Ribosomal RNA Sequence Phylogeny Is Not Congruent with Ascospore Morphology among Species in *Ceratocystis* Sensu Stricto

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The genus *Ceratocystis* sensu stricto includes important fungal pathogens of woody and herbaceous plants. This genus is distinguished from species in *Ceratocystis* sensu lato by the presence of *Chalara* anamorphs. Ascospore shape has been used extensively in delineating *Ceratocystis* taxa, which show a large variety of ascospore shapes. Sequence analysis of one region of the 18S ribosomal RNA subunit and two regions of the 28S ribosomal RNA subunit showed that there was a majority of multiple substitutions at nucleotide sites and that there was a low transition/transversion ratio, $T = 0.72$. Both of these results suggest that these are well established, old species. Ascospore morphology, for the most part, was not congruent with the molecular phylogeny, and the use of morphological characters may be misleading in the taxonomy of these species.

Introduction

Ascomycetes in the genus *Ceratocystis* and related genera are economically important plant pathogens, especially of trees (Seifert et al. 1993), and the identification of species in this group is important for their control. The taxon *Ceratocystis* (Ellis & Halsted) sensu lato includes the genera *Ophiostoma* H & P Sydow, *Ceratocystiopsis* Upadhyay & Kendrick, and *Ceratocystis* sensu stricto de Hoog & Scheffer. Upadhyay (1981), however, recognized only *Ceratocystiopsis* and *Ceratocystis* and based the separation of these two genera on ascospore morphology. *Ceratocystiopsis* included species with falcate ascospores, and all other species were assigned to *Ceratocystis*. Thus Upadhyay (1981) ignored the contention of Weijman and De Hoog (1975) that *Ophiostoma* and *Ceratocystis* sensu stricto are separate taxa distinguishable by cell wall chemistry. De Hoog and Scheffer (1984) reevaluated the generic concepts in this group of fungi and provided sound evidence for treating *Ophiostoma* and *Ceratocystis* as separate genera.

Currently, most authors treat *Ceratocystis* sensu stricto, *Ophiostoma,* and *Ceratocystiopsis* as separate genera (Seifert et al. 1993). *Ceratocystis* sensu stricto is typified by the absence of rhamnose and cellulose in their cells (Weijman and De Hoog 1975). They also all have *Chalara* anamorphs with phialides having ring wall–building conidium development (Minter et al. 1982) and are intolerant to low concentrations of the antibiotic cycloheximide (Harrington 1981). In contrast, species of *Ophiostoma* and *Ceratocystiopsis* have rhamnose and cellulose in their cells, have conidia that develop through apical wall building, and are tolerant to high concentrations of cycloheximide in culture media. *Ophiostoma* and *Ceratocystiopsis* are distinguished only by the presence of falcate ascospores in the latter genus (De Hoog and Scheffer 1984).

Although *Ceratocystis* sensu stricto comprises a uniform assemblage of species in the sense of all having *Chalara* anamorphs, they can be separated by various morphological characteristics. Of these, ascospore shape and size is the most important taxonomic characteristic (Upadhyay 1981; Wolfaardt et al. 1992). In addition to the presence or absence of sheaths on ascospores, species of *Ceratocystis* sensu stricto can have cucullate, ossiform, reniform, or ovoid-shaped spores. This variability in ascospore morphology suggests that *Ceratocystis* sensu stricto may be a polyphyletic taxon.

In this study, we used both direct sequencing of total cellular ribosomal RNA (rRNA) and phenetic and cladistic analyses to infer the phylogeny of nine species of *Ceratocystis* sensu stricto, with *Saccharomyces cerevisiae* as an outgroup. Sequence analysis of rRNA has proved useful, in several phylogenetic studies of other fungi, for solving systematic problems (Blanz and Unseld 1986; Guadet et al. 1989; Bruns and Szaro 1992). We chose one region of 18S rRNA and two regions of 28S rRNA to sequence and estimate a phylogeny.

Key words: *Ceratocystiopsis, Ceratocystis, Ophiostoma,* phylogeny, rRNA sequence analysis, ascospore.

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Previous studies have shown that 28S rRNA can be used to study closely related species or even conspecific strains of fungi (Guadet et al. 1989). The combination of these sequences therefore should provide a good basis for inferring the phylogeny of the species of *Ceratocystis*. The goal of this study was to use a phylogeny based on rRNA sequence variability to assess the value of ascospore morphology in the taxonomy of *Ceratocystis* sensu stricto.

Material and Methods

Isolates and Cultivation

Isolate number and origins of the species used in this study are listed in table 1. These isolates were chosen to represent a diversity of ascospore morphology and hyphal ornamentation. Cultures were grown at 18°C in 500 ml of yeast malt extract broth (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract) on a rotary shaker (170 rpm). All isolates are maintained in the culture collection of the University of the Orange Free State. Cells were harvested after 24–72 h by centrifugation and were freeze-dried for later rRNA extraction.

Table 1
List of *Ceratocystis* Isolates Studied and Their Origin

Species	Abbreviation	Isolate Number[a]
C. adiposa	AD1	CMW0066
C. adiposa	AD2	CMW1622
C. coerulescens	COE	CMW1628
C. fimbriata	FIM	CMW1547
C. laricicola	LAR	CMW1016
C. moniliformis	MON	CMW0100
C. paradoxa	PAR	CMW1546
C. radicicola	RAD	CMW1032
C. virescens	VIR	CMW0067
Saccharomyces cerevisiae	SCER	

[a] Isolate numbers refer to those of M. J. Wingfield and are maintained in the Department of Microbiology, University of the Orange Free State, South Africa. Their origins are as follows: CMW0066 and CMW0067—Forest Pathology Culture Collection, Department of Plant Pathology, University of Minnesota, St. Paul; CMW1622 and CMW1628—culture collection of the Institute for Fermentation, Osaka, IFO 9546 and IFO 8668 respectively; CMW1546 and CMW1547—culture collection of Department of Scientific and Industrial Research, New Zealand, DSIR 05789 and DSIR 08579, respectively; CMW 1016—culture collection of Dr. D. Redfern, Forestry Commission, Northern Research Station, Scotland, number 56.2; CMW0100—culture collection of Dr. T. Hinds, USDA Forest Service, Fort Collins, number C-433; and CMW 1032—Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, number CBS 114.47.

RNA Extraction

Cells were ground in a mortar and pestle, with liquid nitrogen and sand, and the homogenate was suspended in 4 M guanidinium thiocyanate buffer (Chirgwin et al. 1979). rRNA was extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) (Chirgwin et al. 1979). The final rRNA precipitate was suspended in 20 mM Tris (pH 8.5) and was stored at −20°C.

rRNA Sequencing

The isolated rRNA was used as a template for the primer extension–dideoxy termination method of nucleotide sequencing with reverse transcriptase, by following the protocols of Lane et al. (1985). Three oligonucleotide primers (S, L1, and L2) were synthesized by Beckman South Africa. The primer S (5′-ACGGCGGTGTGTAC-3′) is complementary to positions 1627–1641 of the 18S rRNA subunit of *Saccharomyces cerevisiae*, and the primers L1 (5′-GGTCCGTGTTTCAAG-3′) and L2 (5′-TTGGAGACCTGCTGC-3′) are complementary to positions 640–654 and 1844–1858, respectively, of the 28S rRNA subunit of *S. cerevisiae*. Nucleotide fragments were separated on 8% acrylamide, 8 M urea gels and were visualized by autoradiography.

Phylogenetic Inference

Sequences were aligned visually relative to the sequence of *S. cerevisiae*. For a phenetic analysis, we used the program DNADIST (J. Miller) to estimate the average K_{nuc} (number of nucleotide substitutions per nucleotide site between each pair of taxa) with Kimura's (1980) two-parameter statistic $K_{nuc} = -\frac{1}{2} \ln(1-2P-Q)(1-2Q)^{1/2}$, where P and Q are the fractions of nucleotide positions showing transition or transversion substitutions, respectively, between two sequences. This matrix of distances was used to produce phenograms with three clustering algorithms: the unweighted-pair-group method of cluster analysis (UPGMA; Sneath and Sokal 1973), the Fitch-Margoliash (F-M; Fitch and Margoliash 1967) (PHYLIP; J. Felsenstein, University of Washington), and the neighbor-joining method (NJ; Saitou and Nei 1987). The UPGMA assumes equal evolutionary rates along the various branches, whereas the other two algorithms relax this assumption. The F-M algorithm attempts to find the shortest tree consistent with the values of K_{nuc} with a least-squares optimization, and the NJ algorithm progressively finds nearest neighbors that minimize the total length of the tree. The analysis of distance data may or may not lead to trees that reflect the true phylogeny of the taxa, because ancestral similarity is given as much weight as is shared derived similarity among taxa.

For the cladistic trees, the variable nucleotide sites, including autapomorphic states, were analyzed directly with parsimony (PAUP; Swofford 1985), with gaps treated as missing data. We used the branch-and-bound option with Wagner parsimony, which allows reversals of character states on the tree, and used option APOLIST to view nucleotide transitions and transversions at the

branch points of the tree. For multiple parsimonious trees, we used strict consensus (program CONTREE) to produce a single tree. We used bootstrapping (program BOOT, PHYLIP; Felsenstein 1985) with Wagner parsimony to assess the confidence in the tree topologies. A majority-rule consensus tree was constructed from 100 bootstraps, and the percentage of the times that a group of taxa appeared as a clade in the bootstrapped parsimony trees is indicated in the internal nodes of the tree.

Results

The aligned sequences for the S, L1, and L2 primers are shown in figures 1, 2, and 3, respectively. The rRNA sequences of all the *Ceratocystis* species show a gap relative to *Saccharomyces cerevisiae* at positions 1444 and 1454 for the S primer and at positions 400–402 and 468–473 for the L1 primer. There is also an insertion of seven bases in *Ceratocystis,* at position 445 of *S. cerevisiae.* The average K_{nuc} between *S. cerevisiae* and the nine species of *Ceratocystis* was 0.239 (table 2). K_{nuc} between pairs of *Ceratocystis* species ranged from 0.055 to 0.161 and averaged 0.125. The three sequences analyzed separately, however, yielded different levels of differentiation. The average K_{nuc} between pairs of species was the least for the 18S rRNA sequence (mean 0.041), as expected for sequences with low levels of polymorphism. The average K_{nuc} between species for the 28S rRNA primer L1 was 0.211, and for L2 it was 0.1219. The larger number of substitutions for the L1 region is due chiefly to variably sized gaps between positions 466 and 476.

Our first approach to inferring a phylogeny was to use K_{nuc} from the combined sequences, with three phenetic clustering methods. The topology of the UPGMA tree (fig. 4, *top*) (cophenetic coefficient $r = 0.85$) was unlike those of the F-M and the NJ trees (fig. 4, *middle* and *bottom*), which were similar to each other. In the UPGMA tree, AD1 and AD2 were placed to the outside of the other *Ceratocystis* species, but, in F-M and NJ trees, AD1 and Ad2 were placed as a sister group to FIM and LAR within the tree. The groupings of the other taxa were similar in these trees.

Our second approach was to use parsimony to infer a cladogram. We obtained two shortest trees with the combined sequences: one with the same topology as the F-M tree in which AD1-AD2 were placed as a sister group to FIM-LAR (fig. 5, *top*) and another in which AD1-AD2 were placed outside the FIM-LAR and MON-RAD-PAR clades (fig. 5, *middle top*). Both trees had the same length and a consistency index of 0.732. The first tree, however, had a slightly smaller value of Farris's f (3.337) than did the second tree ($f = 3.572$). Hence, the first tree is marginally preferable to the second tree. The strict-consensus tree reflected this ambiguity and

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SMALL SUBUNIT rRNA SEQUENCE

      1421        1431        1441        1451        1461        1471        1481
SCER  ACAGGUCUGU GAUGCCCUUA GAACGUUCUG GGCCGCACGC GCGCUACACU GACGGAGCCA GCGAGUCU-A
COE   NNNNGU.... .......NNN ..U-....U. .G-....... ...CUA.A.. ...AU..... GC......CU
VIR   NNNNGU.... .......NNN ..U-....U. .G-....... ...CUA.A.. ...AC..... GG......CU
MON   NNNNGU.... .......NNN ..U-....U. .--....... ...CUA.A.. ...CC..... GC......UU
PAR   NNNGGU.... .......NNN ..U-....U. .G-....... ...CUA.A.. ...AC..... GC......CU
RAD   NNNGGU.... .......NNN ..U-....U. .G-....... ...CUA.A.. ...AC..... GC......CU
FIM   NNNNGU.... .......NNN ..U-....U. .G-....... ...CUA.A.. ...AC..... GC......CU
LAR   NNNGGU.... .......NNN ..U-....U. .G-....... ...CUA.A.. ...AC..... GC......CU
AD2   NNNGGU.... .......NNN ..U-....U. .G-....... ...-UA.C.. ...AU..... GC......CU
AD1   NNNNG-.... .......NNN ..U-....C. .G-....... ...CUA.A.. ...AC..... GC......CU

       1491        1501        1511        1521        1531        1541        1551
SCER  ACCUUGGCCG .-GAGGUCUU GGUAAUCUUG UGAAACUCCG UCGUGCUGGG GAUAGAGCAU UGUAAUUAUU
COE   U...U.A.A. .GAUGUCCUG GGNA.UC.U. .U.....GUG .C..GCU... .......... U.CN......
VIR   U...-.A.A. .GAUGUCCGG GNGA.UC.U. .U.....GUG .C..GCU... .......... U.CU......
MON   U...U.A.A. .GAUGUCCGG G-NA.CC.U. .U.....GUG .U..GCU... .......... U.CN......
PAR   U...U.A.A. .GAU--CCGG U--G.UC.U. .U.....GUG .C..GCU... .......... U.CN......
RAD   U...U.A.A. .GAUGGCAGG G-AA.CG.A. .U.....GU- .C..GCU... .......... U.CN......
FIM   U...U.A.A. .GAUGUCCGG G-NA.UC.U. .U.....GUG .C..UGC... .......... U.CA......
LAR   U...-.A.A. .GAUGUCCGG G-NA.CC.U. .U.....GUG .C..GCU... .......... A.CN......
AD2   U...U.A.A. .GAUGUCCGG G-NA.UC.U. .U.....GUG .C..GCU... .......... U.CN......
AD1   U...U.A.A. .GAUGUCCGG G-NA.UC.U. .U.....GUG .C..GCU... .......... U.CN......

       1561        1571        1581        1591        1601        1611
SCER  GCUCUUCAAC GAGGAAUUCC UAGUAAGCGC AAGUCAUCAG CUUGCGUUGA UUACGUCCC
COE   ..N...CAAC ..G....C.. .......... A...CA.C.. ..U...G... .U..G....
VIR   ..A...CUAC ..A....C.. .......... N...CA.C.. ..U...U... .U..G....
MON   ..N...CAAC ..N....C.. .......... N...CGUC.. ..U...U... .U..G....
PAR   ..U...CAAC ..G....C.. .......... A...CA.C.. ..U...G... .U..G....
RAD   ..N...CAAC ..G....C.. .......... N...CA.C.. ..A...G... .A..G....
FIM   ..U...CAAC ..G....C.. .......... U...UA.U.. ..U...U... .U..G....
LAR   ..A...CAAC ..G....C.. .......... U...CA.C.. ..U...C... .U..-....
AD2   ..U...UAU- ..G....C.. .......... U...CA.C.. ..U...G... .U..G....
AD1   ..U...UAU- ..G....C.. .......... U...CA.C.. ..U...U... .U..G....
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FIG. 1.—Partial sequences of the 18S rRNA (S region) of *Saccharomyces cerevisiae* and species of *Ceratocystis* sensu stricto. Dots indicate nucleotides identical to those of *S. cerevisiae;* dashes indicate missing nucleotides (gaps); and "N" indicates an undetermined base.

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LARGE SUBUNIT rRNA SEQUENCE1

      381         391         401         411         421         431         441
SCER  UUGAAAAGAG AGUGAAAAAG UACGUGAAAU UGUUGAAAGG GAAGGGCAUU UGAUCAGACA UGGU------
COE   N..... ..... .- --N..... ..... ....U..A.A .GACU....U ..UUUCUGUC
VIR   N..... ..... .- --N..... ..... ....U..A.A .GACU....U ..UUUCUGUC
MON   N..... ..... .- --N..... ..... ....U..A.A .AACG....U ..UUUCUGUC
PAR   N..... ..... .- --N..... ..... ....U..U.A .GACG....U ..UCUCUAUC
RAD   N..... ..... .- --N..... ..... ....U..A.A .GACG....U ..UCUCUAUC
FIM   N..... ..... .- --N..... ..... ....U..A.A .GACC....U ..UUUCUGUC
LAR   N..... ..... .- --N..... ..... ....U..A.A .GCAC....U ..UUUCUGUC
AD2   N..... ..... .- --N..... ..... ....U..U.U .GACU....U ..UCUCUAUC
AD1   N..... ..... .- --N..... ..... ....U..U.U .GACU....U ..UCUCUAUC

                452         462         472         482         492         502
SCER  -GUUUU--GU GCCUCUGCUC CUUGUGGGUA GGGGAAUCUC GCAUUUCACU GGGCCAGCAU CAGUUU
COE   A.....--GC UCG.CU-.GA C.UGU----- -----UU..C -UG.CGGUAC ......ACA. CAG...
VIR   A.....--GC UCG.CU-.GA C.UGUU---- -----NU..C -UG.CGGUAC ......AGA. UAU...
MON   A.....--GG UAG.CU-.GA C.UG------ --CUUA-..C -UG.UGGUCC ......ACA. CAG...
PAR   A.....--GC UAG.CU-.UG G.CUGG---- --UUUAU..C UGG.UGGUCU ......UCA. CAG...
RAD   A.....--GC UAG.CU-.UG G.UGG----- --NUUAU..C UGG.UGGUCU ......ACA. CAG...
FIM   A.....--GG UAG.CU-.GA C.UG------ ----CUU..C UGG.CGGUCU ......AUG. UUG...
LAR   A.....GCUU GUG.CU-.GA C.UG------ ---UUUU..C UGG.CGGUAC ......ACA. CAG...
AD2   A.....--GG UAG.UN-.GA C.UG------ ----CUU..U CUG.UAGUCU ......ACA. CAG...
AD1   A.....--GG UAG.UU-.GA C.UG------ ----CUU..U CUG.UAGUCU ......AGA. UAG...
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FIG. 2.—Partial sequences of the 28S rRNA (L1 region) of *Saccharomyces cerevisiae* and species of *Ceratocystis* sensu stricto. Dots indicate nucleotides identical to those of *S. cerevisiae;* dashes indicate missing nucleotides (gaps); and "N" indicates an undetermined base.

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LARGE SUBUNIT rRNA SEQUENCE2

     1641       1651       1661       1671       1681       1691       1701
SCER ACUGAAUGUG GAGACGUCGG CGCGAGCCCU GGGAGGAGUU AUCUUUUCUU CUUAACAGCU UAUCACCCCG
COE  NN..... ....U.GAGA ..A..ACCCU ....A...CU AU.U..U... U..AA.AGCU GCUG.C..UG
VIR  NN..... ....U.GAGA ..A..ACCCU ....A...CC AU.U..U... U..AA.AGCU UGCG.C..UG
MON  NN..... ....N.GNGG ..A..ACCCU ....A...UU AU.U..A... U..AA.AGCU UGUG.C..AG
PAR  NN..... ....C.GCGG ..A..ACCCU ....A...UU AU.-..A... U..NA.AGCU AGAG.A..AG
RAD  NN..... ....C.GCGG ..A..ACCCU ....A...UU AU.U..A... U..AA.AGCU UGAG.C..UG
FIM  NN..... ....C.NCGG ..G..ACCCU ....A...UC UC.U..U... U..AA.AGUC UACG.C..UG
LAR  NN..... ....C.GCGG ..G..ACCCU ....A...UU CU.U..A... C..AU.GUCU UGCG.C..UG
AD2  NN..... ....C.NCUU ..G..ACCCU ....A...UU AU.U..U... U..AU.GUCU CGUG.C..UG
AD1  NN..... ....C.NCGG ..G..ACCCU ....A...UU AU.U..U... U..AU.GUCU UGUG.C..UG

     1711       1721       1731       1741       1751       1761       1771
SCER GAAUUGGUUU AUCCGGAGAU GGGGUCUUAU GGCUGGAAGA GGCCAGCACC UUUGCUGGCU CCGGUGCGCU
COE  ...UUGGUUU AUCC...GAU GG....UUAC GGCAAG.... GCGCAUGCUC .UCUCUGGGA CCU.UUC..U
VIR  ...UUGGUUU AUCC...GAU GG....UUAC -GCAAG.... GGCCUACACU .CUGCUGGGA CCU.AGC..U
MON  ...UUGGUUU GUCC...GAU GG....GUAU GGCUGG.... GCUCAGCUCC .UUGCUGCGU CUG.UGC..U
PAR  ...UUGGUUU AAUA...GAU AG....GAAU GGCUGG.... GCUCAGCUC- .GUGCUGCCU CUG.UGC..U
RAD  ...UUGGUUU AUCC...GAU AG....GAAU ACGAUA.... GCU-AG-UCU .GUG-UGCGU UCG.UGU..G
FIM  ...UUGGUGU AGUC...AGA GC....UUAC GGCUGG.... GCNCAGCUCU .CUGCUGCGU CUG.UGC..U
LAR  ...UCUUGUU UCCU...GGU GG....UU-C GGCUGG.... GCNUAGCUCU .CUGCUGUGU CUG.UGC..G
AD2  ...CUGGUUU GUCC...GAU AG....UAAC GGCUGG.... GCUCAGCUCU .CUGCUGGGU CCG.UGC..U
AD1  ...UUGGUUN UUGC...GAU GG....UUAC GGCUGG.... GCUCAGCUCU .CUGUCUGGG UCU.GGU..U

     1781       1791       1801       1811       1821
SCER UGUGACGGCC CGUGAAAAUC CACAGGAAGG AAUAGUUUUC AUGCU
COE  CUUACA..A. CU.G....U. C.GGG..G.. ....GUU--C UUGCC
VIR  CUUACA..A. CU.G....U. A.GGG..G.. ....GUU--C UUGCC
MON  CUUAC-..C. CU.G....N. C.GGG..G.. ....GUU--C ACGGC
PAR  CUCGAC..C. CU.G....U. C.GGG..G.. ....GUU--C UCCGC
RAD  CUUGAC..C. CU.G....U. C.GGG..G.. ....GUU--C UCCGC
FIM  CUCGAC..C. CU.G....U. C.GGG..G.. ....GUUU-U UCGCC
LAR  CUCGAC..C. CU.G....U. C.GGG..G.. ....GUU--C UUGCU
AD2  CUCGUC..C. CU.G....U. C.GGG..G.. ....UGCGAC ACUCC
AD1  CUCGAC..C. UU.C....U. C.GGG..G.. ....GGC--C ACGCC
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FIG. 3.—Partial sequences of the 28S rRNA (L2 region) of *Saccharomyces cerevisiae* and species of *Ceratocystis* sensu stricto. Dots indicate identical nucleotides to those of *S. cerevisiae;* dashes indicate missing nucleotides (gaps); and "N" indicates an undetermined base.

placed AD1-AD2, FIM-LAR, and MON-RAD-PAR as trichotomous sister groups (fig. 5, *middle bottom*). The topology of the bootstrap tree was the same as the F-M tree and the topmost tree in figure 5. COE and VIR were placed together, as were AD1 and AD2, in 100% of the bootstrapped trees (fig. 5, *bottom*). PAR, RAD, and MON were placed together in 78% of the trees. The ambiguity in the positions of these groups relative to one other is reflected in a low confidence (42%) at the branchpoint connecting them.

To resolve this ambiguity, we analyzed the sequences from the three regions of rRNA separately. The sequences of the 18S rRNA and of one of the 28S rRNA (L1) regions showed little variability, and several parsimonious trees were found. As noted earlier, the terminal 5′ end of the 28S (L2) region was the most variable of the three regions, and a single parsimony tree was found for this sequence that placed FIM, LAR, AD1, and AD2 into a single group (fig. 6, *top*). The bootstrap tree of this sequence had the same topology, and the latter four species were placed together in 69% of the bootstrapped trees (fig. 6, *bottom*). We thus prefer the topology of the three topmost trees of figure 4 and the topmost and bottommost trees of figure 5, and we use this topology to represent the phylogeny of the species of *Ceratocystis.*

We then used this tree to examine the nature of the nucleotide substitutions among these species. There were 93 (41.7%) transitions and 130 (58.3%) transversions distributed along the branches of the tree. The distributions of these substitutions and the expected numbers of transitions and transversions, based on the frequencies of nucleotides in all the sequences, excluding *S. cerevisiae,* are shown in figure 7. The observed numbers of unpolarized transversions (G-C, A-T, T-G, and A-C) deviated significantly from the expected number ($\chi^2 = 9.48$, degrees of freedom = 3, $P < 0.05$) and were due to an excess of A-T substitutions. The numbers of transitions (A-G and T-C) also significantly exceeded the expected number ($\chi^2 = 64.02$, degrees of freedom = 1, $P < 0.001$) and were due to an excess of A-G substitutions.

To understand this bias, we examined the transition/transversion ratio (T), both at nucleotide sites changing once on the tree and at sites changing more than once. A total of 99 sites changed once, and this group had $T = 0.77$, whereas 124 sites changed more than once and had $T = 0.68$. For each class, except G-T, the number of multiple substitutions exceeded the number of single differences at a nucleotide position. Overall, $T = 0.72$.

Discussion

Phylogeny

Nucleic acid sequences from rRNA subunits have proved useful for inferring phylogenetic trees in several fungal taxa (Bruns and Szaro 1992), because they are orthologous among all organisms, and because rRNAs do not undergo transfer between species, as do some nucleotide sequences (Sogin 1991). Since all rRNAs have the same function, the same parts of the rRNA molecule are under functional and structural constraints in different species. Moreover, different regions of the molecule evolve faster than other parts, because of the functional constraints. Conserved elements are distributed throughout the length of the molecule and are interspersed by regions with high rates of change. rRNA is therefore useful for inferring phylogenies of distantly and closely related taxa. Slowly evolving regions can be used to estimate phylogenetic relationships among distantly related taxa, and rapidly evolving regions can be used to study closely related taxa. Sequence analysis is further facilitated by the interspersion of conserved and nonconserved regions, so that oligonucleotide extension primers to conserved regions can be used to sequence adjoining nonconserved regions. There are, however, several potential sources of error in estimates of phylogenies based on rRNA sequences. One is that the sec-

Table 2
Average Number of Nucleotide Substitutions per Nucleotide Site (Kimura 1980), between *Sacchromyces cerevisiae* and nine species of *Ceratocystis*

	1	2	3	4	5	6	7	8	9	10
1. SCER	0.0									
2. AD1	0.246	0.0								
3. AD2	0.244	0.055	0.0							
4. COE	0.226	0.147	0.133	0.0						
5. FIM	0.251	0.136	0.136	0.141	0.0					
6. LAR	0.245	0.145	0.133	0.136	0.120	0.0				
7. MON	0.205	0.125	0.107	0.090	0.118	0.117	0.0			
8. PAR	0.231	0.145	0.118	0.144	0.129	0.139	0.083	0.0		
9. RAD	0.254	0.148	0.134	0.144	0.150	0.147	0.093	0.070	0.0	
10. VIR	0.245	0.140	0.142	0.053	0.134	0.134	0.101	0.161	0.150	0.0

NOTE.—Distances were calculated from 489 nucleotide sites combined from partial sequences of one region of the 18S and two regions of the 28S rRNA subunits.

ondary structure of the rRNA molecule constrains sequence evolution differently in different parts of the molecule. Wheeler and Honeycutt (1988) found that sequences in stem regions that paired with distant sequences less accurately estimated phylogenies than did nonpairing sequences in loop regions. In this study, we did not attempt to isolate the two kinds of sequences to produce separate trees.

We also combined sequences from different parts of the rRNA molecule, so that there may be conflicting information in the different subsections. Not all of our trees based on the three subsequences were congruent; those based on the less polymorphic 18S rRNA and 28S rRNA (L1 primer) regions showed different topologies than did the others, and we felt that these trees were less reliable than trees based either on the combined data set or on the more polymorphic 28S rRNA (L2 primer) region. Unlike our study, that by Lane et al. (1985), showed that the phylogenetic trees of several widely divergent species, including fungi, constructed with limited regions of 18S rRNA had the same topologies as did those inferred from the complete molecule.

Mode and Tempo of Substitutions

One important result of this study was that there was a significant excess of A-G and G-A transitions on the tree. A similar excess of transitions in rRNA encoded by nuclear genes but not by mitochondrial genes was found by Bruns and Szaro (1992) in 10 species of Boletaceae. In that study, however, the increased number of transitions was due to an excess of C-T substitutions. They suggested that this class of transitions may be due to the deamination of 5-methylcytosine and change to thymine by a process called "repeat induced point mutation" (RIP). Cambareri et al. (1989) found that in *Neurospora,* after RIP produced numerous C-to-T substitutions, replicative repair changed G-T mismatches to A-T. This may be one of the mechanisms bringing about the excess of A-G transitions in the present study. It is not clear, however, why Boletaceae should have an excess of C-T transitions and why *Ceratocystis* should have an excess of A-G transitions.

In the absence of an absolute measure of divergence among taxa that can be correlated with a molecular clock, two pieces of evidence suggest that these *Ceratocystis* species are old. The first is that there were a large proportion of multiple substitutions on the tree. Usually, the initial divergence between two taxa is characterized by the accumulation of single nucleotide substitutions and is followed by an increase in the number of multiple substitutions (Brown et al. 1979).

The second piece of evidence suggesting an old age for *Ceratocystis* species is a large number of transversions. Only about 42% of the total number of substitutions were transitions ($T = 0.72$). In a group of Boletaceae considered to be at a low to moderate level of divergence, Bruns and Szaro (1992) found an overall T of 1.7. The relative proportions of transitions and transversions are related to the amount of divergence between species. For example, De Salle et al. (1987) found that, in the large-subunit mitochondrial rRNA of Hawaiian *Drosophila,* transitions accounted for 90% of the differences between taxa that had been separated for ≤ 1 Myr but accounted for only 40% of the differences between taxa separated for ≥ 10 Myr. The old age of *Ceratocystis* species demonstrated here contrasts with results for some recently evolved species of the similar genus *Ophiostoma* (Brasier 1991). The great phylogenetic distance between these genera has also recently been demonstrated by Hausner et al. (1992, 1993*a*, 1993*b*).

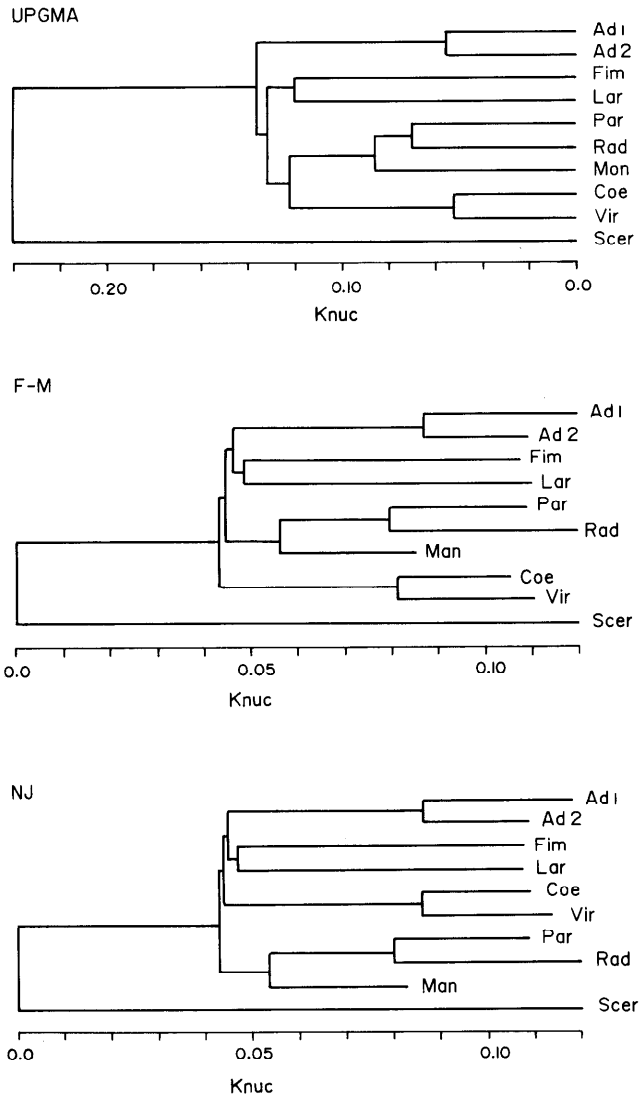


FIG. 4.—Phenetic analyses of K_{nuc}, based on the combined rRNA sequences of *Ceratocystis*.

Taxonomy

On the basis of morphological similarity, *C. coerulescens* and *C. virescens* have been synonymized by Upadhyay (1981). These species, however, have a similar amount of sequence divergence from each other as do other pairs of species in this genus. Although there is no absolute guide to assigning a taxonomic level to a particular level of sequence divergences, the values of K_{nuc} between pairs of the *Ceratocystis* species were strikingly similar and ranged from 0.055 to 0.161. Each of the samples represented in this study appears to be a distinct taxon.

Various aspects of ascospore morphology have been used extensively in the taxonomy of *Ceratocystis* sensu lato (Upadhyay 1981). In our view, there are three basic shapes: hat-shaped and saturnoid ascospores and ascospores with a uniform sheath (Wolfaardt et al. 1992), which do not entirely coincide with the descriptions by Upadhyay (1981). However, many of our observations with a light microscope, especially those for *C. fimbriata,*

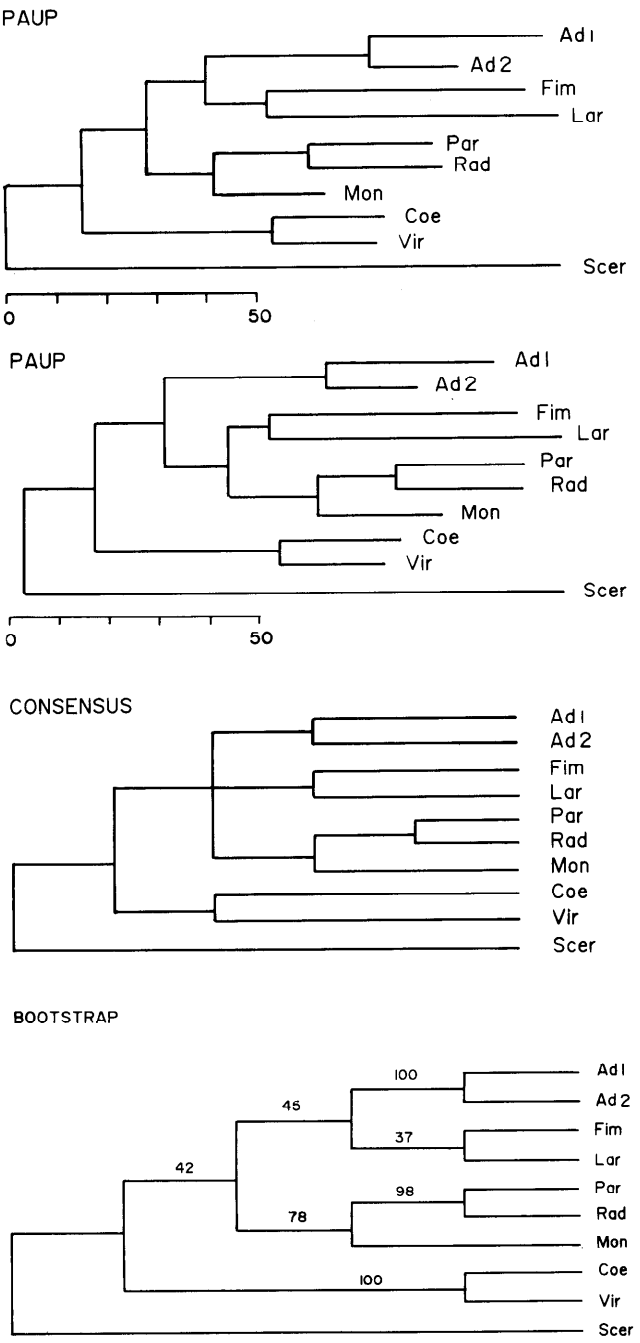
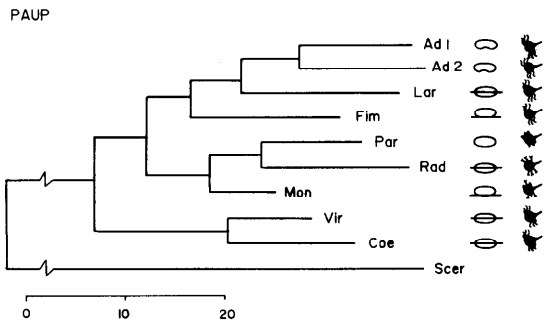


FIG. 5.—Cladistic analyses of the combined rRNA nucleotide sequences of *Ceratocystis*. Strict consensus was used to make a consensus tree of the two equally parsimonious PAUP trees (*two topmost trees*). The bootstrap tree is a majority consensus tree based on 100 bootstraps. The scales represent the number of character-state changes along a branch.

C. moniliformis, and *C. coerulescens,* have been confirmed by electron micrographs (van Wyk and Wingfield 1991).

The distributions of these shapes among the species used in this study are shown in the upper tree in figure 6. Since particular ascospore shapes are not confined to particular clades, specific shapes appear to have originated more than once. This is consistent with recent results reported by Hausner et al. (1992), who found that galeate ascospores have evolved separately in the Ophiostomataceae, Cephaloascaceae, and Endomycetaceae. Hyphal ornamentation, however, appears to be more consistent within groups. Similar ornamentation occurs in both the AD-FIM-LAR clade and the COE-VIR clade and can easily be explained by pleisiomorphic similarity. The spiny and forked appendages in the MON-PAR-RAD group appear to be derived and are consistent with the taxonomies of this group.

Guadet et al. (1989) also found little congruence between an rRNA sequence phylogeny of *Fusarium* and the distributions of morphological characters. In their study, a highly divergent species of *Fusarium* showed convergence of its macroconidium shape with other



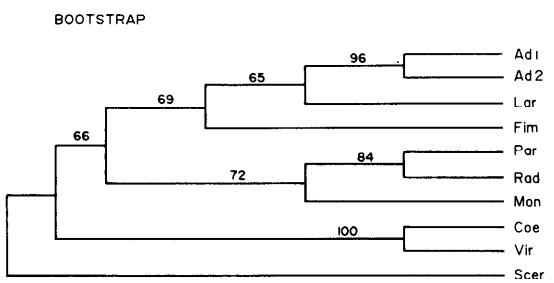


FIG. 6.—Cladistic analyses of the 28S rRNA (L2 region) of *Ceratocystis.* The bootstrap tree is a majority consensus tree based on 100 bootstraps. The scale represents the number of character-state changes. The first column of diagrams indicates ascospore shape, and the second column indicates hyphal ornamentation.

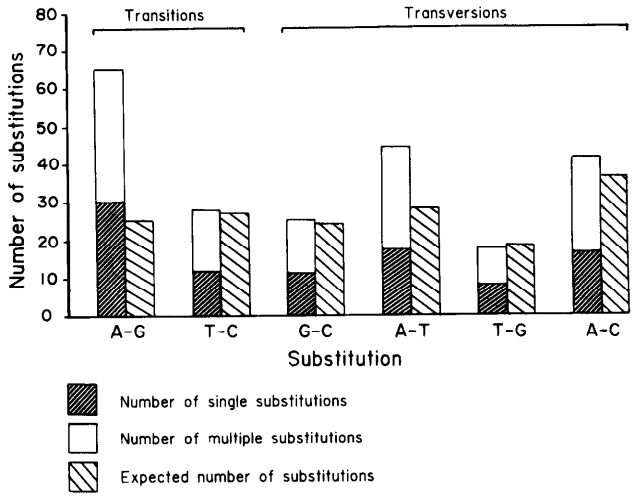


FIG. 7.—Distribution of observed and expected transitions and transversions in the rRNA phylogeny of *Ceratocystis.*

species of *Fusarium.* They also found that the classification of members of the Pyrenomycetes into various genera on the basis of their sexual state resulted in fragmented higher taxa that were genetically closely related. They suggested that perithecial morphology and conidial characters should be used to delineate only subgeneric taxa within the *Fusarium* group. These results, together with those of the present study, indicate that the use of some morphological characters in ascomycete taxonomy should be undertaken with caution and after available molecular data have been taken into consideration.

LITERATURE CITED

BLANZ, P. A., and M. UNSELD. 1986. Ribosomal RNA as a taxonomic tool in mycology. Pp. 247–258 *in* G. S. DE HOOG, M. T. SMITH, and A. C. M. WEIJMAN, eds. The expanding realm of yeast-like fungi. Elsevier Science, Amsterdam.

BRASIER, C. M. 1991. *Ophiostoma novo-ulmi* sp. nov.: causative agent of the current Dutch elm disease pandemics. Mycopathologia **115**:151–161.

BROWN, W. M., M. GEORGE, JR., and A. C. WILSON. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. USA **76**:1967–1971.

BRUNS, T. D., and T. M. SZARO. 1992. Rate and mode differences between nuclear and mitochondrial small-subunit rRNA genes in mushrooms. Mol. Biol. Evol. **9**:836–855.

CAMBARERI, E. B., B. C. JENSEN, E. SCHABTACH, and E. U. SELKER. 1989. Repeat-induced G-C to A-T mutations in *Neurospora.* Science **224**:1571–1575.

CHIRGWIN, J. M., A. E. PRZYBYLA, R. J. MACDONALD, and W. J. RUTTER. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.

DE HOOG, G. S., and R. J. SCHEFFER. 1984. *Ceratocystis* versus *Ophiostoma:* a reappraisal. Mycologia **76**:292–299.

DE SALLE, R., T. FREEDMAN, E. M. PRAGER, and A. C. WILSON. 1987. Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila.* J. Mol. Evol. **26**:157–164.

FELSENSTEIN, J. 1985. Confidence intervals on phylogenies: an approach using the bootstrap. Evolution **39**:783–791.

FITCH, W. M., and E. MARGOLIASH. 1967. Construction of phylogenetic trees. Science **155**:279–284.

GUADET, J., J. JULIEN, J. F. LAFAY, and Y. BRYGOO. 1989. Phylogeny of some *Fusarium* species, as determined by large-subunit rRNA sequence comparison. Mol. Biol. Evol. **6**:227–242.

HAUSNER, G., J. REID, and G. R. KLASSEN. 1992. Do galeate-ascospore members of the Cephaloascaceae, Endomycetaceae and Ophiomataceae share a common phylogeny? Mycologia **84**:870–881.

———. 1993*a*. *Ceratocystiopsis:* a reappraisal based on molecular criteria. Mycol. Res. **97**:625–633.

———. 1993*b*. On the subdivision of *Ceratocystis* s.l., based on partial ribosomal DNA sequences. Can J. Bot. **71**:52–63.

HARRINGTON, T. C. 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis.* Mycologia **73**:1123–1129.

KIMURA, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**:111–120.

LANE, D. J., B. PACE, G. J. OLSEN, D. A. STAHL, M. L. SOGIN, and N. R. PACE. 1985. Rapid determination of 16S ribosomal DNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82**:6955–6959.

MINTER, D. W., P. M. KIRK, and B. C. SUTTON. 1982. Holoblastic phialides. Trans. Br. Mycol. Soc. **79**:75–93.

SAITOU, N., and M. NEI. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**:406–425.

SEIFERT, K. A., M. J. WINGFIELD, and J. F. WEBER. 1993. Methods for studying *Ophiostoma* and *Ceratocystis. Ceratocystis* and *Ophiostoma:* biology, taxonomy and ecology. American Phytopathological Society, St. Paul.

SNEATH, P. H. A., and R. R. SOKAL. 1973. Numerical taxonomy. W. H. Freeman, San Francisco.

SOGIN, M. L. 1991. The phylogenetic significance of sequence diversity and length variations in eukaryotic small subunit ribosomal RNA coding regions. Pp. 175–188 *in* L. WARREN and H. KOPROWSKI, eds. New perspectives on evolution: the Wistar Symposium series. Volume **4.** Wiley-Liss, New York.

SWOFFORD, D. L. 1985. PAUP: phylogenetic analysis using parsimony, version 2.4. Illinois Natural History Survey, Champaign.

UPADHYAY, H. P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis.* University of Georgia Press, Athens.

VAN WYK, P. W. J., and M. J. WINGFIELD. 1991. Ascosporogenesis in *Ophiostoma davidsonii.* Mycol. Res. **95**:725–730.

WEIJMAN, A. C. M., and G. S. DE HOOG. 1975. On the subdivision of the genus *Ceratocystis.* Antonie van Leeuwenhoek **41**:353–360.

WHEELER, W. C., and R. L. HONEYCUTT. 1988. Paired sequence difference in ribosomal RNAs: evolutionary and phylogenetic implications. Mol. Biol. Evol. **5**:90–96.

WOLFAARDT, J. F., M. J. WINGFIELD, and W. B. KENDRICK. 1992. A synoptic key and computer data base to species of *Ceratocystis sensu lato.* S. Afr. J. Bot. **58**:277–285.

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