T...A .......... .......... .T........ ...A...... T.........
18        TC....A..A .......... .......... .T........ .......... T.....CG..
19        .........A ..T.....T. ...T.T.... ......T... .......... T.........
20        AC.--ACCG. T..TG....- G.A-..G.-- ----.CA... ---...C.-- ----------
21        T.....CT.A ..TT.GA.T. ...-----GT. ..-.C.T..C .......... T.........
22        TCT...T... ...GC....T ...A...... .T........ .......... T.........
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          751
 1        AAAAAATTAG AGTGTTCAAA GCAGGCTTAT -GCTCGAAT- ACATTAGCAT GGAATAATAG
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... ......G... .......... ..........
 5        .......... .......... .......... .......... .......... ..........
 6        .......... .......... .......... .......... .......... ..........
 7        .......... .......... .......... ......G... .......... ..........
 8        .......... .......... .......... ......G... .......... ..........
 9        .......... .......... .......... ......G... .......... ..........
10        .......... .......... .......... .......... .......... ..........
11        .......... .......... .......... .......... .......... ..........
12        .......... .......... .......... .......... .......... ..........
13        .......... .......... .......... .......... .......... ..........
14        .......... .......... ........T. .......... .......... ..........
15        .......... .......... .......... .......... .......... ..........
16        .......... ....C..C.G ......A... .......... ..T.C..... ..........
17        .......... ....C..C.G ......C... .........C GT.C-..... ..........
18        ..C....C.. .TC.C.-... .A....C... .........A GT.C..... ..........
19        .......... .......... ......C... .......... .......... ..........
20        .......... ....C..C.G .......... .......... .GT.C..... .........A
21        .......... .......... ......G... T......... .T........ ..........
22        .G........ .......... ......C... ...C...... .......... .........A

          810
 1        AATAGGACGC G-TGGTTCTA TTTTGTTGGT TTCTAGGACC GCCGTAATGA TTAATAGGGA
 2        .......... .......... .......... .......... .......... ..........
 3        .........T .......... .......... .......... .......... ..........
 4        .........T .......... .......... .......... .......... ..........
 5        .........T .......... .......... .......... .......... ..........
 6        .........T .......... .......... .......... .......... ..........
 7        .........T .......... .......... .......... .......... ..........
 8        .........T .......... .......... .......... .......... ..........
 9        .........T .......... .......... .......... .......... ..........
10        .........T .......... .......... .......... .......... ..........
11        .........T .......... .......... .......... .......... ..........
12        .........T .......... .......... .......... .......... ..........
13        .........T .......... .......... .......... .......... ..........
14        .........T .......... .......... .......... .......... ..........
15        .........T .......... .......... .......... .......... ..........
16        .........T .......... .......... .......... .......... ..........
17        .........T .......... .......... .......... .......... ..........
18        .........T .......... .......... .......... .......... ..........
19        .........T .......... .......... .......... .......... ..........
20        .........T ..G....... .......... .......... AG........ .G........
21        .........T T......... .......... .......... AT........ ..........
22        .........T ..C....... .......... ......AGT. .......... ..........

          868                                1546
 1        CAGTCGGGGG CATCAGT..........TAT TGCTCTTCAA CGAGGAATCC CTAGTAAGCG
 2        .......... .......          ... .......... .......... ..........
 3        .......... .......          ... .......... .......... ..........
 4        .......... .......          ... .......... .......... ..........
 5        .......... .......          ... .......... .......... ..........
 6        .......... .......          ... .......... .......... ..........
 7        .......... .......          ... .......... .......... ..........
 8        .......... .......          ... .......... .......... ..........
 9        .......... .......          ... .......... .......... ..........
10        .......... .......          ... .......... .......... ..........
11        .......... .......          ... .......... .......... ..........
12        .......... .......          ... .......... .......... ..........
13        .......... .......          ... .......... .......... ..........
14        .......... .......          ... .......... .......... ..........
15        .......... .......          ... .......... .......... ..........
16        .......... .......          ... .......... .......... ..........
17        .......... .......          ... .......... .......... ..........
18        .......... .......          ... .......... .......... ..........
19        .......... .......          ... ..G....... .......... ..........
20        .......... ....C..          .G. .......... ........T. ..........
21        .G........ ....G..          ... .......... ........T. ..........
22        T...T..... ...T...          ... .......... ........T. ..........
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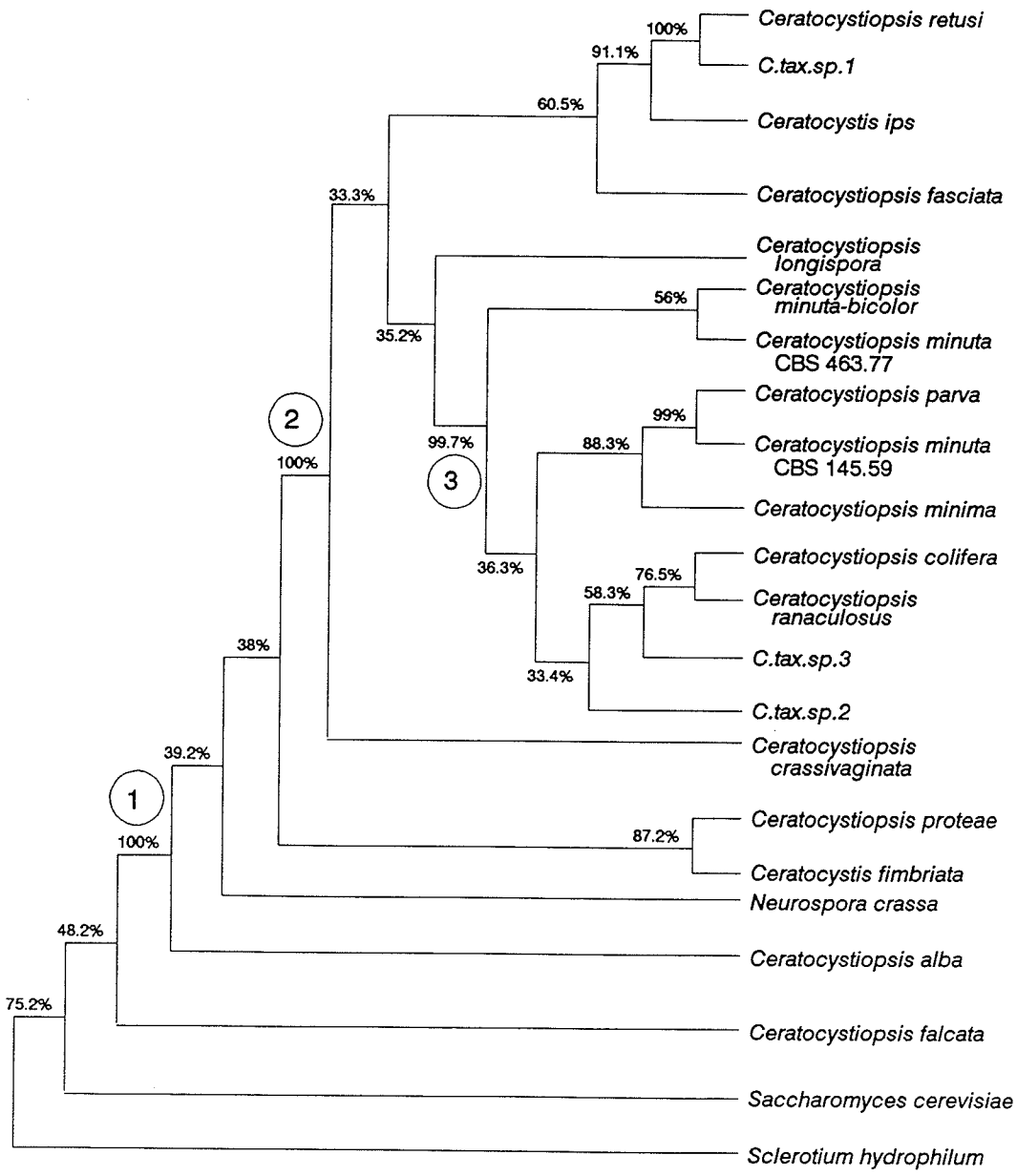
          1579
 1        CAAGTCATCA ACTTGCGCTG ATTACGTCCC -TGCCCTTTG TACACACCGC CCGTCGCTAC
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... .......... ..........
 5        .......... .......T.. .......... .......... .......... ..........
 6        .......... .......T.. .......... .......... .......... ..........
 7        .......... .......T.. .......... .......... .......... ..........
 8        .......... .......T.. .......... .......... .......... ..........
 9        .......... .......T.. .......... .......... .......... ..........
10        .......... .......T.. .......... .......... .......... ..........
11        .......... .......T.. .......... .......... .......... ..........
12        .......... .......T.. .......... .......... .......... ..........
13        .......... .......T.. .......... .......... .......... ..........
14        .......... .......T.. .......... .......... .......... ..........
15        .......... .......T.. .......... .......... .......... ..........
16        .......... G......T.. .......... .......... .......... ..........
17        .......... G......T.. .......... .......... .......... ..........
18        .......... G......T.. .......... .......... .......... ..........
19        .......... .......T.. .......... .......... .......... ..........
20        .......... G......T.. .......... .......... .......... ..........
21        .......... G......T.. .......... .......... .......... .........G
22        TG.......- G..C...T.. .......... C......... .......... ..........

          1638
 1        TACCGATTGA ATGGCTCAGT GAGGCTTCCG GACTGGCCCA GAGGGGTGGG -CAACTACCC
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... .......... ..........
 5        .......... .......... .......... .......... .GA....... .....C....
 6        .......... .......... .......... .......... .GA....... .....C....
 7        .......... .......... .......... .......... .GA....... .....C....
 8        .......... .......... .......... .......... .GA....... .....C....
 9        .......... .......... .......... .......... .GA....... .....C....
10        .......... .......... .......... .......... .GA....... .....C....
11        .......... .......... .......... .......... .GA....... .....C....
12        .......... .......... .......... .......... .GA....... ..........
13        .......... .......... .......... .......... .GA....... .....C....
14        .......... .......... .......... .......... .GA....... ..........
15        .......... .......... .......... .......... .G....C... .....CG...
16        .........G .......... ...A.C.... ........TG ...A..C... .A....G..A
17        .........G .......... ...A.C.... ........G. ...A...... .A........
18        .......... .......... .......... .......... .G.A...C.. .....C...A
19        .......... .......... .......... .......T.T .CATA..... .........TG
20        .......... .......... .....CAT.. ........-T TTTTA.C... ......G.T-
21        .......... ......T... .....C..A. ..TCT..TT. ...AA.G... ......C.AT
22        ....-..... ......T... ....TC.T.. ..T....TTT TG..A.CC.. .....GG.A.

          1697
 1        TCCCGGGCCG GAAAGCTATC CAAACTCGGT CATTTAGAGG AAGTAAAAGT
 2        .T........ .......... .......... .......... ..........
 3        .T........ .......... .......... .......... ..........
 4        .T........ .....T.... .......... .......... ..........
 5        C......... .....T.... .......... .......... ..........
 6        C......... .......... .......... .......... ..........
 7        C......... .....T.... .......... .......... ..........
 8        C......... .....T.... .......... .......... ..........
 9        C......... .....T.... .......... .......... ..........
10        C......... .....T.... .......... .......... ..........
11        C......... .....T.... .......... .......... ..........
12        C......... .....T.... .......... .......... ..........
13        C......... .....T.... .......... .......... ..........
14        C......... .....T.... .......... .......... ..........
15        C......... .G...T.... .......... .......... ..........
16        CT........ .......G.T .......... ....C..... ..........
17        CT.AT..... .......G.T .......... ....C..... ..........
18        C..A...... .......... .......... .......... ..........
19        CG.G.A.... .......... .......... .......... ..........
20        GAGAA..... .....T.G.. .......... .......... ..........
21        CT.A.A..G. AG..TT.GGA ......T... .....G.... ..C.......
22        ..GATT..T. AG...T.GAT ......T... .......... ..........
```

Fig. 2. Majority-rule consensus tree showing the phyletic relationships between Ceratocystiopsis spp. and other members of Ceratocystis s.l. The tree was constructed using the bootstrap procedure (SEQBOOT) in PHYLIP (Felsenstein, 1991) and the DNA distance matrix method contained in the KITSCH program; the percentages are the frequences with which a given branch appeared in 1000 bootstrap replications.


Ceratocystiopsis retusi
C.tax.sp.1
Ceratocystis ips
Ceratocystiopsis fasciata
Ceratocystiopsis longispora
Ceratocystiopsis minuta-bicolor
Ceratocystiopsis minuta CBS 463.77
Ceratocystiopsis parva
Ceratocystiopsis minuta CBS 145.59
Ceratocystiopsis minima
Ceratocystiopsis colifera
Ceratocystiopsis ranaculosus
C.tax.sp.3
C.tax.sp.2
Ceratocystiopsis crassivaginata
Ceratocystiopsis proteae
Ceratocystis fimbriata
Neurospora crassa
Ceratocystiopsis alba
Ceratocystiopsis falcata
Saccharomyces cerevisiae
Sclerotium hydrophilum
100%
91.1%
60.5%
33.3%
56%
35.2%
99.7%
99%
88.3%
36.3%
76.5%
58.3%
33.4%
2
100%
3
38%
39.2%
1
100%
87.2%
48.2%
75.2%

(1990) have already suggested that Ceratocystiopsis should be synonymized with Ophiostoma. However, C. fimbriata did not cluster within this complex, thus supporting placement of Ophiostoma species with Chalara anamorphs in Ceratocystis sensu stricto (von Arx 1974; de Hoog and Scheffer 1984; von Arx and van der Walt 1987).

Cop. proteae, Cop. alba, and Cop. falcata also did not cluster in the Ophiostoma-complex. This suggests that falcate ascospores have evolved more than once in fungi, and that ascospore morphology alone should not be used to define genera in the Ophiostomataceae. The analysis placed Cop. proteae with Ceratocystis s.s. (represented by C. fimbriata), although the bootstrap confidence level is only 87.2%. Cop. falcata, with two distinct Chalara synanamorphs (Hutchison and Reid 1988) and two-celled ascospores, has always been an anomaly in Ceratocystiopsis. The molecular data clearly indicates that it is not related to either the Ophiostoma-complex, nor to Ceratocystis s.s. To assign it to Ceratocystis s.s. (Wingfield et al. 1988b) would be unfortunate, for then that generic circumscription would have to be emended to accommodate both 2-celled and falcate-ascospore forms.

The exclusion of C. alba from Ceratocystiopsis by molecular data (Fig. 2, node 1) is surprising. The only unusual morphological feature reported for this species is colour. Both the perithecial base and neck are hyaline to cream-coloured or pale-yellow, while in most species of

Ceratocystis s.l. the ascocarps are dark brown to black.

The remaining 14 strains of Ceratocytiopsis, plus C. ips, fall within the Ophiostoma-complex as delimited by the rDNA sequence data (Fig. 2, node 2). Olchowecki and Reid (1974) considered C. ips the central species of their Ips-spore group of Ceratocystis s.l.; this group was characterized by species which produce ascospores that are rectangular, ossiform, or pillow-shaped in plan and side view, but never curved. Cop. retusi and C.tax.sp.1 clustered with C. ips within the Ophiostoma-complex in 91% of the bootstrap replicates, and while this is not quite high enough to support an unambiguous grouping of these three species, it again suggests that ascospore morphology alone is insufficient to separate species of Ceratocystis s.l. into sections or genera.

Cop. retusi and C.tax.sp.1 differ from more "typical" species of Ceratocystiopsis in that both have perithecial necks and bases which are pale to ochraceous in colour. Also the ostiolar hyphae of Cop. retusi may be septate, and they are long, flexuous, numerous and divergent, thus giving the ostiolar region a shaggy appearance. In C.tax.sp.1, the ostiolar hyphae are not nearly as long as those of Cop. retusi (up to 42 um vs 80 to 180 um), but they are very abundant, divergent, and may also be septate. Finally, in both species the anamorph appears Allescheriella-like. All of the foregoing factors support the close clustering of Cop. retusi and C.tax.sp.1.

Within the Ophiostoma-complex, nine strains clustered in 99.7% of the bootstrap replicates (Fig. 2, node 3), and these may represent a monophyletic group of species. Comprised of strains of Cop. colifera, Cop. minima, Cop. minuta, Cop. minuta-bicolor, Cop. parva, Cop. ranaculosus, C.tax.sp.2, and C.tax.sp.3, the group can be circumscribed by combining the following criteria: (1) all these species produce elongate ascospores surrounded by gelatinous sheaths, but not all produce falcate ascospores (i.e. Cop. ranaculosus and Cop. colifera); (2) all produce perithecia with very short, tapered perithecial necks except Cop. minuta-bicolor; (3) all their perithecia develop convergent ostiolar hyphae except those of Cop. ranaculosus which lack ostiolar hyphae; (4) all except Cop. ranaculosus are reported to produce a Hyalorhinocladiella-like anamorph or synanamorph; and (5) all are within 0.013 evolutionary distance units (edu, as derived from Kimura's two parameter model) from Cop. minuta, strain CBS 463.77 (Fig. 2). Based on DNA relatedness (evolutionary distances, Fig. 3) of the species tested towards Cop. minuta (CBS 463.77), the inclusion of Cop. retusi (edu = 0.0287), Cop. fasciata (edu = 0.0332), Cop. longispora (edu = 0.0320), and Cop. crassivaginata (edu = 0.0308) within Ceratocystiopsis would also require C. ips (edu = 0.0241) to be transferred to this genus.

Cop. ranaculosus, which produces a Sporothrix anamorph (Bridges and Perry 1987), and Cop. colifera which produces

both Sporothrix and Hyalorhinocladiella-like synanamorphs (Marmolejo and Butin 1990), are clustered together in this analysis by 76% of the bootstrap replicates. These species can also be distinguished from the other Minuta-like species by their adorned perithecia. In Cop. colifera, there is a "collar-like" structure surrounding the upper part of the ascocarp (Marmolejo and Butin 1990), while in Cop. ranaculosus there is a "corona" of globose cells in an approximately corresponding position (Bridges and Perry 1987). Also, though both of these species produce elongate ascospores, in neither species are the ascospores truly falcate.

The rDNA sequence data suggests that Cop. minima and Cop. parva are discrete entities; therefore Upadhyay's (1981) decision to synonymize Cop. parva with Cop. minima is not supported. The data also suggests that there may be a problem with Cop. minuta. The two strains we tested were from different sources, and they exhibited enough sequence diversity to suggest that they represent two different species. This may indicate that an initial misidentification occurred, but because neither produced perithecia in culture, their identity could not be verified. Two morphologically similar species may exist that are being treated under Cop. minuta, a situation similar to that of Cop. minima/Cop. parva (above). However, it should be noted that for the partial rDNA sequences we determined, Cop. minuta (CBS 145.59) differs from Cop. parva by only a single

substitution, and this was found in the fastest evolving segement of the D1 region of the LSrDNA gene. Thus this presumptive Cop. minuta strain may well be a derivative of Cop. parva.

This study failed to resolve the relationships of Cop. crassivaginata, Cop. fasciata, and Cop. longispora within the Ophiostoma-complex. Cop. crassivaginata, unique among Ceratocystiopsis spp. in possessing a Leptographium anamorph, was transferred to Ophiostoma by Harrington (1987). However, isozyme characterizations of a number of Leptographium species, including some with Ophiostoma teleomorphs, failed to show a close affinity between Cop. crassivaginata and the Ophiostoma spp. tested by Zambino and Harrington (1992). The results herein clearly show that it belongs in the Ophiostoma-complex, supporting de Hoog and Scheffer (1984) and Harrington (1987, 1988), but if there is now support for subdividing Ophiostoma into section/groups based on ascospore morphologies (Zambino and Harrington 1992), as proposed by Olchowecki and Reid (1974), Cop. crassivaginata would not fit into any of the current sections/groups when all criteria currently available are considered.

The data do show that although Cop. longispora clearly lies within the Ophiostoma-complex, it is not of the core Minuta-group. It is also separated from this group by possessing a Sporothrix-like anamorph which produces long, fusiform conidia.

Significant morphological criteria which might explain why our rDNA data separates Cop. fasciata from the Minuta-group, as defined herein, are lacking. However, the perithecia of Cop. fasciata do have longer necks than all other species of the Minuta-group except Cop. minuta-bicolor, and it has been the only species of Ceratocystiopsis sensu Upadhyay (1981) reported to produce mycelial endoconidia (Olchowecki and Reid 1974).

At the concentration of cycloheximide tested, the results (Table 2) were similar to those of Fergus (1956) in that four categories of response could be recognized: totally sensitive strains; totally insensitive strains; relatively sensitive strains (area growth of colonies reduced by more than 50% of controls); and relatively insensitive strains (area growth of colonies reduced by less than 50% of controls). However, in this study these four categories are only treated as two; insensitive (I, growth reduction less than 50%) and sensitive (S, growth reduction more than 50%).

The nine Ceratocystiopsis strains that clustered those species which represent a potentially monophyletic group (Fig. 2, node 3) were all cycloheximide sensitive. Conversely, all tested strains of Cop. crassivaginata, Cop. retusi, C.tax.sp.1, Cop. fasciata and C. ips were insensitive; however all 14 of these strains are clustered in the Ophiostoma-complex (Fig. 2, node 2). Thus the sensitivity tests do tend to confirm the possible monophyly

of that group of Ceratocystiopsis spp. centered on Cop. minuta, and they also corroborate with the more distant relationship of this Minuta-group to Cop. crassivaginata and the other insensitive species of the Ophiostoma-group. However the results of these sensitivity tests are at variance with Harrington's (1981) belief that cycloheximide insensitivity might be a characteristic of all true Ophiostoma spp., for if members of the Cop. minuta cluster do belong in Ophiostoma, their cycloheximide sensitivity would be an exception to the rule.

Another problem is that Harrington (1981) reported that the strain of Cop. minuta he tested was cycloheximide insensitive, and at this time this discrepancy cannot be rationalized. However, this study did show that Cop. crassivaginata and Cop. retusi were insensitive, as did Harrington, although the growth of Cop. retusi was slightly reduced by the antibiotic. Similarly, Fergus (1956) reported that his tested strain of Endoconidiophora fimbriata (presumable C. fimbriata) was insensitive to cycloheximide, while the results herein and those of Harrington agree that C. fimbriata is fully sensitive.

Clearly, the use of cycloheximide sensitivity/insensitivity as a taxonomic character must be restricted to cases where the identity of the tested strains is beyond doubt. Thus there is no hesitation in stating that the cycloheximide insensitivity of Cop. falcata would support its molecular separation from Cop. fimbriata;

Wingfield (1990) had suggested that C. falcata more properly belongs in Ceratocystis s.s.

Partial rDNA sequence data suggest that the production of falcate-shaped ascospores is an example of convergent evolution, and that their formation by a group of species should not be used as an indication of relatedness nor to define a genus. It has already been demonstrated (see chapter 3) that C. ips, C. pilifera (= O. piliferum, the type species of Ophiostoma) and C. piceae, along with Cop. minuta and Cop. ranaculosus, can be accommodated in a monophyletic group. And as the present study indicates that other members of Ceratocystiopsis form a monophyletic group that includes C. ips, the data therefore indicates that most of the Ceratocystiopsis species studied, including the type species, should be assigned to Ophiostoma H. & P. Syd. However, only eight of them, including Cop. minuta, would constitute a closely-knit, commonly derived group of species within Ophiostoma.

Therefore, until additional contradictory evidence is presented, those Ceratocystiopsis spp. defined by the data as members of an Ophiostoma-group (Fig. 2, node 2) will be treated as members of Ophiostoma H. & P. Sydow, and Ceratocystiopsis Upadh. & Kendrick (1975) will be reduced to synonymy with Ophiostoma. The necessary new combinations in Ophiostoma to be proposed are as follows:

Ophiostoma coliferum (Marmolejo & Butin) comb. nov.

=Ceratocystiopsis colifera Marmolejo & Butin,

Sydowia **42**, 197-198. 1990. (Basionym)

Ophiostoma fasciata (Olchow. & Reid) comb. nov.
=Ceratocystis fasciata Olchow. & Reid, Can. J. Bot. **52**, 1682. 1974. (Basionym)

Ophiostoma longisporum (Olchow. & Reid) comb. nov.
=Ceratocystis longispora Olchow. & Reid, Can. J. Bot. **52**, 1683. 1974. (Basionym)

Ophiostoma minimum (Olchow. & Reid) comb. nov.
=Ceratocystis minima Olchow. & Reid, Can. J. Bot. **52**, 1684. 1974. (Basionym)

Ophiostoma minuta-bicolor (Davids.) comb. nov.
=Ceratocystis minuta-bicolor Davids., Mycopath. Mycol. appl. **28**, 280-282. 1966. (Basionym)

Ophiostoma parvum (Olchow. & Reid) comb. nov.
=Ceratocystis parva Olchow. & Reid, Can. J. Bot. **52**, 1686. 1974. (Basionym)

Ophiostoma ranaculosum (Bridges & Perry) comb. nov.
=Ceratocystiopsis ranaculosus Bridges & Perry, Mycologia **79**, 631. 1987. (Basionym)

Ophiostoma retusum (Davids. & Hinds) comb. nov.
=Ceratocystis retusum Davids. & Hinds., Mycology **64**, 407. 1972. (Basionym)

This study again indicates that Cop. falcata is only very distantly related to members of Ceratocystis s.l., and that its placement must await additional molecular data; certainly a relationship to Pyxidiophora might be considered. Cop. proteae, on the other hand, may well be

derived from the same lineage as Ceratocystis s.s.

Fig. 3. DNA relatedness based on evolutionary distances as determined by the DNADIST program, using Kimura's two parameter model, between Cop. minuta (CBS 463.77, indicated by central dot) and members of Ceratocystis s.l. The length of the lines connecting with Cop. minuta are proportional to the evolutionary distances between the pair of species based on rDNA sequence divergence.

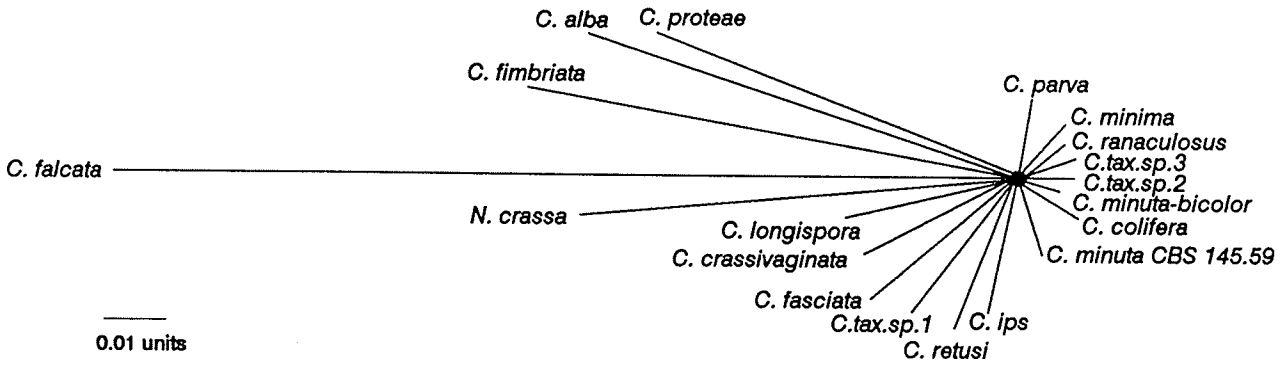

C. alba
C. proteae
C. fimbriata
C. parva
C. minima
C. ranaculosus
C. falcata
C.tax.sp.3
C.tax.sp.2
C. minuta-bicolor
N. crassa
C. colifera
C. longispora
C. minuta CBS 145.59
C. crassivaginata
C. fasciata
C.tax.sp.1
C. ips
C. retusi
0.01 units

Table 3: Cycloheximide tolerance of tested strains

Species	%inhibition of area growth on cycloheximide versus controls	Sensitivity classification[1]
C.alba	100	S
C.colifera	100	S
C.crassivaginata	0	I[3]
C. falcata UM 446	50.7	I
UM 792	31.6	I
UM 793	30.8	I
C.fasciata[2] Trial 1	18	I
Trial 2	17.9	I
C.fimbriata	100	S
C.ips UM 88-58	19.7	I[3]
UM 88-141	17.6	I[3]
C.minima[2] Trial 1	80.8	S
Trial 2	71.7	S
C.minuta CBS 463.77	86.8	S
CBS 145.59	87	S
C.minuta-bicolor		
UM 479[2] Trial 1	86.7	S
Trial 2	79.1	S
UM 480	80.3	S
C.parva	86.7	S
C.proteae	100	S[4]
C.ranaculosus	100	S
C.retusi	16.1	I[3]
C.tax.sp.1	0	I
C.tax.sp.2 UM 110	88.9	S
UM 113	91.4	S
C.tax.sp.3	87.1	S

[1] I = insensitive; S = sensitive
[2] These species were tested on two different occasions, to verify reproducibility.
[3] Results correspond to those of Harrington (1981)
[4] Result corresponds to that of Wingfield et.al. (1988a)

CHAPTER 5

ON THE PHYLOGENY OF *Ophiostoma*, *Ceratocystis*, AND *Microascus*, AND POSSIBLE RELATIONSHIPS WITHIN *Ophiostoma* BASED ON PARTIAL rDNA SEQUENCES.

INTRODUCTION

Historically, a number of authors have included species of Ophiostoma H. and P. Sydow under Ceratocystis Ell. and Halst. sensu lato in monographic studies (e.g. Hunt 1956; Griffin 1968; Olchowecki and Reid 1974; Upadhyay 1981). However others believe that the species of Ceratocystis s.l. with enteroblastic-phialidic anamorphs are unrelated to those Ophiostoma species that have holoblastic anamorphs with precurrent or sympodial proliferations (von Arx 1974; de Hoog 1974; Weijman and de Hoog 1975; Samuels and Müller 1979; de Hoog and Scheffer 1984; Harrington 1981, 1987). Following their detailed reevaluation of this problem, de Hoog and Scheffer (1984) concluded Ophiostoma should be maintained for those species whose anamorphs are not Chalara-like, have rhamnose and cellulose in their cell walls, and are resistant to cycloheximide (Harrington 1981). They also recognized the genus Ceratocystiopsis, which had been erected by Upadhyay and Kendrick (1975) for species of Ceratocystis s.l. whose ascospores are elongate and surrounded by a gel coating.

In the previous chapters using analyses based on partial ribosomal DNA (rDNA) sequences, additional evidence was presented that species of Ceratocystis and Ophiostoma are not closely related. Although species assigned to Ceratocystiopsis do appear to be a natural assemblage

defined by their possessing falcate ascospores, and hardly any intermediate shapes are known between falcate and crescent-shaped ascospores, partial rDNA sequences indicate that Ceratocystiopsis is polyphyletic. Furthermore, molecular citeria placed most members of Ceratocystiopsis within the genus Ophiostoma.

Generally, the molecular data obtained by analyzing a number of species of Ceratocystis s.l. show that only those species with Chalara anamorphs belong in Ceratocystis sensu stricto; all others, regardless of the shape of their ascospores or the nature of their anamorphs, should be transferred to Ophiostoma. Thus Ceratocystis s.s. can be circumscribed very narrowly as follows: its species produce dark-coloured, spherical ascomata with long ostiolar beaks; the ascomata contain small spherical deliquescent asci; and they produce Chalara anamorphs. This leaves over 100 species in Ophiostoma which, apart from having similar cell wall composition (Jewell 1974; Weijman and de Hoog 1975), vary greatly in ascospore shape, conidial states, and ascocarp features. This suggests that the genus Ophiostoma could be polyphyletic. However, to prove that this assemblage of species remaining within Ophiostoma does comprise a discrete genus requires a more vigorous taxon sampling than has been undertaken to date.

As many of these fungi are economically important plant pathogens or blue stain fungi, a revision of this genus that would result in a more natural classification scheme is

desirable. Therefore partial sequences were obtained from the small subunit ribosomal gene (SSrDNA) of 21 of these species to determine whether they represent a monophyletic group. And in a preliminary effort to study both relatedness and evolutionary trends amongst the species of Ophiostoma, the 5' terminal end of the large subunit ribosomal DNA gene (LSrDNA) was determined for 77 strains representing 55 species of Ophiostoma, and the sequence relationships were analyzed.

MATERIALS AND METHODS

The fungal strains sequenced, and their sources, are listed in Table 1.

Sequences of the V3, V4, and V9 regions of the SSrDNA, and the 5' terminal end of the LSrDNA gene (containing domain I) were obtained from strains representing a variety of fungal genera.

The rDNA sequences of Saccharomyces cerevisiae Meyen ex Hansen (Rubstov et al. 1980; Georgiev et al. 1981), Neurospora crassa Shear & B.O. Dodge (Sogin et al. 1986b; Guadet et al. 1989), Kluyveromyces lactis (Domb.) van der Walt (EMBL: X51830), Torulaspora delbrueckii (Lindner) Lindner (GenBank: TOUSRSR), Penicillium notatum Westling (GenBank: PNNDA), Aspergillus fumigatus Fresen. (GenBank: ASNDA). Podospora anserina (Ces.) Niessl (GenBank: PANRRNASS), Fusarium oxysporum (Guadet et al. 1989), Ophiostoma ulmi (Buisman) Nannf., Microascus cirrosus Curzi, Pseudallescheria boydii (Shear) McGinnis et al. (Berbee and Taylor 1992c) were included in this analysis for comparison. In addition, sequences reported in the previous chapters were again included in the analysis.

The nucleotide sequences were aligned initially using the alignment option within the CLUSTAL V package (Higgins and Sharp 1989) and the alignment was then refined by eye with the MASE program. The partial rDNA sequence data were

analyzed using the programs contained within PHYLIP (Felsenstein 1991).

Phylogenetic trees were obtained from the partial SSrDNA data by both parsimony and distance methods. Divergence (or distance) between two sequences was calculated by DNADIST using Kimura's two parameter model (Kimura 1980). The NEIGHBOR program was used to generate unrooted phylogenetic distance networks based on the neighbor-joining method (Saitou and Nei 1987). Confidence intervals on tree topologies were estimated by bootstrap analyses (Felsenstein 1985) by generating 1000 bootstrap replicates with the SEQBOOT program. The majority-rule consensus tree was constructed by the CONSENSE program.

The partial SSrDNA sequences were also analyzed with the FITCH program but, due to the long execution time for this program, it was not possible to evaluate statistically the phyletic output by bootstrap analysis.

The DNAPARS program was used to carry out unrooted parsimony on DNA sequences for estimating phylogenies. Bootstrap analysis was carried out by generating 1000 boostrap replicates with SEQBOOT. Each bootstrap replicate was analyzed by DNAPARS, and a majority-rule consensus tree was generated by CONSENSE.

The NEIGHBOR program was used to construct dendrograms for the 5' terminal LSrDNA sequence data set to reflect DNA sequence similarity, and the clustering of potential operational taxonomic units (OTU) was based on the neighbor-

joining method. As large data sets were being analyzed, the jumble option was used in the DNAPARS and NEIGHBOR programs to randomize the input order of the nucleotide sequences. Both the partial SSrDNA and the partial LSrDNA data set were analyzed three times with the following jumble seed numbers: 7, 11, 19.

TABLE 1. List of fungal strains studied

Species and Isolate No.	Source	Georgraphic origin
Cephaloascus fragrans Hanawa		
ATCC 36174	Not given	Japan
ATCC 60760	*Pseudotsuga menziesii* (Mirb.) Franco	Canada
Ceratocystis ambrosia Bakshi		
CBS 210.64	*Pinus sylvestris* L.	Not given
Ceratocystis californica Devay *et al.*		
ATCC 18392	*Prunus domestica* L.	U.S.A.
Ceratocystis coronata Olchow. & Reid		
WIN(M) 867	*Eucaplyptus* sp.	New Zealand
WIN(M) 868	*Pinus radiata* D. Don.	New Zealand
WIN(M) 71-26	*Pinus banksiana* Lamb.	Canada
Ceratocystis deltoideospora Olchow. & Reid		
WIN(M) 71-26	*Pinus banksiana*	Canada
CBS 187.86	*Pinus resinosa* Aiton	Not given
Ceratocystis hyalothecium Davids.		
ATCC 28825	*Pinus contorta* Douglas ex Loud.	U.S.A.
Ceratocystis introcitrina Olchow. & Reid		
WIN(M) 69-47	*Betula papyrifera* Marsh.	Canada
Ceratocystis novae-zelandiae Hutchison & Reid		
WIN(M) 863	*Podocarpus* sp.	New Zealand
WIN(M) 864	*Pinus radiata*	New Zealand
Ceratocystis ossiformis Olchow. & Reid		
WIN(M) 52	*Abies balsamea* (L.) Mill.	Canada
Ceratocystis ponderosae Hinds. & Davids.		
CBS 496.77	*Pinus*	U.S.A.
Ceratocystis pseudoeurophioides Olchow. & Reid		
WIN(M) 71-13	*Picea mariana* (Mill.) B.S.P.	Canada
Ceratocystis pseudominor Olchow. & Reid		
WIN(M) 460	*Pseudotsuga menziesii*	Canada
Ceratocystis torulosa Butin & Zimmerman		
CBS 770.71	*Fagus sylvatica* L.	Germany
Ceratocystis vesca Davids.		
CBS 800.73	*Picea engelmannii* Parry ex Engelm.	U.S.A.
Endomyces decipiens (Tulasne) Reess		
ATCC 11647	Not given	Not given
Ophiostoma abietinum Marmolejo & Butin		
CBS 125.89	*Abies vejari* Martinez	Mexico
Ophiostoma adjunctum (Davids.) Harrington		
ATCC 34942	*Pinus ponderosa* Laws.	U.S.A.
Ophiostoma araucariae Butin		
CBS 114.68	*Araucaria araucana* K. Koch	Chile
Ophiostoma bacillosporum (Butin & Zimmerman) de Hoog & Scheffer		
CBS 771.71	*Fagus sylvatica* L.	Germany
Ophiostoma bicolor Davids. & Wells.		
ATCC 15007	*Picea engelmannii*	U.S.A.
ATCC 62329	*Picea abies* (L.)H.Karst	Norway
Ophiostoma canum (Münch) H. & P. Sydow		
NFRI 1652/2	*Pinus sylvestris*	Norway

NFRI 60-165	Not given	Norway
Ophiostoma conicolum Marmolejo & Butin		
CBS 127.89	Pinus cembroides Zucc.	Mexico
Ophiostoma cucullatum Solheim		
NFRI 81-83/16	Pinus abies	Norway
Ophiostoma distortum (Davids.) de Hoog & Scheffer		
ATCC 18998	Abies sp.	U.S.A.
Ophiostoma dryocetidis (Kendrick & Molnar) de Hoog & Scheffer		
CBS 376.66	Abies lasiocarpa (Hook.) Nutt.	Canada
Ophiostoma epigloeum (Guerrero) de Hoog		
CBS 573.63	Tremella fuciformis Berk.	Argentina
Ophiostoma europhioides (Wright & Cain) Solheim		
NFRI 80-67/22	Picea abies	Norway
Ophiostoma flexuosum Solheim		
NFRI 81-79/10	Picea abies	Norway
Ophiostoma francke-grosmanniae (Davids.) de Hoog & Scheffer		
ATCC 22061	Quercus sp.	Germany
Ophiostoma gossypinum (Davids.) J. Taylor		
ATCC 18999	Pinus ponderosa	U.S.A.
ATCC 22063	Pinus ponderosa	U.S.A.
Ophiostoma grande Samuels & Müller		
CBS 350.78	Stroma of Diatrype cfr. stigma	Brazil
Ophiostoma longirostellatum (Bakshi) de Hoog		
CBS 134.51	Quercus sp.	U.K.
Ophiostoma microsporum (Davids.) von Arx		
CBS 412.77	Castanea sp.	U.S.A.
Ophiostoma minus (Hedgc.) H. & P. Sydow		
WIN(M) 495	Pinus contorta	Canada
Ophiostoma montium (Rumb.) von Arx		
CBS 151.78	Pinus ponderosa	U.S.A.
WIN(M) 497	Pinus contorta	Canada
ATCC 24285	Pinus contorta	Canada
Ophiostoma nigrum (Davids.) de Hoog & Scheffer		
CBS 163.61	Abies lasiocarpa	U.S.A.
Ophiostoma olivaceum Mathiesen		
CBS 138.51	Picea sp.	Sweden
Ophiostoma penicillatum (Grosmann) Siemaszko		
NFRI 1715/3	Picea abies	Norway
CBS 211.67	Picea abies	Sweden
NFRI 1716/3	Picea abies	Norway
Ophiostoma perfectum (Davids.) de Hoog		
CBS 636.66	Picea engelmannii	Canada
Ophiostoma piceae (Münch) H. & P. Sydow		
CBS 263.65	Pyrus communis L.	Italy
WIN(M) 324	Pinus sylvestris	Norway
Ophiostoma piceaperdum (Rumb.) von Arx		
WIN(M) 82-14b	Pinus nigra Arnold	Norway
Ophiostoma piliferum (Fr.) H. & P. Sydow		
WIN(M) 22	Not given	Sweden
Ophiostoma polyporicola O. Const. & Ryman		

CBS 669.88	Fomitopsis pinicola (Sw.: Fr.) P. Karst.	Sweden
Ophiostoma populina (Hinds. & Davids.) de Hoog & Scheffer		
CBS 212.67	Populus tremuloides Michx.	U.S.A.
Ophiostoma roraimense Samuels & Mueller		
CBS 351.78	Hypoxylon sp.	Brazil
Ophiostoma rostrocoronatum (Davids. & Eslyn) de Hoog & Scheffer		
CBS 434.77	Not given	U.S.A
Ophiostoma tax. sp. I		
SCO132I	Gnathotrichus sulcatus	U.S.A
Ophiostoma tax. sp. II		
WIN(M) 532	Malus sp.	Canada
Ophiostoma tetropii Mathiesen		
NFRI 80-113/9	Picea abies	Norway
Ophiostoma ulmi (Buisman) Nannf.		
WIN(M) 78	Ulmus americana L.	Canada
WIN(M) 895	Ulmus americana	Canada
WIN(M) 902	Ulmus americana	Canada

ATCC, American Type Culture Collection, Rockville, Md., U.S.A.;
CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands;
DAOM, Biosystematics Research Institude, Ottawa, Ont., Canada;
WIN(M), The culture collection of J. Reid, University of Manitoba, Department of Botany, Winnipeg, Manitoba, Canada.

RESULTS

On the phylogeny of ophiostomataceous fungi based on partial SSrDNA sequences

To obtain evidence that species assigned to Ophiostoma represent a monophyletic group, partial SSrDNA sequences from regions V3, V4, and V9 (a total of 711 nucleotide positions) were analyzed; comparable sequences from members of the Basidiomycotina, Endomycetales, Eurotiales, Ophiostomatales, Sordariales, and Microascales were also included. The final aligned dataset of 41 partial sequences (Fig. 1) included sequences obtained from the literature and databanks (GenBank, EMBL), as well as sequences obtained and employed in previous chapters of this study. The latter are identified in the appropriate figure.

Phylogenetic trees generated by both distance and parsimony methods had essentially the same topologies. The estimate of phylogeny based on an unrooted tree generated by the neighbor-joining method (Fig. 2) indicates high levels of confidence (>95%) for internal branches uniting species of the following groupings: (1) the members of the class Hemiascomycetes, order Endomycetales, (2) subclass Plectoascomycetidae (Barr 1990) family Eurotiaceae, (3) the Sordariaceae, (4) species assignable to Ophiostoma (excluding O. roraimense), (5) the Microascaceae, (6) species assignable to Ceratocystis s.s., (7) the

Microascales (members of the Microascaceae, species of Ceratocystis and Ceratocystiopsis proteae), (8) the subclass Parenchymatomycetidae (Barr 1990), (9) the class Hymenoascomycetes, and (10) the subdivision Basidiomycotina.

Parsimony analysis yielded a total of 76 most parsimonious trees, each of which required 685 steps. However most of these trees arose due to alternate arrangements of the branching topologies possible for sequences of Ophiostoma species. In addition, the sequence for O. roraimense in 38 of the parsimony trees was grouped with the Endomycetales sequences, whereas in the remaining trees O. roraimense was placed on a branch that was positioned between the Basidiomycotina and Endomycetales. However, the topologies for the position of the species representing the Microascales, Sordariaceae, Eurotiaceae, Endomycetales, and Basidiomycotina were identical in all phyletic trees generated by DNAPARS.

While parsimony analysis of the sequence data confirmed the potential monophyly for previously stated groupings, the statistical support for some of the internal branches was somewhat lower. For example the presumed monophyly of species assignable to Ophiostoma (excluding O. roraimense) is supported at the 100% level of confidence in the distance analysis, but parsimony analysis supports this at only the 93% level. But when the dataset was reduced to 31 sequences by randomly deleting different sets of 10 Ophiostoma sequences, the level of confidence for the branch uniting

the remaining Ophiostoma sequences rose to levels above 95%. In general, when the data set was further reduced by including only 2 representatives for each grouping that was observed to be monophyletic in the distance analysis, the levels of confidence for all internal branches approached levels observed in the distance analysis. Thus it appears that for the large data set (41 sequences), the partial SSrDNA sequences do not contain enough information for parsimony analysis to generate tree topologies with consistently high levels of statistical support. Admittedly the robustness of some branches, particularly the branch indicating monophyly for members of the Parenchymatomycetidae and that branch supporting the monophyly of the Hymenoascomycetes, might have increased had the entire SSrDNA gene been examined for all taxa. However, overall the parsimony analysis of the data supports the phyletic estimate generated by the distance analysis.

Although both distance and parsimony methods support the idea that species assignable to Ophiostoma represent a valid genus, our partial SSrDNA sequence data does not contain enough information to resolve the branching pattern within the Ophiostoma complex with a high level of confidence. Therefore, branches with a level of confidence <70% in the consensus tree were reduced to polychotomies to reflect the lack of statistical support.

Based on partial SSrDNA, O. cucullatum appears to be the most distant member amongst the species of Ophiostoma

Fig. 1. Alignment for the V3, V4 (positions 402-886), and V9 (positions 1546-1748) regions of the SSrDNA gene. Nucleotide positions correspond to the nucleotide numbering of the SSrDNA of Saccharomyces cerevisiae (Rubstov et al. 1980). 1 = O. penicillatum; 2 = O. grande; 3 = O. abietinum; 4 = O. perfectum; 5 = C. ambrosia; 6 = O. nigrum; 7 = C. torulosa; 8 = C. californica; 9 = O. longirostellatum; 10 = C. hyalothecium; 11 = C. novae-zelandiae; 12 = O. microsporum; 13 = O. rostrocoronatum; 14 = O. polyporicola; 15 = O. cucullatum; 16 = O. minutum; 17 = O. ips; 18 = O. piliferum; 19 = O. ulmi; 20 = O. piceae; 21 = T. delbruecki; 22 = Pen. notatum; 23 = A. fumigatus; 24 = Neo. fischeri; 25 = N. crassa; 26 = Pod. anserina; 27 = S. fimicola; 28 = G. tetrasperma; 29 = C. fimbriata; 30 = C. moniliformis; 31 = K. pachypleura; 32 = Cop. proteae; 33 = Pseud. boydii; 34 = M. cirrosus; 35 = E. decipiens; 36 = Ceph. fragrans; 37 = Sch. commune; 38 = Scl. hydrophilum; 39 = Sacch. cerevisiae; 40 = Kl. lactis; 41 = O. roraimense.

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      402
 1    GGCTACTACA TCCAAGGAAG GCAGCAGGCG CGCAAATTAC CCAATCCCGA TACGGGGAGG
 2    .......... .......... .......... .......... .......... C.........
 3    .......... .......... .......... .......... .......... C.........
 4    .......... .......... .......... .......... .......... CT........
 5    .......... .......... .......... .......... .......... CT........
 6    .......... .......... .......... .......... .......... CT........
 7    .......... .......... .......... .......... .......... CT........
 8    .......... .......... .......... .......... .......... CT........
 9    .......... .......... .......... .......... .......... CT........
10    .......... .......... .......... .......... .......... CT........
11    .......... .......... .......... .......... .......... CT........
12    .......... .......... .......... .......... .......... CT........
13    .......... .......... .......... .......... .......... C.........
14    .......... .......... .......... .......... .......... C.........
15    .......... .......... .......... .......... .......... .T........
16    .......... .......... .......... .......... .......... CG........
17    .......... .......... .......... .......... .......... CT........
18    .......... .......... .......... .......... .......... CT........
19    .......... .......... .......... .......... .......... CT........
20    .......... .......... .......... .......... .......... CT........
21    ......C... .......... .......... .......... ........TA. ...A......
22    ......C... .......... .......... .......... .......... ..........
23    ......C... .......... .......... .......... .......... C.........
24    ......C... .......... .......... .......... .......... C.........
25    .......... .......... .......... .......... .......... C.........
26    .......... .......... .......... .......... .......... C.........
27    .......... .......... .......... .......... .......... C.........
28    .......... .......... .......... .......... .......... C.........
29    ......C..T .TT....... .......... .......... .......... C.........
30    ......C..T .TT....... .......... .......... .......... C.........
31    .......... .......... .......... .......... .......... C.........
32    .......... ..T....... .......... .......... .......... CG........
33    .......... .......... .......... .......... .......... C.........
34    .......... .......... .......... .......... .......... C.........
35    ......C... .......... .......... .......... .......... C.........
36    ......C... .......... .......... .......... .......... C.........
37    ......C... .......... .......... .......... .......... C.........
38    ......C... .......... .......... .......... .......... C.........
39    ......C... .......... .......... .......... .......TA. .T.A......
40    ......C... .......... .......... .......... .......TA. .T.A......
41    ......C... .......... .......... .......... .......... C.........
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      462
 1    TAGTGACAAT AAATACTGAT ACAGGGCTCT TTTGGGTCTT GTAATTGGAA TGAGTACAAT
 2    .......... .......... .......... .......... .......... ..........
 3    .......... .......... .......... .......... .......... ..........
 4    .......... .......... .......... .......... .......... ..........
 5    .......... .......... .......C.. .......... .......... ..........
 6    .......... .......... .......... .......... .......... ..........
 7    .......... .......... .......... .......... .......... ..........
 8    .......... .......... .......... .......... .......... ..........
 9    .......... .......... .......... .......... .......... ..........
10    .......... .......... .......... .......... .......... ..........
11    .......... .......... .......... .......... .......... ..........
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13    .......... .......... .......... .......... .......... ..........
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17    .......... .......... .......... .......... .......... ..........
18    .......... .......... .......... .......... .......... ..........
19    .......... .......... .......... .......... .......... ..........
20    .......... .......... .......... .......... .......... ..........
21    .......... .....AC... .......C.A ..C....... .......... ..........
22    .......... .......... ..G....... .........C .......... ....A.....
23    .......... .......... ..G....... .........C .......... ..........
24    .......... .......... ..G....... .........C .......... ..........
25    .......... .......... .......... .......... .......... ..........
26    .......... .......... .......... .......... .......... ..........
27    .......... .......... .......... .......... .......... ..........
28    .......... .......... .......... .......... .......... ..........
29    .......... .......... .......... .A........ .....C.... ..........
30    .......... .......... .......... .A........ .....C.... ..........
31    .......... .......... .......... .......... .....C.... ..........
32    .......... .......... .......... .......... .....C.... ..........
33    .......... .......... .......... .......... .....C.... ..........
34    .......... .......... .......... ..A....... .....C.... ..........
35    .......... .....AC... .......C.A .......... .......... ..........
36    .......... .....AC... ..G...-..C A....A...C .-........ ....A.....
37    .......... .....ACA.. .T........ ..C.....C. A......... ..........
38    .......... .....ACA.. .T.....G.C .......... .......... ..........
39    .......... .....AC... .......C.A ..C....... .......... ..........
40    .......... .....AC... .......C.A ..C-...... .......... ..........
41    .......... .....ACA.. G......C.. .......... .......... ..........
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          522
 1        TTAATTCCCT TAACGAGGAA CAATTGGAGG GCAAGTCTGG TGCCAGCAGC CGCGGTAATT
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... .......... ..........
 5        .......... .......... .......... .......... .......... ..........
 6        .......... .......... .......... .......... .......... ..........
 7        .......... .......... .......... .......... .......... ..........
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13        .......... .......... .......... .......... .......... ..........
14        .......... .......... .......... .......... .......... ..........
15        .......... .......... .......... .......... .......... ..........
16        .......... .......... .......... .......... .......... ..........
17        .......... .......... .......... .......... .......... ..........
18        .......... .......... .......... .......... .......... ..........
19        .......... .......... .......... .......... .......... ..........
20        .......... .......... .......... .......... .......... ..........
21        G...A.A... .......... .......... .......... .......... ....-.....
22        ....A..... .......... .......... .......... .......... ..........
23        C...A..... .......... .......... .......... .......... ..........
24        C...A..... .......... .......... .......... .......... ..........
25        ....A..... .......... .......... .......... .......... ..........
26        ....A..... .......... .......... .......... .......... ..........
27        ....A..... .......... .......... .......... .......... ..........
28        ....A..... .......... .......... .......... .......... ..........
29        ....A..... .......... .......... .......... .......... ..........
30        ....A..... .......... .......... .......... .......... ..........
31        ....A..T.. .......... .......... .......... .......... ..........
32        ....A..... .......... .C........ .......... .......... ..........
33        ....A..... .......... .......... .......... .......... ..........
34        ....A..... .......... .......... .......... .......... ..........
35        ....A.A... .......... .......... .......... .......... ..........
36        ....A-A... .......... ...G.A.... .......... .......... ..........
37        ....A..... .........T .......... .......... .......... ..........
38        ....A..... .........T .......... .......... .......... ..........
39        G...A.A... .......... .......... .......... .......... ..........
40        G...A.A... .......... ...C...... .......... .......... ....-.....
41        ....A..... .......... .G........ .......... .......... ..........
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          582
 1        CCAGCTCCAA TAGCGTATAT TAAAGTTGTT GCAGTTAAAA AGCTCGTAGT TGAACCTT-G
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... .......... ..........
 5        .......... .......... .......... .......... .......... ..........
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 9        .......... .......... .......... .......... .......... ..........
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14        .......... .......... .......... .......... .......... ..........
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16        .......... .......... .......... .......... .......... ..........
17        .......... .......... .......... .......... .......... ..........
18        .......... .......... .......... .......... .......... ..........
19        .......... .......... .......... .......... .......... ..........
20        .......... .......... .......... .......... .......... ..........
21        .......... .......... .......... .......... .......... .....T....
22        .......... .......... .......... .......... .......... ..........
23        .......... .......... .......... .......... .......... ..........
24        .......... .......... .......... .......... .......... ..........
25        .......... .......... .......... .AG....... .......... ..........
26        .......... .......... .......... .AG....... .......... ..........
27        .......... .......... .......... .......... .......... ..........
28        .......... .......... .......... .AG....... .......... ..........
29        .......... .......... .......... .TG....... .......... ..........
30        .......... .......... .......... .TG....... .......... ..........
31        .......... .......... .......... .TG....... .......... C.........
32        .......... .......... .......... .TG....... .......... C.........
33        .......... .......... .......... .TG....... .......... ..........
34        .......... .......... .......... .TG....... .......... C.........
35        .......... A......... .......... .......... .......... ..........
36        .......G.C G......... .......... .......... .......... ....A.....
37        .......... .......... .......... .......... .......... .........CA
38        .......... .......... .......... .......... .......... .........CA
39        .......... .......... .......... .......... .......... .....T....
40        .........G .......... .......... .......... .......... .....T....
41        .......... .......... .......... .......... .......... ....GT....
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      641
 1    GGCCTGGCTG G-CCGGTCCG CCTCACCGCG TGCACT--GG -TCCGG--CC GGGCCTTTC-
 2    .......... .......... .......... .......... .......... ...T......
 3    .......... .......... .......... .......... .......... ..........
 4    .......... .......... .......... .......... .......... ...T......
 5    .......... .......... .......... .......... .C........ ...T......
 6    .......... .......... .......... .......... .......... ..........
 7    .......... .......... .......... .......... .......... ...T......
 8    .......... .......... .......... .......... .......... ...T......
 9    .......... .......... .......... .......... .......... ...T......
10    .......... .......... .......... .......... .......... ...T......
11    .......... .......... .......... .......... .......... ...T......
12    .......... .......... .......... .......... .......... ..........
13    .......... .......... .......... .......... .......... ..........
14    .......... .......... .......... .......... .......... ...T......
15    .......... .......... .......... .......... .......... ..........
16    .......... .......... .......... .......... .......... ..........
17    .......... .......... .......... .......... .......... ...T......
18    .......... .......... .......... .......... .......... ...T......
19    .......... .......... .......... .......... .......... ...T......
20    .......... .......... .......... .......... .......... ...T......
21    .......T.. .......... AT.TTTT-.. ..T....... ..TTCCAA.. ..........
22    ..T....... .......... .......... A.T....... .........T ..A.......
23    ..T....... .......... .......... A.T....... .........T ..A.......
24    ..T....... .......... .......... A.T....... .........T ..A.......
25    ...TC...C. -.T....... .......... .........A .CTG....T. ........T.
26    ....C...CC T........C ........G. .......... .CT....... ..........
27    ....CA..C. .......... .......... .......... .ATA....TT ..........
28    ....C...CC ..T....... .......... .........A .CTG....T. ..........
29    .......... .........C ........G. .......... T......... ...T......
30    .......... .........C ........G. .......... ..T.C.G... ...T......
31    .......... .........C ........G. .........A ....A..... ..........
32    .......... .........C ........G. .......... .......... ..........
33    ........C. ...T.....C ........G. .........A ....A..... ..........
34    ........C. .........C ........G. .........A .......... ..........
35    ...T...T.. A..T...... .T.TTTG..- A.T.......- .-A.TCAA.. .A........
36    .--...TT.. ..TG...... .T.AGT-... A.-.....C- .-AG.CGAA. .A......A.
37    ........C. ..G.....T. ...A..G.TA ..T.......- ...T...... ...T...A..
38    .......T.. ..G.....T. ......G.TA ..T.......- .....A.... ...T...A..
39    ....C..T.. .......... AT.TTTT-.. ..T....... .ATTTCCAA. ...G.C..TC
40    ..T....T.. T......... A..TTAT.-T C..G.ACT.. ..TTTCAA.. ..AT......
41    .T.TC..AC. C.TG....T. .T.A.TT..A ..T...T..A .--...T... .A.A...-..
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          696
 1        CCTCTGG--G GAACCG-CAT GCCCTT---- -CACTGGGCG TGTCGGGGAA CCAGGACTTT
 2        ......... ..G..... ......... ......... ..C..... .......G..
 3        ......... ..G..... ......... .........T. ......... .........
 4        ......... ..G..... ......... ......... ......... .......A..
 5        ......... ..G..... ......... ......... ......... .........
 6        ......... ..G..... ..TT..... .T.T.AA... ......... .........
 7        ......... ..G..... ......... ......... ......... .......A..
 8        ......... ..G..... ......... ......... ......... .......A..
 9        ......... ..G..... ......... ......... ......... .......A..
10        ......... ..G..... ......... ......... ..C..... .......A..
11        ......... ..G..... ......... ......... ......... .......A..
12        ......... ..G..... ......... ......... ..C..... .......G..
13        ......... ......... ......... ......... ..C..... .........
14        ......... ..G..... ......... ......... ......... .........
15        ......... ..G..... ......... ..G..... ......... .........
16        ......... ..G..... ......... ......... ......... .........
17        ......... ..G..... ......... ......... ......... .......A..
18        ......... ..G..... ......... ..G..... ......... .......A..
19        ......... ..G..... ......... ......... ......... .......A..
20        ......... ..G..... ......... ......... ......... .......A..
21        .T.......C T....T.TGG .T..... .--G...C.C .TG--.C... .........
22        .T..... ..... .T.... .G..... .......CT. ..GG...-.. .........
23        .T..... ..... .T.... .G..... .......CT. ..GG...-.. .........
24        .T..... ..... .T.... .G..... .......CT. ..GG..... .........
25        TTC.....A ......... ......... .........T. ......... .........
26        .T.......A ......... ......... ......... ..C..... .........
27        .T.......A ......... ......... .........T. ......... .........
28        TT.......A ......... ......... .........T. ......... .........
29        ......T... ......... ......... .........T. ......... A.........
30        .........- -......... C..... .........T. ......... A.........
31        ......T... .....C.... .G..... .......CT. ..CG..... A.........
32        ......T... ....T..... ......... .T..... ...-A..... A.........
33        ......T... .....C.... .G..... .......C.. ..G..... A.........
34        ......T... .....C.... .G..... .......CT. ..CG..... A.........
35        .T.......C TTC-T..GC. A.T...C..T CTTTC...A. .T---.A... .........
36        AT.TG..... ..-----.GTG TGTT..ATTT ATTGA.A.-. CA.--AC... G.CAA..A..
37        .TCT.....T ......G.G. ..T..... .T..... C.....C... .........
38        .TCT.....T ..G...G... ..T..... .A......T. ......... .........
39        .T.......C T....T.TGA .T..... .--G...CTC .TG--.C... .........
40        .T.......C T....T.GTA CT..... .--G....T. CAG--.C... .........
41        .T...T.GTC ...-..G.-C .....C.... .----...T. -...-C.... ........A.
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          745
 1        TACTTTGAAA AAATTAGAGT GTTCAAAGCA GGC-TTATGC TCGGATA-CA TTAGCATGGA
 2        .......... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        .......... .......... .......... .......... ...A...... ..........
 5        .......... .......... .......... .......... .......... ..........
 6        .......... .......... .......... .......... ...A...... ..........
 7        .......... .......... .......... .......... ...A...... ..........
 8        .......... .......... .......... .......... ...A...... ..........
 9        .......... .......... .......... .......... ...A...... ..........
10        .......... .......... .......... .......... ...A...... ..........
11        .......... .......... .......... .......... ...A...... ..........
12        .......... .......... .......... .......... .......... ..........
13        .......... .......... .......... .......... ...A...... ..........
14        .......... .......... .......... .......... .......... ..........
15        .......... .......... .......... .......... .......... ..........
16        .......... .......... .......... .......... ...A...... ..........
17        .......... .......... .......... .......... ...A...... ..........
18        .......... .......... .......... .......... ...A...... ..........
19        .......... .......... .......... .......... ...A...... ..........
20        .......... .......... .......... .......... ...A...... ..........
21        .......... .......... .......... ...G.AT... ...A....T. ..........
22        ....G..... .......... .......... ....C.T... ...A...... ..........
23        ....G..... .......... .......... ....C.T... ...A...... ..........
24        ....G..... .......... .......... ....C.T... ...A...... ..........
25        ...CG....C ....C...TC .C......A. ....C..... ...A..G.T. C.........
26        ....C....C ........TC .C.T....A. ....C..... ...A....GT C.........
27        ....C....C ........TC .C.T....A. ....C..... ...A...... ..........
28        ...CG....C ....C...TC .C.T....A. ....C..... ...A..-GT. C.........
29        .......... .......... .C..C.G... ....C..... ...A..CGT. C-........
30        .......... .......... .C..C.G... ....C..... ...A...GT. C-........
31        ....G..... .......... .C..C.G... ....C..... ...A...... ..........
32        .......... .......... .C..C.G... ....A..... ...A.....T .C........
33        .......... .......... .C..C.G... ....C..... ...A...... ..........
34        ....G..... .......... .C..C.G... ....C..... ...A...... ..........
35        .......... .......... .......... ....C.T... ...A....T. ..........
36        .......... .......... .......... ....C.T... ...A....T. ..........
37        ...C....G. .......... .......... .......C.. C..A...... ..........
38        ........G. .......... .......... ....C..... C..A...... ..........
39        .......... .......... .......... ...G.AT... ...A....T. ..........
40        .......... .......... .......... ....GA.A.. ...A....T. ..........
41        .......... .......... .......... ...CA..G.. C..A....T. ..........
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          804
 1        ATAATAGAAT AGGACGT-GC GGTTCTATTT TGTTGGTTTC TAGGACCGCC GTAATGATTA
 2        .....G.... .......... .......... .......... .......... ..........
 3        .......... .......... .......... .......... .......... ..........
 4        ......A... ......C..T .......... .......... .......... ..........
 5        .......... .......... .......... .......... .......... ..........
 6        .......... .......... .......... .......... .......... ..........
 7        .......... .......C.. .......... .......... .......... ..........
 8        .......... .......C..T .......... .......... .......... ..........
 9        .......... .......C..T .......... .......... .......... ..........
10        .......... .......C... .......... .......... .......... ..........
11        .......... .......C... .......... .......... .......... ..........
12        .......... .......C... .......... .......... .......... ..........
13        .......... .......... .......... .......... .......... ..........
14        .......... .......... .......... .......... .......... ..........
15        .......... .......... .......... .......... .......... ..........
16        .......... ..........T .......... .......... .......... ..........
17        .......... ..........T .......... .......... .......... ..........
18        .......... ..........T .......... .......... .......... ..........
19        .......... .......C... .......... .......... .......... ..........
20        .......... .......C... .......... .......... .......... ..........
21        .......... .........TT .......... .......... .......AT. ..........
22        .......... ..........T .......... .......... .......... ..........
23        .......... .......... .......... .......... .......... ..........
24        .......... .......... .......... .......... .......... ..........
25        .......... ..........T .......... .......... .......... ..........
26        .....G.... ..........T .......... .......... .......... ..........
27        .......... ..........T .......... .......... .......... ..........
28        .......... ..........T .......... .......... .......... ..........
29        .......... ..........T .......... .......... .......... ..........
30        .......... ..........T .......... .......... .......... ..........
31        .......... ..........T .......... .......... .......... ..........
32        .......... ..........T .......... .......... .......... ..........
33        .......... ..........T .......... .......... .......... ..........
34        .......... .......C..T .......... .......... .......... ..........
35        .......... ........TAT .......... .......... .......AT. ..........
36        .......... .........AT .......... .......... .........T. ..........
37        ......A... .......... ...C...... .......... .....T.... ..........
38        ......A... .......... .......... .......... ...AGT.... ..........
39        .......... .........TT .......... .......... .......AT. ..........
40        .....G.... .........TT .......... .......... .......AT. ..........
41        ....C..... .......... .......... .......... ...A..T... ..........
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      862                                  1546
 1    ATAGGGACAG TCGGGGGCAT CAGT-----T ATTGCTCTTC AACGAGGAAT CCCTAGTAAG
 2    .......... .......... ....     . .......... .......... ..........
 3    .......... .......... ....     . .......... .......... ..........
 4    .......... .......... ....     . .......... .......... ..........
 5    .......... .......... ....     . .......... .......... ..........
 6    .......... .......... ....     . .......... .......... ..........
 7    .......... .......... ....     . .......... .......... ..........
 8    .......... .......... ....     . .......... .......... ..........
 9    .......... .......... ....     . .......... .......... ..........
10    .......... .......... ....     . .......... .......... ..........
11    .......... .......... ....     . .......... .......... ..........
12    .......... .......... ....     . .......... .......... G.........
13    .......... .......... ....     . .......... .......... ..........
14    .......... .......... ....     . .......... .......... ..........
15    .......... .......... ....     . .......... .......... ..........
16    .......... .......... ....     . .......... .......... ..........
17    .......... .......... ....     . .......... .......... ..........
18    .......... .......... ....     . .......... .......... ..........
19    .......... .......... ....     . .......... .......... ..........
20    .......... .......... ....     . .......... .......... ..........
21    .........G. .......... ....     . .......... .......... T.........
22    .......T.. .........G. ....     . .......... .......... G.......G.
23    .......T.. .........G. ....     . .......... .......... G.......G.
24    .......... .........G. ....     . .......... .......... G.......G.
25    .......... .......... ....     . .......... .......... ..........
26    .......... .......... ....     . .......... .......... T.........
27    .......... .......... ....     . .......... .......... ..........
28    .......... .......... ....     . .......... .......... ..........
29    .......... .......... ....     . .......... .......... ..........
30    .......... .......... ....     . .......... .......... ..........
31    .......... .......... ....     . .......... .......... ..........
32    .......... .......... ....     . .......... .......... ..........
33    .......... .......... ....     . .......... .......... ..........
34    .......... .......... ....     . .......... .......... ..........
35    .........G. .......... ....     . .......... .......... T.........
36    .......... .......... ....     . .......... .......... ..........
37    .......T.. .T........ TG..     . .......... .......... A.........
38    .......T.. .T........ T...     . .......... .......... T.........
39    .........G. .......... .G..     . .......... .......... T.........
40    .........G. .......... ....     . .......... .......... T.........
41    .A........ .......... ....     . .......... .......... ..........
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      1577
 1    CGCAAGTCAT CAACTTGCGC TGATTACGTC CCTGCCCTTT GTA-CACACC GCCCGTCGCT
 2    .......... .........T .......... .......... .......... ..........
 3    .......... .........T .......... .......... .......... ..........
 4    .......... .......... .......... .......... .......... ..........
 5    .......... .........T .......... .......... .......... ..........
 6    .......... .........T .......... .......... .......... ..........
 7    .......... .........T .......... .......... .......... ..........
 8    .......... .......... .......... .......... .......... ..........
 9    .......... .......... .......... .......... .......... ..........
10    .......... .......... .......... .......... .......... ..........
11    .......... .......... .......... .......... .......... ..........
12    .......... .........T .......... .......... .......... ..........
13    .......... .........T .......... .......... .......... ..........
14    .......... .........T .......... .......... .......... ..........
15    .......... .........T .......... .......... .......... ..........
16    .......... .........T .......... .......... .......... ..........
17    .......... .......... .......... .......... .......... ..........
18    .......... .......... .......... .......... .......... ..........
19    .......... .......... .......... .......... .......... ..........
20    .......... .......... .......... .......... .......... ..........
21    .......... ..G......T .......... .......... .......... ..........
22    .A.G...... ..G..C.T.. C......... .......... .......... ..........
23    .A.G...... ..G..C.T.. C......... .......... .......... ..........
24    .A.G...... ..G..C.T.. C......... .......... .......... ..........
25    .......... ..G......T .......... .......... .......... ..........
26    .......... ..G....... .......... .......... .......... ..........
27    .......... ..G......T .......... .......... .......... ..........
28    .......... ..G......T .......... .......... .......... ..........
29    .......... ..G......T .......... .......... .......... ..........
30    .......... ..G......T .......... .......... .......... ..........
31    .......... ..G......T .......... .......... .......... ..........
32    .......... ..G......T .......... .......... .......... ..........
33    .......... ..G......T .......... .......... .......... ..........
34    .......... ..G......T .......... .......... .......... ..........
35    .......... ..G......T .......... .......... .......... ..........
36    .......... ..G......T .......... .......... .......... ..........
37    ..TG...... ..G..C...T .......... .......... .......... ..........
38    ..TG...... .-G..C...T .......... .......... .......... ..........
39    .......... ..G......T .......... .......... .......... ..........
40    .......... ..G......T .......... .......... .......... ..........
41    .......... ..G......T ...C...... .......... ...T...... ..........
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          1636
 1        ACTACCGATT GAATGGCTCA GTGAGGCTTC CGGACTGGCC CAGGGGGG-C GGG-CAACCC
 2        .......... .......... .......... .......... ...A.....T .........A
 3        .......... .......... .......... .......... ...A.....T .......TA
 4        .......... .......... .......... .......... ...A.....T .......TA
 5        .......... .......... .......... .......... ....A....T .......TA
 6        .......... .......... .........T .......... ...A.....T .......TA
 7        .......... .......... .......... .......... ...A.....T .......TA
 8        .......... .......... .......... .......... ....A...GT .......TA
 9        .......... .......... .......... .......... ...A.....T .......TA
10        .......... .......... .......... .......... ...A.....T .......TA
11        .......... .......... .......... .......... ...A.....T .......TA
12        .......... .......... .......... .......... .........T .........A
13        .......... .......... .........T .......... ...A.....T ....A...TA
14        .......... .......... .......... .......... ...A.....T .......TA
15        .......... .......... ........CT .......... .......... .........A
16        .......... .......... .......... .......... ....A....T .........A
17        .......... .......... .......... .......... ...A.....T .......TA
18        .......... .......... .......... .......... ...A.....T .......TA
19        .......... .......... .......... .......... ...A.....T .......TA
20        .......... .......... .......... .......... ...A.....T .......TA
21        .G........ .........T. .......C.. A...TCT..T T..A.AA..G .........T.
22        .......... .......... .......C.T G...T....T T...A....T T.......GA
23        .......... .........G .......C.T .........T ......A..T T.......GA
24        .......... .........G .......C.T .........T ......A..T T.......GA
25        .......... .......... .......... .......... .....A...T C.......GA
26        .......... .......... .......... .......... .....A...T C.......G.
27        .......... .......... .......... .......... .....A...T C.......GA
28        .......... .......... .......... .......... .....A...T C.......GA
29        .......... .G........ .....A.C.. .......... G..A.A...T ....A...TA
30        .......... .G........ .....A.C.. .......... G..A.A...T ....A...TA
31        .......... .......... .....A.C.. .......... ...A.A...T ........TA
32        .......... .G........ .....A.C.. .......... TG.A.A.... ....A...TG
33        .......... .......... .......C.. .......... ...A.A...T ........TA
34        .......... .......... .......C.T .......... ...A.A.... ........TG
35        .......... .........T. ......AC.. ....T...TT ....A.TA.G A..G....TT
36        .........C .........T. .......... ....T..A.T GTTTA-...A ....AG..TT
37        .......... .........T. ......TC.T ....TC...T TT....A..A A........GG
38        .......... .........T. ......TC.T ....T....T TTT...A... C........GG
39        .G........ .........T. .......C.. A...TCT..T T..A.AA..G .........T.
40        .G........ .........T. .......C.. A...T.T..T T..A.AA..G .........T.
41        .......... .........T. ......AC.T G...-GA.TA ..TC.....- -A.C..G.AA
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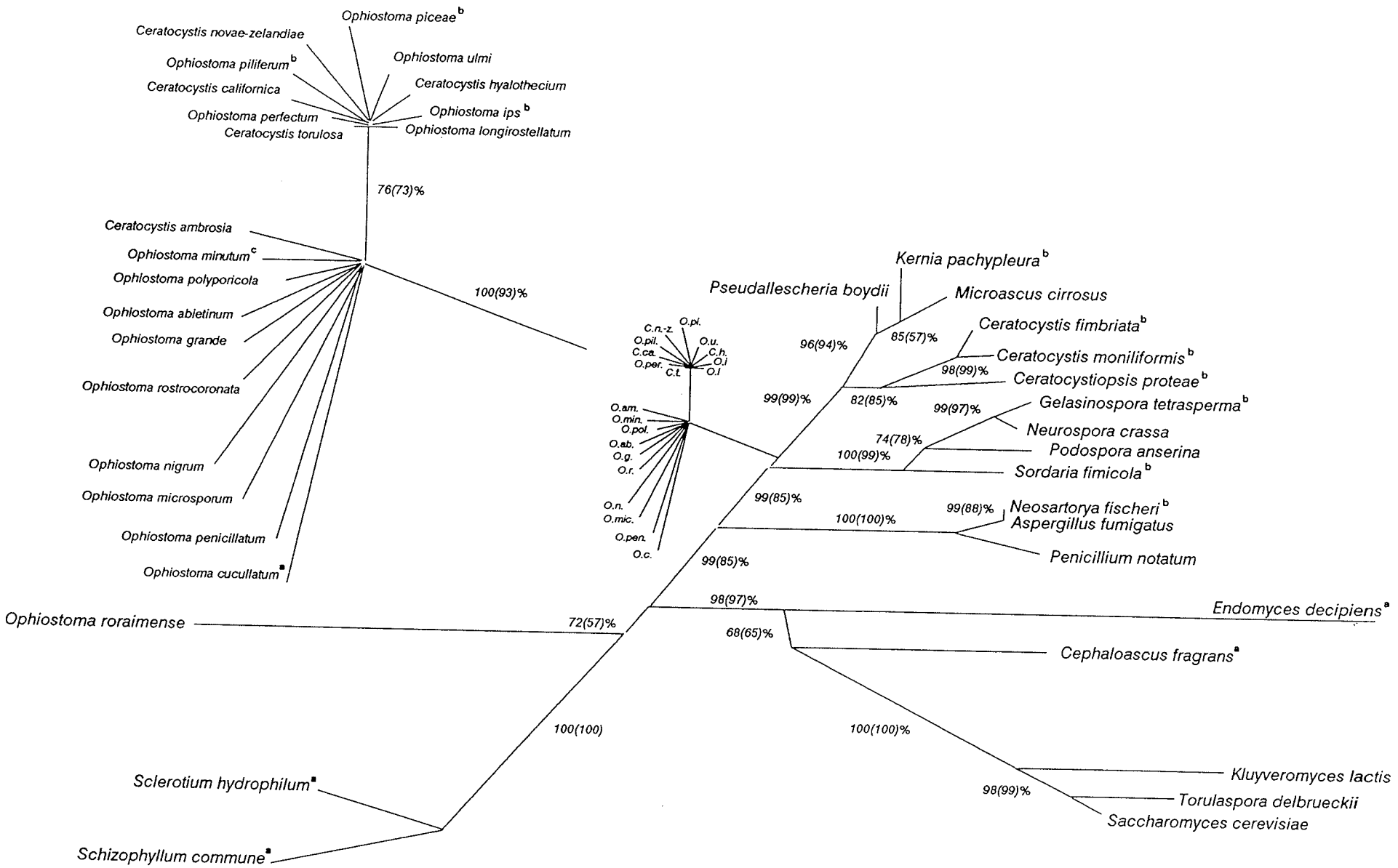
	1694						
1	CCCCCCCAGG	CCG--GAAAG	TTATCCAAAC	TC-GGTCATT	TAGAGGAAGT	AAAAGT	
2	..T....G..	....	C.........	....	....	......	
3	...TT..G..	....	....	....	....	......	
4	...TT..G..	....	C.........	....	....	......	
5	.......G..	....	....	....	....	......	
6	...TT.....	....	....	....	....	......	
7	...TT..G..	....	C.........	....	....	......	
8	...TT..G..	....	C.........	....	....	......	
9	...TT..G..	....	C.........	....	....	......	
10	...TT..G..	....	C.........	....	....	......	
11	...TT..G..	....	C.........	....	....	......	
12	.......G..	.G........	....	....	....	......	
13	...TT..G..	....	....	....	....	......	
14	...TT..G..	....	C.........	....	....	......	
15	.......G..	.G....G...	....	....	....	......	
16	.......G..	....	C.........	....	....	......	
17	...TT..G..	....	C.........	....	....	......	
18	...T...G..	....	C.........	....	....	......	
19	..TT...G..	....	C.........	....	....	......	
20	...TT..G..	....	C.........	....	....	......	
21	.AT.T.AGA.	.G...AG..T	C.GGT.....	.T........	........C.	......	
22	......AGA.	.....A...C	..GGT.....	....	....	......	
23	.T....AGA.	....	..GGT.....	C.........	....	......	
24	.T....AGA.	....	..GGT.....	C.........	....	......	
25	..A...AG..	....	C.........	....	....	......	
26	.AA...AG..	....	C.........	....	....	......	
27	.A...AGG.A	GG.CG.....	C.........	....	....	......	
28	.A...-AG..	....	C.........	....	....	......	
29	....T.AT..	....	C.G.T.....	....	C.........	......	
30	....T.AT..	....	C...T.....	C	....	......	
31	GGA.T.AG..	....	..G.T.....	....	....	......	
32	..A.T..G..	....	C.G.T.....	....	C.........	......	
33	..A.T.AG..	....	..G.......	....	....	......	
34	..A.T.AG..	....	..G.......	....	....	......	
35	.AA.TTGGAA	.....-....	C.G.T.....	.T........	....	......	
36	TTTAGA.GA.	AT...AG...	C..GT.....	.T........	....	......	
37	.A..T.ATT.	.T...AG...	..GAT.....	.T........	....	......	
38	.A.T.GATT.	.T...AG...	..GAT.....	.T........	....	......	
39	.AT.T.AGA.	.G...AG..T	..GGA.....	.T........	........C.	......	
40	.AT.T.AGA.	.GA..AG..T	C.GGT.....	.T........	........C.	......	
41	-------T..	..ACCCTG.-	CGGCT.....	..TT......	....	......	

Figure 2. Unrooted neighbor-joining network showing the phyletic relationships between species assignable to Ophiostoma and other ophiostomataceous and ascomycetous fungi. The network was generated by the analysis of the partial SSrDNA data (about 700 positions) with the NEIGHBOR and DNAPARS program. The branch lengths are those as determined by the NEIGHBOR program. The percentages along the branches are an indication of the level of confidence for major branches as determined by bootstrap analysis. The percentages given in brackets are the bootstrap support for major branches when the data was analyzed by DNAPARS. Branches with a level of confidence less then 70% were reduced to polychotomies.

[a]Chapter 2; [b]Chapter 3; [c]Chapter 4.


Ophiostoma piceae b
Ceratocystis novae-zelandiae
Ophiostoma piliferum b
Ceratocystis californica
Ophiostoma perfectum
Ceratocystis torulosa
Ophiostoma ulmi
Ceratocystis hyalothecium
Ophiostoma ips b
Ophiostoma longirostellatum
76(73)%
Ceratocystis ambrosia
Ophiostoma minutum c
Ophiostoma polyporicola
Ophiostoma abietinum
Ophiostoma grande
Ophiostoma rostrocoronata
Ophiostoma nigrum
Ophiostoma microsporum
Ophiostoma penicillatum
Ophiostoma cucullatum a
100(93)%
C.n.-z.
O.pi.
O.pil.
O.u.
C.ca.
C.h.
O.per.
O.i
C.t.
O.l
O.am.
O.min.
O.pol.
O.ab.
O.g.
O.r.
O.n.
O.mic.
O.pen.
O.c.
Kernia pachypleura b
Pseudallescheria boydii
Microascus cirrosus
96(94)%
85(57)%
Ceratocystis fimbriata b
Ceratocystis moniliformis b
98(99)%
Ceratocystiopsis proteae b
99(99)%
82(85)%
99(97)%
Gelasinospora tetrasperma b
Neurospora crassa
74(78)%
Podospora anserina
100(99)%
Sordaria fimicola b
99(85)%
99(88)%
Neosartorya fischeri b
Aspergillus fumigatus
100(100)%
Penicillium notatum
99(85)%
98(97)%
Endomyces decipiens a
Ophiostoma roraimense
72(57)%
68(65)%
Cephaloascus fragrans a
100(100)
100(100)%
Kluyveromyces lactis
98(99)%
Torulaspora delbrueckii
Saccharomyces cerevisiae
Sclerotium hydrophilum a
Schizophyllum commune a

tested. The evolutionary distance values between O. cucullatum and other Ophiostoma species range from 0.0147 (O. penicillatum and O. microsporum) to 0.0282 (O. perfectum). This compares with evolutionary distances of 0.0177 between K. pachypleura and M. cirrosus, 0.0284 between Sordaria fimicola and P. anserinia, or 0.0103 between G. tetrasperma and N. crassa.

Inferred relatedness of 55 Ophiostoma species based on partial LSrDNA sequence data

To obtain an overview of the evolutionary trends amongst species assigned to Ophiostoma, the 5' terminal region of the LSrDNA gene (about 260 nucleotide positions) was determined for 99 strains of fungi. These included 22 strains (representing 18 species) which served as outgroups, and 77 strains representing 55 species assignable to Ophiostoma (Fig. 3). The 5' terminal domain of the LSrDNA contains a variety of segments that differ in their rates of nucleotide substitution, thereby providing a set of internal indicators that are suited for the estimation of both short- and long-range phylogenetic relationships (Qu et al. 1988). As well, the 5' terminal end of the LSrDNA gene containing Domain I has already been shown to be useful for studying the phylogeny of Fusarium species (Guadet et al. 1989).

The NEIGHBOR program (neighbor-joining) was chosen for the 5' terminal LSrDNA data set as this method generated identical dendrograms for each analysis run even when the

sequences were entered in a different input order (jumble option). The FITCH program generated dendrograms similar to those produced by the NEIGHBOR program, but the topology of the FITCH distance networks was influenced by the input order of the sequences. The partial LSrDNA data set was also run through the bootstrap option (200 reiterations) contained within the CLUSTAL V program. However for such a large dataset (low sequence character to taxa ratio), the level of confidence for most branches within the neighbor-joining distance network is expected to be low i.e. <90% (Gee 1992). Therefore the dendrogram (Fig. 4) should be viewed only as a preliminary effort to study DNA relatedness and evolutionary trends within Ophiostoma.

Using the basidiomycetous fungus S. hydrophilum as an outgroup, the dendrogram generated grouped the sequences in a pattern similar to that observed in the phylogenetic estimate based on SSrDNA genes. Again O. roraimense is placed as an intermediate between the Basidiomycotina and the Hemiascomycotina, while N. fischeri is placed outside of node 4 that unites all of the members of the Parenchymatomycetidae. The members of the Sordariaceae are grouped together (node 9) and both K. pachypleura and F. oxysporum appear to be intermediate between the Sordariaceae and the genus Ceratocystis s.s. And, as observed in the phylogenetic estimate based on the partial SSrDNA data, Cop. proteae groups next to the node that unites all the members of Ceratocystis s.s. (node 11). All strains assignable to

Ophiostoma (except O. roraimense) are derived from one branch (node 6), supporting the findings of the analysis of the SSrDNA data set which suggested that Ophiostoma represents a monophyletic grouping.

All the Ophiostoma strains tested are within a range of 0.0171 - 0.0947 evolutionary distance units (edu, as derived from Kimura's two parameter model) from a strain of O. piliferum, WIN(M) 932; this is the type species of Ophiostoma. However, the data suggests that there may be a problem with O. piliferum. The two strains we tested were from different sources (New Zealand and Sweden), and they exhibited enough sequence divergence to suggest that they may represent two different species. O. piliferum WIN(M) 932 and WIN(M) 22 have diverged by 0.0308 edu, but the neighbor-joining dendrogram groups the two O. piliferum strains together as a potential operational taxonomic unit. Evolutionary distances were calculated for the sequences with respect to O. piliferum, as these distances are more relevant when discussing relationships within Ophiostoma. Based on an arbitrary decision, we have used O. piliferum strain WIN(M) 932 as the reference organism for our data.

The distance between O. piliferum WIN(M) 932 and the tested species of the Sordariaceae and Ceratocystis s.s., ranges from 0.1147 to 0.1216 and 0.1161 to 0.1455 edu, respectively. Therefore one may assume that O. piliferum is as distant from species of Neurospora, Gelasinospora, and Sordaria as it is from species of Ceratocystis s.s.; this

provides further evidence for separating Ceratocystis s.l. into Ophiostoma and Ceratocystis s.s. (de Hoog and Scheffer 1984). The majority of strains assignable to Ophiostoma are within 0.05 edu of O. piliferum WIN(M) 932, with the most distant member assignable to Ophiostoma being C. pseudoeurophioides (edu 0.0947). Two species of the genus Togninia are grouped next to the branch that unites members of the Ophiostoma (node 7). Morphological and molecular evidence has already been presented, which distinguishes between Togninia and Ophiostoma (Eyjólfsdóttir 1990; Hausner et al. 1992), however the extensive survey of partial LSrDNA sequences presented herein suggests that Togninia could be closely related to Ophiostoma. Therefore a more extensive study to determine the phylogenetic position of other members of the Calosphaeriaceae to Ophiostoma might be warranted.

Guadet et al. (1989) did not observe sequence divergence in the 5' terminal end of the LSrDNA gene within morphologically homogeneous groups of Fusarium. Such results suggest that, although no criteria for species assignment based on rDNA sequence divergence is yet available, one should not expect sequence divergence to occur between strains supposedly representing a single species. No sequence divergence was observed between the six strains of O. ips tested, although they differed in geographical origin (Norway and New Zealand). Nor were nucleotide differences observed within the following species when more then one

strain was included for analysis: O. gossypinum (ATCC 18999 and ATCC 22063); C. novae-zelandiae (WIN(M) 863 and WIN(M) 864); O. bicolor (ATCC 1500 and ATCC 62329); O. canum (NFRI 1652/2 and NFRI 60-165); O. penicillatum (CBS 212.67, NFRI 1715/3 and NFRI 1716/3); O. ulmi (WIN(M) 780, 895, 902); and C. deltoideospora (CBS 187.86 and WIN(M) 71-26). Therefore sequence divergence within the 5' terminal region of the LSrDNA gene could be an indication of speciation.

However, a lack of sequence divergence between two or more species within the 5' terminal region of the LSrDNA should not be viewed as absolute evidence for synonymizing them, since, as Bruns et al. (1991) have noted, the significance of sequence divergence may be very different between different taxonomic groups. It must be recognized that it requires thorough sampling of taxa to make taxonomic decisions from molecular data, and even then they will be arbitrary. But a lack of sequence divergence between two or more species would probably suggest they are closely related. If so, then the data indicates that O. longirostellatum, O. conicolum, and C. ponderosae are closely related taxonomic units as are O. ulmi and O. piceae, although one strain of O. piceae (CBS 263.35) differed by one substitution from the O. ulmi and O. piceae (WIN(M) 324) sequence. In general few differences could be detected at the rDNA sequence level between any of the species clustered by node 24 (O. minus to C. tetropii, including O. ulmi). In addition, a lack of sequence

divergence was also observed between O. bicolor and C. ossiformis, and the data shows that O. gossypinum and O.tax.sp.I differ by only one substitution. The latter example shows the utility of partial LSrDNA sequences in placing an undescribed species within a genus near potentially closely related species.

The data also indicate that the strains of C. coronata and O. montium tested could both be heterogenous assemblages. Of the three strains identified as C. coronata, two strains (WIN(M) 867 and 868) differed by only one substitution, but the third strain (WIN(M) 71-26) grouped apart to form a potential OTU with O. rostrocoronatum. Hutchinson and Reid (1988) already questioned whether their putative C. coronata strains from New Zealand (WIN(M) 867 and 868) should be asigned to C. coronata, but they felt that the morphological variation expressed in these New Zealand strains was insufficient to justify the designation of a new species. Similarily, two strains of O. montium (CBS 151.78 and WIN(M) 497) lack sequence divergence, but strain ATCC 24285 appears to be unrelated, and it grouped with O.tax.sp.II and O. tetropii.

Although O. ips and O. adjunctum are viewed as synonyms by Upadhyay (1981), they differed by the deletion of one nucleotide position. The significance of this finding is debatable, but it was shown previously (Chapter 1) that both O. montium and O. adjunctum can be readily distinguished from O. ips by RFLP patterns of mitochondrial DNA and rDNA.

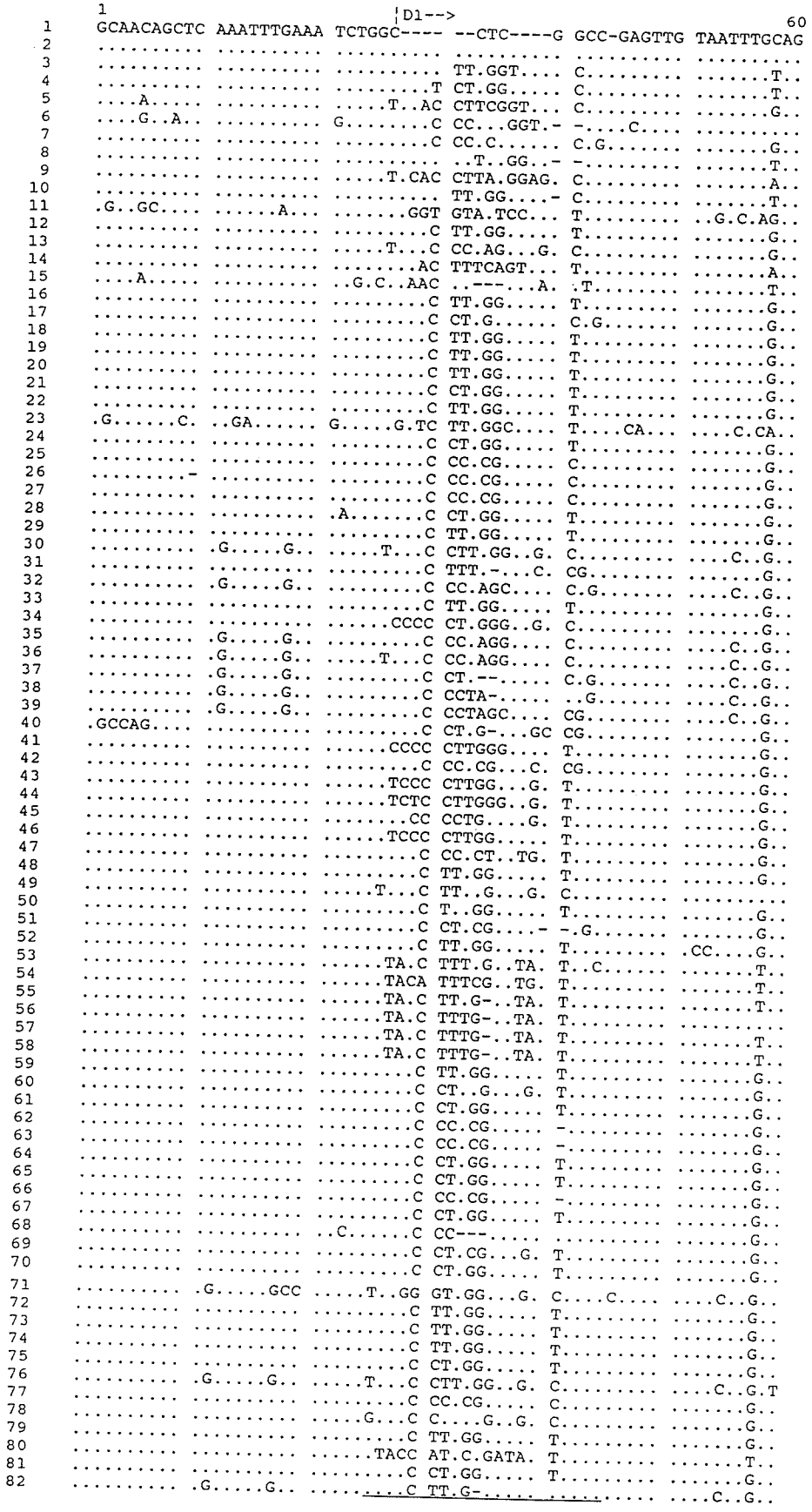
The partial LSrDNA data also suggests that the following synonymies proposed by Upadhyay (1981) might not be valid, and therefore the morphological criteria used for making these combinations should be revaluated: O. distorta (=C. torulosa), O. montium (=O. adjunctum), O. olivaceum (=C. vesca), O. populinum (=C. ponderosae), O. penicillatum (=C. pseudoeurophioides), O. piliferum (=C. longirostellata), O. piceaperdum (=O. europhioides), and O. piliferum (=C. ambrosia); this also applies to C. californica (=O. longirostellatum) (de Hoog 1974).

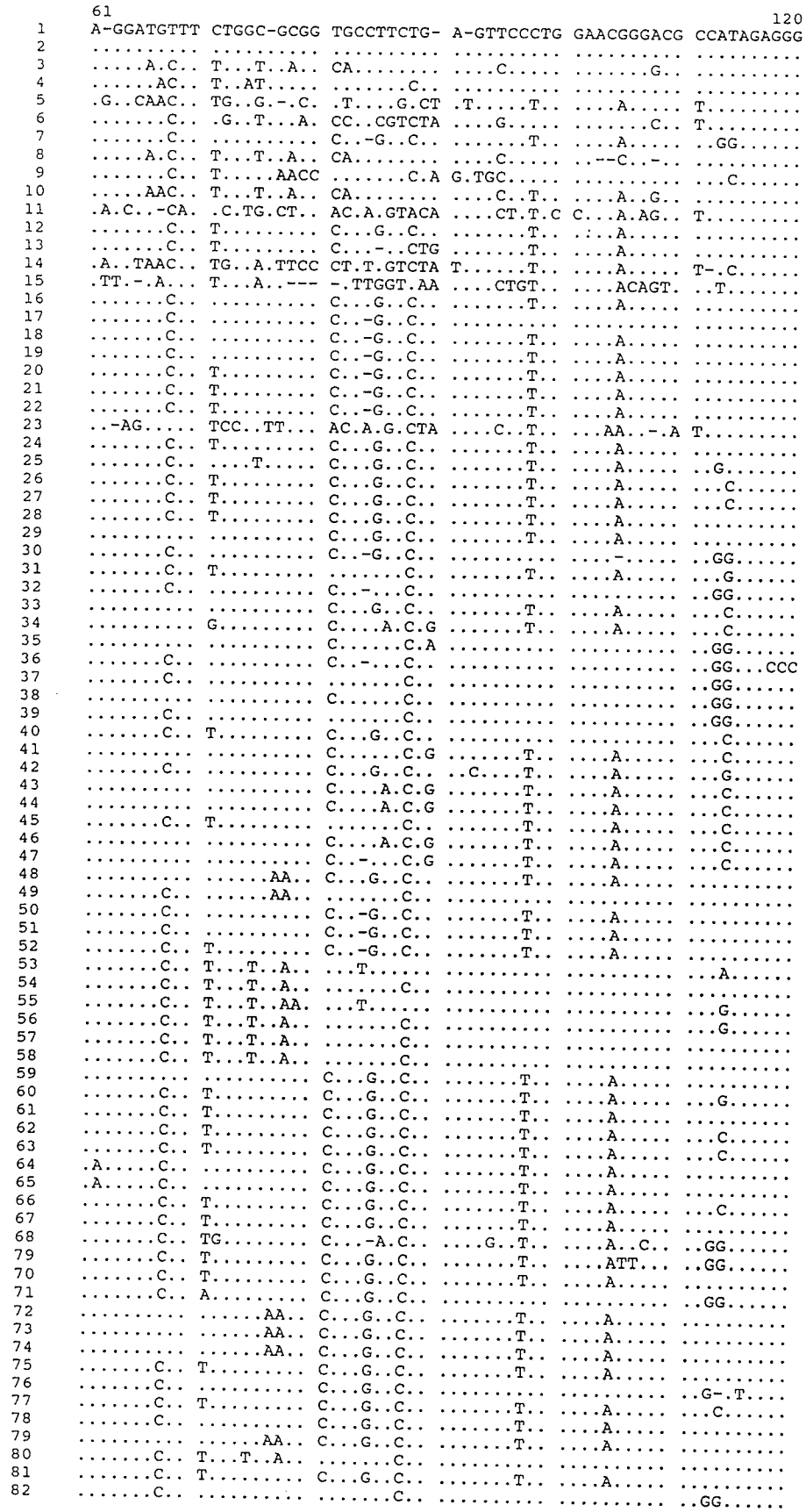
The clustering obtained by analyzing partial LSrDNA sequences could not be correlated precisely with either the type of anamorph produced by the tested species, or the shape of its ascospores. But subgroups within Ophiostoma can be distinguished based on such features; in particular groupings can be recognized based on ascospore shape as proposed by Olchowecki and Reid (1974) for Ceratocystis s.l. For example node 18 groups 14 species, including O. piliferum, which are characteristic of the Pilifera-spore group of Olchowecki and Reid (1974). This grouping by node 18 also contains a strain of O. montium (887) which, if accurately identified, would pose a problem because it has Ips-type spores. However this strain may have been misidentified from the beginning. It does not now fruit in culture, so its true identity cannot be checked. Five species (including O. ips) assignable to the Ips-spore group of Olchowecki and Reid (1974) are grouped together by node

22. Six falcate ascospore species which would be members of the Minuta-spore group (including O. minutum) are clustered together by node 20, and nine species which could be assigned to their Fimbriata-spore group are clustered together by node 12. The latter include Ophiostoma species with Phialographium-, Phialocephala-, and Leptographium-like anamorphs and, in addition, this section includes the most distant members of Ophiostoma with respect to the type species, with the evolutionary distance values ranging from 0.0572 to 0.0947.

O. microsporum (edu 0.0819), O. nigrum (edu 0.0671), O. longisporum (edu 0.0887), and C. deltoideospora (edu 0.0887) hold an intermediate position between the above species with Fimbriata-type spores (node 12) and the remaining species of Ophiostoma. The placement of the remaining Ophiostoma species by partial LSrDNA data shows no correlation with either the type of anamorphs or the shape of the ascospores produced. For example, species with falcate-type ascospores also group with species which lack such ascospores; O. retusum is placed near O. conicolum, and O. crassivaginatum is grouped with species producing Fimbriata-type spores but conidial states assignable to Leptographium.

Fig. 3. Alignment of the 5' terminal end of the LSrRNA gene. Nucleotide positions 1-265 of the alignment correspond to positions 102-360 in the LSrRNA of Saccharomyces cerevisiae (Guadet et al. 1989). Positions that are underlined were excluded from the analysis as it was difficult to obtain reliable alignments.




61
120
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2 ....
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4 ......AC.. T..AT..... .....C.. ....
5 .G..CAAC.. TG..G.-.C. .T....G.CT .T.....T.. ....A..... T.....
6 .....C.. .G..T...A. CC..CGTCTA ....G..... .....C.. T.....
7 .....C.. ..... C..-G..C.. .....T.. ....A..... ..GG.....
8 .....A.C.. T...T..A.. CA..... ....C..... ..--C..-.. .....
9 .....C.. T.....AACC .....C.A G.TGC..... ....C.....
10 .....AAC.. T...T..A.. CA..... ....C..T.. ....A..G.. .....
11 .A.C..-CA. .C.TG.CT.. AC.A.GTACA ....CT.T.C C...A.AG.. T.....
12 .....C.. T..... C...G..C.. .....T.. ...A..... .....
13 .....C.. T..... C...-..CTG .....T.. ....A..... .....
14 .A..TAAC.. TG..A.TTCC CT.T.GTCTA T.....T.. ....A..... T-.C.....
15 .TT.-.A... T...A..--- -.TTGGT.AA ....CTGT.. ....ACAGT. ..T.....
16 .....C.. ..... C...G..C.. .....T.. ....A..... .....
17 .....C.. ..... C..-G..C.. ....
18 .....C.. ..... C..-G..C.. .....T.. ....A..... .....
19 .....C.. ..... C..-G..C.. .....T.. ....A..... .....
20 .....C.. T..... C..-G..C.. .....T.. ....A..... .....
21 .....C.. T..... C..-G..C.. .....T.. ....A..... .....
22 .....C.. T..... C..-G..C.. .....T.. ....A..... .....
23 ..-AG..... TCC..TT... AC.A.G.CTA ....C..T.. ...AA..-.A T.....
24 .....C.. T..... C...G..C.. .....T.. ....A..... .....
25 .....C.. ....T..... C...G..C.. .....T.. ....A..... ..G.....
26 .....C.. T..... C...G..C.. .....T.. ....A..... ...C.....
27 .....C.. T..... C...G..C.. .....T.. ....A..... ...C.....
28 .....C.. T..... C...G..C.. .....T.. ....A..... .....
29 .... C...G..C.. .....T.. ....A..... .....
30 .....C.. ..... C..-G..C.. ....-..... ..GG.....
31 .....C.. T..... .....C.. .....T.. ....A..... ...G.....
32 .....C.. ..... C..-...C.. ....GG.....
33 .... C...G..C.. .....T.. ....A..... ...C.....
34 ..... G..... C....A.C.G .....T.. ....A..... ...C.....
35 .... C......C.A ....GG.....
36 .....C.. ..... C..-...C.. ....GG...CCC
37 .....C.. ....C.. ....GG.....
38 .... C......C.. ....GG.....
39 .....C.. ....C.. ....GG.....
40 .....C.. T..... C...G..C.. ....C.....
41 .... C......C.G .....T.. ....A..... ...C.....
42 .....C.. ..... C...G..C.. ..C....T.. ....A..... ...G.....
43 .... C....A.C.G .....T.. ....A..... ...C.....
44 .... C....A.C.G .....T.. ....A..... ...C.....
45 .....C.. T..... .....C.. .....T.. ....A..... ...C.....
46 .... C....A.C.G .....T.. ....A..... ...C.....
47 .... C..-...C.G .....T.. ....A..... ...C.....
48 ....AA.. C...G..C.. .....T.. ....A..... .....
49 .....C.. .....AA.. .....C.. ....
50 .....C.. ..... C..-G..C.. .....T.. ....A..... .....
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52 .....C.. T..... C..-G..C.. .....T.. ....A..... .....
53 .....C.. T...T..A.. ...T..... ....A.....
54 .....C.. T...T..A.. .....C.. ....
55 .....C.. T...T..AA. ...T..... ....G.....
56 .....C.. T...T..A.. .....C.. ....G.....
57 .....C.. T...T..A.. .....C.. ....
58 .....C.. T...T..A.. .....C.. ....
59 .... C...G..C.. .....T.. ....A..... .....
60 .....C.. T..... C...G..C.. .....T.. ....A..... ...G.....
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65 .A.....C.. ..... C...G..C.. .....T.. ....A..... .....
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67 .....C.. T..... C...G..C.. .....T.. ....A..... .....
68 .....C.. TG..... C...-A.C.. ....G..T.. ....A..C.. ..GG.....
79 .....C.. T..... C...G..C.. .....T.. ....ATT... ..GG.....
70 .....C.. T..... C...G..C.. .....T.. ....A..... .....
71 .....C.. A..... C...G..C.. ....GG.....
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74 ....AA.. C...G..C.. .....T.. ....A..... .....
75 .....C.. T..... C...G..C.. .....T.. ....A..... .....
76 .....C.. ..... C...G..C.. ....G-.T....
77 .....C.. T..... C...G..C.. .....T.. ....A..... ...C.....
78 .....C.. ..... C...G..C.. .....T.. ....A..... .....
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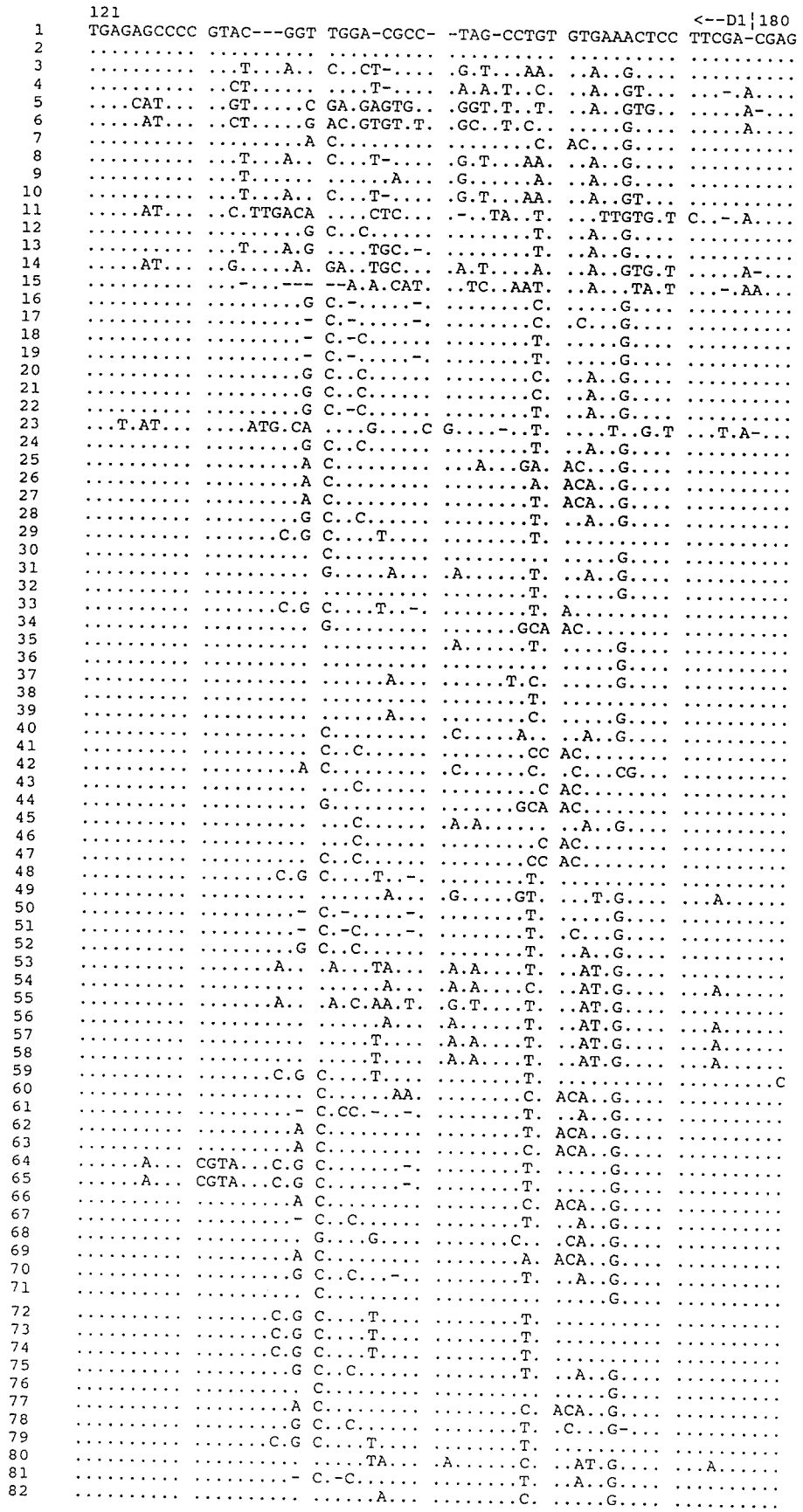
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```
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  3   .......... .......... .......... .......A.. ..C..C.... .........T
  4   .......... .......... ....T..... .......... ..C..C.... ..........
  5   ......T... .........A ....T..G.. ..T....A.. TCCA.C.... .........T
  6   ......T... .........A ....T..... ..T....A.. T.CA.C.... ..........
  7   .......... .......... .......... .......A.. T.C..C.... ..........
  8   .......... .......... .......... .......A.. T.C..C.... ..........
  9   .......... .......... .......... .......A.C CCC..C.... .........T
 10   .......... .......... .......... .......A.. T.C..C.... .........T
 11   ......T... .........A .......... ..T....A.. TCCA.C.... .......ATA
 12   .......... .......... .......... .......A.. T.C..C.... ..........
 13   .......... .......... .......... .......A.. T.C..C.... ..........
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 18   .......... .......... .......... .......A.. T.C..C.... ..........
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 22   .......... .......... .......... .......A.. T.C..C.... ..........
 23   ......T... .........A .......... ..T....A.. .CCA.C.... G........T
 24   .......... .......... .......... .......A.. T.C..C.... ..........
 25   .......... .......... .......... .......A.. T.C..C.... ..........
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 29   .......... .......... .......... .......A.. T.C..C.... .........T
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 32   .......... .......... .......... .......A.. T.C..C.... ..........
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 36   .......... .......... .......... .......A.. T.C..C.... ..........
 37   .......... .......... .......... .......A.. T.C..C.... .........T
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 80   .......... .......... ....T..... .......... C.C..C.... .........T
 81   .......... .......... .......... .......A.. T.C..C.... ..........
 82   .......... .......... .......... .......A.. T.C..C.... .........T
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     241
1    C-GGCCAGAG ACCGATAGCG CACAA Togninia fraxinopensylvanica
2    T......... .......... ..... Togninia novae-zealandiae
3    T......... .......... ..... Neurospora crassa
4    .......... .......... ..... Fusarium oxysporum
5    T....G.... .......... A.... Saccharomyces cerevisiae
6    T.....G... .......... ..... Neosartorya fischeri
7    .......... .......... ..... Ceratocystis ambrosia
8    .......... .......... ..... Gelasinospora tetrasperma
9    T......... ...-...... ..... Kernia pachypleura
10   A......... .......... ..... Sordaria fimicola
11   TT...G.... .......... A.... Sclerotium hydrophilum
12   .......... .......... ..... Ophiostoma bacillosporum
13   .......... .......... ..... Ceratocysits introcitrina
14   T....G.... .......... A.... Cephaloascus fragrans
15   TT...G.... .......... A.... Endomyces decipiens
16   .......... .......... ..... Ophiostoma montium
17   .......... .......... ..... Ceratocystis hyalothecium
18   .......... .......... ..... Ophiostoma flexuosum
19   .......... .......... ..... Ophiostoma adjuncti
20   T......... .......... ..... Ophiostoma minus
21   T......... .......... ..... Ceratocystis pseudominor
22   .......... .......... ..... Ophiostoma ulmi & O. piceae 324
23   T...GG.... .......... A.... Ophiostoma roraimense
24   .......... .......... ..... Ophiostoma canum
25   .......... .......... ..... Ophiostoma epigloeum
26   .......... .......... ..... Ophiostoma abietinum
27   .......... .......... ..... Ophiostoma polyporicola
28   .......... .......... ..... Ceratocystis torulosa
29   T......... .......... ..... Ceratocystis californica
30   .......... .......... ..... Ceratocystis vesca
31   .......... .......... ..... Ophiostoma nigrum
32   .......... .......... ..... Ophiostoma piceaperdum
33   T......... .......... ..... Ophiostoma perfectum
34   .......... .......... ..... Ophiostoma minuta-bicolor
35   .......... .......... ..... Ophiostoma crassivaginatum
36   .......... .......... ..... Ophiostoma francke-grossmaniae
37   T......... .......... ..... Ophiostoma dryocetidis
38   .......... .......... ..... Ophiostoma europhioides
39   T......... .......... ..... Ophiostoma pseudoeurophioides
40   .......... .......... ..... Ophiostoma microsporum
41   T......... .......... ..... Ophiostoma parvum
42   .......... .......... ..... Ophiostoma fasciatum
43   .......... .......... ..... Ophiostoma coliferum
44   .......... .......... ..... Ophiostoma minutum
45   T......... .......... ..... Ophiostoma longisporum
46   .......... .......... ..... Ophiostoma ranaculosum
47   T......... .......... ..... Ophiostoma minimum
48   T......... .......... ..... Ophiostoma retusum
49   .......... .......... ..... Ceratocystiopsis proteae
50   .......... .......... ..... Ophiostoma ips
51   .......... .......... ..... Ophiostoma piliferum WIN(M)932
52   .......... .......... ..... Ophiostoma tax. sp. II
53   A....T.... .......... ..... Ceratocystis fagacearum
54   A....T.... .......... ..... Ceratocystis moniliformis
55   A....T.... .......... ..... Ceratocystis adiposa
56   .....T.... .......... ..... Ceratocystis coerulescens
57   .....T.... .......... ..... Ceratocystis radicicola
58   A....T.... .......... ..... Ceratocystis paradoxa
59   T......... .......... ..... Ophiostoma populinum
60   .......... .......... ..... Ophiostoma rostrocoronatum
61   .......... .......... ..... Ophiostoma araucariae
62   .......... .......... ..... Ceratocystis coronata WIN(M) 867
63   .......... .......... ..... Ceratocystis coronata WIN(M) 868
64   .......... .......... ..... Ophiostoma bicolor
65   .......... .......... ..... Ceratocystis ossiformis
66   .......... .......... ..... Ophiostoma gossypinum
67   .......... .......... ..... Ophiostoma distortum
68   T......... .......... ..... Ceratocystis deltoideospora
69   .......... .......... ..... Ceratocystis coronata WIN(M) 71-26
70   .......... .......... ..... Ophiostoma montium ATCC 24285
71   .......... .......... ..... Ophiostoma olivaceum
72   T......... .......... ..... Ophiostoma longirostellatum
73   T......... .......... ..... Ophiostoma conicolum
74   T......... .......... ..... Ceratocystis ponderosae
75   .......... .......... ..... Ophiostoma tetropii
76   .......... .......... ..... Ophiostoma cucullatum
77   .......... .......... ..... Ophiostoma tax. sp. I
78   .......... .......... ..... Ophiostoma piliferum WIN(M) 22
79   T......... .......... ..... Ceratocystis novae-zelandiae
80   A....T.... .......... ..... Ceratocystis fimbriata
81   .......... .......... ..... Ophiostoma piceae CBS 263.65
82   T......... .......... ..... Ophiostoma penicillatum
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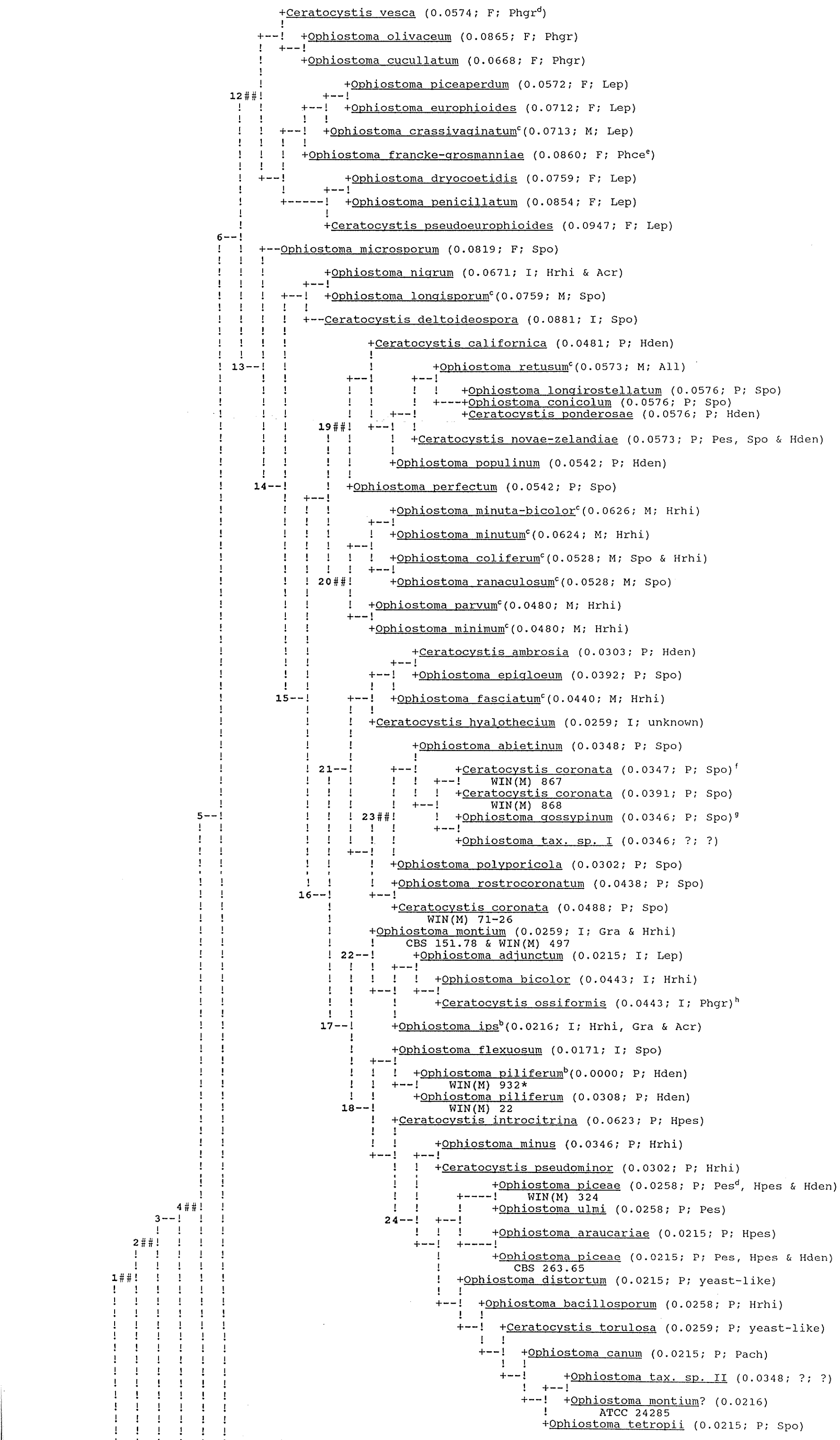
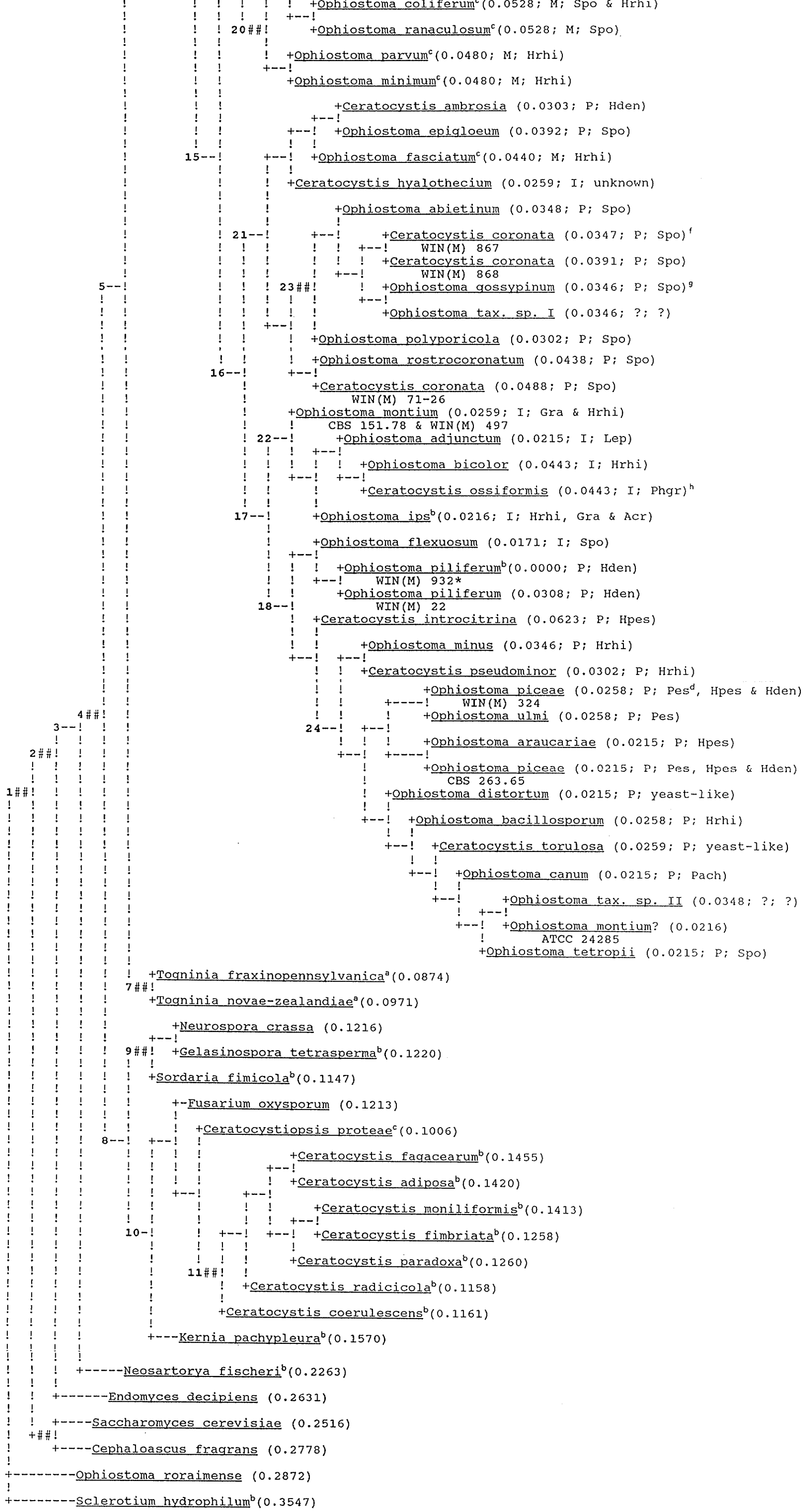
Fig. 4. Dendrogram based on a majority rule consensus tree obtained from analysing the partial LSrDNA data set with the NEIGHBOR program (neighbor-joining option) showing DNA sequence relatedness and clustering of potential operational taxonomic units for Ophiostoma species. Nodes defined by ## have >75% level of confidence based on bootstrap analysis (200 replicates). Branch lengths are not drawn to scale. Following the name of the Ophiostoma species the brackets contain the following information: (i) the evolutionary distance with respect to O. piliferum WIN(M) 932 as calculated by DNADIST; (ii) ascospore type as defined by Olchowecki and Reid (1974), i.e. Fimbriata (F) type, Minuta (M) type, Ips (I) type, and Pilifera (P) type; (iii) Hyphomycete genera which most closely resemble the anamorph (Acr = Acremonium; All = Allescheriella; Gra = Graphilbum; Hden = Hyalodendron; Hpes = Hyalopesotum; Hrhi = Hyalorhinocladiella; Lep = Leptographium; Pac = Pachnodium; Pes = Pesotum; Phce = Phialocephala; Phgr = Phialographium; Spo = Sporothrix).

[a]Chapter 2; [b]Chapter 3; [c]Chapter 4; [d]Wingfield et al. (1991) consider Pesotum and Phialographium to be synonyms of Graphium; [e]Wingfield et al. (1989) and Mouton et al. (1992) argue that the anamorph of Ophiostoma francke-grosmanniae should be placed in Leptographium; [f]Ceratocystis tenella Davids. (= C. coronata fide Upadhyay 1981); [g]Ceratocystis stenoceras (Robak) C. Moreau (= Ophiostoma gossypinum fide Upadhyay 1981); [h]Ceratocystis columnaris Olchow. & Reid (= C. ossiformis fide Upadhyay 1981).


+Ceratocystis vesca (0.0574; F; Phgr[d])
+Ophiostoma olivaceum (0.0865; F; Phgr)
+Ophiostoma cucullatum (0.0668; F; Phgr)
+Ophiostoma piceaperdum (0.0572; F; Lep)
12##
+Ophiostoma europhioides (0.0712; F; Lep)
+Ophiostoma crassivaginatum[c](0.0713; M; Lep)
+Ophiostoma francke-grosmanniae (0.0860; F; Phce[e])
+Ophiostoma dryocoetidis (0.0759; F; Lep)
+Ophiostoma penicillatum (0.0854; F; Lep)
+Ceratocystis pseudoeurophioides (0.0947; F; Lep)
6
+Ophiostoma microsporum (0.0819; F; Spo)
+Ophiostoma nigrum (0.0671; I; Hrhi & Acr)
+Ophiostoma longisporum[c](0.0759; M; Spo)
+Ceratocystis deltoideospora (0.0881; I; Spo)
+Ceratocystis californica (0.0481; P; Hden)
+Ophiostoma retusum[c](0.0573; M; All)
13
+Ophiostoma longirostellatum (0.0576; P; Spo)
+Ophiostoma conicolum (0.0576; P; Spo)
+Ceratocystis ponderosae (0.0576; P; Hden)
19##
+Ceratocystis novae-zelandiae (0.0573; P; Pes, Spo & Hden)
+Ophiostoma populinum (0.0542; P; Hden)
14
+Ophiostoma perfectum (0.0542; P; Spo)
+Ophiostoma minuta-bicolor[c](0.0626; M; Hrhi)
+Ophiostoma minutum[c](0.0624; M; Hrhi)
+Ophiostoma coliferum[c](0.0528; M; Spo & Hrhi)
20##
+Ophiostoma ranaculosum[c](0.0528; M; Spo)
+Ophiostoma parvum[c](0.0480; M; Hrhi)
+Ophiostoma minimum[c](0.0480; M; Hrhi)
+Ceratocystis ambrosia (0.0303; P; Hden)
+Ophiostoma epigloeum (0.0392; P; Spo)
15
+Ophiostoma fasciatum[c](0.0440; M; Hrhi)
+Ceratocystis hyalothecium (0.0259; I; unknown)
+Ophiostoma abietinum (0.0348; P; Spo)
21
+Ceratocystis coronata (0.0347; P; Spo)[f]
WIN(M) 867
+Ceratocystis coronata (0.0391; P; Spo)
WIN(M) 868
5
23##
+Ophiostoma gossypinum (0.0346; P; Spo)[g]
+Ophiostoma tax. sp. I (0.0346; ?; ?)
+Ophiostoma polyporicola (0.0302; P; Spo)
+Ophiostoma rostrocoronatum (0.0438; P; Spo)
16
+Ceratocystis coronata (0.0488; P; Spo)
WIN(M) 71-26
+Ophiostoma montium (0.0259; I; Gra & Hrhi)
CBS 151.78 & WIN(M) 497
22
+Ophiostoma adjunctum (0.0215; I; Lep)
+Ophiostoma bicolor (0.0443; I; Hrhi)
+Ceratocystis ossiformis (0.0443; I; Phgr)[h]
17
+Ophiostoma ips[b](0.0216; I; Hrhi, Gra & Acr)
+Ophiostoma flexuosum (0.0171; I; Spo)
+Ophiostoma piliferum[b](0.0000; P; Hden)
WIN(M) 932*
+Ophiostoma piliferum (0.0308; P; Hden)
WIN(M) 22
18
+Ceratocystis introcitrina (0.0623; P; Hpes)
+Ophiostoma minus (0.0346; P; Hrhi)
+Ceratocystis pseudominor (0.0302; P; Hrhi)
+Ophiostoma piceae (0.0258; P; Pes[d], Hpes & Hden)
WIN(M) 324
+Ophiostoma ulmi (0.0258; P; Pes)
4##
3
24
+Ophiostoma araucariae (0.0215; P; Hpes)
2##
+Ophiostoma piceae (0.0215; P; Pes, Hpes & Hden)
CBS 263.65
1##
+Ophiostoma distortum (0.0215; P; yeast-like)
+Ophiostoma bacillosporum (0.0258; P; Hrhi)
+Ceratocystis torulosa (0.0259; P; yeast-like)
+Ophiostoma canum (0.0215; P; Pach)
+Ophiostoma tax. sp. II (0.0348; ?; ?)
+Ophiostoma montium? (0.0216)
ATCC 24285
+Ophiostoma tetropii (0.0215; P; Spo)


+Ophiostoma coliferum[c](0.0528; M; Spo & Hrhi)
20##
+Ophiostoma ranaculosum[c](0.0528; M; Spo)
+Ophiostoma parvum[c](0.0480; M; Hrhi)
+Ophiostoma minimum[c](0.0480; M; Hrhi)
+Ceratocystis ambrosia (0.0303; P; Hden)
+Ophiostoma epigloeum (0.0392; P; Spo)
15--
+Ophiostoma fasciatum[c](0.0440; M; Hrhi)
+Ceratocystis hyalothecium (0.0259; I; unknown)
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21--
+Ceratocystis coronata (0.0347; P; Spo)[f]
WIN(M) 867
+Ceratocystis coronata (0.0391; P; Spo)
WIN(M) 868
5--
23##
+Ophiostoma gossypinum (0.0346; P; Spo)[g]
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+Ophiostoma rostrocoronatum (0.0438; P; Spo)
16--
+Ceratocystis coronata (0.0488; P; Spo)
WIN(M) 71-26
+Ophiostoma montium (0.0259; I; Gra & Hrhi)
CBS 151.78 & WIN(M) 497
22--
+Ophiostoma adjunctum (0.0215; I; Lep)
+Ophiostoma bicolor (0.0443; I; Hrhi)
+Ceratocystis ossiformis (0.0443; I; Phgr)[h]
17--
+Ophiostoma ips[b](0.0216; I; Hrhi, Gra & Acr)
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+Ophiostoma piliferum[b](0.0000; P; Hden)
WIN(M) 932*
+Ophiostoma piliferum (0.0308; P; Hden)
18--
WIN(M) 22
+Ceratocystis introcitrina (0.0623; P; Hpes)
+Ophiostoma minus (0.0346; P; Hrhi)
+Ceratocystis pseudominor (0.0302; P; Hrhi)
+Ophiostoma piceae (0.0258; P; Pes[d], Hpes & Hden)
WIN(M) 324
4##
+Ophiostoma ulmi (0.0258; P; Pes)
3--
24--
+Ophiostoma araucariae (0.0215; P; Hpes)
2##
+Ophiostoma piceae (0.0215; P; Pes, Hpes & Hden)
CBS 263.65
1##
+Ophiostoma distortum (0.0215; P; yeast-like)
+Ophiostoma bacillosporum (0.0258; P; Hrhi)
+Ceratocystis torulosa (0.0259; P; yeast-like)
+Ophiostoma canum (0.0215; P; Pach)
+Ophiostoma tax. sp. II (0.0348; ?; ?)
+Ophiostoma montium? (0.0216)
ATCC 24285
+Ophiostoma tetropii (0.0215; P; Spo)
+Togninia fraxinopennsylvanica[a](0.0874)
7##
+Togninia novae-zealandiae[a](0.0971)
+Neurospora crassa (0.1216)
9##
+Gelasinospora tetrasperma[b](0.1220)
+Sordaria fimicola[b](0.1147)
+-Fusarium oxysporum (0.1213)
+Ceratocystiopsis proteae[c](0.1006)
8--
+Ceratocystis fagacearum[b](0.1455)
+Ceratocystis adiposa[b](0.1420)
+Ceratocystis moniliformis[b](0.1413)
10-
+Ceratocystis fimbriata[b](0.1258)
+Ceratocystis paradoxa[b](0.1260)
11##
+Ceratocystis radicicola[b](0.1158)
+Ceratocystis coerulescens[b](0.1161)
+---Kernia pachypleura[b](0.1570)
+-----Neosartorya fischeri[b](0.2263)
+------Endomyces decipiens (0.2631)
+----Saccharomyces cerevisiae (0.2516)
+##
+----Cephaloascus fragrans (0.2778)
+--------Ophiostoma roraimense (0.2872)
+--------Sclerotium hydrophilum[b](0.3547)

DISCUSSION

Ophiostoma, Ceratocystis s.s., and Microascus

Both the phylogenetic estimates based on the partial SSrDNA sequences, and the branching order noted within the dendrogram derived from the partial LSrDNA data support the view that Ophiostoma, as defined herein, represents a monophyletic taxon. The partial SSrDNA data set could provide a statistically valid framework for the assignment of strains to Ophiostoma based on the neighbor-joining method when definitive morphological characterizations cannot be obtained. With the application of PCR technology to fungal systematics, very little mycelium is required for analysis, and sequences can be determined quite rapidly from PCR amplification products that represent specific target regions (White et al. 1990; Bruns et al. 1991). For example, this study shows unequivocally that there is a problem with the strain analyzed under the name O. roraimense. Its sequence failed to group with other Ophiostoma sequences and, since the strain is supposedly derived from the type specimen, either O. roraimense is not an Ophiostoma, or the original culture represents an isolation error. The final disposition of this species must await the analysis of fresh teleomorph material.

Although Ophiostoma appears to be heterogeneous, the diverse morphological variations exhibited by its species

could be an indicator of continuous coevolution with their insect vectors and/or host plants. Therefore in Ophiostoma characters such as ascospore shape, conidial states, and features of the ascocarps (pigmentation, length of perithecial necks, presence or absence of ostiolar hyphae), although very useful for delimiting species, may not represent phylogenetic diversity. It appears, then, that the presence of cellulose and rhamnose in the hyphal walls of Ophiostoma species could be an important diagnostic feature. However, more species and representatives of other genera will have to be examined to evaluate the phylogenetic significance of these hyphal wall components.

Evolutionary relationships within the ophiostomataceous fungi are poorly understood due both to their simple and frequently convergent morphologies, and their great diversity. The study of evolutionary trends of morphological features based on molecular evolution should provide valuable insight into the phylogenetic significance of certain morphological characters. The results reported in chapters 2 and 4 have already demonstrated that both falcate and galeate (hat- or helmet-shaped) ascospores are found in unrelated groups of fungi, and are therefore examples of convergent evolution. The genus Ceratocystiopsis is based primarily on a morphological character that is subject to convergence, and therefore it should not be accepted. This is supported by the partial LSrDNA data which also confirms that members of Ceratocystiopsis should not be segregated

from Ophiostoma species. Nor does the data support the expanded concept of the Endomycetaceae proposed by Redhead and Malloch (1977); they submerged members of the Ophiostomataceae within the Endomycetaceae based on their belief that galeate ascospores are not likely to have had multiple originations during evolution.

The sequence data clearly shows that Ophiostoma and Ceratocystis s.s. do not form a monophyletic group. Thus similarities in the teleomorph states of their species are also due to convergence, as the species independently evolved mechanisms to facilitate insect dispersal of their ascospores. Wright and Cain (1961) noted that in species of Ceratocystis s.l. there appears to be a trend towards a completely irregular arrangement of globose, evanescent asci, and they observed that many species in this complex show transitional stages in the shape and disposition of the asci. They also argued that irregularly arranged asci increase the efficiency of fungi in oozing ascospores from the ascocarp for insect dispersal. This plectascaceous perithecial centrum was a character used to unite the members of the Ophiostomataceae (Luttrell 1951; Upadhyay 1981). However, recent work by Berbee and Taylor (1992b; 1992c), and this study, show that this type of centrum arrangement has arisen in unrelated groups of fungi, and therefore may be of little value in defining higher order relationships.

The genus Microascus Zukal was originally assigned to

the Ophiostomataceae by Nannfeldt (1932) because its species also produce small evanescent asci that appear to be scattered irregularly within the ascocarp. Luttrell (1951) noted that in Microascus species the centrum tissue was filamentous, but in Ophiostoma species it was parenchymatous. Subsequently the interpretation of the significance of centrum features has led to much confusion in proposing ordinal relationships for the ophiostomataceaous fungi, and for members of the Microascaceae (Benny and Kimbrough 1980; Upadhyay 1981; Barr 1990). The presented partial SSrDNA data show that Ophiostoma is not closley related to Microascus, and therefore the concept of the Microascales as proposed by Luttrell (1951), Upadhyay (1981), and Barr (1990) represents a polyphyletic arrangement. However the data also show that Ceratocystis s.s. has a kinship with the Microascaceae rather than the Ophiostomataceae, as both parsimony and distance analysis support the branch uniting the members of the Microascaeae with species of Ceratocystis s.s. and Cop. proteae at the 99% confidence level.

Wingfield et al. (1989) noted that Cop. proteae was a rather unusual Ceratocystiopsis species because it shared with species of Ceratocystis s.s. such characters as long perithecial necks, divergent ostiolar hyphae, and cycloheximide sensitivity. The present analysis also places Cop. proteae with Ceratocystis s.s., although the bootstrap confidence level based on distance and parsimony analysis

was only 82 and 85% resepectively. Cop. proteae was originally collected from insect-infested Protea repens in southern Africa, and therefore based on its ecology it also appears similar to species of Ceratocystis s.s. which include temperate and tropical species that grow on a wide variety of herbaceous and woody plants. By comparison, most Ophiostoma species are found on woody plants of temperate forests. Cop. proteae, with its unique anamorph, clearly needs to be accommodated within a new genus which, along with Ceratocystis s.s., would have its affinities within the Microascales.

Probably the most universally accepted concept of the Microascaceae is based on the work of Malloch (1970), and the most striking feature of the fungi assigned to this family is the dextrinoid character of the immature ascospores. The phyletic estimate based on the partial SSrDNA data supports the monophyly of the Microascaceae, and this includes species with ostiolate (M. cirrosus) and non-ostiolate (Kernia pachypleura, Pseudallescheria boydii) ascocarps; this is in agreement with the findings of Berbee and Taylor (1992c).

Based on partial SSrDNA sequences, the rDNA phylogeny has provided new insights into the phylogenetic relationships of ophiostomataceous fungi, and their position within the Ascomycotina. This sequence data indicates that Ophiostoma should remain the sole genus of the Ophiostomataceae, and this should be the sole family within

the Ophiostomatales, while Ceratocystis s.s. is best disposed of within the Microascales. Morphological features such as ascospore shape and the irregular arrangement of evanescent asci within the ascocarp, are noted as being subject to convergent evolution; therefore they probably are of limited use in proposing higher order relationships. However, ultimately more intensive taxon sampling, including representatives of many other ascomycetous genera, will be required to propose definitive ordinal relationships for the ophiostomataceous fungi.

Relatedness within the genus Ophiostoma

Ascospore shape, considered to be an important taxonomic character, has been used to delimit species and genera in many fungal groups, including the Ophiostomataceae (Redhead and Malloch 1977; Upadhyay 1981; von Arx and van der Walt 1987). Earlier workers (Wright and Cain 1961; Griffin 1968; Olchowecki and Reid 1974) noted that ascospore morphology appears to be a stable character in Ceratocystis s.l., and it was argued that the genus could be divided into sections based on ascospore morphology (Olchowecki and Reid 1974; Upadhyay 1981). For example Upadhyay and Kendrick (1975) segregated members of Ceratocystis s.l. with falcate ascospores, irrespective of anamorphs, into their new genus Ceratocystiopsis. More recently Zambino and Harrington (1992) studied isozyme variation in both species of the anamorphic genus Leptographium, and Ophiostoma species with

Leptographium anamorphs; they found there was some justification for subdividing Ophiostoma into sections or groups based on ascospore morphology as proposed by Olchowecki and Reid (1974). This could be construed as support for generic status for Ceratocystiopsis and, possibly, the segregating out of additional genera based on ascospore shape. However, the analysis of the partial LSrDNA data failed to support the subdivision of Ophiostoma into sections based on either ascospore shape or the nature type of the conidial state. Nontheless, there are smaller groupings within Ophiostoma that appear to be correlated with ascospore shape.

In addition, the branching pattern found within the partial LSrDNA sequence dendrogram does suggest that complex ascospore shapes such as those found in Ophiostoma species referable to the Fimbriata section of Olchowecki and Reid (1974), and elaborate conidial structures (Leptographium, Phialocephala, Phialographium), could represent ancestral characters within Ophiostoma. And there does appear to be a trend towards simpler ascospore shapes (e.g. the Pilifera spore group of Olchowecki and Reid 1974) and less complex and smaller conidial states (Hyalorhinocladiella, Hyalodendron, and Sporothrix) in this genus. The rDNA RFLP analysis presented in chapter 1 also indicates that Ophiostoma species with Leptographium anamorphs (O. pieceaperdum and O. penicillatum) are not closely related to species grouping with O. piliferum and O. ips.

Samuels (1992), in presenting arguments for distinguishing between Ceratocystis and Ophiostoma, suggested that those species of Ceratocystis s.l. with Phialographium and Phialocephala anamorphs could represent a distinct genus. However, the SSrDNA data for O. cucullatum, which has a Phialographium anamorph, indicates that its affinity is with Ophiostoma. The LSrDNA data set also groups species with Phialographium anamorphs (O. cucullatum, C. vesca, O. olivaceum) and Phialocephala anamorphs (O. francke-grosmannia) with other Ophiostoma species; in particular with species producing Leptographium anamorphs.

This study shows that there are still many species listed in Ceratocystis that belong in Ophiostoma. The necessary new combinations in Ophiostoma to be proposed are as follows:

Ophiostoma ambrosium (Bakshi) comb. nov.

= Ceratocystis ambrosia Bakshi, Trans. Brit. Mycol. Soc. **33**: 116. 1950. (Basionym).

Ophiostoma californicum (Devay, Davids. & Moller) comb. nov.

= Ceratocystis californica Devay, Davids. & Moller, Mycologia **60**: 639. 1968. (Basionym).

Ophiostoma deltoideosporum (Olchow. & Reid) comb. nov.

= Ceratocystis deltoideospora Olchow. & Reid, Can. J. Bot. **52**: 1691. 1974. (Basionym).

Ophiostoma hyalothecium (Davids.) comb. nov.

= Ceratocystis hyalothecium Davids., Mem. New. York. Bot. Gard. **28**: 47. 1976. (Basionym).

Ophiostoma introcitrinum (Olchow. & Reid) comb. nov.

= Ceratocystis introcitrina Olchow. & Reid, Can. J. Bot. **52**: 1706. 1974. (Basionym).

Ophiostoma novae-zelandiae (Hutchison & Reid) comb. nov.

= Ceratocystis novae-zelandiae Hutchison & Reid, N.Z.J. Bot. **26**: 70. 1988.

Ophiostoma ponderosae (Hinds & Davids.) comb. nov.

= Ceratocystis ponderosae Hinds & Davids., Mycologia **67**: 715. 1975. (Basionym).

Ophiostoma pseudoeurophioides (Olchow. & Reid) comb. nov.

= Ceratocystis pseudoeurophioides Olchow. & Reid, Can. J. Bot. **52**: 1700-1701. 1974. (Basionym).

Ophiostoma pseudominus (Olchow. & Reid) comb. nov.

= Ceratocystis pseudominor Olchow. & Reid, Can. J. Bot. **52**: 1708. 1974. (Basionym).

Ophiostoma torulosum (Butin & Zimmerman) comb. nov.

= Ceratocystis torulosa Butin & Zimmerman, Phytopath. Zeitschrift **74**: 284. 1972. (Basionym).

Ophiostoma vescum (Davids.) comb. nov.

= Ceratocystis vesca Davids., Mycologia **50**: 666-667. 1958. (Basionym).

CHAPTER 6

APPLICATION OF MOLECULAR TAXONOMY TO SPECIES OF *Ceratocystis s.s.*, *Togninia*, AND *Beauveria*.

INTRODUCTION

This chapter deals with three specific problems that arose during this study, but which are all related as they concern the use of ITS1 sequences to determine interspecific relationships. The first section shows that in species of Ceratocystis s.s., ITS1 sequences could be suitable for studying interspecific relationships. The remaining two sections deal with fungi that were isolated along with members of Ceratocystis s.l. from bark-beetle galleries in standing and fallen trees or cut timber. The latter two sections are based on certain aspects of the study by Eyjólfsdóttir (1990) where molecular data were obtained to evaluate taxonomic decisions on designating new species based on morphological criteria.

I. INFERRED RELATEDNESS OF FIVE SPECIES OF *Ceratocystis sensu stricto* BASED ON ANALYSIS OF THE RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACER I SEQUENCES

INTRODUCTION

During these studies on the phylogenetic relationships within the ophiostomataceous fungi, it was noted that the analysis of sequences from both the small subunit ribosomal DNA (SSrDNA) and large subunit ribosomal DNA (LSrDNA) were well suited for resolving relationships above the genus level. Based on either partial SSrDNA or LSrDNA sequences it was demonstrated (see chapters 2 and 4) that species of *Ceratocystis s.s.*, as defined by de Hoog and Scheffer (1984), are monophyletic, and only distantly related to those assigned to *Ophiostoma*. However the sequences from the rDNA coding region did not vary sufficiently to allow one to obtain statistically valid phyletic estimates amongst the species studied. Therefore, the internal transcribed spacer I (ITS1) region, which separates the SSrDNA gene from the 5.8s gene, was examined in five species of *Ceratocystis s.s.* to determine what level of interspecific phylogenetic resolution could be obtained.

It has been shown that ITS1 sequences exhibit low intraspecific variability in isolates of both *Armillaria* species (Anderson and Stasovski 1992) and *Phytophthora*

species (Lee and Taylor 1992), as well as in isolates of Fusarium sambucinum (O'Donnell 1992). Therefore ITS1 sequences could prove to be quite valuable for designing taxon-specific probes, for confirming anamorph/teleomorph connections, and also for distinguishing species which are very similar morphologically.

MATERIALS AND METHODS

DNA was extracted from the following strains: Ceratocystis adiposa (Butler) C. Moreau, CBS 127.27 (CBS = Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands); Ceratocystis coerulescens (Munch) Bakshi, WIN(M) 98 (J.R. collection); Ceratocystis major (von Beyma) C. Moreau, CBS 138.34; Ceratocystis paradoxa (Dade) C. Moreau, CBS 107.22; and Ceratocystis radicicola (Bliss.) C. Moreau, CBS 114.47. The polymerase chain reaction (PCR) amplification protocol used to generate sequencing templates, and the sequencing of the double-stranded PCR products were as described previously. The ITS1 region was amplified and sequenced in both directions with the SS3 (ACAAGGTCTCCGTTGGTG) and 5.8B (CTGCGTTCTTCATCGAT) primers.

Nucleotide sequences were aligned with the CLUSTAL V program (Higgins and Sharp 1989), and the aligned data set (Fig. 1) was then analyzed using programs contained within PHYLIP (version 3.4; Felsenstein 1991). Divergence (or distance) between two sequences was calculated by DNADIST (using Kimura's two parameter model) (Kimura 1980) and phyletic estimates were generated by the following programs FITCH, DNAML, DNAPARS, DNAPENNY and DNACOMP. Confidence intervals on tree topologies were estimated by bootstrap analysis (Felsenstein 1985), by generating 100 bootstrap replicates with either SEQBOOT for the FITCH, DNAML, and DNAPENNY programs or DNABOOT for parsimony analysis.

RESULTS AND DISCUSSION

Upadhyay (1981) considered C. major to be a synonym of C. adiposa, a decision that is supported by the sequence data because the tested strains have identical ITS1 sequences (Fig. 1). Based on evolutionary distance, the DNA relatedness suggests that C. coerulescens is the most distant of the species examined (Table 1), while C. paradoxa and C. radicicola appear to be more closely related to each other than either one is to C. coerulescens or C. adiposa. Phyletic estimates based on the distance matrix method (FITCH), maximum likelihood method (DNAML), parsimony method (DNAPARS and DNAPENNY), and the DNA compatibility program (DNACOMP) which selects a tree based on the maximum number of sites that require the minimum number of substitutions, all generated identical unrooted phyletic estimates (Fig. 2a and 2b). Thus the results indicate that the ITS1 region appears to contain sufficient sequence divergence to study interspecific relatedness within Ceratocystis s.s. by either distance or parsimony methods.

DNA relatedness did not correlate with ascospores shape, although the latter has been considered an important taxonomic character within this genus (Olchowecki and Reid 1974; Wolfaardt et al. 1992b). For example the ascospores of C. coerulescens superficially resemble those of C. radicicola (Upadhyay 1981), but DNA relatedness places C. radicicola near C. paradoxa. This suggests that ascospore

shape, although very useful for species delimitation, should not be construed as a character of phylogenetic significance above the species level. Similar findings were obtained by Wolfaardt et al. (1992a), who analyzed partial SSrRNA and LSrRNA sequences and concluded that diversity of ascospore shape in Ceratocystis s.s. is not indicative of polyphyly. DNA relatedness, however, may have some correlation with host and geographic origin. C. coerulescens, the most distant member in our analysis, occurs on conifers and hardwoods in temperate forests, whereas the other species tested are common in warmer areas and occur on a diverse variety of tropical plants.

As pathogens, species of Ceratocystis s.s. may be of economic significance: C. paradoxa causing diseases in palms and sugar cane; C. adiposa, black rot of sugar cane; and C. coerulescens, maple streak. Therefore ITS sequences could be very useful in confirming pathogen identity, in addition to containing enough variability to be useful for phylogenetic investigations. ITS sequences are flanked by conserved gene sequences, and are thus easy targets for PCR amplification. Ultimately, ITS sequences might prove to be more valuable for studying interspecific relatedness than the more highly conserved rDNA gene sequences (Baura et al. 1992). DNA sequences from the rDNA repeat unit have the potential to resolve relationships where reliable taxonomic characters are missing or have been misinterpreted due to convergence. However, sequences have to be obtained from appropriate

regions of the rDNA repeat unit in order to detect enough variability to permit statistical evaluation of the phyletic estimate. This will require studies to identify these regions for the taxa being investigated, as in different taxa different regions of the rDNA might have to be analyzed (Bruns et al. 1991). The results presented here indicate that future work within the genus Ceratocytsis might best be served by analyzing the ITS regions of its member species.

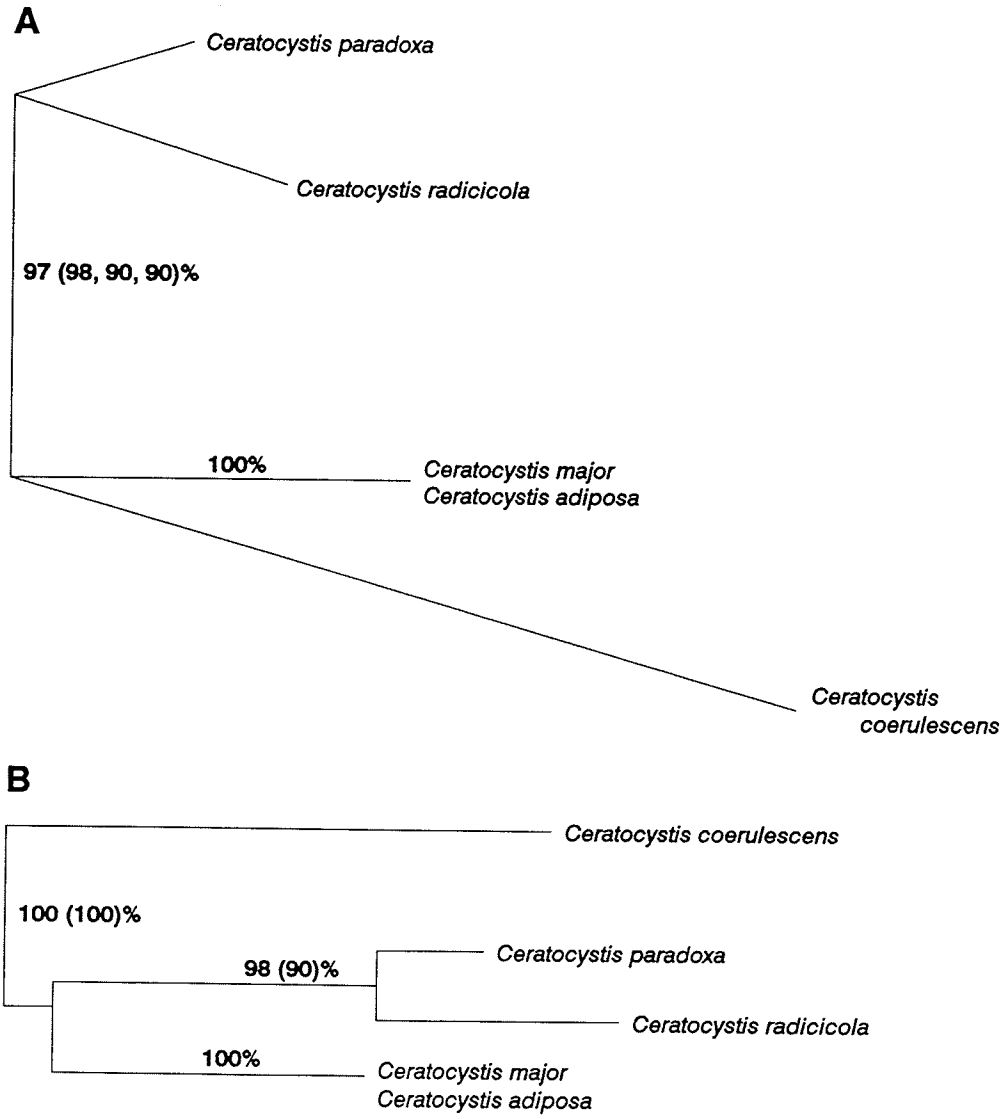
Fig. 1. Alignment of the ITS1 sequences of five Ceratocystis species (C.m. = C. major, C.a. = C. adiposa, C.r. = C. radicicola, C.p. = C. paradoxa, C.c. = C. coerulescens). The presumtive beginning and end of the ITS1 regions are indicated; these were determined by their homologies with published sequence data for Saccharomyces cerevisiae and Neurospora crassa (Chambers, Dutta & Crouch, 1986).

```
                ¦ITS1--->                                                50
C.m.    GATCATTACT GAGTTTTCAA CTCTAAAACC ATTTGTGAAC ATACCTATCT
C.a.    GATCATTACT GAGTTTTCAA CTCTAAAACC ATTTGTGAAC ATACCTATCT
C.r.    GATCATTATC GAGTTTCTAA CTCTTAAACC ATTTGTGAAC TTACCT-TTT
C.p.    GATCATTATC GAGTTTTTAA CTCTTAAACC ATTTGTGAAC TTACCT-TCT
C.c.    GATCATTACT CACTTTTTAA CTCTTAAACC ATATGTGAAC ATACCT--TT

                                                                 100
C.m.    TTAGCTGCTT TGGCGTGGTC TTCGGG-AT- --TGTGCCGG TAGTATTTAC
C.a.    TTAGCTGCTT TGGCGTGGTC TTCGGG-AT- --TGTGCCGG TAGTATTTAC
C.r.    TTAGCTGCTT TGGCAGTTAC TTCGGGGAA- --CCTGCCAG CAGTATAAAC
C.p.    --AGCTGCTT TGGCAGGT-C TTCGGG-A-- --TTTGCCGG TAGCACAAAC
C.c.    TTAGCTGCTT TGGCAGGCTT GGTAAAAACA AGTCTGCCGG TAGTATTTAA

                                                                 150
C.m.    AAA---CTCT TTTA-ATTTC TAGAGAATT- ATT--CATTG CTGAGTTGCA
C.a.    AAA---CTCT TTTA-ATTTC TAGAGAATT- ATT--CATTG CTGAGTTGCA
C.r.    AAA---CTCT TTTT-ATTTC TAGAGAATTT ATT--CATTG CTGAGTGGCA
C.p.    AAA---CTCT TTAT-ATTTC TATAGAATT- ATT--CATTG CTGAGTGGCA
C.c.    AAAAAACTCT TTAATATTTC TAGAGAATTT ATTTTCATTG CTGAGTGGCA

                          ¦5.8s--->          188
C.m.    TTTA-ACAAA TA-GTTAAAA CTTTCAACAA CGGATCTC
C.a.    TTTA-ACAAA TA-GTTAAAA CTTTCAACAA CGGATCTC
C.r.    TTAAC-TAAA TAAGTTAAAA CTTTCAACAA CGGATCTC
C.p.    TTAACATAAA TAAGTTAAAA CTTTCAACAA CGGATCTC
C.c.    TTAAACATAA TAAGTTAAAA CTTTCAACAA CGGATCTC
```

Table 1: Evolutionary distances for the ITS1 sequences of five Ceratocystis species

Organism	C. major	C. adiposa	C. radicicola	C. paradoxa
C. adiposa	0.0000			
C. radicicola	0.1513	0.1513		
C. paradoxa	0.1327	0.1327	0.0674	
C. coerulescens	0.1678	0.1678	0.2021	0.1928

Fig. 2. Unrooted distance network (A) showing the phyletic relationships between five Ceratocystis species. The branch lengths were drawn proportional and were determined by the DNAML program (maximum likelihood method). Percentages along the branches of the network are an indication of the level of confidence for branches as determined by bootstrap analysis (N = 100). The data was analyzed by 4 programs and boostrap support for the topology of the networks major branch are listed in the following order: (1) DNAML; (2) FITCH; (3) DNAPARS; (4) DNACOMP. A minimum-length phylogenetic tree (B) was obtained by parsimony (DNAPENNY) and distance method (FITCH). The most parsimonious tree required 69 steps and the branch lengths shown were calculated by the Fitch program. Sums of squares for this tree were 0.00146 with an average percentage standard deviation of 1.216. Level of confidence for branches given for (1) the FITCH analysis and (2) analysis by the DNAPENNY program.


A
Ceratocystis paradoxa
Ceratocystis radicicola
97 (98, 90, 90)%
100%
Ceratocystis major
Ceratocystis adiposa
Ceratocystis
coerulescens
B
Ceratocystis coerulescens
100 (100)%
98 (90)%
Ceratocystis paradoxa
Ceratocystis radicicola
100%
Ceratocystis major
Ceratocystis adiposa

II. ON TWO SPECIES OF *Togninia* AND THEIR TAXONOMIC POSITION WITH RESPECT TO *Ceratocystis s.l.*

INTRODUCTION

The placement of *Ceratocystis fraxinopennsylvanica* Hinds apud Hinds and Davidson (1975) in the genus *Ceratocystis s.l.* was questioned by Upadhyay (1981). He felt that this species only superficially resembled species of *Ceratocystis s.l.* and therefore proposed the new combination *Calosphaeria fraxinopennsylvanica* (Hinds) Upadh. In her study of potential wood-staining fungi isolated from bark-beetle galleries Eyjólfsdóttir (1990) described a new species that closely resembles *C. fraxinopennsylvanica*. However, based on morphological and cultural data, Eyjólfsdóttir felt that both her new species and *C. fraxinopennsylvanica* are distinct, but very closely related species of the genus *Togninia* Berl. Therefore after comparisons with specimens of *Togninia minima* (Tul. and C. Tul.) Berl., these two additional species were assigned to *Togninia* as *Togninia novae-zealandiae* n. sp. and *Togninia fraxinopennsylvanica* (Hinds) n. comb. Partial rDNA sequences were obtained in support of segregating *T. fraxinopennsylvanica* from *Ceratocystis s.l.*, and to distinguish between the two new species of *Togninia*.

MATERIALS AND METHODS

DNA was extracted from the following strains according to the procedures described in the Materials and Methods: T. novae-zealandiae WIN(M) 82-89 bi and 82-113 bi; and T. fraxinopennsylvanica (as Ceratocystis fraxinopennsylvanica ATCC 26664). The 5' terminal region of the LSrDNA was obtained for all three strains of Togninia, however the ITS1 was determined only for T. novae-zealandiae 82-89 bi and the strain of C. fraxinopennsylvanica. For the data analysis, sequences were added from the 5' terminal of the LSrDNA gene (containing Domain 1) of the following strains: O. ips WIN(M) 82-58; O. piliferum WIN(M) 932; C. moniliformis CBS 773.77; C. fagacearum ATCC 24789; Gelasinospora tetrasperma ATCC 11345; Neosartorya fischeri CBS 525.65. The corresponding sequences for Neurospora crassa (Guadet et al. 1989) and Saccharomyces cerevisiae (Georgiev et al. 1981) were obtained from the literature.

Nucleotide sequences of the 5' terminal of the LSrDNA were aligned with the CLUSTAL V and MASE programs, and then the data was analyzed with programs contained within PHYLIP (Felsenstein 1991). The sequence alignment is contained within Fig. 3, chapter 5. A majority rule consensus tree was contructed based on bootstrap analysis (100 replicates) of the aligned data set combined with distance (FITCH) and parsimony (DNABOOT) analysis.

The ITS1 sequences were aligned with the NALIGN program

(IntelliGenetics Inc.) using the following computational parameters: open gap cost, 10; and unit gap cost, 10.

RESULTS AND DISCUSSION

Eyjólfsdóttir's (1990) belief that T. fraxinopennsylvanica and T. novae-zealandiae are distinct species was based on the following criteria: (i) ascospore size and shape; (ii) ascus breadth; (iii) perithecial size and degree of ornamentation; (iv) neck length; and (v) culture growth rates. However, it might be argued that the degree of morphological variation observed between these two proposed new species of Togninia merely reflected what would be expected among strains of a single species taken from different locations. In order to determine if there is enough sequence divergence to warrant separate species assignment, the ITS1 sequence was determined for both Togninia species. The ITS1 of T. fraxinopennsylvanica has 197 bp and that of T. novae-zealandiae has 178 (Fig. 3). Allowing for gaps, 176 positions of the two sequences could be aligned, with 116 positions conserved. The 60 variable positions consisted of 24 transitions and 36 transversions. Although no criteria for species assignment based on the ITS1 have been established, the differences in size and sequence of the ITS1 could be indicative of speciation. Therefore the molecular data appears to support the separation of the two Togninia species which was initially only based on morphological criteria.

Eyjólfsdóttir (1990) argued that species of the genus Togninia are separable from those of Ceratocystis s.l. in:

(i) having more persistent asci; (ii) the manner of their ascus formation; (iii) the fact they possess paraphyses; and (iv) in the nature of their perithecial necks. Superficially, however, the dark coloured and long necked ascocarps of these two Togninia species, which at maturity have spore masses at the tips of their beaks, do resemble those found in species of Ceratocystis s.l. The majority rule consensus tree (Fig. 4), based on the analysis of the partial LSrDNA data by parsimony and distance methods, groups the sequences of the two Togninia species together at the 100 and 96% levels of confidence respectively, but appart from species of Ceratocystis s.l.. Indeed, the sequences for the two Togninia species differ by only two substituion. However the partial LSrDNA sequence data do suggest that Togninia could be derived from the same evolutionary line as species of Ophiostoma. The potential monophyly of Ophiostoma and Togninia is supported at the 70-71% level of confidence based on parsimony and distance methods. This level of support is certainly not sufficient to prove monophyly, but there does appear to be a trend in the data which suggests that Ophiostoma and Togninia share a common phylogeny. The more extensive survey of partial LSrDNA sequences presented in chapter 5 also placed the two Togninia sequences beside the branch that groups 55 Ophiostoma species.

In order to resolve the taxonomic postion of Togninia with respect to Ophiostoma more sequence data will have to

be obtained in order to gain phyletic estimates with higher levels of confidence. Togninia is considered to be a genus of the Calosphaeriaceae, order Calosphaeriales (Barr 1985, 1990), but unfortunately, little is known about these fungi, although members of this family are often found on wood beneath loosened periderm. None of the species have been studied developmentally (Barr 1985), and their relationships have not been assessed by other than classical criteria. Authentic material of Togninia minima, the type of Togninia, no longer exists, thus a more detailed study of Togninia and its position within the Calosphaeriales is problematic. However, a study focussed on the evolutionary position of other genera of the Calosphaeriales with respect to Ophiostoma might be warranted.

As more data on Ophiostoma species accumulates, it is very likely that this genus will be considered a derived rather than a primitive genus. Should there be an evolutionary connection of Ophiostoma with fungi having paraphyses and more persistent asci arranged in spicate clusters that line the centrum, such as members of the Calosphaeriales (Barr 1990), the boundries of the Ophiostomatales would have to be reassessed.

Fig. 3. Comparison of the sequences of ITS1 of T. fraxinopennsylvanica and T. novae-zealandiae. Positions are numbered from the 5' end of the molecule and identical bases are joined by a vertical line (|) and deleted bases by a dash (-). The presumptive end of the SSrDNA gene and the beginning of the 5.8S rDNA gene are indicated; the latter were determined by their homologies with published sequence data for S. cerevisiae and N. crassa (Chambers et al. 1986).

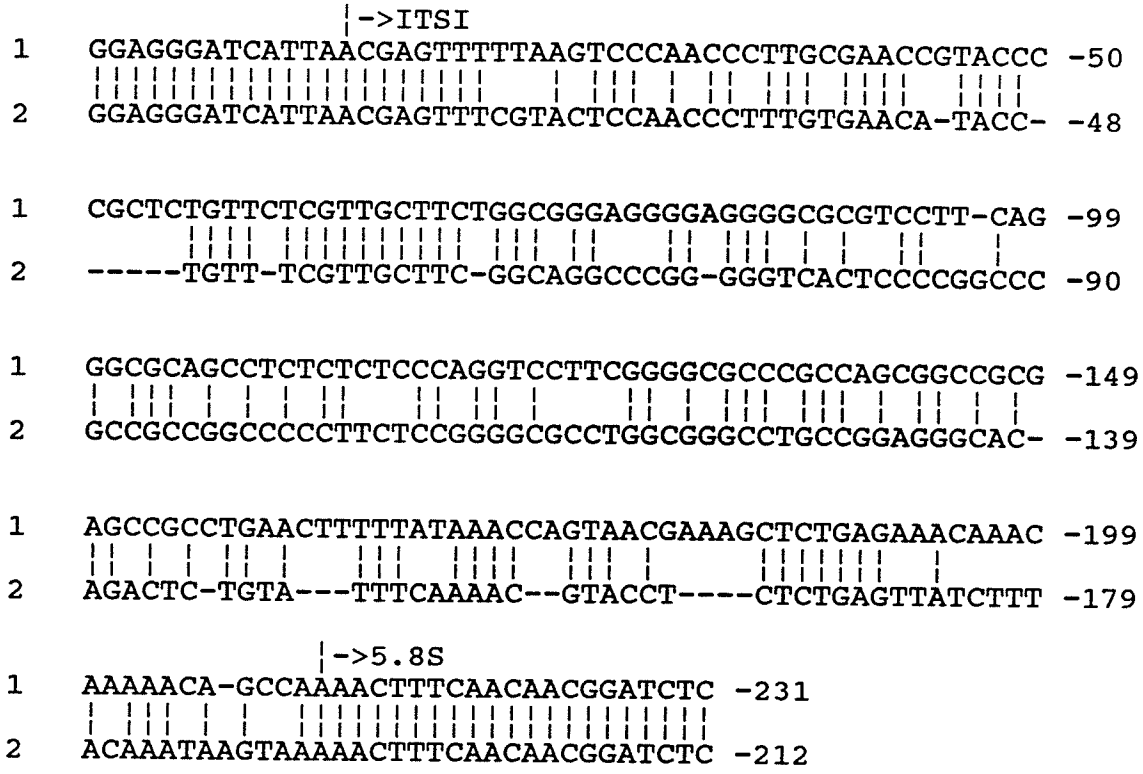
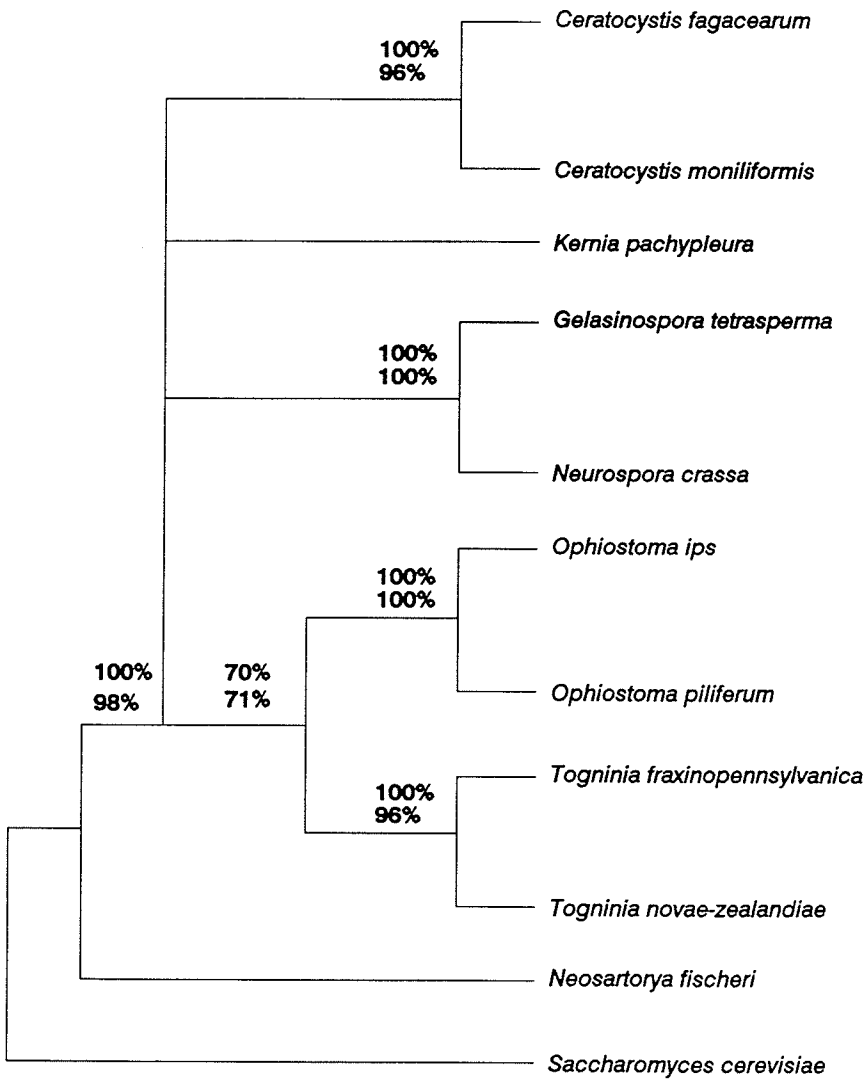

->ITSI
1 GGAGGGATCATTAACGAGTTTTTAAGTCCCAACCCTTGCGAACCGTACCC -50
2 GGAGGGATCATTAACGAGTTTCGTACTCCAACCCTTTGTGAACA-TACC- -48
1 CGCTCTGTTCTCGTTGCTTCTGGCGGGAGGGGAGGGGCGCGTCCTT-CAG -99
2 -----TGTT-TCGTTGCTTC-GGCAGGCCCGG-GGGTCACTCCCCGGCCC -90
1 GGCGCAGCCTCTCTCTCCCAGGTCCTTCGGGGCGCCCGCCAGCGGCCGCG -149
2 GCCGCCGGCCCCCTTCTCCGGGGCGCCTGGCGGGCCTGCCGGAGGGCAC- -139
1 AGCCGCCTGAACTTTTTATAAACCAGTAACGAAAGCTCTGAGAAACAAAC -199
2 AGACTC-TGTA---TTTCAAAAC--GTACCT----CTCTGAGTTATCTTT -179
->5.8S
1 AAAAACA-GCCAAAACTTTCAACAACGGATCTC -231
2 ACAAATAAGTAAAAACTTTCAACAACGGATCTC -212

Fig. 4. A 60% majority rule consensus tree, based on 100 boostrap replicates (SEQBOOT), showing the phyletic relationships between species of Togninia and members of Ceratocystis s.l.. The phyletic estimate is based on the partial LSrDNA sequence data (see Fig. 3, Chapter 5 for the alignment) which was analyzed with the FITCH and DNAPARS programs. Bootstrap support for the major nodes are given with the level of confidence for the DNAPARS analysis given first and listed below that number is the level of confidence derived from the FITCH analysis.


Ceratocystis fagacearum
100%
96%
Ceratocystis moniliformis
Kernia pachypleura
Gelasinospora tetrasperma
100%
100%
Neurospora crassa
Ophiostoma ips
100%
100%
100%
98%
70%
71%
Ophiostoma piliferum
Togninia fraxinopennsylvanica
100%
96%
Togninia novae-zealandiae
Neosartorya fischeri
Saccharomyces cerevisiae

III. MOLECULAR RELATEDNESS BETWEEN *Beauveria confusa* sp. nov., *B. amorpha*, *B. brongniartii*, AND *Engyodontium album*.

INTRODUCTION

During Eyjólfsdóttir's (1990) studies of possible blue-stain fungi isolated from bark-beetle galleries in standing and fallen trees or cut timber, interesting organisms other than members of the genus *Ceratocystis s.l.* were isolated. Two such species from New Zealand were possibly entomogenous and actually pathogenic on the bark beetles in the original samples. They were initially identified as *Beauveria amorpha* (Hohn.) Samson & Evans and *Engydodontium album* (Linder) de Hoog, neither of which has been reported previously from New Zealand. However, as *Beauveria* species are difficult to identify, Eyjólfsdóttir compared her strains in culture with strains of both *B. amorpha* and *B. brongniartii* (Sacc.) Petch. obtained from the Central Bureau voor Schimmelcultures, Baarn. Ultimately, based on spore shape and size, it was decided the isolates thought to represent *B. amorpha*, should be assigned to a new species *Beauveria confusa*.

In conjunction with morphological and culture characters (Eyjólfsdóttir 1990), molecular characters were employed herein to seeking confirmation for the decision to describe the New Zealand strains as a new species. Partial rDNA sequence data were also obtained for two New Zealand

strains of E. album in an attempt to resolve whether this species should be assigned to Beauveria; it has been treated in both Engyodontium de Hoog (1978) and Beauveria Vuill. (Saccas 1948; de Hoog 1972) in the past, and its final placement is still in doubt.

MATERIALS AND METHODS

DNA was extracted from the following isolates: B. confusa WIN(M) 82-168c, and WIN(M) 82-168d; B. brongniartii (Sacc.) Petch, CBS 410.34 and CBS 128.53; B. amorpha (Hohn.) Samson & Evans, CBS 604.80; E. album (Limber) de Hoog, WIN(M) 82-79 and WIN(M) 82-136b. The oligonucleotide primers for PCR and DNA sequencing for obtaining sequences for both ITS1 and the LSrDNA gene region containing Domain 1 have been described previously. Corresponding sequences from Gelasinospora tetrasperma (ATCC 11345) and N. crassa (Chambers et al. 1986) were included in the analysis to serve as outgroups. The B. brongniartii isolates were included in this study as Samson (personal communication) felt that the New Zealand isolates were assignable to this species.

Nucleotide sequences were aligned with the aid of the CLUSTAL V and MASE programs (Fig. 5a, b), and the aligned data sets were then analyzed using programs contained within PHYLIP (Felsenstein 1991). In both cases, the B. confusa strains had identical sequences, and therefore only one of the sequence was used in the analysis. As the rate and mode of evolution for the ITS1 and Domain regions are probably different, both distance (FITCH, KITCH) and parsimony methods (DNAPARS) were used to analyze the two regions independently and as a combined dataset. In the case of independent treatment, majority rule consensus trees based

on 1000 bootstrap replicates (SEQBOOT) were constructed by CONSENSE based on FITCH and DNAPARS analysis. The combined data set was analyzed with the FITCH, KITSCH, and DNAPARS programs and bootstrap analysis (400 replicates) was used to assess the level of confidence in the branches of the resulting phyletic estimates.

RESULTS AND DISCUSSION

There was general agreement between the phylogeny estimates which were derived using the partial LSrDNA sequence and those based on ITS1 sequences (Fig. 6). However, branches within the phyletic estimates based on the ITS1 sequences had higher levels of confidence than branches in the phyletic estimates based on the partial LSrDNA data set. In the phyletic estimates based on the combined data set (about 500 positions), four major nodes are evident, each supported by >99% confidence in bootstrap analysis (Figs. 7 and 8). The high levels of confidence support the monophyly of of Beauveria species tested, but the two E. album strains, while grouped together, cluster apart from the Beauveria cluster. Members of the Sodariaceae (N. crassa and G. tetrasperma) are grouped together but outside of the node that unites the Engyodontium strains and the Beauveria cluster. Both the distance and parsimony analysis on the combined data set segregate B. confusa from the other two Beauveria species tested. However, the distinction between B. brongniartii and B. amorpha is not as clear since parsimony analysis generated two most parsimonious trees: one distinguishes the two B. brongniartii isolates from B. amorpha; the second groups B. amorpha with B. brongniartii CBS 410.34.

Based on this limited sequence sample, B. confusa appears to be a valid species which should not be merged

with either B. amorpha or B. brongniartii. This finding would confirm the concept that in Beauveria shape of the conidia is the most reliable character by which species can be identified. De Hoog (1972) noted that in Beauveria a lack of stable characters has made it very difficult to separate them into different species. Clearly, the species of the genus Beauveria would benefit from a more detailed molecular analysis to determine phylogenetic relationships within this group. ITS sequences could be useful in assigning isolates to a particular species, but first the amount of sequence divergence occurring within isolates of a single species must be determined. This will require the analysis of many authentic isolates representing one accepted species of the genus.

While Engyodontium differs from Beauveria species in lacking an inflated basal portion to the conidiogenous cell, the mode of conidiogenesis and basic colony morphology of Engyodontium album are similar to that found in Beauveria species. Molecular characters appear to segregate the New Zealand strains of E. album from the three Beauveria species included in this study. This would support de Hoog's (1978) removal of E. album from Beauveria, although Mungai et al. (1989) included E. album in Beauveria. However the latter authors did note that "B. album shows considerable biochemical separation from B. bassiana (type of Beauveria), and can be distinguished from all other taxa by its enzyme spectrum". Thus the present limited study, plus the

observation that E. album does not appear to be entomogenous (Beauveria species usually are), would suggest that E. album should be treated as an Engyodontium.

Eyjólfsdóttir (1990) did note some differences between the two E. album strains. For example in comparison with isolate WIN(M) 82-79, isolate WIN(M) 82-136 sporulated less, had longer and thinner sympodulae, and produced subglobose to short-elliptical conidia. Sequence divergence was also noted between these two strains, however without other Engyodontium sequences available for analysis, no comment can be made on the taxonomic significance of this observation. Again more ITS sequences have to be collected from many isolates of authentic Engyodontium species before species assignment based on sequence divergence is possible.

Fig. 5. (a) Alignment of partial rDNA sequences which include the ITS1. The presumptive beginning and end of the ITS1 region are indicated; these were determined by their homologies with published sequence data for S. cerevisiae and N. crassa (Chambers et al. 1986).

Fig. 5. (b) Alignment of partial LSrDNA sequences which contain Domain 1. Positions are numbered from the 5' end of the molecule and Domain 1 is flagged. Nucleotide positions 1- 252 of the alignment correspond to nucleotide positions 102-360 of S. cerevisiae (Guadet et al. 1989).

Note: UM numbers equals WIN(M) numbers.

```
      1  3'end of SSrDNA gene   |ITS1-->
1     GGTGAACCAG CGGAGGGATC ATTACAGAGT TGCAAAACTC CCACAAACCA TGGCGAATCT
2     .......... .......... .......... .......... .......... .C........
3     .......... .......... .....C.... .T-TC..... ...-..C..T .TTGTG.A.C
4     .......... .......... .....C.... .T-TC..... ..T-..C..T .ATGTG.A.C
5     .......... .......... .....C.... .T-TC..... ..T-..C..T .CTGTG.A.C
6     .......... .......... .....C.... .T-TC..... ..T-..C..T .CTGTG.A.C
7     .......... .......... .....C.... .T-.C..... ...-..C... .--GTG.A.A
8     .......... .......... .....C.... .T-.C..... ...-..C... .--GTG.A.A

      61
1     TACCCGTACG GTTGCCTCGG CGCTGGCGGT CCGGAA-GGC CCTCGGGCC- CCCGGATCCT
2     .......... .......... .......... ......A... .T.......T ..........
3     ....TA.-.. -....T.... ..--.A.--. .-.---.--. ..-.A.---. ..........
4     ....TA.-T. -....T.... ..--.A.--. ..---.--. ..-.A.---. ..........
5     ....TA.-.. -....T.... ..--.A.--. ..---.--. ..-.A.---. ..........
6     ....TA.-T. -....T.... ..--.A.--. .-.---.--. ..-.A.---. ..........
7     ....TT.... -....T.... ..--.A---G ..---.--. ..-.....G ......A...
8     ....TT.... -....T.... ..--.A---G ..---.--. ..-.....G ......A..G

      121
1     CG--GGTCTC CCGCTCG--- -CGGGAGGCT GCCCGCCGGA GTGCCGAAAC -CAAACTCTT
2     .......... .......... ....CT.C.C ...G.----. .......... T.........
3     --..-----. G..G..TGGA C.A.CG.-.C ...G.----G .AC..----- T.........
4     --..-----. G..GAGTGGA C.A.CG.-.C ...G.----G .AC..----- T.........
5     --..-----. G..GA.TGGA C.A.CG.-.C ...G.----G .AC.------ T.......C.
6     --..-----. G..GA.TGGA C.A.CG...C ...G.----G .AC..----- ..........
7     .-TC...T.. G..GC.CGGA A.AA.GC..C CG.G.----. .GC.------ A.........
8     .TTC...T.. G..GC.CGGA A.CA.GC..C CG.G.----. .GC.------ A.........

      181                                                    <--ITS1|
1     GATATTTTAT --GTCTCT-C TGAGTAAAC- ---------- TTTTAAATAA GTCAAAACTT
2     .......... AT........ .......... .......... .......... ..........
3     ..--.--.C CA.CA.G.T. ...A..CG.C GCAAGGCAA. -AAC...--. T.-.......
4     .-.....-.. CA.CA...T. ...A..CG.C GCAAGGCAA. -AAC...... A.........
5     .-....-C-- CA.CAG..T. ...A..CG.C GCAAGGCAA. AAAC....G. A.........
6     -....--C.. CA.CAC..T. ...A..CG.C GTAAGGCAA. -AAC...... A.........
7     -C.GC..CTA CA..T...T. .....GTG.C GCAAGGCAA. ..AC....G. A.........
8     -T.G....TA CA..T...T. .....GTG.C GCAAGGCAAA A.AC....G. A.........

      241
1     TCAACAACGG ATCT  (Gelasinospora tetrasperma)
2     .......... ....  (Neurospora crassa)
3     .......... ....  (Beauveria confusa)
4     .......... ....  (Beauveria brongniartii CBS 128.53)
5     .......... ....  (Beauveria brongniartii CBS 410.34)
6     .......... ....  (Beauveria amorpha)
7     .......... ....  (Engyodontium album UM 82-79)
8     .......... ....  (Engyodontium album UM 82-136d)
```

```
      1                                |D1-->
1     GCAACAGCTC AAATTTGAAA TCTGGC-TTC GG--CC-CG- AGTTGTAATT TGTAGAGGAA
2     .......... .......... .......... ..TG...... .......... ..........
3     .......... .......... ......CC.. ..GT...-.. .......... .........T
4     .......... .......... ......CC.. ..GT...-.. .......... .........T
5     .......... .......... ......TC.. A.GG...... .......... .........T
6     .......... .......... ......CC.. A.....G..G .......... .........T
7     .......... .......... .....TCCC. A.GG...... .......... .........T
8     .......... .......... .....TCCC. A.GG...... .......... .........T

      61
1     GCTTTTGGTG AGGCACCTTC TGAGTCCCCT GGAACGGGGC GCCATAGAGG GTGAGAGCCC
2     .......... .......... .......... .......... .......... ..........
3     ........C. ...TG..... C....T.... ........A. .......... ..........
4     ........C. ...TG..... C....T.... ........A. ....C..... ..........
5     ........C. ...TG..... C....T.... ........A. ....C..... ..........
6     ........C. ...TG..... C....T.... ........A. ....C..... ..........
7     ........C. ....G.-... C....T.... ........A. ....C..... ..........
8     ........C. ....G.-... C....T.... ........A. ....G..... ..........

      121                                        <--D1|
1     CGTATAGTCG GATGCCGATC CAATGTAAAG CTCCTTCGAC GAGTCGAGTA GTTTGGGAAT
2     .......... .C........ .......... .......... .......... ..........
3     ...C.G.... ..CA....G. .TC....... .......... .......... ..........
4     .....G.... ..CA....G. .TC....... .......... .......... ..........
5     .....G.... ..CA....G. .TC....... .......... .......... ..........
6     .....G.... ..CA....G. .TC....... .......... .......... ..........
7     ...C.G.... ......A.G. .T........ .......... .......... ..........
8     ...C.G.... ......A.G. .T........ .......... .......... ..........

      181
1     GCTGCTCAAA A-TGGGAGGT AAATTTCTTC TAAAGCTAAA TACCGGCCAG AGACCGATAG
2     .......... .......... ....G..... .......... ..TT...... ..........
3     .......... .......... .T..G..... .......... ...T...... ..........
4     .......... .......... .T..G..... .......... ..TT...... ..........
5     .......... .A........ .T..G..... .......... ..TT...... ..........
6     .......... .......... .T..G..... .......... ...T...... ..........
7     .......... .......... .T..G..... .......... .......... ..........
8     .......... .......... .T..G..... .......... .......... ..........

      241
1     CGCACAAGTA GA  (Gelasinospora tetrasperma)
2     .......... ..  (Neurospora crassa)
3     .......... ..  (Beauveria confusa)
4     .......... ..  (Beauveria brongniartii CBS 128.53)
5     .......... ..  (Beauveria brongniartii CBS 410.34)
6     .......... ..  (Beauveria amorpha)
7     .......... ..  (Engyodontium album UM 82-79)
8     .......... ..  (Engyodontium album UM 82-136d)
```

Fig. 6. Majority rule consensus trees showing the phyletic relationships between three species of Beauveria and Engyodontium album based on the analysis of 1000 boostrap replicates (SEQBOOT) with the DNAPARS (a and b) and the FITCH (c and d) programs. Phyletic estimates are based on the individual analysis of the partial LSrDNA data set (a and c) and the ITS1 data set (b and d). The percentages at the nodes are an indication of the level of confidence for the branches as determined by boostrap analysis.

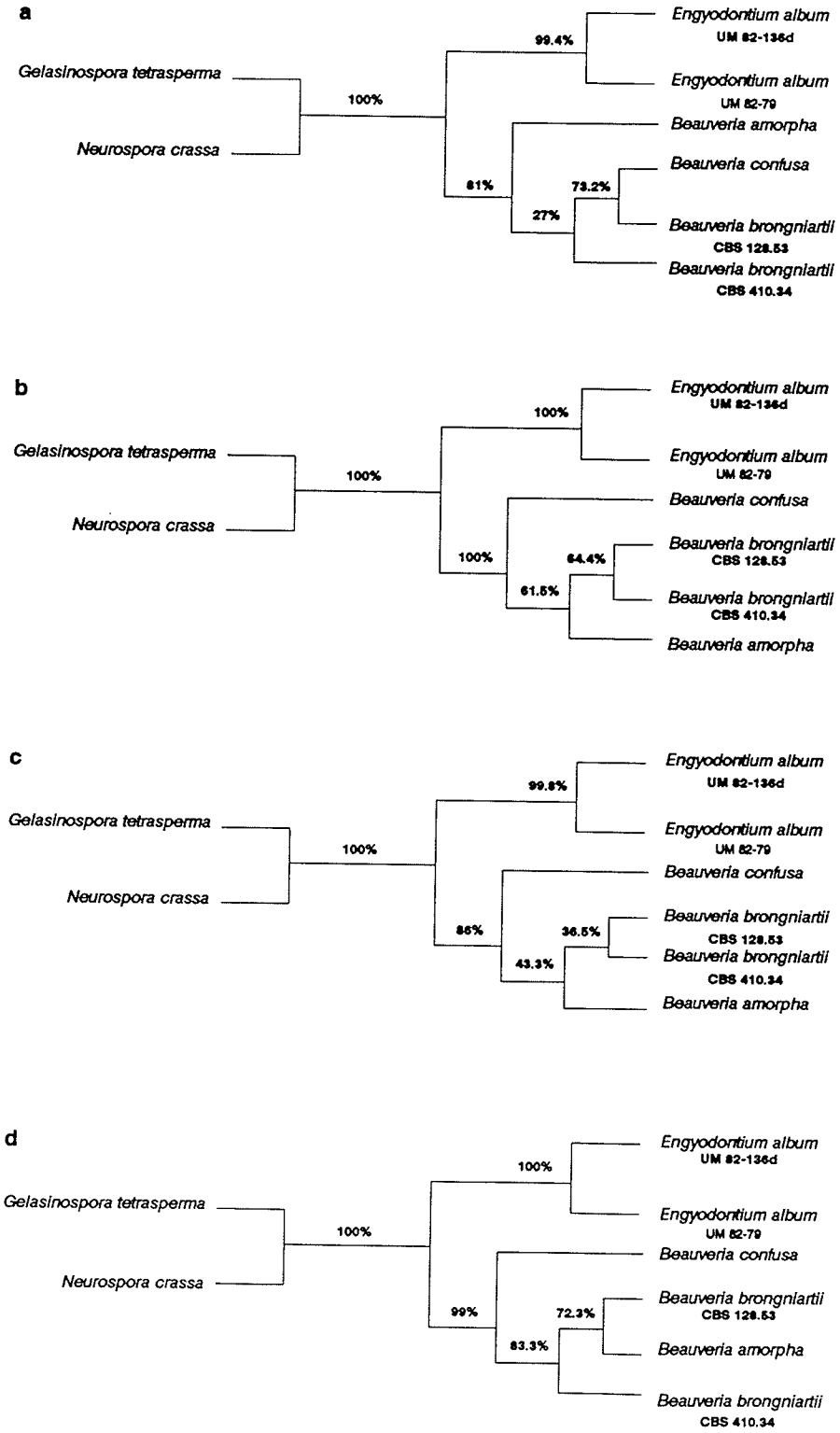

a
Gelasinospora tetrasperma
Neurospora crassa
100%
99.4%
81%
27%
73.2%
Engyodontium album
UM 82-136d
Engyodontium album
UM 82-79
Beauveria amorpha
Beauveria confusa
Beauveria brongniartii
CBS 128.53
Beauveria brongniartii
CBS 410.34
b
Gelasinospora tetrasperma
Neurospora crassa
100%
100%
100%
64.4%
61.5%
Engyodontium album
UM 82-136d
Engyodontium album
UM 82-79
Beauveria confusa
Beauveria brongniartii
CBS 128.53
Beauveria brongniartii
CBS 410.34
Beauveria amorpha
c
Gelasinospora tetrasperma
Neurospora crassa
100%
99.8%
86%
43.3%
36.5%
Engyodontium album
UM 82-136d
Engyodontium album
UM 82-79
Beauveria confusa
Beauveria brongniartii
CBS 128.53
Beauveria brongniartii
CBS 410.34
Beauveria amorpha
d
Gelasinospora tetrasperma
Neurospora crassa
100%
100%
99%
83.3%
72.3%
Engyodontium album
UM 82-136d
Engyodontium album
UM 82-79
Beauveria confusa
Beauveria brongniartii
CBS 128.53
Beauveria amorpha
Beauveria brongniartii
CBS 410.34

Fig. 7. Unrooted Fitch-Margoliash network showing the phyletic relationships between species of Beauveria and Engyodontium album, based on the analysis of the combined ITS1 and partial LSrDNA data set (Figs. 5a and 5b) with the KITSCH program. The percentages along the nodes are an indication of the level of confidence for the major branches as determined by bootstrap analysis (N = 400).

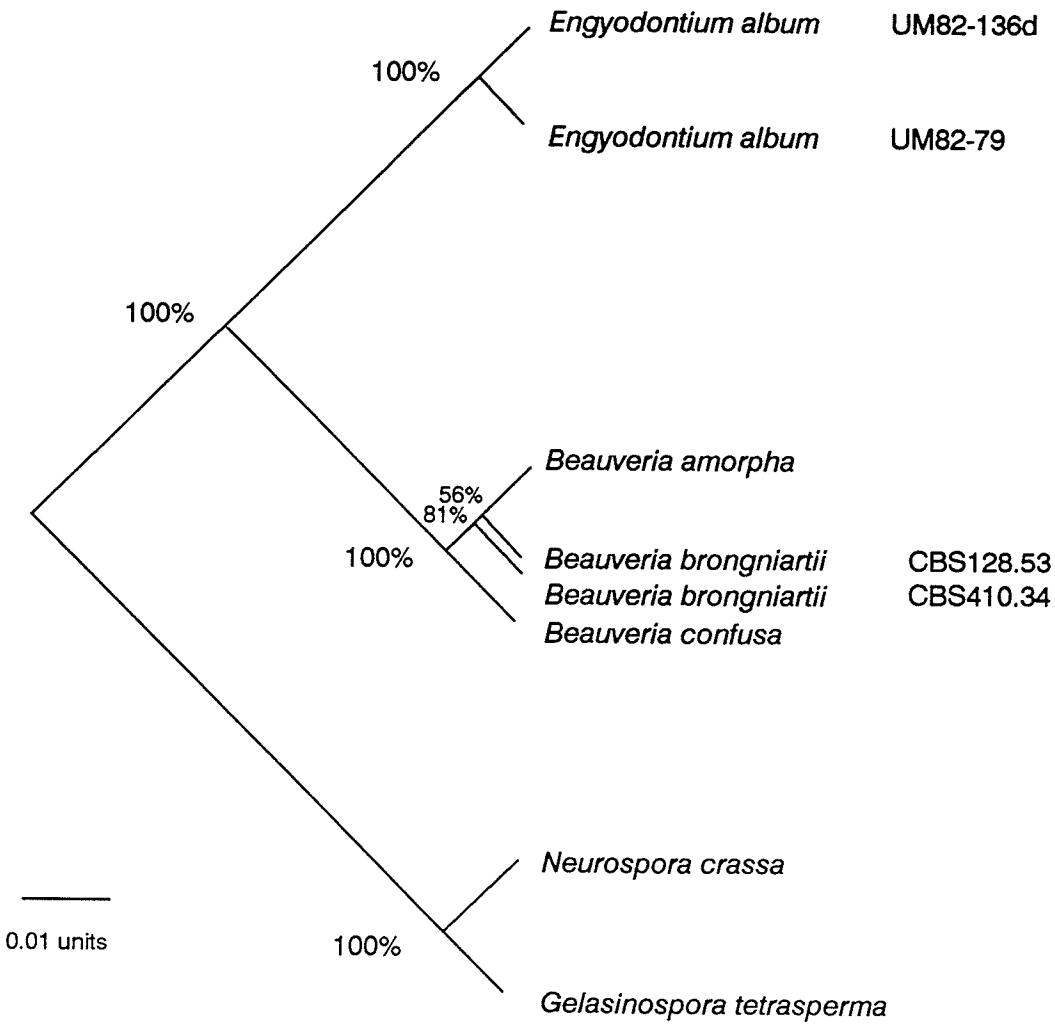
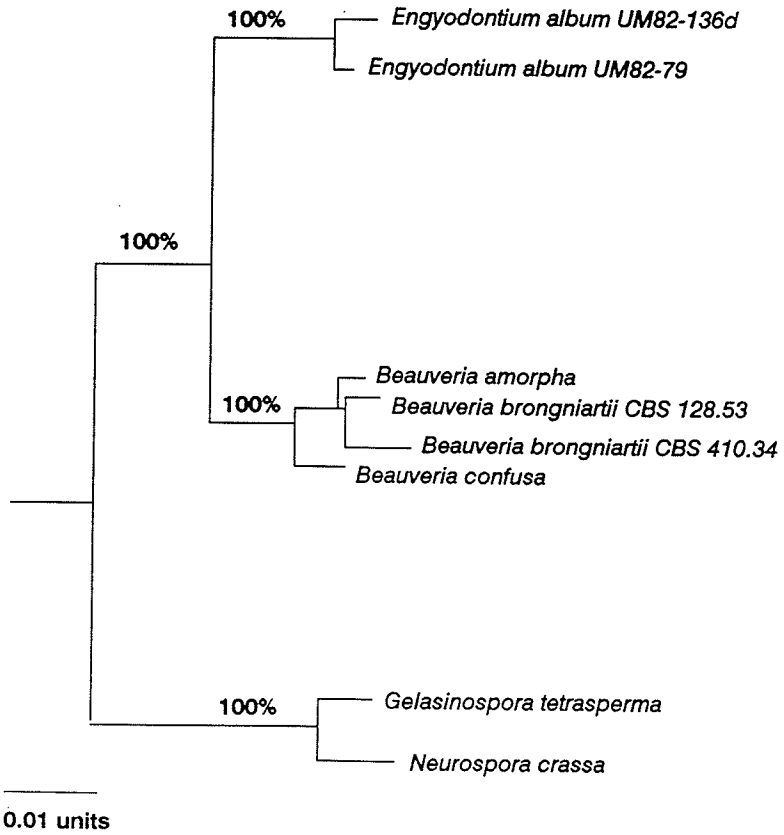

Engyodontium album
UM82-136d
100%
Engyodontium album
UM82-79
100%
Beauveria amorpha
56%
81%
100%
Beauveria brongniartii
CBS128.53
Beauveria brongniartii
CBS410.34
Beauveria confusa
Neurospora crassa
0.01 units
100%
Gelasinospora tetrasperma

Fig. 8. A minimum-length phylogenetic tree obtained by parsimony (DNAPARS) and distance methods (FITCH). The two most parsimonious trees each required 225 steps and the branch lengths shown are those determined by the FITCH program. The second most parsimonious tree (not shown) grouped B. amorpha with B. brongniartii CBS 410.34. The boostrap support based on DNAPARS analysis of 400 boostrap replicates (SEQBOOT) is indicated for major nodes with levels of confidence > 75%.


100%
Engyodontium album UM82-136d
Engyodontium album UM82-79
100%
Beauveria amorpha
100%
Beauveria brongniartii CBS 128.53
Beauveria brongniartii CBS 410.34
Beauveria confusa
100%
Gelasinospora tetrasperma
Neurospora crassa
0.01 units

CHAPTER 7

AN UNUSUALLY COMPACT RIBOSOMAL RNA GENE CLUSTER IN *Sphaeronaemella fimicola*

INTRODUCTION

In most eukaryotes the primary product of RNA polymerase I is a large precursor containing the 16-18S, 5.8S, and 23-28S rRNA genes, as well as the external (ETS) and internal (ITS) transcribed spacer sequences. Internal transcribed spacers (ITS1 and ITS2) are rapidly evolving regions that differ greatly in size and nucleotide sequence between different taxa. For example, the ITS1 and ITS2 of Giardia lamblia are 41 bp and 55 bp long respectively, whereas in mouse they are quite large with ITS1 being 999 bp, and ITS2 1089 bp (Boothroyd et al. 1987; Michot et al. 1983). Within the fungi, the smallest ITS sequences reported so far are from Yarrowia lipolytica where, based on R-loop mapping, the combined length of the putative ITS1 and ITS2 regions was estimated to be about 150 bp (Heerikhuizen et al. 1985), and the largest are from Schizosaccharomyces pombe (ITS1: 412-420 bp and ITS2: 300 bp) (Schaak et al. 1982). One approach to determining the role of spacer sequences is to examine the shortest existing spacers to see what has been retained during evolution and is thus presumably indispensible.

Originally the investigation began as an attempt to study sequence divergence of the ITS regions within species of Ophiostoma and Ceratocystis, and the use of such as an aid in taxonomy. However, although DNA fragments containing the ITS regions could be generated readily by PCR

amplification, no reliable sequences could be obtained from Ophiostoma species, and therefore this study was not extended. But it was noted that for isolates of Sphaeronaemella fimicola Marchal, the PCR fragments thought to contain the ITS regions were unusually short. As there is considerable interest in the function of ITS regions in the processing of the primary RNA transcript within eukaryotes, this study was extended to determine the nature of the rRNA gene arrangement within this fungus. In addition the unusually compact rRNA gene arrangement of Sph. fimicola is another character that sets this species apart from members of Ceratocystis s.l.

In the present investigation the ribosomal DNA repeat unit of the ascomycetous fungus Sph. fimicola is characterized, and it appears to have very short internal spacers based on DNA sequence homology with other known spacers. The nucleotide sequences for the central portion of the rDNA repeat unit, including the 5.8S RNA gene and flanking regions, were determined in a preliminary effort to understand the nature of these ITS regions and their potential role in the processing of the primary RNA transcript.

METHODS AND MATERIALS

Six strains of Sphaeronaemella fimicola (WIN(M) 815, 816, 817, 818, 819, 827), all isolated from Pinus radiata in different localities of New Zealand, were examined. The culturing methods, DNA extraction protocols, endonuclease digestion of DNA, and Southern hybridization procedures were as described previously.

The polymerase chain reaction (PCR) protocol used to generate sequencing templates (Saiki et al. 1988) and the sequencing of double-stranded PCR products were as described previously. Oligonucleotide primers utilized for amplification and DNA sequencing are listed in Figures 1 and 2.

Computer analysis of the sequencing data was performed with NALIGN for homology searches, and RNA-fold for secondary structure predictions. Both of these programs are components of the PC/gene software package (IntelliGenetics Inc., Mountain View, Cal.).

RESULTS AND DISCUSSION

The physical map of the rDNA repeat unit of Sph. fimicola 827 (Fig. 1) was constructed by analysis of single and double digestion profiles of genomic DNA which had been probed with pMF2, a plasmid containing the ribosomal genes of N. crassa (Free et al. 1979). The SSrRNA and LSrRNA genes were also successfully amplified with primer pairs whose sequences were based on published terminal sequences of these genes (SSJ-SSm for the SSrRNA gene and LS1-LSM for the LSrRNA gene), and restriction site maps of the PCR products agreed with the genomic map. The rDNA repeat was found to be quite large (13.7 kb) compared to those of other Ascomycotina, but the gene cluster, tentatively identified on the basis of conserved restriction sites (Verbeet et al. 1984; Russel et al. 1984), appeared unusually compact, presumably due to the presence of short internal spacers.

The polymerase chain reaction was performed with primers SSZ and LS4 to generate an 850 bp product that included the 3' end of the SSrRNA gene, the 5.8S gene and the 5' end of the LSrRNA gene. This product was obtained from two strains of Sph. fimicola (827, 818) and sequenced in both directions (Fig. 2). No differences were found between the two strains. The sequence was compared to the corresponding region in N. crassa (Chambers et al. 1986; Sogin et al. 1986; Kelly and Cox 1981) to find the putative gene termini and spacer locations. The ends of the mature

RNA products of this gene region will have to be characterized to establish the reality of the postulated spacers, but due to the slow growth rate of Sph. fimicola, sufficient mycelium could not be obtained for RNA sequencing and characterization. If the gene termini of Sph. fimicola are homologous with those of N. fimicola, and with those of all of the other members of the Ascomycotina reported in the literature, then both of the internal spacers are indeed exceptionally short. The putative ITS1 (40 bp) of Sph. fimicola could represent the shortest ITS1 yet reported for any eukaryote, and the putative ITS2 (62 bp), the shortest ITS2 recorded so far for any fungus. An attempted alignment of the ITS regions of Sph. fimicola with those of N. crassa (Chambers et al. 1986) and Saccharomyces cerevisiae (Veldman et al. 1980) by the NALIGN computer program, indicated a general lack of homology between the ITS regions of these taxa.

To show that short internal spacers are typical within the species, the central gene region of the 6 strains of Sph. fimicola was amplified with two primer pairs, SSGH3-LS2 and SSZ-5.8B (Fig. 3) (For primer locations see figures 1 or 2). The first product contains both spacers and the second only ITS1. All 6 strains produced products of the same size, whose sizes are predicted from the determined sequence (Fig. 2).

The compact nature of the ITS regions in Sph. fimicola could simplify the search for processing signals in the

precursor ribosomal RNA molecules. Muster et al. (1990) noted that in yeast the deletion of the central regions of the ITS1 prevented the accumulation of functional 40S subunits; thus ITS1 is required for the production of mature 17S rRNA molecules. Yeh et al. (1990), using a combination of enzymatic and chemical structure probes, concluded that the recognition signals for the processing enzymes involved in rRNA splicing lie more in the higher order structure of ITS1 rather than its actual nucleotide sequences. Van der Sande et al. (1992) have demonstrated that in S. cerevisiae the secondary structure of the ITS2 appears to be very important for its efficient removal from the primary transcript. The hypotheses concerning the nature of processing signals and possible RNA/RNA interactions involved in the maturation of the RNA transcript could be tested by the generation of deletion mutants or by in vitro mutagenesis of the short spacer segments that flank the 5.8S RNA gene of Sph. fimicola. In Sph. fimicola, the secondary structure of the ITS2 region, as predicted by RNA-fold, could include a recognition signal (Fig. 4). A potential hairpin loop located at the 5' end of the putative ITS2 region contains the last 4 bases of the 5.8S RNA and the first 31 bases of the spacer-like element. Thus exactly half of the potential ITS2 region is involved in this secondary structure, and this could be a significant feature for placing potential cleavage site(s) into close proximity with one another and exposing these sites to nucleases.

The sequence of the 5.8S rRNA gene of Sph. fimicola has several interesting characteristics. Its secondary structure (Fig. 5) was deduced from the rDNA sequence and drawn according to the structures proposed by Nazar et al. (1975), as modified by Pace et al. (1977). This model leaves the 5' and 3' ends of the 5.8S rRNA as single stranded tails; such have been implicated in the hydrogen bonding of the 5.8S rRNA to the LSrRNA in the 60S ribosomal subunit (Veldman et al. 1981). The 5.8S RNA of Sph. fimicola can also be arranged to fit the unmodified model of Nazar et al. (1975), in which the 5' and the 3' ends of the 5.8S RNA are permitted to base pair. As in A. alternata (Tsuge et al. 1989) this base pairing is probably weak, and this secondary structure likely represents only an intermediate stage during the maturation of the ribosomal precursor RNA. However, possible recognition sites which flank the 5.8S RNA and control processing events may be brought into close proximity by this structure, thus facilitating the proper splicing of the 5.8S RNA. In addition, this structure might serve to stabilize free 5.8S RNA, and thus prevent degradation by nucleases (Schaak et al. 1982).

The length of the 5.8S gene appears to be conserved within the fungi (157-158 bases). In Sph. fimicola four positions appear to be deleted (positions 79, 80, 81, 85). However a small four bp insert occurs near the 3' end of the gene, thus compensating for the deletions. The 3' end of the

5.8S gene of Sph. fimicola also reveals an extended complementarity with the 5' end of the LSrDNA, thus the 5.8S RNA can interact with the LSrRNA through hydrogen bonds (Fig. 5). This interaction has also been well documented in mouse (Michot et al. 1983), Dictyostelium discoideum (Olsen and Sogin 1982), S. cerevisiae (Veldman et al. 1980), and N. crassa (Chambers et al. 1986), and the maintenance of this complementarity during evolution via compensatory base changes suggests the importance of the 5.8S/LSrRNA interaction in the formation of the 60S ribosomal subunit. In Sph. fimicola the four bp insert in the 3' end of the 5.8S gene is compensated for by a complementary insert in the 5' end of the LSrRNA gene, thus two insertions 81 bases apart may have evolved to maintain the interaction between the 5.8S RNA and LSrRNA.

The characterization of the rDNA of Sph. fimicola yielded some surprising features. The ribosomal repeat unit of Sphaeronaemella fimicola is quite large (about 14 kb) compared to that reported for other members of the Ascomycotina (Russel et al. 1984; Verbeet et al. 1984), and its NTS comprises about 8.8 kb of the repeat unit . Although the NTS is large, the internal transcribed spacers are quite reduced in size, and therefore could represent the vestigial remnants of larger spacer segments. The ITS1 (40 bp) of Sph. fimicola represents the shortest ITS1 yet reported for any eukaryote, and the ITS2 (62 bp) represents the shortest ITS2

recorded so far for any fungus. Recent studies in the maturation of the primary rRNA transcription product in a variety of organisms suggest that the ITS regions are important in the proper splicing of the ribosomal precursor RNA (Muster et al. 1990; Yeh et al. 1990). However, the exact cleavage sites and potential secondary structures which could be involved in the maturation process have not yet been elucidated. The compact nature of the ITS regions of Sph. fimicola might potentially simplify the search for processing signals in these spacers. Thus Sph. fimicola could serve as a useful model in the investigation of structure and function of precursor rRNA molecules.

Fig. 1. Physical map of the rDNA repeat unit of Sph. fimicola (strain 827). Restriction sites are indicated by symbols above the map (B: BglII, C: ClaI, D: HindIII, H: HincII, K: KpnI, M: BamHI, R: EcoRI, S: SmaI, X: XbaI). Oligonucleotide primer sites are indicated below the map with arrows giving 5'-3' orientation. Sequences of the oligonucleotides are as follows: SSJ: (dCTGGTTGATCCTGCCAGTAG); SSZ: (dATAACAGGTCTGTGATG); SSm: (dTAATGATCCGTTGGTG); LS1: (dAGTACCCGCTGAACTTAAG); LS4: (dTTGTGCGCTATCGGTCTC); LSM: (dGATTCTGACTTAGAGGCGT).

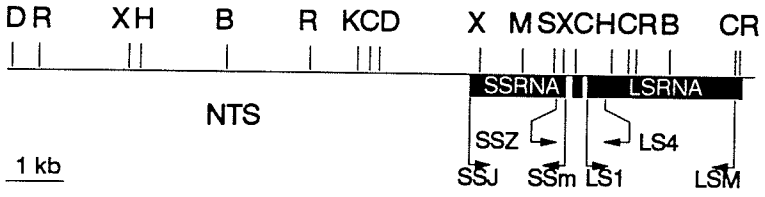

DR
XH
B
R
KCD
X
MSXCHCRB
CR
SSRNA
LSRNA
NTS
SSZ
LS4
1 kb
SSJ
SSm
LS1
LSM

Fig. 2. The nucleotide sequence of the Sph. fimicola (Sf) SSZ-LS4 PCR product (EMBL accession number Z12124) and corresponding sequences from Neurospora crassa (Nc) (Chambers et al. 1986). The (*) denotes the position of identical bases. The positions of the ITS1 and ITS2 regions are indicated along with the postion and sequence of primers SSA, SS3, 5.8B, LS2. The complementary stem regions of a potential hairpin loop which could form at the rRNA sequence level within the 5' half of the ITS2 region are underlined.

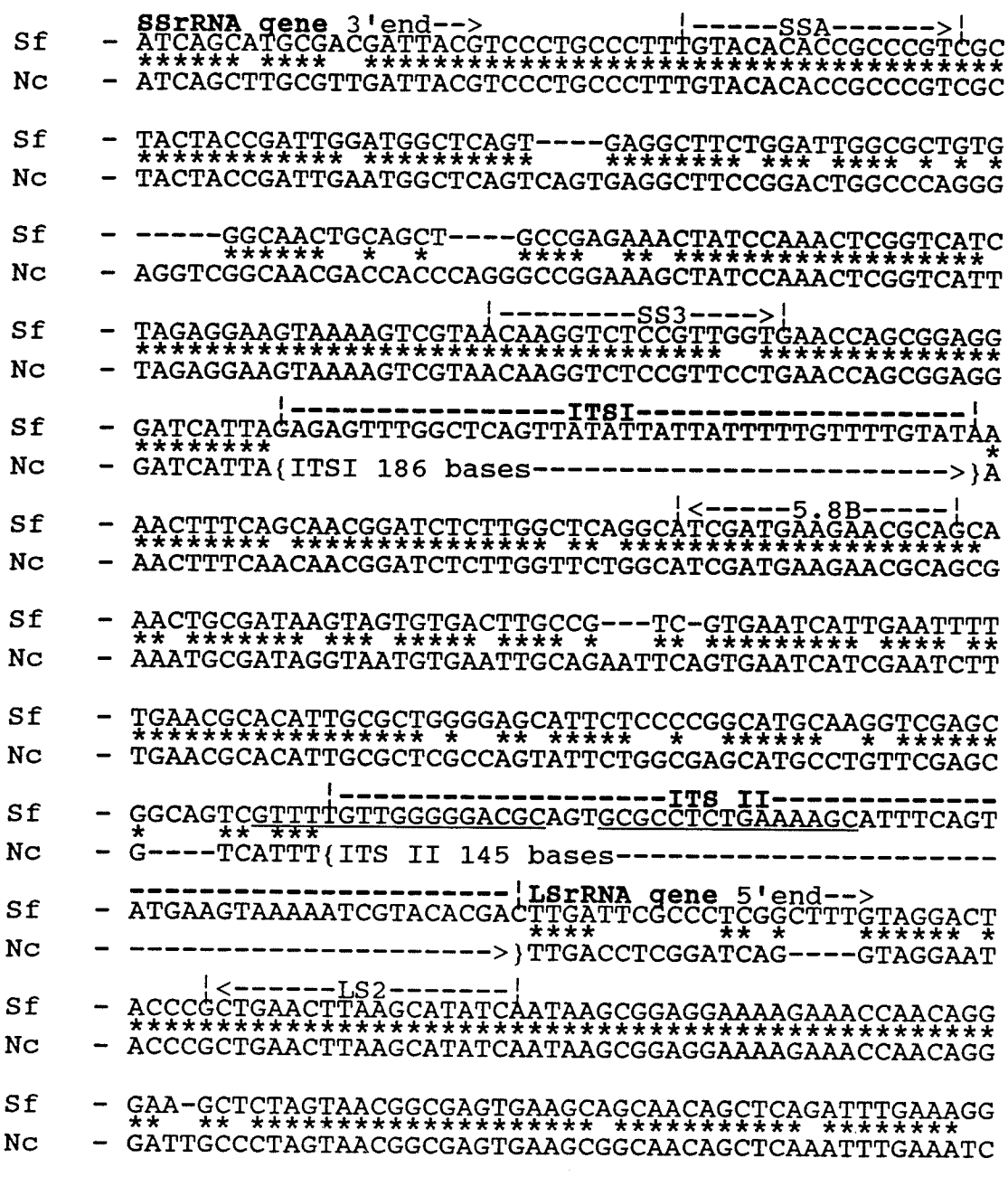

SSrRNA gene 3'end-->
|-----SSA------>|
Sf - ATCAGCATGCGACGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGC
Nc - ATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGC
Sf - TACTACCGATTGGATGGCTCAGT----GAGGCTTCTGGATTGGCGCTGTG
Nc - TACTACCGATTGAATGGCTCAGTCAGTGAGGCTTCCGGACTGGCCCAGGG
Sf - -----GGCAACTGCAGCT----GCCGAGAAACTATCCAAACTCGGTCATC
Nc - AGGTCGGCAACGACCACCCAGGGCCGGAAAGCTATCCAAACTCGGTCATT
|--------SS3---->|
Sf - TAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGG
Nc - TAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTCCTGAACCAGCGGAGG
|-----------------ITSI-------------------|
Sf - GATCATTAGAGAGTTTGGCTCAGTTATATTATTATTTTTGTTTTGTATAA
Nc - GATCATTA{ITSI 186 bases----------->}A
|<-----5.8B-----|
Sf - AACTTTCAGCAACGGATCTCTTGGCTCAGGCATCGATGAAGAACGCAGCA
Nc - AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG
Sf - AACTGCGATAAGTAGTGTGACTTGCCG---TC-GTGAATCATTGAATTTT
Nc - AAATGCGATAGGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
Sf - TGAACGCACATTGCGCTGGGGAGCATTCTCCCCGGCATGCAAGGTCGAGC
Nc - TGAACGCACATTGCGCTCGCCAGTATTCTGGCGAGCATGCCTGTTCGAGC
|-------------------ITS II-------------
Sf - GGCAGTCGTTTTGTTGGGGGACGCAGTGCGCCTCTGAAAAGCATTTCAGT
Nc - G----TCATTT{ITS II 145 bases-------------------
----------|LSrRNA gene 5'end-->
Sf - ATGAAGTAAAAATCGTACACGACTTGATTCGCCCTCGGCTTTGTAGGACT
Nc - ---------->}TTGACCTCGGATCAG----GTAGGAAT
|<------LS2-------|
Sf - ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGG
Nc - ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGG
Sf - GAA-GCTCTAGTAACGGCGAGTGAAGCAGCAACAGCTCAGATTTGAAAGG
Nc - GATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATC

Fig. 3. PCR amplification products indicating the compact ribosomal RNA gene clusters in six Sph. fimicola strains (all from WIN(M) collection). Lane L: BRL 1 kb ladder; lanes 1 to 6 PCR products from SS3-LS2 amplifications; lanes 7 to 12 PCR products from SSZ-5.8B amplifications; lanes 1 and 7 isolate 815; lanes 2 and 8 isolate 816; lanes 3 and 9 isolate 817; lanes 4 and 10 isolate 818; lanes 5 and 11 isolate 819; lanes 6 and 12 isolate 827.

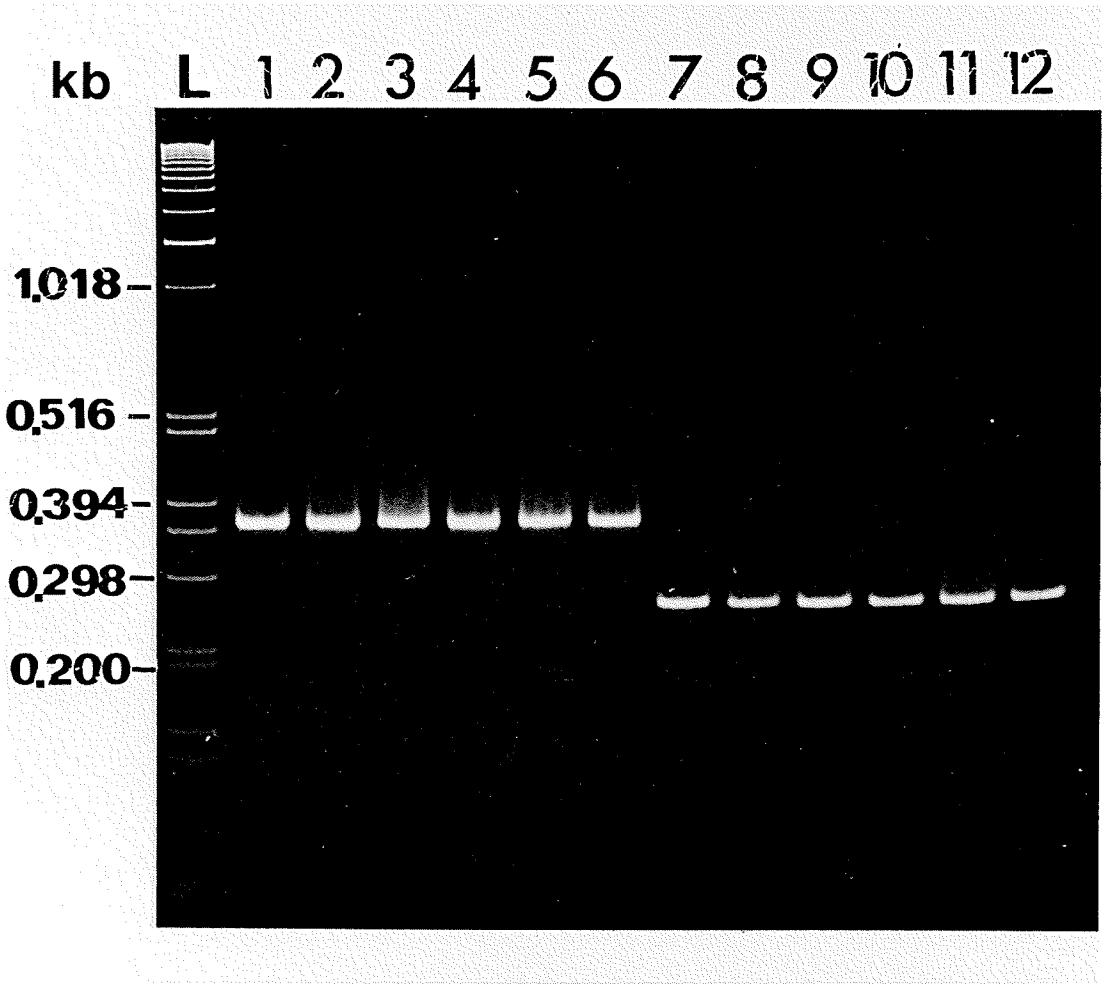

kb
L
1
2
3
4
5
6
7
8
9
10
11
12
1,018
0,516
0,394
0,298
0,200

Fig. 4. Predicted secondary structure of the 5' half of the ITS2 region. The rRNA sequence was deduced from the rDNA sequence.

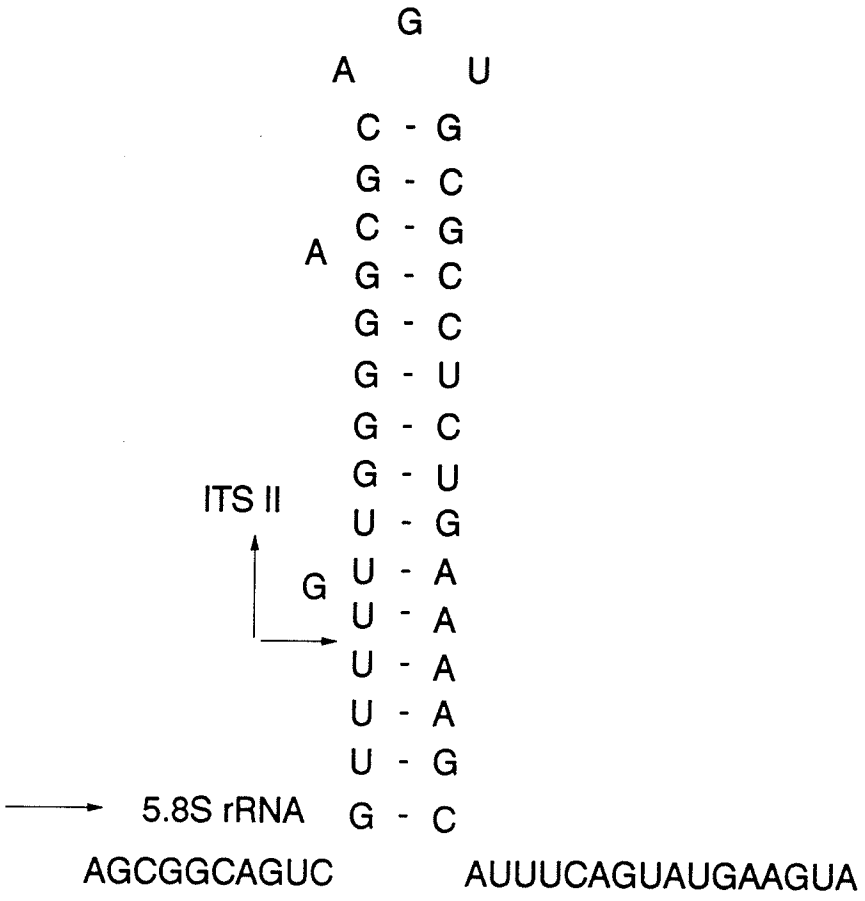
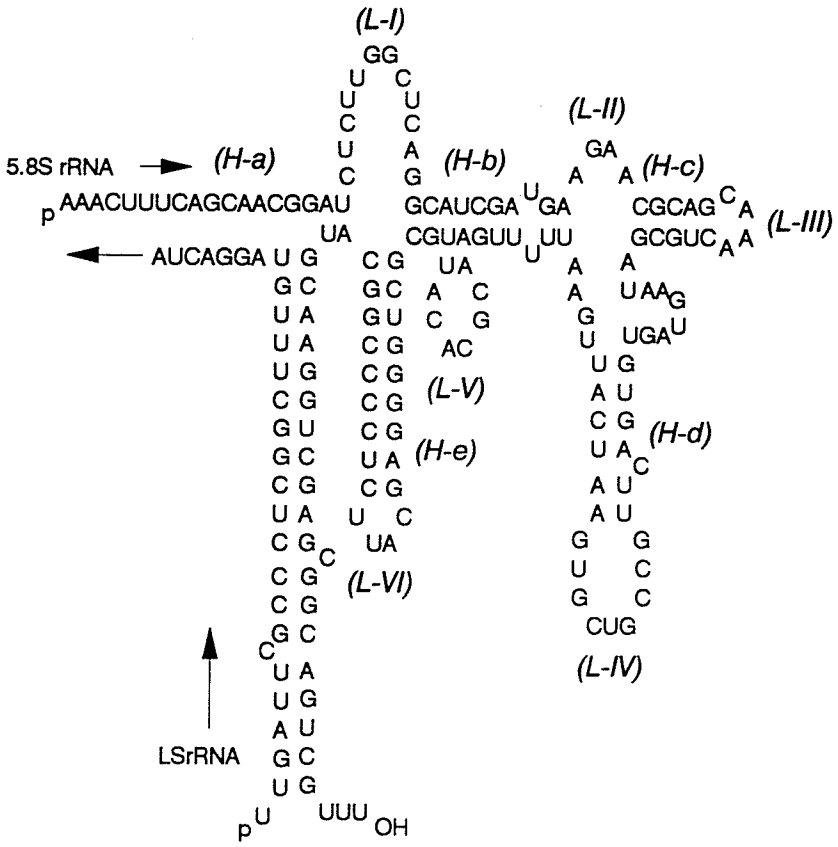

G
A U
C - G
G - C
C - G
A
G - C
G - C
G - U
G - C
G - U
ITS II
U - G
U - A
G
U - A
U - A
U - A
U - G
5.8S rRNA
G - C
AGCGGCAGUC
AUUUCAGUAUGAAGUA

Fig. 5. Predicted secondary structure of Sph. fimicola 5.8S rRNA based on the model of Nazar et al. (1975), with modifications as proposed by Pace et al. (1977). The 5.8S rRNA sequence was deduced from the rDNA sequence. The nomenclature of helical (h) and loop (l) regions are according to Kelly and Maden (1980).


(L-I)
(L-II)
5.8S rRNA
(H-a)
(H-b)
(H-c)
(L-III)
(L-V)
(H-e)
(H-d)
(L-VI)
(L-IV)
LSrRNA
OH

CONCLUDING REMARKS

Restriction fragment analysis

A total of 52 fungal strains representing 27 species that comprised various aspects of previous groupings within the Ophiostomatales were analyzed by restriction fragment analysis of the rDNA. The results indicate that such rDNA analysis could be of diagnostic value for strains of O. ips, since only one common RFLP phenotype was obtained following treatment with each "four cutter" endonuclease for all 22 isolates tested. In addition, strains of O. adjunctum and O. montium, species which Upadhyay (1981) synonymized with O. ips, were segregated from O. ips, and from each other, by their RFLP phenotypes. In general, however, rDNA restriction patterns were found to be highly variable between most of the species of Ceratocystis s.l. tested, thus this approach was not useful for establishing generic limits within this group of fungi.

Although the rDNA restriction patterns obtained for Sph. fimicola, Cop. falcata, C. autographa, Cephaloascus fragrans, and Endomyces decipiens appeared to be quite different from those obtained from either C. fimbriata or O. piliferum, a common ancestry for all of these fungi cannot be precluded from these results. Similarily although rDNA analysis did group together species assignable to Ceratocystis s.s. (except C. autographa), whether this

grouping should be viewed as evidence for sectional status within Ceratocystis s.l., or separate generic status for species of Ceratocystis s.l. with Chalara anamorphs was not resolved. Overall, although restriction fragment analysis of rDNA from species of Ceratocystis s.l. did suggest that this genus is heterogeneous, this method of analysis probably is most useful for identifying groups of closely related species, and for providing additional evidence for placing strains into a particular fungal species.

Mitochondrial DNA RFLPs obtained for strains of O. ips and related species indicate that this molecule might be useful for resolving taxonomic questions below the species level, and for DNA typing of strains, but once again taxonomic resolution above the species level was limited. Based on strains originating from Scandinavia and New Zealand, within O. ips mtDNA restriction phenotypes appear to correlate with geographic origins. Therefore mtDNA variation could be useful in studying geographic distribution of various strains within O. ips.

Overall, restriction analysis failed to provide definitive answers to the major taxonomic questions concerning Ceratocystis s.l. Therefore this study was expanded to include rDNA sequence analysis in the hope that generic and ordinal relationships within the Ophiostomatales could be more effectively addressed by this technique.

Ribosomal DNA sequence analysis

Analysis of partial rDNA sequences from both the small and large subunit genes of species of Ceratocystis s.l., and presumed related genera, did provide a more suitable approach for resolving generic concepts within the Ophiostomataceae. From such sequences, statistically evaluated phyletic estimates were obtained by DNA distance matrix and DNA parsimony analysis of the aligned data sets. Therefore a number of taxonomic questions were apparently resolved based on the phylogeny of the fungi deemed assignable to the Ophiostomataceae. Apparently resolved, because conclusions based on rDNA phylogenies are only based on an assumption that the molecular evolution of the ribosomal DNA corresponds to the evolutionary history of the organism as a whole; this has not been proven. One of the great advantages of rDNA sequence analysis is that sequence data obtained by others can be included in later studies, and compared directly during analysis. Thus the current investigation also provides data sets that can be incorporated into future studies that are based on the same regions of the SSrDNA or LSrDNA as examined herein. Or, more conveniently, this study could be extended by adding sequences derived from presumptive new strains of Ophiostoma or Ceratocystis to confirm the taxonomic disposition of such strains.

The sequence data analyzed during this study provide strong support for restricting the use of the generic name

Ceratocystis to species with Chalara anamorphs (Ceratocystis s.s.), while all other members of Ceratocystis s.l. should be assigned to Ophiostoma. Phyletic estimates based on partial SSrDNA sequences also demonstrated that Ceratocystiopsis and Europhium should be considered synonyms of Ophiostoma. Ceratocystis s.l., as presently understood, is polyphyletic, and includes unrelated species which superficially resemble one another as a consequence of convergent evolution. The sequence data also suggests that the species of Ceratocystis s.s. are derived from the same evolutionary lineage as are members of the Microascaceae.

The position of Ophiostoma within the Parenchymatomycetidae was not resolved in this study, but must await the availability of more sequence data from other pyrenomycete groups that could be related to Ophiostoma. However, a possible connection of Ophiostoma species to members of the Calosphaeriales was indicated, and this possibility should be investigated further. Nonetheless this study did confirm that both Ceratocystis and Ophiostoma are comprised of derived fungal species that, as groups, are not closely related to one another. The phylogenies inferred from rDNA sequence data also show that ascospore shape, lack of an ostiole, and ascus arrangement and shape, are probably not sufficiently reliable characters upon which to propose phylogenetic classification schemes.

The correct taxonomic dispositions of Cop. falcata and Sph. fimicola were also not resolved. However, based on rDNA

data they can be excluded from both Ceratocystis and Ophiostoma. Similar findings were made for Cop. alba, O. roraimense, and C. autographa which appear to have little relation to members of either Ceratocystis or Ophiostoma. Cop. proteae, however, appears to be best disposed of in a new genus that would be aligned with Ceratocystis within the Microascales.

Although molecular criteria would suggest that the genus Ophiostoma is monophyletic, based on morphological criteria it is heterogeneous; therefore efforts to subdivide this genus into smaller groupings will continue. In fact the partial LSrDNA sequence data (see chapter 5) did suggest that within Ophiostoma there does appear to be a trend towards simpler ascospore shapes and less complex and smaller conidial states. However within this phyletic estimate no strict groupings based on ascospore morphology or the nature of the anamorphs could be observed. In addition, the statistical support for the major branches of the phyletic estimate were insufficient to propose distinct evolutionary lines within Ophiostoma. Therefore future studies on species of Ophiostoma should concentrate on the more rapidly evolving segments of the rDNA such as the internal transcribed spacers and the intergenic regions. These might provide more informative sites towards phylogenetic reconstruction and therefore more robust phyletic estimates could be obtained.

It is very unlikely that species groupings within

Ophiostoma will be found that are related to either their ascospore morphologies or the nature of their anamorphs. Ascospore morphology actually appears to be a highly convergent character, and therefore it is probably not a very reliable basis upon which to propose relationships. For example, molecular data failed to support either the expanded concept of the Endomycetaceae presented by Redhead and Malloch (1977) or the circumscription of Ceratocystiopsis (Upadhyay and Kendrick 1975); both of these were based on ascospore characteristics.

The current taxonomic treatment for the anamorphic states of the Ophiostoma species is still unresolved because the exact method of conidium formation for many Ophiostoma species is still unknown. Only a few detailed studies on condial development in Ophiostoma have been undertaken to date (Wingfield 1985; Wingfield et al. 1989; 1991) but their results suggest that the anamorphic states of Ophiostoma species can be accommodated in considerably fewer genera of the Hyphomycetes than was thought previously (Upadhyay and Kendrick 1975). However, with many species having two or more distinct synanamorphs, it is unlikely that in Ophiostoma conidial states will yield stable characters suitable for establishing phylogenetic links.

Recently Zambino and Harrington (1992) studied isozyme variation in species of the anamorphic genus Leptographium, and in Ophiostoma species with Leptographium anamorphs. They concluded that there is some justification for subdividing

Ophiostoma into sections based on the four ascospore morphologies employed by Olchowecki and Reid (1974). Their conclusion was based on the low relatedness between the four branches of their dendrogram which linked O. adjunctum, O. crassivaginatum, O. penicillatum and other Ophiostoma spp. with Leptographium anamorphs. However the LSrDNA sequence data presented herein suggests that Ophiostoma species with Leptographium anamorphs could form a distinct group and that both O. crassivaginata and O. penicillatum cluster within this group; this is in contrast to the findings of Zambino and Harrington. Isozymes are potentially useful in resolving intraspecific relationships, and for distinguishing between closely related species, however, within Ophiostoma, a genus exhibiting a high degree of variability, isozyme characterization will probably not be useful in resolving more distant relationships.

Van Wyk and Wingfield (1991c) stated that ultrastructural studies of centrum and ascospore development could be useful in establishing generic concepts in Ceratocystis s.l. and in the placement of this group at the family and ordinal level. Their studies do appear to distinguish between members of Ceratocystis s.s. and Ophiostoma with similar ascospore morphology (Van Wyk and Wingfield 1991a; 1991b). However, based on the rDNA sequence data, it would appear to be more relevant to compare the ultrastructure of ascoma and ascospore development between Ceratocystis s.s. and members of the Microascaceae. Within

Ophiostoma, based on the current studies by Van Wyk and Wingfield (1991c, 1992), centrum organization in species and, to a somewhat lesser extent, ascosporogenesis both appear to be variable. These features might therefore only be useful for taxonomy at the species level. Thus while Van Wyk and Wingfield (1991c) noted that based on differences in the centrum organization and structure of ascospores wall layers, O. minus and O. distortum might not be congeneric, analysis of partial LSrDNA sequences placed both O. minus and O. distortum near O. piliferum. Thus centrum and ascospore development data have to be interpreted with caution when used as taxonomic criteria.

At the moment, DNA sequence analysis is probably the most effective approach for studying the phylogeny of fungi. And future studies on Ophiostoma and Ceratocystis will certainly benefit should additional sequences from both the rDNA and other suitable regions within the genome be forthcoming. Ultimately such molecular taxonomy could potentially resolve the phylogenetic relationships that generally exist among the genera of the Ascomycotina, and, as a consequence, fungi traditionally placed in the Ophiostomatales due to their convergent morphology could be arranged within more appropriate taxonomic groupings.

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