Mycologia, 92(3), 2000, pp. 447–452.

Comparison of isozymes, rDNA spacer regions and *MAT*-2 DNA sequences as phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex

R. Corli Witthuhn[1]

Department of Food Science, Private Bag X1, University of Stellenbosch, Matieland, 7602, South Africa

Thomas C. Harrington
Joseph P. Steimel

Department of Plant Pathology, 351 Bessey Hall, Iowa State University, Ames, Iowa, USA 50011

Brenda D. Wingfield

Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

Michael J. Wingfield

Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

Abstract: The genus *Ceratocystis* sensu stricto includes species that are insect-vectored, plant pathogens, occurring mainly on angiosperm hosts. Species in the *Ce. coerulescens* complex form a morphologically distinct group that are pathogens on gymnosperms (Pinaceae) and angiosperms. In recent years, species in the *Ce. coerulescens* complex have been intensively studied based on their morphology and isozymes, as well as on their molecular characteristics. The aim of this study was to compare the phylogeny of these species inferred from the *MAT*-2 HMG box DNA sequences to the phylogenies inferred from partial rDNA sequences and isozyme data. Part of the *MAT*-2 HMG box of species in the *Ce. coerulescens* complex was amplified using PCR. The PCR fragments were sequenced and phylogenetically analysed using parsimony, with *Ce. fimbriata* as the outgroup taxon. Species in the *Ce. coerulescens* complex on gymnosperms formed a single clade, inferred to be a sister-taxon to the clade that included the species on angiosperm hosts, thus supporting the hypothesis that the adaptation to gymnosperms was a single evolutionary event in the history of *Ceratocystis*. The phylogeny inferred from the *MAT*-2 HMG box sequences was found to be similar to the phylogeny based on the internal transcribed spacer (ITS) regions of the rDNA. However, the *MAT*-2 HMG box sequences enabled the separation of *Ce. laricicola* from *Ce. polonica*, as well as the separation of the *Ce. virescens* isolates on *Acer* and *Liriodendron* from those on *Fagus* and *Quercus*. Similarly, isozyme data separated these morphologically similar sister species, but isozyme analysis did not appear to reflect a true phylogeny of the species in the *Ce. coerulescens* complex.

Key Words: Ascomycetes, ITS, HMG box, Microascales

Accepted for publication November 22, 1999.
[1] Email: rcwit@maties.sun.ac.za

INTRODUCTION

The ascomycete genus *Ceratocystis* is comprised of several species complexes of insect-dispersed plant pathogens. Interfertility, host range, vector relationships, morphology, isozyme electromorphs, and rDNA sequences have been utilized to delimit 11 species in the *Ceratocystis coerulescens* complex (Harrington et al 1998, Harrington and McNew 1998, Harrington and Rizzo 1999, Harrington and Wingfield 1998, Witthuhn et al 1998, 1999). This complex of closely related species provides a good model to compare phenotypic and genotypic characters in species evolution.

Ceratocystis coerulescens (Münch) Bakshi s. l. causes bluestain on spruce (*Picea*), larch (*Larix*), Douglas-fir (*Pseudotsuga*) and pine (*Pinus*) sapwood across the Northern Hemisphere. Harrington and Wingfield (1998) recognized five morphological variants of *Ce. coerulescens* on the Pinaceae: *Ce. coerulescens* s. s. (on spruce, larch and pine in North America, Europe and Asia), *Ce. pinicola* Harrington & Wingfield (on pine in Great Britain), *Ce. douglasii* (Davidson) Wingfield & Harrington (on Douglas-fir in western North America), *Ce. rufipenni* Wingfield, Harrington & Solheim (on spruce in North America), and *Ce. resinifera* Harrington & Wingfield (on spruce and pine in Europe and North America). *Ceratocystis laricicola* Redfern & Minter and *Ce. polonica* Siemaszko are Eurasian species that are morphologically similar to *Ce. coerulescens* and form part of the *Ce. coerulescens* complex; *Ce. laricicola* occurs on larch and is associated with the bark beetle *Ips cembrae* (Redfern et al 1987, Yamaoka et al 1998), and *Ce. polonica* occurs on spruce infested with the bark beetle *Ips typographus* (Christiansen and Solheim 1990, Yamaoka

TABLE I. Isolates of *Ceratocystis*

Species	Isolate[a]	Host	Origin	*MAT*-2 GenBank
Ce. coerulescens	C301, ATCC12859, CBS100198	*Pinus banksiana*	Minnesota, USA	AF164190
	C1423	*Larix kaempferi*	Japan	AF164189
Ce. pinicola	C490, CMW1323	*Pinus* sp.	England	AF164193
	C795	*Pinus nigra*	England	AF164194
Ce. rufipenni	C608, CBS100209	*Picea engelmannii*	BC, Canada	AF164185
	C613	*Picea glauca*	BC, Canada	AF164186
Ce. resinifera	C662, CBS100202	*Picea abies*	Norway	AF164188
	C1308	*Pinus radiata*	California, USA	AF164187
Ce. laricicola	C178	*Larix* sp.	Scotland	AF164195
	C181	*Larix* sp.	Scotland	AF164196
Ce. polonica	C123	*Picea abies*	Norway	AF164197
	C731	*Picea abies*	Norway	AF164198
Ce. virescens	C69	*Fagus americanum*	New Hampshire, USA	AF164182
	C70, CMW0447	*Unknown*	New Hampshire, USA	AF164183
	C74, CMW0460	*Quercus* sp.	New York, USA	AF164184
	C252	*Acer saccharum*	New York, USA	AF164176
	C256	*Acer saccharum*	New York, USA	AF164177
	C257	*Acer saccharum*	New York, USA	AF164178
	C259	*Acer saccharum*	New York, USA	AF164179
	C261	*Acer saccharum*	New York, USA	AF164180
	C262	Unknown	New Hampshire, USA	AF164181
Ce. douglasii	C324, CBS556.97	*Pseudotsuga menziesii*	Colorado, USA	AF164191
	C1179	*Pseudotsuga menziesii*	Washington, USA	AF164192
Ce. eucalypti	C639	*Eucalyptus sieberi*	Victoria, Australia	AF164170
	C641	*Eucalyptus globoidea*	Victoria, Australia	AF164172
	C644	*Eucalyptus sieberi*	Victoria, Australia	AF164171
Ch. neocaledoniae	C694, CBS149.83	*Coffea robusta*	New Caledonia	AF164173
Ch. australis	C448	*Nothofagus cunninghamii*	Tasmania, Australia	AF164175
	C631	*Nothofagus cunninghamii*	Victoria, Australia	AF164174
Ce. fimbriata	C1099, CMW1547	*Ipomoea batatas*	Papua New Guinea	AF164169

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

et al 1997). These seven species of the *Ce. coerulescens* complex are the only species in the genus that occur primarily on gymnosperms (Pinaceae) (Harrington et al 1996).

Four other species in the *Ce. coerulescens* complex are known from angiosperms. *Ceratocystis virescens* (Davidson) Moreau, a pathogen of hardwoods in eastern USA, has been considered a synonym of *Ce. coerulescens* (Hunt 1956, Upadhyay 1981) but has been shown to be distinct (Harrington et al 1996, Witthuhn et al 1998, 1999). *Ceratocystis eucalypti* Yuan & Kile, isolated from *Eucalyptus* in Australia (Kile et al 1996), together with two asexual Australasian species from hardwoods, *Chalara neocaledoniae* Kiffer & Delon and *Ch. australis* Walker & Kile, also form part of the *Ce. coerulescens* complex (Harrington et al 1996, 1998, Witthuhn et al 1998).

The aim of this study was to determine the phylogenetic relationships among the species in the *Ce. coerulescens* complex based on the *MAT*-2 HMG box DNA sequences (Turgeon 1998) and to determine whether the mating type genes are taxonomically useful in delineating species of *Ceratocystis*. The *MAT*-2 HMG box gene phylogeny was compared to the previously generated phylogenies based on the ITS rDNA sequences (Witthuhn et al 1998) and isozyme data (Harrington et al 1996).

MATERIALS AND METHODS

The collection information on most of the studied isolates is found in Harrington et al (1996). Most isolates (TABLE I) were grown in 20 g/L malt extract and 10 g/L yeast extract for 10–14 d at room temperature (approximately 21 C) before DNA extraction. However, isolates of *Ce. douglasii*, *Ce. rufipenni* and *Ch. australis* were grown at 19 C. Mycelium was harvested, and the DNA was extracted using the method described by DeScenzo and Harrington (1994).

The initial PCR reactions for the amplification of the *MAT*-2 HMG box were performed using degenerate primers (HMG-1 and HMG-2) as described by Arie et al (1997).

After analyses of these sequences, we designed a number of specific primers internal to HMG-1 and HMG-2 to amplify a portion of the *MAT*-2 HMG box in the different *Ceratocystis* species. The *Ce. eucalypti MAT*-2 specific primers EUM2-1 (5'-GACATCAAGCCGTCAAGACCG-3') and EUM2-2 (5'-GTCTTTTTGTATGCTTCGGCC-3') were used for the amplification of the *MAT*-2 HMG box in *Ce. eucalypti, Ce. virescens, Ch. neocaledoniae* and *Ch. australis*. The *Ce. pinicola MAT*-2 specific primers COER2-1 (5'-GACACCAAGACGTCAAAGCC-3') and COER2-2 (5'-GCTTTTCTTGTAAGTTTCAGC-3') were used for the amplification of the *MAT*-2 HMG box in *Ce. pinicola* and *Ce. douglasii*. The primers used for *Ce. laricicola* and *Ce. polonica* were LMAT2-F (5'-CGGAAAGACAGACACCAAGAC-3') and LMAT2-R (5'-GGTTGTAACGGTAGTGGGGATA-3'). Amplification of the *MAT*-2 HMG box in *Ce. coerulescens* utilized the primer pair HMG-1 and COER2-2. For *Ce. resinifera* and *Ce. rufipenni*, we used the primer pairs HMG-1 and HMG-2 or COER2-1 and HMG-2. *Ceratocystis fimbriata* Ellis & Halsted, was amplified using the primers CFM2-1 (5'-GCTACATTTTGTATCGCAAAGAC-3') and CFM2-2 (5'-TAGTGGGGATATGTCAACATG-3').

The 100-μL PCR reactions included 200 μM of dNTPs, 0.5 μM of the degenerate primers or 0.25 μM of the specific primers, 4 mM $MgCl_2$, 1X PCR buffer (supplied with the enzyme), 2.5 units *Taq* polymerase (Promega, Madison, Wisconsin) or Expand High Fidelity *Taq* DNA polymerase (Boehringer Mannheim, Germany), and 10–50 ng of extracted DNA. Most PCR products were generated using an initial denaturation of 60 s at 95 C, followed by 35 cycles of 95 C for 35 s, annealing at 54 C (degenerate primers) or 58 C (specific primers) for 60 s, and elongation at 72 C for 60 s. A final elongation of 10–30 min was added to ensure an "A" overhang on the 3' ends of the PCR product for cloning.

Amplified products of the expected size were purified using the QIAquick PCR Purification Kit (Qiagen Inc., USA) for direct sequencing if it appeared that there was a single PCR product after gel electrophoresis. When multiple bands were found, as was the case when degenerate primers were used, the band of the expected size was eluted from the agarose using the Geneclean II Kit (Bio 101, USA). Both template and coding strands of the PCR products were sequenced at the Iowa State University DNA Sequencing and Synthesis Facility or at the University of the Orange Free State, using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, USA).

The *MAT*-2 HMG box nucleotide sequences were manually aligned and all the characters were phylogenetically analyzed using the heuristic search option (optimality criterion = maximum parsimony, gaps treated as missing, branch-swapping algorithm = tree-bisection reconnection, 100 replicates) in PAUP (version 3.1.1) (Swofford 1993). *Ceratocystis fimbriata,* isolated from sweet potato, was used as the outgroup taxon (Witthuhn et al 1998) in all the analyses. Confidence intervals were determined on all the characters using 1000 bootstrap replicates (Felsenstein 1985). A partition homogeneity test was performed to determine whether the *MAT*-2 and ITS data sets could be combined into a single analysis.

RESULTS

At least 210 bp of the *MAT*-2 HMG box were successfully amplified and sequenced from all of the eleven species forming the *Ce. coerulescens* complex and from the outgroup taxon, *Ce. fimbriata*. Most nucleotides were unambiguously aligned, except for a 30 bp region of the single intron, which differed greatly between the taxa in the angiosperm-associated and Pinaceae-associated clades. Parsimony analysis of the manually aligned *MAT*-2 HMG box DNA sequence data (103 parsimony-informative characters of the 199 bp total DNA sequence) was performed after all the ambiguously aligned intron sequences (30 characters in total) were removed from the data set. A single most parsimonious tree (tree length = 207, CI = 0.792, HI = 0.208, RI = 0.940) was inferred (FIG. 1). The topology of this tree did not differ from the topology of the tree when all the characters were included in the data set (tree length = 243, CI = 0.802, HI = 0.198 and RI = 0.948) or when gaps were treated as an additional character (data not shown). Based on the partition homogeneity test the *MAT*-2 and ITS data sets could not be combined ($P = 0.01$).

Ceratocystis eucalypti, Ch. neocaledoniae, Ch. australis and *Ce. virescens,* all from angiosperm hosts, formed a well supported clade (100% bootstrap value), sister to the clade formed by the species from Pinaceae (86% bootstrap value) in the *MAT*-2 gene tree. The *Ce. eucalypti, Ch. neocaledoniae* and *Ch. australis* isolates formed a clade (66% bootstrap value) sister to the clade formed by the *Ce. virescens* isolates (99% bootstrap value). The *Ce. virescens* isolates from *Acer* and *Liriodendron* formed a clade (83% bootstrap value) sister to the *Ce. virescens* isolates from *Fagus* and *Quercus* (95% bootstrap value).

The species in the *Ce. coerulescens* complex isolated from Pinaceae formed two sister groups. The weakly supported clade of *Ce. rufipenni* and *Ce. resinifera* was sister to the clade formed by *Ce. coerulescens, Ce. pinicola, Ce. douglasii, Ce. laricicola* and *Ce. polonica* (80% bootstrap value). The sequences of *Ce. laricicola* and *Ce. polonica* were distinct but similar in nucleotide composition, and these taxa formed a clade distinct from the other species from conifers (80% bootstrap value). *Ceratocystis coerulescens, Ce. pinicola* and *Ce. douglasii* formed a weakly supported clade (58% bootstrap value).

DISCUSSION

Phylogenetic analysis of *MAT*-2 HMG box DNA sequences from species in the *Ce. coerulescens* complex inferred a phylogeny similar to that based on the ITS

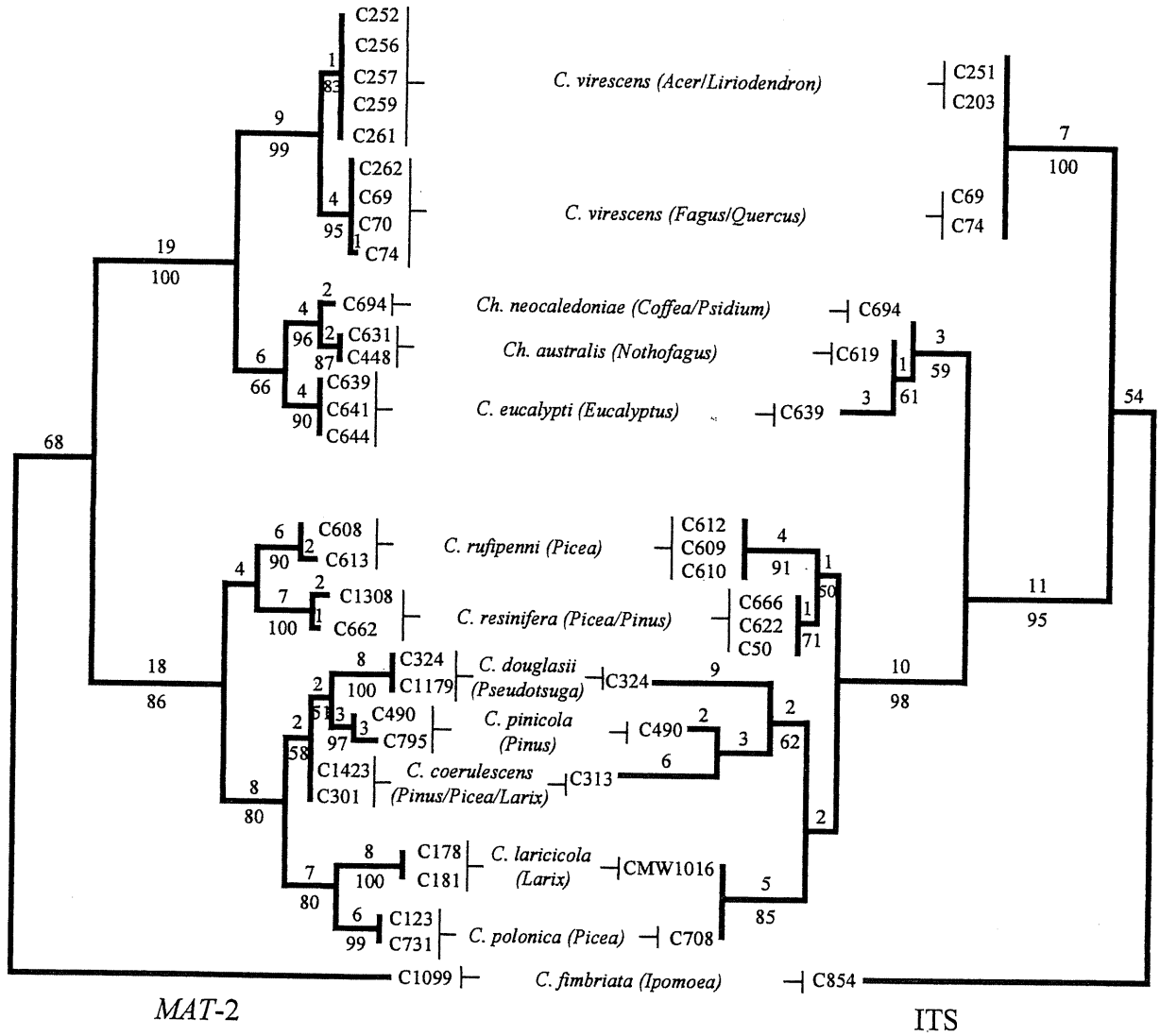

FIG. 1. Comparison of two phylograms (based on ITS and *MAT*-2 DNA sequences) of species in the *Ceratocystis coerulescens* complex. The single most parsimonious tree (tree length = 207) inferred from the DNA sequences of a portion of the *MAT*-2 HMG box (199 bp in total, excluding 30 ambiguously aligned characters) is on the left. One of two most parsimonious trees (tree length = 124) produced from the analysis of the aligned DNA sequences (565 bp) of the ITS and 5.8S regions of the ribosomal RNA operon (Witthuhn et al 1998) is on the right. The number of base substitutions are indicated above the branch points, and the bootstrap values (1000 replicates) are indicated below the branches.

rDNA sequences in many respects. Similar to the ITS rDNA gene phylogeny, the phylogram based on *MAT*-2 HMG box DNA sequences inferred the monophyletic origin of the *Ceratocystis* species found on the Pinaceae. Unlike the ITS sequences, the *MAT*-2 HMG box phylogeny distinguished *Ce. polonica* from *Ce. laricicola* and the *Ce. virescens* isolates on *Fagus* and *Quercus* from those on *Acer* and *Liriodendron*. The isozyme data separated all the species in the *Ce. coerulescens* complex, but phylogenetic relationships among the species in the complex did not appear to be reflected by the isozyme analysis (Harrington et al 1996).

Based on the parsimony analysis of *MAT*-2 HMG box DNA sequences, the species from angiosperms formed a single clade, which was not the case in the ITS rDNA phylogeny. Based on ITS rDNA sequences, the Australasian species *Ch. australis, Ch. neocaledoniae* and *Ce. eucalypti* formed a single clade, with the North American *Ce. virescens* basal to all the other species in the *Ce. coerulescens* complex (FIG. 1, Witthuhn et al 1998). The phylogeny inferred from the *MAT*-2 HMG box DNA sequences suggests a monophyletic origin of the four species from angiosperms, all of which have similar conidiophore characteristics (Harrington et al 1998). We believe that the angiosperm and Pinaceae lineages are distinct and that the most recent common ancestor for these lineages was

adapted to angiosperm hosts (Witthuhn et al 1998). The isozyme data (Harrington et al 1996) did not distinguish the angiosperm group from the Pinaceae group.

The specific primers, EUM2-1 and EUM2-2, designed from the DNA sequence of the *MAT*-2 HMG box in *Ce. eucalypti,* amplified the *MAT*-2 HMG box from the MAT-2 strains of only the *Ceratocystis* species from angiosperms, namely, *Ce. eucalypti, Ce. virescens, Ch. neocaledoniae,* and *Ch. australis. Chalara australis* and *Ch. neocaledoniae* have no known teleomorph but behave as MAT-2 strains in interspecific crosses (Kile et al 1996, Harrington et al 1998). It was, therefore, not surprising that the *MAT*-2 HMG box was amplified from these two *Chalara* species.

In the phylogenetic analysis of the *MAT*-2 HMG box DNA sequences, the species from Pinaceae hosts (namely, *Ce. pinicola, Ce. douglasii, Ce. rufipenni, Ce. resinifera, Ce. coerulescens, Ce. laricicola,* and *Ce. polonica*) formed a sister taxon to the clade comprised of species from angiosperm hosts. The same relationship of the Pinaceae-associated species was observed when a phylogenetic analysis was performed using ITS rDNA sequences. In both trees, *Ce. rufipenni* and *Ce. resinifera* are phylogenetically closely related, *Ce. polonica* and *Ce. laricicola* are phylogenetically closely related and the remaining three Pinaceae-inhabiting species are phylogenetically closely related, although the bootstrap support for these clades was weak. Isozyme analysis grouped *Ce. rufipenni* and *Ce. resinifera,* as well as *Ce. polonica* and *Ce. laricicola,* but the other relationships among the angiosperm- and Pinaceae-associates were incongruent with the DNA sequence analyses.

The species of *Ceratocystis,* especially the four species in the angiosperm host clade of the *Ce. coerulescens* complex, are known primarily as wound colonizers of living sapwood. The Pinaceae-associated clade may be derived from an ancestor that adapted to gymnosperm hosts and had a biology similar to *Ce. resinifera,* a wound colonizer of spruce in Europe (Harrington and Wingfield 1998, Roll-Hansen and Roll-Hansen 1980). The closely related species *Ce. rufipenni* also colonizes spruce trees wounded by its vector, the bark beetle *Dendroctonus rufipennis,* in North America (Solheim and Safranyik 1997). It has been speculated that *Ce. rufipenni* has the derived characters of loss of conidiophore production and loss of insect-attracting volatiles. These were thought to be adaptations for bark beetle dispersal rather than dispersal by fungal feeding insects (e.g., Nitidulidae), which is more typical for *Ceratocystis* species (Harrington et al 1996). *Ceratocystis laricicola* and *Ce. polonica,* though distantly related to *Ce. rufipenni,* share these putatively derived characters and are known as tree pathogens dispersed by bark beetles in Eurasia (Harrington et al 1996, Harrington and Wingfield 1998). It is not clear if these bark beetle adaptations in the *Ce. coerulescens* complex are from a single event in the evolutionary history of the complex. Alternatively the similarities of *Ce. rufipenni* and *Ce. laricicola/Ce. polonica* could be a result of convergent evolution.

Three species in the complex (*Ce. coerulescens, Ce. pinicola* and *Ce. douglasii*) appear to be closely related and are unusual for the genus in that they are not known to colonize living trees but, rather, cause bluestain in the sapwood of dead trees. The ITS and *MAT-2* sequence data suggest that this saprophytic tendency is a derived character. Of the three saprophytic species, *Ce. coerulescens* is the most widely distributed and has the broadest host range; it is known from Europe, Japan and North America on pine, spruce, and larch. The other species may be more recently derived because they are more specialized to hosts and have a more limited geographic distribution; *Ce. pinicola* is known from pine in Great Britain and *Ce. douglasii* from Douglas-fir in western North America.

Harrington and Wingfield (1998) were unable to separate *Ce. laricicola* from *Ce. polonica* based on morphological differences, although Yamaoka et al (1998) found slight differences in the size of perithecial bases and conidiophores in Japanese isolates of these two species. These species occur on different hosts, and crosses between the two species give rise to no perithecia or perithecia with non-viable ascospores (Harrington and McNew 1998). Surprisingly, the ITS DNA sequences for the two species are identical (Witthuhn et al 1998). The isozyme data did, however, separate *Ce. laricicola* from *Ce. polonica* based on a single isozyme locus (Harrington et al 1996). Phylogenetic analysis of the DNA sequences of the *MAT*-2 HMG box also separate *Ce. laricicola* and *Ce. polonica* into distinct clades.

The *MAT*-2 HMG box DNA sequences separated the *Ce. virescens* isolates on *Acer* and *Liriodendron* from the isolates on *Fagus* and *Quercus,* consistent with the isozyme data (Harrington et al 1996). These two groups appear to be intersterile (Harrington et al 1998) and should be recognized as distinct species. The failure of the ITS sequences to separate these two apparently host-specialized groups (Witthuhn et al 1998) is another example of the sequences of rDNA spacer regions failing to separate sister species of fungi that are sympatric (Harrington and Rizzo 1999).

The *MAT*-2 HMG box DNA sequences (103 parsimony-informative characters of the 199 bp total DNA sequence) of species of *Ceratocystis* are considerably

more variable than the ITS and 5.8S rDNA sequence data (89 parsimony-informative characters of the 565 bp total DNA sequences). The intron sequences of the *MAT*-2 HMG box are especially divergent. The *MAT*-2 HMG box sequences are, therefore, more useful in comparing closely related species from different hosts, such as *Ce. laricicola* and *Ce. polonica,* than the ITS rDNA sequences. For more distantly related species, there was generally good congruence between the *MAT*-2 HMG box tree and the ITS tree. The isozyme data, as phenotypic characters, do not appear to infer a true phylogeny of the species in the complex, but such physiological characters are useful in delimiting morphologically similar, host-specialised species in the complex. Thus, phenotypic characters may be more useful than certain genotypic characters (e.g., ITS sequences) in delimiting closely related species of plant pathogens.

ACKNOWLEDGMENTS

Financial support for this study was provided by the members of the Tree Pathology Co-operative Programme (TPCP), the National Research Foundation (NRF), South Africa, the United Nations Education, Science and Cultural Organisation (UNESCO), the United States Department of Agriculture (USDA/FAS/ICD/Research & Scientific Exchanges, Agreement No. 58-3148-6-019), and the National Science Foundation (DEB-9870675). We gratefully acknowledge the various people, including Hayato Masuya, who have generously contributed cultures that were used in this and earlier studies.

LITERATURE CITED

Arie T, Christiansen SK, Yoder OC, Turgeon BG. 1997. Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG Box. Fungal Gen Biol 21:118–130.

Christiansen E, Solheim H. 1990. The bark beetle-associated blue-stain fungus *Ophiostoma polonicum* can kill various spruces and Douglas fir. Eur J For Pathol 20:436–446.

DeScenzo RA, Harrington TC. 1994. Use of $(CAT)_5$ as a DNA fingerprinting probe for fungi. Phytopathology 84:534–540.

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

Harrington TC, McNew DL. 1998. Partial interfertility among the *Ceratocystis* species on conifers. Fungal Gen Biol 25:44–53.

———, Rizzo DM. 1999. Defining species in the fungi. In: Worrall JJ, ed. Structure and Dynamics of Fungal Populations. Dortrecht, The Netherlands: Kluwer Academic Press. p 43–71.

———, Steimel JP, Kile GA. 1998. Genetic variation in three *Ceratocystis* species with outcrossing, selfing and asexual reproductive strategies. Eur J For Pathol 28:217–226.

———, ———, Wingfield MJ, Kile GA. 1996. Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex. Mycologia 88:104–113.

———, Wingfield MJ. 1998. The *Ceratocystis* species on conifers. Can J Bot 76:1446–1457.

Hunt J. 1956. Taxonomy of the genus *Ceratocystis*. Lloydia 19:1–58.

Kile GA, Harrington TC, Yuan ZQ, Dudzinski MJ, Old KM. 1996. *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. Mycol Res 100:571–579.

Redfern DB, Stoakley JT, Steele H, Minter M. 1987. Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. Pl Pathol 36:467–480.

Roll-Hansen F, Roll-Hansen H. 1980. Microorganisms which invade *Picea abies* in seasonal stem wounds. II. Ascomycetes, Fungi Imperfecti, and bacteria. General discussion, Hymenomycetes included. Eur J For Pathol 10: 396–410.

Solheim H, Safranyik L. 1997. Pathogenicity to Sitka spruce of *Ceratocystis rufipenni* and *Leptographium abietinum,* blue-stain fungi associated with the spruce beetle. Can J For Res 27:1336–1341.

Swofford DL. 1993. PAUP Phylogenetic Analysis using Parsimony Version 3.1.1. Champaign, Illinois: Illinois Natural History Survey.

Turgeon BD. 1998. Application of mating type gene technology to problems in fungal biology. Annu Rev Phytopathol 36:115–137.

Upadhyay HP. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. Athens, Georgia: The University of Georgia Press. 176 p.

Witthuhn RC, Wingfield BD, Wingfield MJ, Harrington TC. 1999. PCR based identification and phylogeny of species of *Ceratocystis sensu stricto*. Mycol Res 103:743–749.

———, ———, ———, Wolfaardt M, Harrington TC. 1998. Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. Mycologia 90:96–101.

Yamaoka Y, Wingfield MJ, Ohsawa M, Kuroda Y. 1998. Ophiostomatoid fungi associated with *Ips cembrae* in Japan and their pathogenicity to Japanese larch. Mycoscience 39:367–378.

———, ———, Takahashi I, Solheim H. 1997. Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* in Japan. Mycol Res 101:1215–1227.