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Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data

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Abstract: *Ceratocystis* sensu stricto includes numerous species of insect-vectorred, wood-staining and plant pathogenic fungi. Among these, *Ceratocystis coerulescens* is a well-known cause of blue-stain in spruce and pine. Previous investigations, using morphological characteristics and isozyme comparisons, have shown that *C. coerulescens* encompasses at least five morphological types. The aim of this study was thus to compare isolates of *C. coerulescens* sensu lato and morphologically similar species, including *C. laricicola*, *C. polonica*, *C. virescens*, *C. eucalypti*, *Chalara australis* and *Ch. neocaledoniae*, on the basis of DNA sequence data. Using the polymerase chain reaction (PCR), a 600 base pair fragment within the ribosomal DNA operon was amplified, and the PCR products were sequenced. The analyzed sequence included the 5.8S rRNA gene and the internal transcribed spacers (ITS) 1 and 2. Relationships were determined by parsimony analysis. Using *C. fimbriata* as the outgroup taxon, the five morphological types previously known as *C. coerulescens* and the two other taxa from conifers formed a strongly-supported monophyletic group that includes all the *Ceratocystis* species occurring primarily on conifers. The species from hardwood trees, *C. eucalypti*, *Ch. australis* and *Ch. neocaledoniae*, also formed a monophyletic group, sister to the conifer group. The fourth species from hardwoods, *C. virescens*, formed a group basal to the two sister groups.

Key Words: ITS sequence, phylogeny, ribosomal DNA

INTRODUCTION

Ceratocystis sensu stricto Ellis & Halsted includes many economically important species, which are pathogens on a wide variety of plants, including forest and fruit trees, sweet potatoes, rubber and sugar cane (Kile, 1993). Most *Ceratocystis* species occur on angiosperms, but *Ceratocystis coerulescens* (Münch) Bakshi, first described from Germany (Münch, 1907), is the cause of sapstain on spruce and pine. Based on isozyme analysis Harrington et al. (1996) demonstrated five morphologically distinct species previously known as *C. coerulescens*. The *C. coerulescens* complex thus includes species A, B and C from logs or wounds on *Picea* or *Pinus* (Harrington et al., 1996) and two newly recognized species, *C. rufipenni* Wingfield, Harrington and Solheim and *C. douglasii* (Davidson) Wingfield and Harrington (Wingfield et al., 1997). *Ceratocystis douglasii* causes a stain in wood of Douglas-fir (*Pseudotsugae menziesii*) (Davidson, 1953; Wingfield et al., 1997), and *C. rufipenni* occurs on spruce attacked by the bark beetle *Dendroctonus rufipennis* (Davidson, 1955; Wingfield et al., 1997).

Ceratocystis laricicola Redfern and Minter and *C. polonica* (Siemaszko) Moreau also occur on conifers and are morphologically similar to *C. coerulescens* but have smaller and more ellipsoid ascospores (Harrington et al., 1996). *Ceratocystis polonica* is associated with the bark beetle *Ips typographus* on spruce in Europe (Siemaszko, 1938; Christiansen and Solheim, 1990) and Japan (Yamaoka et al., 1997). Harrington et al. (1996) found *C. laricicola*, which occurs on larch infested with the bark beetle *Ips cembrae* in Europe (Redfern et al., 1987) and Japan (Visser et al., 1995), to be morphologically indistinguishable from *C. polonica*. Comparisons of these species using DNA sequence data also suggest that they may be synonyms (Visser et al., 1995), although they differ at a single isozyme locus (Harrington et al., 1996) and are not sexually compatible (Harrington and McNew, unpublished).

Ceratocystis virescens (Davidson) Moreau, described by Davidson (1944) from hardwood lumber, has been considered a synonym of *C. coerulescens* (Hunt, 1956; Upadhyay, 1981). More recent studies have shown that it is restricted to hardwood hosts, as a pathogen on maple or as a saprophyte, and is morphologically distinct from *C. coerulescens* (Harrington et al., 1996;

TABLE I. Isolates of *Ceratocystis* used for DNA sequencing, their origins and GenBank Accession Numbers of ITS sequence of DNA

Isolate number ^a	Species	Host	Origin	GenBank Accession Number
C490, CMW1323	<i>C. coerulescens</i> sp. A	<i>Pinus</i> sp.	England	U75614
C313, CBS140.37	<i>C. coerulescens</i> sp. B	<i>Picea abies</i>	Germany	U75615
C50, CMW0451	<i>C. coerulescens</i> sp. C	<i>Picea engelmannii</i>	USA	U75616
C662	<i>C. coerulescens</i> sp. C	<i>Picea abies</i>	Norway	U75617
C666	<i>C. coerulescens</i> sp. C	<i>Picea abies</i>	Norway	U75618
C609	<i>C. rufipenni</i>	<i>Picea engelmannii</i> / <i>D. rufipennis</i> ^b	Canada	U75619
C610	<i>C. rufipenni</i>	<i>Picea engelmannii</i> / <i>D. rufipennis</i> ^b	Canada	U75620
C612	<i>C. rufipenni</i>	<i>Picea engelmannii</i> / <i>D. rufipennis</i> ^b	Canada	U75621
CMW1016	<i>C. laricola</i>	<i>Larix decidua</i> / <i>I. cembra</i> ^b	Scotland	U75622
C708, CMW0672, CBS228.83	<i>C. polonica</i>	<i>Picea abies</i> / <i>I. typographus</i> ^b	Norway	U75623
C74, CMW0460	<i>C. virescens</i>	<i>Quercus</i> sp.	USA	U75624
C251	<i>C. virescens</i>	<i>Acer</i> sp.	USA	U75625
C69	<i>C. virescens</i>	<i>Fagus americanum</i>	USA	Sequence = U75625
C203, ATCC11066	<i>C. virescens</i>	<i>Liriodendron tulipifera</i>	USA	Sequence = U75625
C324, CBS142.53	<i>C. douglasii</i>	<i>Pseudotsuga menziesii</i>	USA	U75626
C639	<i>C. eucalypti</i>	<i>Eucalyptus sieberi</i>	Australia	U75627
C694, CBS149.83	<i>Chalara neocaledoniae</i>	<i>Coffea robusta</i>	New Caledonia	U75628
C619	<i>Chalara australis</i>	<i>Nothofagus cunninghamii</i>	Australia	U75629
C854	<i>C. fimbriata</i>	<i>Ipomoea batatas</i>	USA	AF007749

^a CMW—Culture collection of M. J. Wingfield. C—Culture collection of T. C. Harrington. CBS—Centraal Bureau voor Schimmelcultures, Baarn, Netherlands. ATCC—American Type Culture Collection.

^b Bark beetle associates, where known, are in the genera *Ips* and *Dendroctonus*.

Kile and Walker, 1987). *Ceratocystis eucalypti* Yuan and Kile is morphologically similar to *C. virescens* and colonizes wounds in *Eucalyptus* in Australia (Kile et al., 1996). Two *Chalara* species, namely *Ch. neocaledoniae* Kiffer and Delon and *Ch. australis* Walker and Kile, have no known teleomorphs, but their anamorphs are similar in morphology to the anamorphs of *C. virescens* and *C. eucalypti* (Harrington et al., 1996) and are thus included in the present study of the *C. coerulescens* complex. Both these *Chalara* species cause diseases in hardwoods that are similar to sap streak of maple caused by *C. virescens* (Kile, 1993). *Chalara australis* occurs in Australia on *Nothofagus cunninghamii* and *Ch. neocaledoniae* was described from coffee and guava in New Caledonia (Kile et al., 1996).

Sequence data from the ribosomal RNA genes has successfully been used in determining the phylogenetic relationships among species of *Ceratocystis* (Hausner et al., 1993; Wingfield et al., 1994; Visser et al., 1995; Wingfield et al., 1996). High variability in DNA sequence exists among these species in the internal transcribed spacer (ITS) regions of the ribosomal RNA genes (Visser et al., 1995; Wingfield et al., 1996). The aim of this study was, therefore, to

compare species of the *Ceratocystis coerulescens* complex using the DNA sequence from the variable ITS regions in the ribosomal RNA operon.

MATERIALS AND METHODS

All isolates (TABLE I) were grown on malt extract agar (20 g/L ME and 20 g/L agar) in Petri dishes at room temperature for 10 d. Template DNA for amplification was obtained by scraping the mycelium with a pipette tip (Harrington and Wingfield, 1995). Amplifications were performed using the primers ITS1 and ITS4 (White et al., 1990) or ITS1F (Gardes and Bruns, 1993) and ITS4. The amplified fragments include the 3' end of the small subunit (SSU) rRNA gene, the 5.8S rRNA gene, part of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) regions 1 and 2. The PCR reaction mixture included 2.5 units of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany), the buffer supplied with the enzyme, 250 μ M dNTPs, 6.25 mM MgCl₂ and 0.5 μ M of each primer. Initial denaturation was performed at 96 C for 60 s, followed by 35 cycles of primer annealing at 55 C for 30 s, chain elongation at 72 C for 60 s and denaturation at 92 C

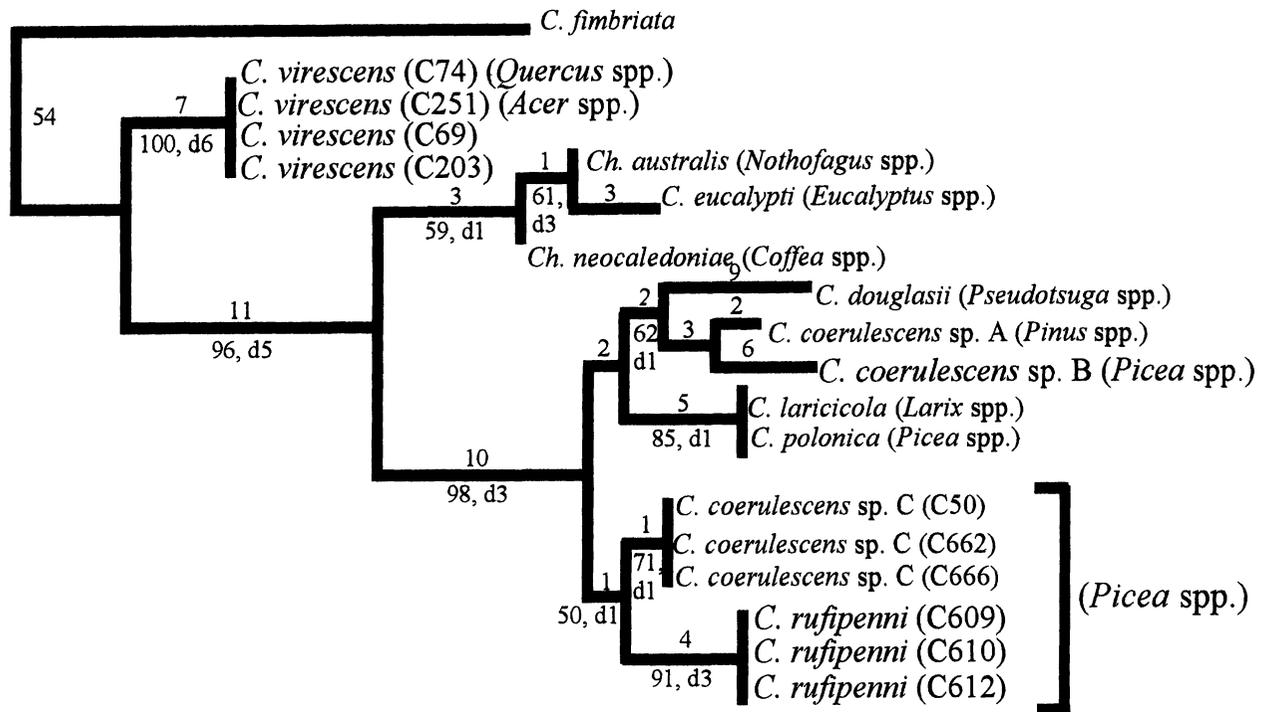


FIG. 1. One of the two most parsimonious trees produced from analysis of the DNA sequence of part of the ribosomal RNA operon (565 bp in size) using PAUP 3.1.1. Tree length = 124. The number of base substitutions are indicated above the branches and the bootstrap percentages (100 bootstrap replicates) and decay index (100 replicates) are indicated below the branches. Host species are indicated in brackets.

for 60 s. Final chain elongation took place at 72 C for 5 min. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized using UV light.

The 600 base pair PCR fragments were purified using Wizard PCR Mini-Preps (Promega Corporation, USA) or Microcon Microconcentrators (Amicon, Inc., USA). Both strands of the PCR products were sequenced using the *fmol* DNA Sequencing Kit (Promega Corporation., USA). Seven of the isolates were sequenced using the ABI PRISM 377 DNA sequencer and ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, USA) at the DNA Sequencing Facility at Iowa State University. The primers ITS1, ITS1F, ITS2, ITS3 and ITS4 were used in the sequencing reactions. The DNA sequence data were deposited in GenBank (TABLE I). The nucleotide sequences were manually aligned, then analyzed using PAUP (Phylogenetic Analysis Using Parsimony) 3.1.1 (Swofford, 1993). The ITS sequence of *Ceratocystis fimbriata* Ellis & Halsted, isolated from sweet potatoes, was used as an outgroup. Confidence intervals of the branch points were determined using the bootstrap technique (Felsenstein, 1985) and decay indexes (AUTODECAY 2.9.4., a computer program developed by Torsten and Eriksson in 1996).

Sequence alignments and cultures are available from the corresponding author.

RESULTS

The PCR amplifications from living fungal mycelium of isolates studied consistently produced amplification products of approximately 600 bp, and the DNA sequences of the species in the *C. coerulescens* complex were found to be similar. It was possible to align the data manually by inserting gaps in the sequence data. With *C. fimbriata* as the outgroup taxon, two most parsimonious trees of 124 steps were produced from the aligned 565 bp sequence data, including ITS 1 and 2 and the 5.8S gene, using the Heuristic search option of PAUP (FIG. 1) (CI=0.887, HI=0.113, RI=0.899, RC=0.797). Gaps were treated as missing data. The two trees differed only in the relationship between *C. coerulescens* species A and B and *C. douglasii*. One tree (FIG. 1) grouped *C. coerulescens* species A and B, while the second tree grouped *C. coerulescens* species B and *C. douglasii*. The same topology was obtained when midpoint rooting was used.

All the species associated with conifers, that is, *C. coerulescens* spp. A, B and C, *C. douglasii*, *C. laricicola*,

C. polonica and *C. rufipenni*, formed a single clade, supported by a bootstrap value of 98% and a decay index of d3. Among the conifer species, three isolates of *C. rufipenni* grouped closely together into one well supported subclade (91%, d3). A second distinctive subclade was formed by the three isolates of *C. coerulescens* species C (71%, d1). *C. laricicola* and *C. polonica* had identical sequences and grouped together (85%, d1), forming a subclade sister to the clade formed by *C. coerulescens* sp. A and sp. B and *C. douglasii* (62%, d1). The three Australasian species from hardwoods, *C. eucalypti*, *Ch. neocaledoniae* and *Ch. australis*, grouped in a clade sister to the conifer clade (59%, d1). The fourth species from hardwoods, namely *C. virescens*, represented by four isolates with identical sequence, was basal to the conifer and Australasian subgroups (100%, d6). Numerous gaps were created to achieve the alignment and there were regions of ambiguity, particularly in aligning the outgroup with the ingroup. The same tree topology was observed when ten ambiguously aligned regions (157 bases in total) were removed from the data set.

Since most of the alignment ambiguity was between the ingroup and the outgroup, *C. fimbriata*, the data set was realigned and reanalyzed after the removal of the *C. fimbriata* sequence. *C. virescens* was defined as the outgroup and two ambiguously aligned regions (52 bases in total) near the large subunit gene were removed, with the gaps treated as a fifth character (gap-mode=newstate). Two parsimonious trees of 90 steps were produced from the aligned 477 bp sequence data (CI=0.811, HI=0.189, RI=0.919, RC=0.776). Both these trees reflected the same topology as found when *C. fimbriata* was used as an outgroup, with *C. coerulescens* species A and B grouping together (69%, d3). In the one tree *C. coerulescens* species C was basal to the rest of the conifer species (95%, d3). The conifer subclade (98%, d4), the Australasian subclade (92%, d2), as well as the subclade including *C. coerulescens* species A and B, *C. douglasii*, *C. laricicola* and *C. polonica* (99%, d5) were better supported after the removal of *C. fimbriata* than in the analysis with *C. fimbriata* as the outgroup taxon.

DISCUSSION

The phylogenetic analyses show that species of *Ceratocystis* from conifers form a distinct clade, suggesting that these species are monophyletic. This is despite the fact that they differ somewhat in their morphology and more noticeably in their ecology, except that they all occur on conifers in the family *Pinaceae*. In fact these are the only *Ceratocystis* species that commonly occur on conifers. It appears that the adaptation to conifers is a derived character in *Ceratocystis*, which otherwise

occur on dicots and monocots. This change in host range may have evolved only once in the genus.

In this study it was possible to determine a phylogenetic relatedness of species in the *C. coerulescens* complex based on DNA sequence data from the variable region in the ribosomal RNA operon. The results confirmed the recent delineation of species in this complex based on morphology and isozyme analyses (Harrington et al., 1996). There was less variation than expected in the DNA sequence data of the ITS regions among species in the *C. coerulescens* complex. Little or no variation exists between these species in the highly conserved large subunit rRNA gene (authors, unpublished), which indicates close relationships in the complex as a whole.

Ceratocystis coerulescens has been recognized as a causal agent of blue-staining of conifers in Europe and in North America (Kile, 1993). In the years subsequent to its description, this species had become a repository for many wound and lumber colonizing fungi that generally resemble the fungus originally described by Münch (1907). Species such as *C. virescens* were reduced to synonymy with *C. coerulescens* (Hunt, 1956; Upadhyay, 1981), and the taxa recently described as *C. douglasii* and *C. rufipenni* (Wingfield et al., 1997) were treated under the name *C. coerulescens*. In a recent study based on isozyme analyses, Harrington et al. (1996) showed that these species and three additional species, designated *C. coerulescens* species A, B and C, represent distinct taxa. The sequence data support these delimitations.

Our results indicate that *C. coerulescens* sp. A and *C. coerulescens* sp. B are closely related but distinct, and these species form a clade with *C. douglasii*. *C. douglasii* was originally described as *C. coerulescens* f. *douglasii* by Davidson (1953), who noted differences in the anamorphs of *C. douglasii* and *C. coerulescens*. He also noted that the former causes stain in Douglas-fir rather than spruce and pine. The isolates of *C. laricicola* and *C. polonica* had identical ITS sequences. Harrington et al. (1996) and Visser et al. (1995) showed that these fungi are very similar and may be conspecific. These bark beetle associates differ from the other conifer species in the *C. coerulescens* complex in ascospore morphology (Harrington et al., 1996) but form a weakly-supported clade with *C. coerulescens* sp. A, sp. B and *C. douglasii*. Isozyme analyses also showed similarity between *C. laricicola* and *C. polonica* and *C. coerulescens* species A and B (Harrington et al., 1996).

Included in the conifer clade were *C. coerulescens* sp. C and *C. rufipenni*, which is associated with the bark beetle *Dendroctonus rufipennis*, which attacks spruce in North America. The three isolates of *C. coerulescens* species C, which have perithecial necks shorter than those of *C. coerulescens* sp. A but other-

wise are morphologically similar (Harrington et al., 1996), form a distinct clade separate from the other conifer species. Relationships among *C. rufipenni*, *C. coerulescens* sp. C and the other conifer species are not well resolved.

Ceratocystis eucalypti appears to be very closely related to *Ch. australis* and *Ch. neocaledoniae*. These fungi are common to Australasia, and their apparent relatedness is not surprising. It has also been shown that *Ch. australis* is partially interfertile with MAT-1 strains of *C. eucalypti* (Kile et al., 1996). *C. virescens*, also on hardwoods, has a morphology similar to that of *C. eucalypti* (except the latter has much larger ascospores) and was found to be basal to the Australasian hardwood species and to the conifer species.

The phylogenetic relationships as determined here differ somewhat from the distance analysis derived from isozyme data (Harrington et al., 1996). Our phylogenetic approach shows taxa on conifers to be monophyletic, possibly evolving from a hardwood ancestor. The hardwood species, *Ch. neocaledoniae*, *Ch. australis* and *C. eucalypti*, grouped more closely with each other based on the DNA sequence data than on the isozyme markers. Also, *C. douglasii* differed substantially from all other species in the complex based on isozymes but was near *C. coerulescens* sp. A and sp. B based on the ITS sequence data. In both analyses, *C. laricicola* and *C. polonica* were closer to *C. coerulescens* sp. A and sp. B than to *C. coerulescens* sp. C and *C. rufipenni*.

In this study we have shown that it is possible to distinguish taxa in *Ceratocystis* based on DNA sequence data from the ribosomal RNA operon. The fact that various species that we have considered have been compared using other techniques with very similar results gives us substantial confidence in this approach. The data pointed to the importance of host range in the evolution of *Ceratocystis* species. We believe that there is considerable opportunity to compare other species of *Ceratocystis* and related fungi based on sequence data of the ribosomal RNA operon and expect that this will result in a considerable improvement in our understanding of the relationships of the group.

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