PCR-based identification and phylogeny of species of *Ceratocystis sensu stricto*

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Most species of *Ceratocystis sensu stricto* are virulent pathogens of a wide variety of plants including forest and fruit trees, sweet potato, pineapple and sugar cane. Confusion exists regarding the taxonomy of the species in this genus. The aim of this study was to develop a rapid and reliable PCR-based RFLP identification method and to consider phylogenetic relationships among the better-known species of *Ceratocystis*. A 1·6 kb fragment within the ribosomal DNA operon was directly amplified from living fungal tissue, without extracting DNA. The amplified fragment included part of the small (SSU) and large (LSU) sub-unit rRNA genes, the 5·8S rRNA gene and the internal transcribed spacers (ITS) 1 and 2. The PCR fragments were digested with eighteen restriction enzymes. Four of these (*Alu* I, *Dra* I, *Hae* III and *Rsa* I) produced RFLPs that separated the species of *Ceratocystis*. The amplification products from the best-known species were sequenced, and the delimitation of taxa based on this phylogenetic analysis generally agreed with results of previous studies using isozymes and rDNA sequence analysis. This study provides an extended understanding of the relationships among species of *Ceratocystis* and will form a sound foundation for further taxonomic studies of the group.

Species of the so-called ophiostomatoid fungi are found in four genera, *Ceratocystis sensu stricto* Ellis & Halst., *Ophiostoma* Syd. & P. Syd., *Ceratocystiopsis* H. P. Upadhyay & W. B. Kendr. and *Gondwanamyces* Marais & M. J. Wingf. These fungi are adapted to dispersal by insects, and *Ceratocystis* includes many economically important plant pathogens (Christiansen & Solheim, 1990; Teviotdale & Harper, 1991; Kile, 1993; Morris, Wingfield & de Beer, 1993) *Ceratocystis coerulescens*, the cause of sapstain on spruce and pine, is considered to be a weak pathogen. In contrast *C. fagacearum* and *C. fimbriata* are aggressive primary pathogens. *C. fagacearum* causes oak wilt disease (Bretz, 1952; Sinclair, Lyon & Johnson, 1987), while *C. fimbriata* causes vascular stain and cankers on various hosts, including plane (Grosclaude & Oliver, 1988), mango (Ribeiro *et al*., 1986), and rubber (Olson & Martin, 1949).

Ceratocystis species have more than one means of dispersal (Kile, 1993). Some are closely associated with bark beetles (Coleoptera: Scolytidae), such as *C. polonica* (Siemaszko, 1938; Christiansen & Solheim, 1990), *C. laricicola* (Redfern *et al*., 1987) and *C. rufipenni* (Wingfield, Harrington & Solheim, 1997). Fungal- and sap-feeding insects are also recognized as vectors of *Ceratocystis* species; for example, picnic beetles (Coleoptera: Nitidulidae) are recognized as direct vectors of *C. paradoxa* (Chang & Jensen, 1974) and *C. fagacearum* (Himelick & Curl, 1958; Juzwik & French, 1983). *Ceratocystis* species may also be dispersed in soil or in frass of ambrosia beetles, or the spores may be splashed by water (Grosclaude & Oliver, 1988; Vigouroux & Stojadinovic, 1990; Kile, 1993).

DNA sequence data from the ribosomal RNA genes have been used effectively to determine the phylogenetic relationships among ophiostomatoid fungi (Hausner, Reid & Klassen, 1992, 1993*a–c*; Spatafora & Blackwell, 1994; Wingfield *et al*., 1994). These phylogenetic analyses suggest that ascospore morphology is an unreliable taxonomic character (at the genus level) for this group (Hausner *et al*., 1993*b*; Spatafora & Blackwell, 1994; Wingfield *et al*., 1994). Wingfield *et al*. (1994) used large sub-unit ribosomal RNA sequences to determine the phylogenetic relationships among eight species of *Ceratocystis* and found this region to be conserved for this genus.

The more variable ITS regions were used to determine the relationships between *C. fimbriata* and *C. albofundus* (Wingfield *et al*., 1996), between *C. polonica* and *C. laricicola* (Visser *et al*., 1995) and among species within the *C. coerulescens* complex (Witthuhn *et al*., 1998), but the phylogenetic relationships among these groups of species, as well as their relationship to other *Ceratocystis* species, remain poorly defined. Furthermore, insufficient attention has been given to the taxonomy of *Ceratocystis sensu stricto*. This has become especially evident in recent studies (Visser *et al*., 1995; Harrington *et al*., 1996; Wingfield *et al*., 1996; Witthuhn *et al*., 1998) that have shown that species regarded as single entities in fact represent species complexes.

The aim of this study was to develop a rapid and reliable method for the identification of *Ceratocystis* species. Fur-

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Table 1. Isolates and origins of species of *Ceratocystis* studied and the RFLP fragment sizes using the restriction enzymes *Alu* I, *Dra* I, *Hae* III and *Rsa* I. The RFLP patterns for the four restriction enzymes were uniform within species

Isolate numbers	Origin	Host	*Alu* I	*Dra* I	*Dra* I/*Hae* III double digests	*Rsa* I
C. fagacearum (Bretz) J. Hunt			440, 400, 200, 180, 150			
CMW2037	MN, U.S.A.	*Quercus* sp.				
CMW2038	MN, U.S.A.	*Quercus* sp.				
CMW2039	MN, U.S.A.	*Quercus* sp.				
CMW2651 (AFO43598)*	U.S.A.	*Quercus* sp.				
CMW2658	U.S.A.	*Quercus* sp.				
C. moniliformis (Hedgc.) C. Moreau			400, 350, 260, 220, 150			
CMW1626, IOF8667	Japan	Unknown				
CMW4458	China	*Hevea* sp.				
CMW3782 (AFO43597)	South Africa	*Erythrina* sp.				
C. fimbriata Ellis & Halst. from *Platanus*			720, 220, 180, 150	1600		
CMW1894	Switzerland	*Platanus* sp.				
CMW1895	Switzerland	*Platanus* sp.				
CMW1896	Switzerland	*Platanus* sp.				
CMW2218	France	*Platanus* sp.				
CMW2219, PREM51642 (AFO43604)	France	*Platanus* sp.				
CMW2220, PREM51644	France	*Platanus* sp.				
CMW2228, PREM51830	Sicily	*Platanus* sp.				
CMW2242, PREM51831	Italy	*Platanus* sp.				
CMW2324	Switzerland	*Platanus* sp.				
C. fimbriata from *Populus* and *Prunus*			560, 220, 180, 150			
CMW1270, C89	SD, U.S.A.	*Populus* sp.				
CMW2901, C685	Quebec, Canada	*Populus* sp.				
CMW0078	CO, U.S.A.	*Populus* sp.				
CMW2911, C578	CA, U.S.A.	*Prunus* sp.				
CMW2902, C686	CA, U.S.A.	*Prunus* sp.				
C. albofungus M. J. Wingf. De Beer & Michael Morris			720, 220, 180, 150	1200, 320, 150		
CMW2473, PREM51639	South Africa	*Acacia* sp.				
CMW2475, PREM51641 (AFO43605)	South Africa	*Acacia* sp.				
C. adiposa (E. J. Butler) C. Moreau			400, 340, 200, 180, 150			
CMW0066	Unknown	Unknown				
CMW0071	Unknown	Unknown				
CMW0121	Unknown	Unknown				
CMW1622, IOF9546 (AFO43606)	Japan	Unknown				
CMW2573, CBS136.34	Japan	*Saccharum* sp.				
CMW2575, CBS600.74	Japan	*Pinus* sp.				
CMW3307, C299	U.S.A.	Wood chips				
C. paradoxa (Dade) C. Moreau			400, 340, 200, 180, 150			
CMW1546 (AFO43607)	Unknown	*Musa* sp.				
C. radicicola (Bliss) C. Moreau			400, 280, 200, 180, 150			
CMW3186, CBS114.47	CA, U.S.A.	*Phoenix* sp.				
CMW3191, CBS146.59 (AFO43599)	CA, U.S.A.	Unknown				
C. coerulescens (Münch) B. K. Bakshi complex:						
C. pinicola T. C. Harr. & M. J. Wingf.			400, 280, 200, 180, 150		620, 550, 280	700, 600, 250
C488	England	*Pinus* sp.				
C489	England	*Pinus* sp.				
CMW1323, C490 (AFO43602)	England	*Pinus* sp.				
CMW3759, C798	England	*Pinus* sp.				
C. coerulescens			400, 280, 200, 180, 150		620, 550, 280	700, 600, 250
CMW3231, C313	Germany	*Picea* sp				
CMW3235, C321	Netherlands	Unknown				
C. resinifera T. C. Harr. & M. J. Wing.			400, 280, 200, 180, 150		620, 550, 280	700, 600, 250
CMW3227, C276	Norway	*Picea* sp.				
CMW3229, C278	Norway	*Picea* sp.				
C. rufipenni M. J. Wingf. T. C. Harr. & H. Solheim			400, 280, 200, 180, 150		620, 550, 280	700, 600, 250
CMW3247, C609	Canada	*Picea* sp.				
C. virescens (R. W. Davidson) C. Moreau			400, 280, 200, 180, 150		620, 550, 280	600, 480, 250
C70	Unknown		Unknown			
CMW0460, C74 (AFO43603)	NY, U.S.A.	*Quercus* sp.				

Table 1. (*Cont.*)

Isolate numbers	Origin	Host	*Alu* I	*Dra* I	*Dra* I/*Hae* III double digests	*Rsa* I
C252	NY, U.S.A.	*Acer* sp.				
C253	NY, U.S.A.	*Acer* sp.				
CMW3225, C254	NY, U.S.A.	*Acer* sp.				
C256	WI, U.S.A.	*Acer* sp.				
C262	NH, U.S.A.	*Acer* sp.				
C. laricocola Redfern & Minter			400, 280, 200, 180, 150		550, 510, 360	700, 600, 250
CMW1016 (AFO 43600)	Scotland	*Larix* sp.				
CMW3212, C177	Scotland	*Larix* sp.				
CMW3214, C178	Scotland	*Larix* sp.				
CMW3217, C179	Scotland	*Larix* sp.				
CMW3219, C180	Scotland	*Larix* sp.				
CMW3221, C181	Scotland	*Larix* sp.				
C. polonica Siemaszko			400, 280, 200, 180, 150		550, 510, 360	700, 600, 250
CMW3208, C123	Norway	*Picea* sp.				
CMW3235, C321	Norway	*Picea* sp.				
CMW0672, C322, CBS133.38 (AFO43601)	Poland	*Picea* sp.				

CMW – Culture collection of M. J. Wingfield; C – Culture collection of T. C. Harrington; PREM – National Collection of Fungi, South Africa; CBS – Centraal Bureau voor Schimmelcultures, Netherlands, ATCC – American Type Culture Collection, USA; IOF – Institute for Fermentation, Japan.

* GenBank Accession no. in parenthesis for those isolates selected for sequencing.

thermore, DNA sequence data of the ribosomal RNA genes of the best-known species were compared in order to resolve phylogenetic relationships.

MATERIALS AND METHODS

Isolates

Isolates of *Ceratocystis* spp. used in this study were obtained from a wide range of geographical areas and diverse sources (Table 1). These were grown on malt extract agar (20 g l^{-1} malt extract and 20 g l^{-1} agar) in Petri-dishes at room temperature for 10 d.

Polymerase chain reaction

PCR reactions were performed directly from the mycelium of the isolates without extracting DNA (Harrington & Wingfield, 1995). A part of the ribosomal DNA operon was amplified using the primers ITS1 and LR6 (Table 2). The amplified fragment included the 3′ end of the small sub-unit (SSU) rRNA gene, the 5.8S rRNA gene, part of the large sub-unit (LSU) rRNA gene and the internal transcribed spacer (ITS) regions 1 and 2. The PCR reactions were performed as described by Witthuhn *et al*. (1998). The PCR products were electrophoresed in 15 g l^{-1} agarose gels, using 0·5 × TBE electrophoresis buffer, stained with ethidium bromide, and visualised using uv light. Amplification reactions were repeated at least twice for each isolate.

Restriction fragment length polymorphisms

Eighteen restriction enzymes (*Alu* I, *Cfo* I, *Dde* I, *Dra* I, *EcoR* I, *Hae* III, *Hind* II, *Hind* III, *Hinf* I, *Hpa* II, *Pst* I, *Pvu* II, *Rsa* I, *Sau*3A, *Sau*96 I, *Scr*F I, *Taq* I, *Xba* I) were tested for RFLPs of the PCR products. The digested PCR products were separated on 20 g l^{-1} agarose gels, using 0·5 × TBE electrophoresis buffer, stained with ethidium bromide, and visualized using uv light. The sizes of the restriction products were determined against a 100 bp ladder. No fragments smaller than 150 bp were scored.

Table 2. Primers used for the generation and sequencing of the PCR products

	Sequence (5′-3′)	Source
ITS1	TCCGTAGGTGAACCTGCGG	White *et al*., 1990
ITS2	GCTGCGTTCTTCATCGATGC	White *et al*., 1990
ITS3	GCATCGATGAAGAACGCAGC	White *et al*., 1990
ITS4	TCCTCCGCTTATTGATATGC	White *et al*., 1990
LR1	GGTTGGTTTCTTTTCCT	Vilgalys & Hester, 1990
LR3	GGTCCGTGTTTCAAGAC	Vilgalys & Hester, 1990
LR5	ATCCTGAGGGAAACTTC	Vilgalys & Hester, 1990
LR6	CGCCAGTTCTGCTTACC	Vilgalys & Hester, 1990
LR1R	AGGAAAAGAAACCAACC	Complement of LR1
LR3R	GTCTTGAAACACGGACC	Complement of LR3
LR5R	GAAGTTTCCCTCAGGAT	Complement of LR5
404x*	CCCTTTCAACAATTTCAC	Authors, unpublished
L1	GGTCCGTGTTTCAAG	Wingfield *et al*., 1994

* Binds to position 404 (5′–3′) of the large sub-unit ribosomal RNA gene of *Saccharomyces cerevisiae*.

DNA sequencing

One isolate of each of eleven species of *Ceratocystis* was selected for sequencing based on the results of the RFLP study. These isolates, with their GenBank Accession no. are listed in Table 1. *Petriella setifera* (J. C. Schmidt) Curzi (ATCC26490, GenBank Accession no. AFO43596) was used as the outgroup taxon (Spatafora & Blackwell, 1994). In the case of *C. coerulescens*, it is recognized that this represents a complex of at least five species (Harrington *et al*., 1996;

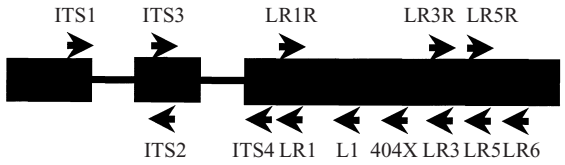

Fig. 1. Diagrammatic representation of the 1600 bp PCR fragment of the species of *Ceratocystis* used in this study. The positions of the 13 sequencing primers are indicated with arrows.

Wingfield *et al*., 1997; Witthuhn *et al*., 1998; Harrington & Wingfield, 1998), but only one, *C. pinicola*, was selected to represent the complex.

The PCR fragments were purified using Wizard PCR Preps (Promega Corporation, U.S.A.) or Microcon Microconcentrators (Amicon, Inc., U.S.A.). Both strands of the PCR products of nine of the 12 isolates were sequenced with 13 primers (Table 2, Fig. 1) using the *f*mol DNA sequencing kit (Promega Corporation, U.S.A.). Three of the isolates were sequenced using an ABI PRISM 377 DNA sequencer (Perkin-Elmer, U.S.A.) at the DNA sequencing facility at Iowa State University. The DNA sequence data were submitted to GenBank. The nucleotide sequences were manually aligned. Phylogenetic relationships among species were determined using the heuristic search option in PAUP, with gaps treated as missing data (Swofford, 1993). Bootstrap values (Felsenstein, 1985) were determined from 100 replicates.

RESULTS

RFLPs

The PCR amplifications of the species of *Ceratocystis* under consideration produced PCR products that were 1·6 kb in size. Of the 18 restriction enzymes tested *Alu* I, *Dra* I, *Hae* III and *Rsa* I produced RFLP patterns that were used to distinguish the species. The restriction enzyme maps in Figs 2 and 3 are based on the actual DNA sequences.

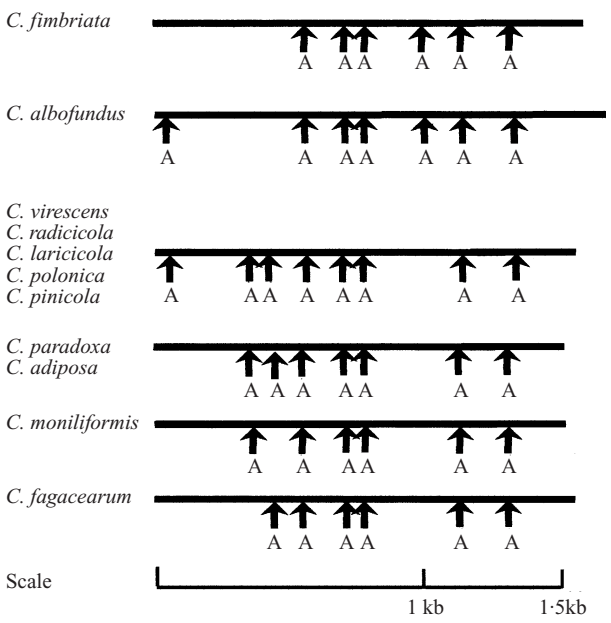

Fig. 2. *Alu* I (A) restriction maps, inferred from sequence analysis, of the PCR fragments amplified from all the *Ceratocystis* species studied.

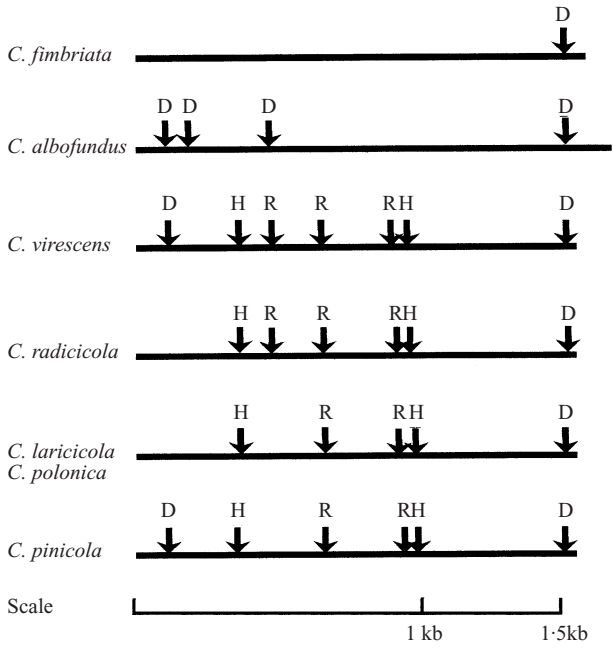

Fig. 3. Restriction maps of the restriction enzymes (*Dra* I, *Hae* III and *Rsa* I), inferred from sequence analysis, used to distinguish closely related *Ceratocystis* species. D – *Dra* I, H – *Hae* III, R – *Rsa* I.

Restriction digests using *Alu* I (Table 1, Fig. 2) produced unique restriction patterns for *C. moniliformis*, *C. fagacearum* and *C. fimbriata* isolates from *Populus* and *Prunus* (Table 1). The groups of species that had the same RFLP patterns after *Alu* I digests are: *C. fimbriata* isolates from *Platanus* spp. and *C. albofundus*, *C. adiposa* and *C. paradoxa*, and species in the *C. coerulescens* complex and *C. radicicola*.

Ceratocystis adiposa and *C. paradoxa* could not be separated based on the RFLPs produced by any of the restriction enzymes tested. Many of the other closely related species that had the same RFLP patterns using *Alu* I were, however, separated from each other based on the RFLPs produced by *Dra* I, *Hae* III and *Rsa* I (Table 1, Fig. 3). The restriction patterns produced after a digestion with *Dra* I enabled distinction between *Platanus* isolates of *C. fimbriata* and *C. albofundus*. Double digestions using the enzymes *Dra* I and *Hae* III were used to separate *C. coerulescens* and *C. virescens* from *C. laricicola*, *C. polonica* and *C. radicicola*. *Ceratocystis coerulescens* isolates were distinguishable from *C. virescens* isolates based on *Rsa* I digests. *Rsa* I was also used to distinguish *C. radicicola* from *C. laricicola* and *C. polonica*. The recently described *C. pinicola*, *C. resinifera* and *C. rufipenni* could not be distinguished from *C. coerulescens* based on the RFLP analyses.

DNA sequencing

The aligned DNA sequences of the representative species of *Ceratocystis* were 1731 bp in size after gaps were inserted to achieve the alignment. Within the ITS region, high variability was observed between the DNA sequence of the various

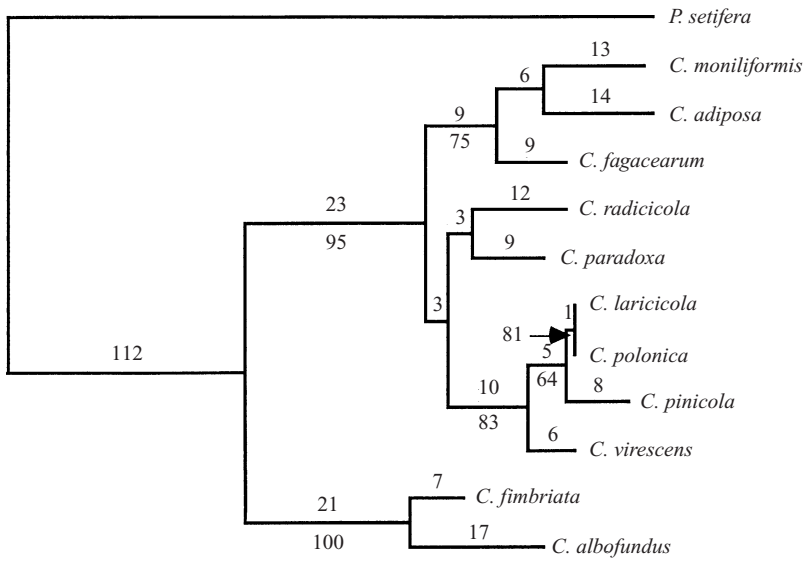

Fig. 4. The most parsimonious tree (tree length = 288) produced from the DNA sequence of part of the large sub-unit ribosomal RNA gene. Bootstrap values (100 replicates) greater than 50% are indicated at the bottom of the branches and the number of base substitutions are indicated at the top of the branches.

species, with numerous insertions–deletions, which made the alignment of the sequences in this region very difficult. In contrast, the large sub-unit rRNA gene (1087 bases in total) was found to be relatively conserved.

A heuristic search from the aligned DNA sequence data (1081 characters) of the large sub-unit rRNA gene produced one most parsimonious tree (Fig. 4) of 288 steps (CI = 0·788, HI = 0·212, RI = 0·667). The tree was rooted to *Petriella setifera*, the outgroup species. Two major clades were found within *Ceratocystis: C. fimbriata* and *C. albofundus* grouped together (100% bootstrap value), sister to the clade (95% bootstrap value) formed by the other nine *Ceratocystis* species under consideration. Relationships among the nine other species were not clear, but *C. moniliformis*, *C. fagacearum* and *C. adiposa* formed a single, weakly supported clade (75% bootstrap value). The *C. coerulescens* complex (*C. laricicola*, *C. polonica*, *C. pinicola* and *C. virescens*) formed another weakly supported (83% bootstrap value) clade.

Much of the alignment of the DNA sequence data within the ITS1 and ITS2 regions proved to be ambiguous for all the species studied. A second analysis was performed on the DNA sequence data of the ITS and LSU regions after all characters of ambiguous alignment were removed (378 of the 1731 characters removed), with most of the removed characters in the ITS1 and ITS2 regions. A single most parsimonious tree of 420 steps (CI = 0·800, HI = 0·200, RI = 0·648) was produced (data not shown), and the topology was found to be similar to the tree produced when only the LSU sequence data was analysed (Fig. 4). *Petriella setifera* was again defined as the outgroup. *Ceratocystis fimbriata* and *C. albofundus* grouped together (100% bootstrap value) and formed a clade sister to the clade formed by all the other *Ceratocystis* species studied (96% bootstrap value). *C. moniliformis*, *C. fagacearum* and *C. adiposa* formed a single clade (60% bootstrap value). The members of the *C. coerulescens* complex formed a clade (89% bootstrap value), and there was support (92% bootstrap value) for the clade of species that occur on conifers (*C. pinicola*, *C. polonica* and *C. laricicola*).

DISCUSSION

In this study, the best known species of *Ceratocystis* have been characterized based on sequence data and RFLP analyses. The results of the sequence analyses generally support those of earlier studies (Hausner, Reid & Klassen, 1993*a*, *c*; Visser *et al*., 1995; Harrington *et al*., 1996; Wingfield *et al*., 1996). The RFLP comparisons of a large number of isolates has shown that it is possible to distinguish most of the species using this reliable and quick technique.

Ceratocystis fimbriata is a well known pathogen on a wide variety of hosts, including sweet potatoes, from which it was first described (Halsted, 1890). *Ceratocystis albofundus* is a pathogen of *Acacia mearnsii* in South Africa (Morris, Wingfield & de Beer, 1993) and was recently shown by ITS sequence analysis and morphology to represent a distinct taxon similar to, but quite distinct from, *C. fimbriata* (Wingfield *et al*., 1996). The RFLP and LSU analyses provide additional support for this distinction. Based on the RFLP analyses, isolates of *C. fimbriata* from *Platanus* could be separated from isolates from *Populus* and *Prunus*, suggesting that *C. fimbriata* represents a species aggregate, such as was previously proposed by Webster & Butler (1967). The RFLPs of *C. albofundus* are closer to the *Platanus* isolates than to the *Prunus* isolates of *C. fimbriata*.

Ceratocystis coerulescens, *C. laricocola*, *C. polonica* and *C. virescens* are known to be very similar and related fungi, in the *C. coerulescens* complex (Harrington *et al*., 1996). The seven species in the complex that occur on conifers appear to be monophyletic and form a strongly supported clade based on ITS sequence analysis (Witthuhn *et al*., 1998). Three of these conifer species (*C. pinicola*, *C. laricicola* and *C. polonica*) also grouped together based on LSU data, further suggesting that

this clade arose through an adaptation to conifers. These species and *C. virescens* are not easily distinguished based on RFLP data presented here. Although *C. polonica* and *C. laricicola* can be distinguished from the other species based on *Dra* I/*Hae* III digestions, these two species cannot be separated from each other. Evidence from sequence analyses (Visser *et al*., 1995; Witthuhn *et al*., 1998) and isozyme analysis (Harrington *et al*., 1996) has led us to believe that *C. polonica* and *C. laricicola* are very similar, and LSU sequence analysis further shows the similarity between these two species.

The analyses of the LSU DNA sequence data loosely grouped *C. fagacearum*, *C. adiposa* and *C. moniliformis*. SSU analysis (Hausner, Reid & Klassen, 1993*a*, *c*) showed similarity between *C. fagacearum* and *C. adiposa*. *C. moniliformis* is, however, more similar to *C. fimbriata* than to *C. adiposa* and *C. fagacearum* based on the SSU data. *C. fimbriata*, *C. albofundus* and *C. moniliformis* are the only *Ceratocystis* species with hat shaped ascospores, and a closer phylogenetic relationship between *C. moniliformis* and *C. fimbriata*, as shown by the analyses of the SSU data (Hausner *et al*., 1993*a*, *c*), seems more probable than the close relationship between *C. moniliformis*, *C. adiposa* and *C. fagacearum* based on LSU data.

Although the statistical support was not strong, *C. radicicola* and *C. paradoxa* appeared to be phylogenetically related. These two species are morphologically similar and their separation is based on their asexual states. *C. radicicola* was isolated from data palms in the U.S.A. (Bliss, 1941), while *C. paradoxa* has been isolated from a wide host range of monocotyledonous hosts, including palms (Kile, 1993). The similarity of the hosts, morphology and DNA sequence data supports the contention that they are phylogenetically closely related.

Ceratocystis species included in this study are those that are best known and for which cultures are readily available. The isolates used were chosen based on careful morphological comparisons and we believe that the results contribute to our further understanding of this important group of fungi. Results of this study will lay a firm foundation for the description and characterisation of new species in the future. The RFLP results will also provide a rapid means to accurately identify most of these species.

We thank the Foundation for Research Development (FRD), South Africa, members of the Tree Pathology Co-operative Programme (TPCP), South Africa, the United States Department of Agriculture (USDA/FAS/ICD/Research & Scientific Exchanges, Agreement No. 58-3148-6-019) and UNESCO for financial support. We further thank Dr Meredith Blackwell for supplying DNA from *P. setifera*. Marianne Wolfaardt and Cassi Myburg are thanked for their assistance in dealing with the large collection of DNA sequence data.

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(*Accepted 3 September 1998*)

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