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Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex

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Abstract: Nineteen electrophoretic phenotypes (unique combinations of electromorphs) were found among 98 isolates of *Ceratocystis coerulescens* and morphologically similar species using 10 isozymes. Analysis of the isozyme data and morphological comparisons suggested that there are five variants of *C. coerulescens* found on conifers: three are associated with blue-stain of *Picea* or *Pinus*, one (*C. coerulescens* f. *douglasii*) with blue-stain of *Pseudotsuga*, and one associated with the bark beetle *Dendroctonus rufipennis* on *Picea*. *Ceratocystis polonica* and *C. laricicola*, associated with bark beetle species in the genus *Ips* on *Picea* and *Larix*, respectively, had similar isozymes, are morphologically indistinguishable from each other, and should probably be synonymized. *Ceratocystis virescens*, cause of stain of hardwoods and sapstreak disease of *Acer saccharum*, is distinct from the conifer species of *Ceratocystis* in isozyme electromorphs and anamorph morphology. Isozymes of *C. virescens* show some similarity to those of two Australian species, an undescribed species of *Ceratocystis* from *Eucalyptus* and *Chalara australis*. The *Chalara* states of these three hardwood species and *Chalara neocaledoniae* are morphologically similar.

Key Words: *Acer*, blue-stain, *Chalara*, *Dendroctonus*, *Eucalyptus*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*

INTRODUCTION

Ceratocystis sensu stricto (excluding *Ophiostoma*) is a relatively small genus of often insect-vectored ascomycetes, comprised primarily of plant pathogens and wood-staining fungi (Kile, 1993). In spite of their economic importance, species limits within the genus are poorly defined, particularly in the group of taxa referred to here as the *C. coerulescens* complex. *Ceratocystis coerulescens* (Münch) Bakshi was described as a cause of blue-stain in the wood of spruce (*Picea*) and pine (*Pinus*) in Europe (Münch, 1907). The anamorph state may be *Chalara ungeri* Sacc. (Nag Raj and Kendrick, 1975).

Our examinations of *C. coerulescens* isolates suggest that there are up to five morphological variants of this species on conifers. Two of these variants from western North America were recognized by Davidson (1953, 1955), one as *C. c.* f. *douglasii* on wood of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] and the other as an associate of the spruce beetle, *Dendroctonus rufipennis* (Kirby) (Coleoptera: Scolytidae).

Two other conifer taxa related to *C. coerulescens* are also bark beetle associates. *Ceratocystis polonica* (Siemaszko) Moreau was described from spruce attacked by *Ips typographus* L. (Siemaszko, 1939) and *C. laricicola* Redfern & Minter from larch (*Larix*) attacked by *Ips cembrae* Heer (Redfern et al., 1987). The *Ips typographus* associate had been known as *Ophiostoma polonicum* Siemaszko but recent studies (Visser et al., 1994) have shown that the species belongs in *Ceratocystis*. With this recognition, *C. laricicola* is difficult to distinguish morphologically from *C. polonica*.

Davidson (1935) and Verrall (1939) reported *C. coerulescens* as a cause of stain in hardwood lumber in the southeastern United States, and Davidson (1944) later described the hardwood fungus as new: *C. virescens* (Davidson) Moreau. Others (Hunt, 1956; Upadhyay, 1981) have considered this hardwood species as a synonym of *C. coerulescens*, although this was not supported by Kile and Walker (1987). The hardwood species is recognized as a cause of sapstreak of maple (*Acer*) in the northeastern United States, and similar diseases on angiosperms in New Caledonia and Australia (Kile, 1993) have been associated with *Chalara* species; *Chalara neocaledoniae* Kiffer & Delon was reported on coffee and guava and *Ch. australis* Walker & Kile on *Nothofagus*. The anamorph of *C. virescens* is similar to the two *Chalara* species and also to the anamorph of a weak pathogen on *Eucalyptus*, an undescribed species of *Ceratocystis*. Ascospores of the *Eucalyptus* fungus are much larger than those of *C. virescens* or *C. coerulescens* (Kile et al., 1994).

Isozymes have proved useful in delimiting species

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and infra-specific taxa in other fungal groups (e.g., *Leptographium*, Zambino and Harrington, 1989; 1992). Before describing new taxa or synonymizing species based on morphology, we chose to apply isozyme analysis to the *C. coerulescens* complex. Besides the five variants of *C. coerulescens*, we studied *C. polonica*, *C. laricicola*, *C. virescens*, the undescribed *Ceratocystis* species from *Eucalyptus*, and two morphologically similar species with no known teleomorphs, *Chalara australis* and *Ch. neocaledoniae*.

MATERIALS AND METHODS

Ninety-eight isolates of *Ceratocystis* and *Chalara* from Australia, New Caledonia, Japan, North America and Europe were tested for isozyme variation (TABLE I). Many of the isolates were obtained from recognized collections (ATCC and CBS), and others were supplied H. Roll-Hansen and H. Solheim, Norwegian Forest Institute, Norway; D. Redfern, Forestry Commission, Northern Research Station, Edinburgh, U.K.; J. Gibbs, Forestry Commission Research Station, Alice Holt Lodge, Surrey, England; Y. Yamaoka, Department of Plant Pathology, University of Tsukuba, Ibaraki, Japan; D. Houston, U.S.D.A. Forest Service, Hamden, Connecticut; and T. Hinds, U.S.D.A. Forest Service, Ft. Collins, Colorado.

Fresh mycelium for enzyme extraction was grown in 30 ml of liquid medium (20 g Difco malt extract and 2 g yeast extract per liter of water) in 125 ml Erlenmeyer flasks and incubated at room temperature for approximately two weeks. Buffers and protocols for extraction of the enzymes from mycelia onto paper wicks were as previously described (Zambino and Harrington, 1989; 1992). Wicks were frozen at-70 C until electrophoresis. Starch gels (12 %) were prepared as described by Marty et al. (1984) and poured into gel forms such as those described by Cardy et al. (1983). Buffers and electrophoresis conditions are shown in TABLE II. Staining for FUM and G6PD activity followed the procedures of Marty et al. (1984), but staining for other isozymes followed the procedures of Cardy et al. (1983). At least two independent extractions from each isolate were tested for isozyme activity.

Among the isozymes tested, only 10 gave consistent results for all 98 isolates. For each isolate, only one band was evident for most of the 10 isozymes, but second bands were inconsistently seen in some isolates when tested for DIA, G6PD, and PGD activity. With these latter three isozymes, only the consistently produced band was scored. Electromorphs of representative isozymes are shown in FIG. 1. For each isozyme, electromorphs were designated by letters in order of decreasing anodal migration.

Electrophoretic phenotypes were defined as unique combinations of electromorphs for the 10 isozymes.

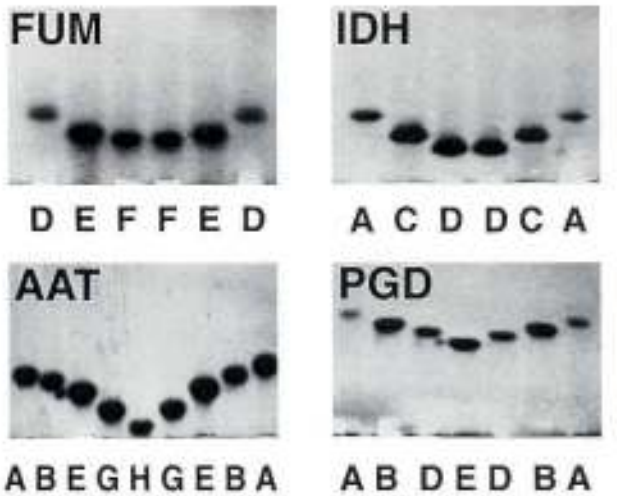

FIG. 1. Representative electromorphs, designated by letter in order of decreasing anodal migration, for fumerase, isocitrate dehydrogenase, aspartate aminotransferase, and phosphogluconate dehydrogenase.

The electromorphs were used to cluster the ETs into putative taxonomic units. A matrix was developed using Rogers' genetic distance (NTSYS-PC program, Rohlf, 1990) or Nei's genetic distance (Felsenstein, 1993) and a phenogram was generated using neighbor-joining (Felsenstein, 1993).

RESULTS

Nineteen electrophoretic phenotypes (ETs) were identified among the 98 isolates of *Ceratocystis* and *Chalara* tested (TABLE III). One to four unique ETs were seen in each of 11 putative taxonomic units. The ETs within a taxonomic unit generally varied for only one or two isozymes, but the 10 tested isolates from *Eucalyptus* showed variation in electromorphs for four of the ten isozymes. The isozymes AAT, DIA and GDH were the most variable of the enzymes, with eight electromorphs for each found among the 98 isolates (TABLE II). In contrast, only two electromorphs were seen for G6PD.

Genetic distances among the electrophoretic phenotypes were determined by both Nei's and Rogers' methods, and neighbor-joining analyses of these matrixes gave similar topologies. The single isolate of *C. c.* f. *douglasii* represented a phenotype quite distinct from the others, and principal component analyses (Rohlf, 1990) indicated that this phenotype had little affinity to the others. It was used as an outgroup in generating the tree in FIG. 2. The other phenotypes fell into two clusters, one primarily of the isolates from hardwoods and the other of conifer isolates only.

Analysis of the isozyme data grouped similar ETs into what appear to be natural taxonomic units or

TABLE 1. Isolate numbers, substrate, origin and electrophoretic phenotype of isolates of *Ceratocystis* and *Chalara* examined for isozyme variation

Species	Isolate No.[a]	Other No./Collector[b]	Substrate	Country of origin	Electrophoretic phenotype
C. coerulescens sp. A	C487	CMW1383, 0-905/Gibbs	*Pinus*	England	I
	C488	CMW1311, 0-907/Gibbs	*Pinus sylvestris*, tree	England	I
	C489	CMW1312, 0-906/Gibbs	*Pinus nigra*, log	England	I
	C490	CMW1323, 0-901/Gibbs	*Pinus*	England	II
C. coerulescens sp. B	C693	CBS489.80/A. Lilja	*Pinus* or *Picea*, log	Finland	III
	C313	CBS140.37	*Picea abies*, wood	Germany	III
	C301	ATCC12859, 3/Campbell	*Pinus banksiana*, wood	Minnesota, U.S.A.	III
	C321	CBS137.34/Kneteman	Moist timber	Netherlands	III
C. coerulescens sp. C	C50	CMW451, CO381/Hinds	*Picea engelmannii*, wood	New Mexico, USA	IV
	C278	NFRI86-434-9	*Picea abies*, log	Norway	IV
	C662	NFRI86-434-15	*Picea abies*, wound	Norway	IV
	C666	NRFI1750-2/Roll-Hansen	*Picea abies*, log	Norway	IV
	C276	ATCC44993, NFRI66-157-21/Roll-Hansen	*Picea abies*, wound	Norway	V
C. coerulescens sp. D	C608	252-8/Solheim	*Picea engelmannii, Dendroctonus rufipennis*	B.C., Canada	VI
	C609	258-6/Solheim	*Picea engelmannii, Dendroctonus rufipennis*	B.C., Canada	VI
	C610	280-7/Solheim	*Picea engelmannii, Dendroctonus rufipennis*	B.C., Canada	VI
	C611	285-8/Solheim	*Picea engelmannii, Dendroctonus rufipennis*	B.C., Canada	VI
	C612	290-9/Solheim	*Picea engelmannii, Dendroctonus rufipennis*	B.C., Canada	VI
	C613	404-2/Solheim	*Picea engelmannii, Dendroctonus rufipennis*	B.C., Canada	VI
C. coerulescens f. *douglasii*	C324	CBS142.53, 70703/Davidson	*Pseudotsuga menziesii*, lumber from Oregon	Colorado, U.S.A.	VII
C. polonica	C123	NCC1714-1-1	*Picea abies*	Norway	VIII
	C320	CBS228.83, Solheim	*Picea abies, Ips typographus*	Norway	VIII
	C322	CBS133.38/Siemaszko	*Picea abies, Ips typographus*	Poland	VIII
	C750	CMW2272, YCC115/Yamaoka	*Picea jezoensis, Ips typographus*	Japan	VIII
	C755	CMW2273, YCC067/Yamaoka	*Picea jezoensis, Ips typographus*	Japan	VIII
	C757	CMW2274, YCC118/Yamaoka	*Picea jezoensis, Ips typographus*	Japan	VIII
	C731	CMW1164, ATCC62335, 80-53-7/Solheim	*Picea abies, Ips typographus*	Norway	VIII
	C788	CMW2441, 83-14-40/Solheim	*Picea abies, Ips typographus*	Norway	VIII
	C789	CMW2442, 83-43-4/Solheim	*Picea abies, Ips typographus*	Norway	VIII
	C791	CMW2444, 90-120-181/Solheim	*Picea abies, Ips typographus*	Norway	VIII
C. laricicola	C741	CMW1953, L31A/Yamaoka	*Larix, Ips cembrae*	Japan	IX
	C738	CMW1952, L35A/Yamaoka	*Larix, Ips cembrae*	Japan	IX

TABLE I. Continued.

Species	Isolate No.[a]	Other No./Collector[b]	Substrate	Country of origin	Electrophoretic phenotype
	C745	CMW1954, L30A/Yamaoka	*Larix, Ips cembrae*	Japan	IX
	C746	CMW1955, L20A/Yamaoka	*Larix, Ips cembrae*	Japan	X
	C177	55-9/Redfern	*Larix decidua, Ips cembrae*	Scotland	XI
	C178	56-13/Redfern	*Larix decidua, Ips cembrae*	Scotland	XI
	C179	56-2/Redfern	*Larix decidua, Ips cembrae*	Scotland	XI
	C180	56-12/Redfern	*Larix decidua, Ips cembrae*	Scotland	XI
	C181	56-10/Redfern	*Larix decidua, Ips cembrae*	Scotland	XI
C. virescens	C68	Mantle1-1/Houston	*Acer saccharinum*	New York, U.S.A.	XII
	C203	ATCC11066, Forintek 272A, 94256/Hepting	*Liriodendron tulipifera*, veneer	North Carolina, U.S.A.	XII
	C251	Mantle1-G25/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C252	Mantle60/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C253	Mantle68/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C254	Mantle95/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C255	Mantle128/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C256	3MTE/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C257	Lybdaker1/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C258	Lybdaker2/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C259	FFT#3/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C260	FFT#1/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C261	Yancy/Houston	*Acer saccharum*, sapstreak	New York, U.S.A.	XII
	C69	Houston	*Fagus americanum*, log	New Hampshire, U.S.A.	XIII
	C70	UM50, CMW447/Shigo			XIII
	C74	RWD-431, CMW460, CO462/Hinds	*Quercus*, wood	New York, U.S.A.	XIII
	C262	Houston	*Acer saccharum?*, sapstreak?	New Hampshire, U.S.A.	XIII
Ceratocystis sp. from *Eucalyptus*	C449	VIC1/Kile	*Eucalyptus*	Victoria, Australia	XIV
	C450	VIC8/Kile	*Eucalyptus*	Victoria, Australia	XIV
	C639	V1/Kile	*Eucalyptus sieberi*	Victoria, Australia	XIV
	C640	V2/Kile	*Eucalyptus globoidea*	Victoria, Australia	XIV
	C641	V3/Kile	*Eucalyptus globoidea*	Victoria, Australia	XIV
	C646	V12/Kile	*Eucalyptus sieberi*	Victoria, Australia	XIV
	C642	V5/Kile	*Eucalyptus sieberi*	Victoria, Australia	XV
	C644	V6/Kile	*Eucalyptus sieberi*	Victoria, Australia	XV
	C643	V8/Kile	*Eucalyptus sieberi*	Victoria, Australia	XVI
	C645	V7/Kile	*Eucalyptus sieberi*	Victoria, Australia	XVI
	C457	YEP/Kile	*Eucalyptus regnans*	Tasmania, Australia	XVII
Chalara australis	C448	1.91/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C451	BR2/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C452	BR5/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C453	ESP3/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII

TABLE I. Continued.

Species	Isolate No.[a]	Other No./Collector[b]	Substrate	Country of origin	Electrophoretic phenotype
	C454	ESP6/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C455	LFRHeaped4/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C456	LFRDrought6/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C614	1.55/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C615	X1/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C616	Blackwater/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C617	2.81/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C618	2.85/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C619	T.50/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C620	BR1/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C621	BR8/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C622	2.90/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C623	L1/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C624	2.89/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C625	2.88/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C626	Esp5/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C627	Esp11/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C628	BR11/Kile	*Nothofagus cunninghamii*	Tasmania, Australia	XVIII
	C631	N1/Kile	*Nothofagus cunninghamii*	Victoria, Australia	XVIII
	C632	N2/Kile	*Nothofagus cunninghamii*	Victoria, Australia	XVIII
	C633	N3/Kile	*Nothofagus cunninghamii*	Victoria, Australia	XVIII
	C634	N4/Kile	*Nothofagus cunninghamii*	Victoria, Australia	XVIII
	C635	N5/Kile	*Nothofagus cunninghamii*	Victoria, Australia	XVIII
	C636	N7/Kile	*Nothofagus cunninghamii*	Victoria, Australia	XVIII
	C637	N6/Kile	*Nothofagus cunninghamii*	Victoria, Australia	XVIII
	C638	1.66/Kile	*Nothofagus cunninghamii*	Victoria, Australia	XVIII
C. neocaledoniae	C694	CBS149.83, LCPM1055/Kiffer	*Coffea robusta*	New Caledonia	XIX

[a] Isolate numbers from the collection of the senior author.

[b] Collectors and their institutions are listed in the Materials and Methods.

TABLE II. Abbreviations, buffer systems and number of electromorphs found for 10 enzymes tested for variation in the *Ceratocystis coerulescens* complex

Enzyme	Abbreviation	EC Number[a]	Buffer system/ Dilutions[b]	Number of electromorphs
Aspartate aminotransferase	AAT	2.6.1.1	E/1:1	8
NADH diaphorase	DIA	1.6.4.3	E/1:15	8
Fumerase	FUM	4.2.1.2	E/1:3	6
Glucose-6-phosphate dehydrogenase	G6PD	1.1.1.49	E/none	2
Glucosephosphate isomerase	GPI	5.3.1.9	B/1:3	5
Glutamate dehydrogenase (NADP)	GDH	1.4.1.3	B/1:3	8
Isocitrate dehydrogenase	IDH	1.1.1.42	E/1:3	4
Malate dehydrogenase	MDH	1.1.1.37	B/1:3	4
Phosphoglucomutase	PGM	5.4.2.2	B/1:3	3
Phosphogluconate dehydrogenase	PGD	1.1.1.43	E/1:1	5

[a] Nomenclature Committee of the International Union of Biochemistry.

[b] Buffer B was a continuous histine citrate system, adjusted to pH 5.7, run at 9 watts constant wattage for 4 hours. Buffer E was a continuous morpholine citrate system, adjusted to pH 8.1, run at 15 watts constant wattage for 6 hours, except for diaphorase, which was run at 9 watts for 6 hours.

species (FIG. 2). The single ET of *C. polonica* clustered with the three ETs of *C. laricicola*. Two ETs of *C. virescens* differed by only a single isozyme and were distinct from all other taxonomic units. The four ETs of the undescribed species from *Eucalyptus* clustered together. No variation was found among the 30 isolates of *Ch. australis*, which grouped nearest to *Ch. neocalidoniae*.

Within *C. coerulescens*, five clusters of ETs could be seen: sp. A, sp. B, sp. C, sp. D and f. *douglasii*. The two ETs of sp. A differed by only one enzyme, as did the two ETs of sp. C (TABLE III). Two of the variants

TABLE III. Isozyme electromorphs for 19 electrophoretic phenotypes among 98 isolates of the *Ceratocystis coerulescens* complex

Species	Electrophoretic phenotype	Number of isolates	Isozymes									
			AAT	DIA	FUM	G6PD	GPI	GDH	IDH	MDH	PGM	PGD
C. coerulescens sp. A	I	3	B[a]	E	E	B	C	B	C	D	B	D
	II	1	B	G	E	B	C	B	C	D	B	D
C. coerulescens sp. B	III	4	A	E	E	B	D	A	C	B	B	B
C. coerulescens sp. C	IV	4	E	D	D	B	C	C	A	C	A	B
	V	1	E	H[b]	D	B	C	C	A	C	A	B
C. coerulescens sp. D	VI	6	H	B	D	B	B	G	A	C	A	B
C. coerulescens f. *douglasii*	VII	1	A	C	F	A	E	B	D	D	C	A
C. polonica	VIII	10	E	B	D	B	D	B	C	B	B	E
C. laricicola	IX	3	E	E	D	B	D	B	C	B	B	E
	X	1	G	E	D	B	D	B	C	B	B	E
	XI	5	E	F	D	B	D	B	C	B	B	E
C. virescens	XII	13	F	B	D	B	C	D	A	A	A	B
	XIII	4	D	B	D	B	C	D	A	A	A	B
C. sp. from *Eucalyptus*	XIV	6	F	C	A	B	B	E	A	A	A	B
	XV	2	F	B	A	B	A	E	A	A	A	B
	XVI	2	F	C	A	B	B	E	A	A	A	C
	XVII	1	F	C	A	B	B	H	A	A	A	B
Chalara australis	XVIII	30	F	D	C	B	B	D	B	A	C	B
Chalara neocalidoniae	XIX	1	C	A	B	B	A	F	B	A	C	B

[a] Letters represent isozyme electromorphs for each isozyme in order of decreasing anodal migration.

[b] No activity for DIA.

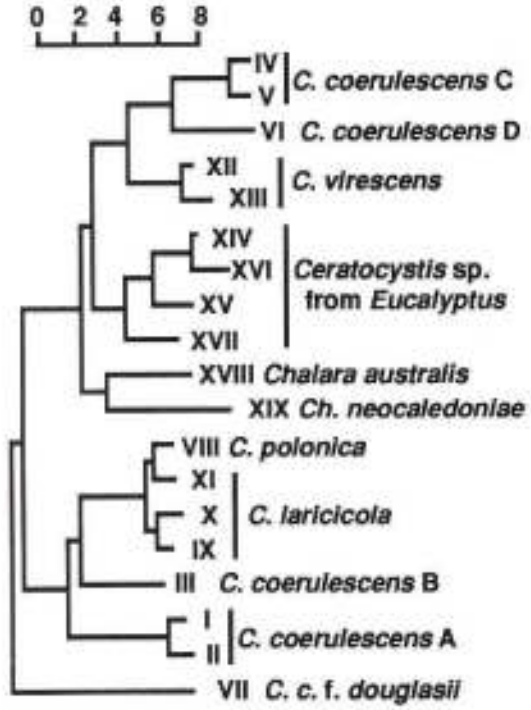

FIG. 2. Phenogram (Neighbor-joining analysis) based on isozyme electromorphs of 19 electrophoretic phenotypes (Roman numerals) in the *Ceratocystis coerulescens* complex.

(sp. A and sp. B) grouped most closely to *C. polonica* and *C. laricicola*, and sp. C and D grouped most closely to *C. virescens* (FIG. 2). *C. c.* f. *douglasii* was represented by only one isolate, which had unique electromorphs for five of the ten isozymes tested and did not cluster with the other phenotypes.

DISCUSSION

Analysis of the isozyme data clustered morphologically similar isolates and supported delineation of many species within the *C. coerulescens* complex. Although the number of isozymes tested was too low to confidently compare the relative relatedness among all the tested taxa, two broadly defined groups were suggested: one centered around the hardwood species *C. virescens* and another centered around *C. coerulescens sensu stricto* (sp. A). The isolate of *C. c.* f. *douglasii* did not cluster closely with other isolates.

Hardwood species.—Four taxa on hardwoods (*C. virescens, Ceratocystis* sp. from *Eucalyptus, Ch. australis* and *Ch. neocalidoniae*) share many isozyme electromorphs and have similar conidiophore states: tapering and proliferating phialides as opposed to the non-tapering phialides typically found in the conifer species of the *C. coerulescens* complex. Three of the hardwood species are primary pathogens and are believed to be similar in pathogenesis (Kile, 1993), but the species on *Eucalyptus* is only weakly pathogenic (Kile, unpublished).

The degree of within-species variation in the hardwood group may be a reflection of the sexual compatibility systems of these respective taxa. Among all the species studied, the *Eucalyptus* fungus had the greatest variation in isozyme electromorphs (four ETs among 10 isolates). This species is heterothallic (Kile et al., 1994), and assuming that ascospores are the primary dispersal propagule, an isolate would be forced to cross to be dispersed. Substantially less variation (two ETs among 17 isolates) was found in *C. virescens*. Aside from the species on *Eucalyptus*, the *Ceratocystis* species studied here, including *C. virescens*, are homothallic, perhaps due to a mechanism of mating type switching (Harrington and McNew, unpublished). Thus, ascospores can be produced by selfing in *C. virescens*, and an intermediate level of variation might be expected. *Chalara australis* showed no variation in isozyme electromorphs and has no known teleomorph; it is apparently dispersed as asexual propagules, most probably in the frass of ambrosia beetles (Kile, 1993). This low level of isozyme polymorphism might be expected in such an asexual fungus (Zambino and Harrington, 1989; 1992). The data would suggest that there has been little sexual recombination since speciation or since introduction of *Ch. australis* to Australia, but the species is not known outside southeastern Australia.

Conifer species.—Isozyme variation supports distinguishing five morphological variants of *C. coerulescens* as distinct taxa. Differences in electromorphs between any two of these variants was substantial, greater than that found within the other *Ceratocystis* species studied. Two of the variants of *C. coerulescens* showed similarity to *C. polonica* and *C. laricicola*, two were closer to the hardwood species *C. virescens*, and the single isolate of *C. c.* f. *douglasii* differed markedly from all of the other isolates in isozyme electromorphs. An isolate of *C. coerulescens* examined by Wingfield et al. (1994) for rRNA homology had greater similarity to *C. virescens* than to *C. laricicola*. It is not clear to which of the five variants of *C. coerulescens* their isolate belongs, but the isozyme data presented herein would suggest that their isolate was *C. coerulescens* sp. C.

Among the variants of *C. coerulescens*, the isolates of sp. A are morphologically closest to Münch's (1907) concept of the species. The four isolates we studied originated from stained wood of *Pinus* spp. in England. Perithecia produced by pairing isolate C490 with any of the three other isolates (C487, C488, or C489) were large and robust with long necks, fitting the dimensions given by Münch (1907) and as recognized by Lagerberg et al. (1927), Siemaszko (1939), and Bakshi (1951).

Only one isolate (C301) representing sp. B produces perithecia in culture, and these perithecia are smaller than those of sp. A. However, Campbell (1957), who originally isolated C301, reported dimensions of perithecia for *C. coerulescens* similar to those reported by Münch (1907), and crossing C301 with C693 results in perithecia with necks comparable to those of species A. Our species B isolates were from both pine and spruce in Europe and North America. Similarity in biology and paucity of perithecial material for morphological comparisons preclude a clear separation of sp. A from sp. B.

Perithecia of sp. C produced in culture have shorter necks than the perithecia of sp. A. Isolates were obtained from Europe and North America. At least two of the isolates representing sp. C were from wounds in spruce. As a wound colonizer of living trees, this species may be weakly pathogenic, which is also true for the most electrophoretically similar variant, sp. D.

Species D appears to be the variant recognized by Davidson (1955) from beetle-attacked spruce in Colorado. Our isolates of sp. D grow slowly at room temperature and produce perithecia only at 20 C or less. Ascospores of this variant are larger than those of the other conifer species. Our isolates of sp. D all originated from *Dendroctonus rufipennis* or from *Picea engelmannii* attacked by this bark beetle in Canada. It appears to be pathogenic to spruce (Solheim, 1994) and is likely to be an important symbiont of this bark beetle. Its pathogenicity would tend to coincide with the pathogenicity of the other species with which it shared isozyme electromorphs (i.e., sp. C and the hardwood species of the complex).

Davidson's (1953) recognition of isolates of *C. coerulescens* from Douglas-fir as a separate form is well supported by the isozyme data, although *C. c.* f. *douglasii* was represented in this study by only one isolate. Its sensitivity to warm temperatures and the two types of conidiophores described by Davidson (1953), particularly the long, tapering phialides, further distinguish this fungus. Although treated as a synonym of *C. coerulescens* in the past (Hunt, 1956; Upadhyay, 1981) we believe that *C. c.* f. *douglasii* should be recognized at the rank of species.

The other examined species from conifers, *C. polonica* and *C. laricicola*, are noteworthy for their ascospores, which are relatively small, more broadly ellipsoid with broadly-thickened sheaths (outer walls), and have two distinct guttules at each end. The ascospores of the other *Ceratocystis* species studied are considerably longer and have sheaths that are most obvious at their terminal ends. Conidiophores are scarce in *C. polonica* and *C. laricicola* and often found only as hairs or ornamentation on the base of perithecia. When *C. laricicola* was described (Redfern et al., 1987), it was not compared to *C. polonica*, which was then believed to be an *Ophiostoma* species with a *Leptographium* anamorph. Now that *C. polonica* is recognized to have a *Chalara* anamorph (Visser et al., 1994), the morphological distinction between *C. laricicola* and *C. polonica* is difficult to discern. Isolates of *C. polonica* from Poland (an isolate from the holotype), Norway and Japan had identical isozyme electromorphs, and these differed only slightly from the ETs of *C. laricicola*, which originated from Japan and Scotland. These two taxa are strongly associated with closely related species of *Ips*, perhaps co-evolving with their insect symbionts.

Bark beetle associations.—Several characteristics of *C. polonica* and *C. laricicola* are also found in *C. coerulescens* sp. D, the other bark beetle associate in this study. These three taxa produce few conidiophores and generally lack strong, fruity odors, features important to spermatization of mats with conidia carried by fungal-feeding insects. Because the protoperithecia of these species are mostly confined to the galleries of bark beetles, the opportunity for cross-fertilization would be less than in the other *Ceratocystis* species, which tend to produce mats with protoperithecia on exposed wood. Because mats would not be exposed for acquisition and dispersal of spermatizing conidia, the bark beetle associates might be expected to suppress conidiophore production and the production of attractive volatiles (such as isobutyl acetate). They may also respond strongly to wounding of the mycelium (feeding by bark beetles) as a cue to producing many perithecia with ascospores, and cultures of these species on malt agar do tend to produce perithecia in abundance after wounding. Because the *Ips*-associated species (*C. polonica* and *C. laricicola*) and *Dendroctonus*-associated species (sp. D) showed dissimilar isozyme electromorphs, it may be that suppression of conidiophore production, the low level of odors and the wound response evolved separately in the *Ips* and the *Dendroctonus*-associated *Ceratocystis* species.

Although the association of *Ophiostoma* species with bark beetles has been much discussed (most recently by Harrington, 1993), the association of *Ceratocystis* species with conifer-infesting bark beetles has only been recognized recently. Depending on whether *C. laricicola* and *C. polonica* turn out to represent distinct taxa or not, we are now aware of two or perhaps three species of *Ceratocystis* carried by conifer infesting bark beetles. From pathogenicity tests with *C. polonica* and *C. laricicola* (Christiansen and Solheim, 1990; Redfern et al., 1987; Solheim, 1993) and also with *C. coerulescens* sp. D (Solheim, 1994), indications are that the *Ceratocystis* species are considerably more virulent than *Ophiostoma* species to the tree hosts and of more importance to the beetle in killing mass-attacked trees. In contrast, the *Ophiostoma* species might be relatively

unimportant and even a hindrance to the beetles, as proposed by Harrington (1993). As tree pathogens, these few *Ceratocystis* species may have evolved a mutualistic symbiosis with bark beetles, where both partners benefit from the relationship.

Many of the fungi studied here are believed to be dispersed by insects, which has apparently led to substantial convergence in morphological characteristics (Blackwell, 1994; Hausner et al, 1993; Wingfield et al., 1994). This convergence and limited variation has made species delimitation based on morphology alone difficult. However, variation in isozyme patterns within *C. coerulescens* and among related species generally corresponded well with the limited morphological variation in the group. The technique proved useful in helping to delineate taxa of common biology, and this information will be applied to revise the taxonomy of this important complex.

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