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Genetic relationships among *Leptographium terebrantis* and the mycangial fungi of three western *Dendroctonus* bark beetles

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Abstract: Morphology, mitochondrial DNA (mtDNA) restriction fragment polymorphisms (RFLPs) and nuclear DNA (nDNA) fingerprinting were used to clarify relationships among the morphologically similar *Ophiostoma* and *Leptographium* species associated with mycangia of three *Dendroctonus* bark beetles (*Ophiostoma clavigerum* associated with both *D. ponderosae* and *D. jeffreyi*, and *L. pyrinum* associated with *D. adjunctus*), as well as a closely related nonmycangial bark beetle associate (*L. terebrantis*). Most isolates of *O. clavigerum* form long (40–70 µm), septate conidia, while all isolates of *L. terebrantis* and *L. pyrinum* form conidia less than 17.0 µm in length. The conidia of *L. pyrinum* are pyriform, with truncate bases, while the conidia of the other species form only slightly truncate bases. Conidial masses of *L. terebrantis* are creamy yellow, while the conidial masses of the other species are white. Nuclear DNA fingerprints resulting from probing *Pst*I restrictions with the oligonucleotide probe (CAC)₅ and *Hae*III and *Msp*I restrictions of mtDNA, exhibited three major clusters. In the dendrogram developed from mtDNA RFLPs, the *L. pyrinum* isolates formed one cluster, while the majority of *O. clavigerum* isolates, including all *D. jeffreyi* isolates, formed another. A third cluster was composed of all *L. terebrantis* isolates, as well as several *O. clavigerum* isolates from *D. ponderosae*. The inclusion of some *O. clavigerum* isolates in the *L. terebrantis* cluster suggests that horizontal transfer of mtDNA has occurred among these fungi. The nDNA dendrogram also ex-

hibited three clusters, and most isolates of *L. pyrinum*, *L. terebrantis* and *O. clavigerum* grouped separately; however, one isolate of *O. clavigerum* grouped with the *L. terebrantis* isolates, while one isolate of *L. terebrantis* grouped with *O. clavigerum*. No genetic markers were found that distinguished between *O. clavigerum* associated with *D. ponderosae* and *O. clavigerum* associated with *D. jeffreyi*. *Ophiostoma clavigerum* might be a recently diverged morphological variant of *L. terebrantis*, with special adaptations for grazing by young adults of *D. jeffreyi* and *D. ponderosae*. The anamorph of *O. clavigerum*, *Graphiocladiella clavigerum*, is transferred to *Leptographium*.

Key words: *Dendroctonus adjunctus*, *D. jeffreyi*, *D. ponderosae*, DNA fingerprinting, *Leptographium pyrinum*, *L. terebrantis*, mitochondrial DNA, mycangial fungi, *Ophiostoma clavigerum*, RFLP, Scolytidae

INTRODUCTION

Several bark beetles (Scolytidae: Coleoptera) in the genus *Dendroctonus* Erichson possess mycangia formed by invaginations of the exoskeleton that function in the dissemination of symbiotic fungi. Many associations among *Dendroctonus* bark beetles and mycangial fungi are believed to be mutualistic due to the presence of these specialized structures, which clearly are involved in the maintenance of the association. These associations appear to be obligate; the fungi are highly adapted to dissemination by insects (Malloch and Blackwell 1993) and apparently are dependent wholly on the host beetle for dispersal. Likewise, the beetles appear to be dependent upon at least some mycangial fungi for successful development and reproduction (Whitney 1971, Barras 1973, Bridges 1983, Goldhammer et al 1990, Coppedge et al 1995, Six and Paine 1998, Ayres et al 2000).

Among the many ascomycetes associated with *Dendroctonus* mycangia are several blue-staining fungi in the genus *Ophiostoma* H. & P. Sydow and the asexual genus *Leptographium* Lagerb. & Melin. (many species of *Ophiostoma* possess *Leptographium* anamorphs) (Harrington 1988). *Dendroctonus jeffreyi* Hopkins and *D. ponderosae* Hopkins carry *Ophiostoma clavigerum* (Robinson-Jeffrey & Davidson) Harrington in their mycangia (Whitney and Farris 1970, Six and Paine 1997), while *D. adjunctus* Blandford carries *Leptogra-*

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TABLE I. Origin and morphological characteristics of isolates of *Leptographium pyrimum*, *L. terebrantis*, and *Ophiostoma clavigerum* used to assess genetic relationships

Species	Isolate No.	Origin	Beetle associate	Tree species	Color of conidial mass	Conidium length (μm)	Number of conidial septa
<i>L. pyrimum</i>	^a C96	New Mexico	Unknown	Unknown	White	10-16	0
	C831	Sacramento Mtns., New Mexico	<i>Dendroctonus adjunctus mycangia</i>	Unknown	White	12-17	0
	C832	Sacramento Mtns., New Mexico	<i>D. adjunctus mycangia</i>	Unknown	White	12-16	0
	C833	Twilight Camp, Pinaleno Mtns., AZ	<i>D. adjunctus mycangia</i>	<i>Pinus arizonica</i>	White	12-14	0
	C834	Riggs Flat, Pinaleno Mtns., AZ	<i>D. adjunctus mycangia</i>	<i>P. monticola</i>	White	10-12	0
	C835	Turkey Flat, Pinaleno Mtns., AZ	<i>D. adjunctus mycangium</i>	<i>P. arizonica</i>	White	10-13	0
	ATCC58565	British Columbia, Canada	Unknown	<i>P. contorta</i>	Creamy-yellow	8-11	0
	C25	Blodgett, CA	Associated with <i>D. brevicornis</i>	<i>P. ponderosa</i>	Creamy-yellow	6-8	0
	C681	Trout Creek, CA	From <i>D. valens</i>	Unknown	Creamy-yellow	8-11	0
	C814	Burns, OR	From <i>D. valens</i>	Unknown	Creamy-yellow	8-12	0
C815	Burns, OR	From <i>D. valens</i>	Unknown	Creamy-yellow	10-12	0	
C86	Teton Nat'l Forest, WY	Unknown	<i>P. contorta</i>	White	10-13	0	
C186	California	Associated with <i>D. ponderosae</i>	<i>P. ponderosa</i>	White	32-50	0-2	
C187	Yosemite Valley, CA	Associated with <i>D. ponderosae</i>	<i>P. ponderosa</i>	White	42-64	0-2	
C293	Bally River, Alberta, Canada	Beetle gallery	<i>P. contorta</i>	White	10-13	0	
C295	Invermero, British Columbia, Canada	Associated with <i>D. ponderosae</i>	<i>P. contorta</i>	White	10-14	0	
C813	Burns, OR	From <i>D. valens</i>	Unknown	White	12.0-16.0	0	
C836	Inyo Craters, Sierra Nevada Mtns., CA	<i>D. ponderosae mycangia</i>	<i>P. contorta</i>	White	11.0-16.0	0	
C837	Truckee, Sierra Nevada Mtns., CA	<i>D. ponderosae mycangia</i>	<i>P. contorta</i>	White	32-50	0-3	
C838	Deerlick, San Bernardino Mtns., CA	<i>D. ponderosae mycangia</i>	<i>P. lambertiana</i>	White	38-70	0-3	
C839	Deerlick, San Bernardino Mtns., CA	<i>D. ponderosae mycangia</i>	<i>P. lambertiana</i>	White	44-56	0-2	
C840	Deerlick, San Bernardino Mtns., CA	<i>D. ponderosae mycangia</i>	<i>P. lambertiana</i>	White	Not measured	Not measured	
C842	Meyers, Sierra Nevada Mtns., CA	<i>D. jeffreyi mycangia</i>	<i>P. jeffreyi</i>	White	12-16	0	

TABLE I. Continued

Species	Isolate No.	Origin	Beetle associate	Tree species	Color of conidial mass	Conidium length (μm)	Number of conidial septa
	C843	Monitor Pass, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	44-70	0-2
	C844	Meyers, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	14-32	0-1
	C845	Monitor Pass, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	12-44	0-1
	C846	Indiana Summit, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	48-63	0-2
	C847	Meyers, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	40-50	0-4
	C848	Lassen, Cascade Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	47-72	0-2
	C849	Greenhorn, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	48-54	0-2
	C850	Smokey Bear Flats, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	48-60	0-2
	C851	Arrowbear, San Bernardino Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	14-22	0-1
	C852	Bell Meadows, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	46-65	0-1
	DLS608	Monitor Pass, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	Not measured	Not measured
	C841	Deadman Summit, Sierra Nevada Mtns., CA	<i>D. jeffreyi</i> mycangia	<i>P. jeffreyi</i>	White	40-50	0-2

^a Isolates beginning with C are from the culture collection of T. C. Harrington. Isolates beginning with DLS are from the culture collection of D. L. Six.

phium pyrinum Davidson (Six and Paine 1996). The anamorph of *Ophiostoma clavigerum* and *L. pyrinum* are similar but morphologically distinct, and the two fungi exhibit high genetic identity with one another based on isozyme markers (Zambino and Harrington 1992, Six and Paine 1999a). *Leptographium terebrantis* also is similar morphologically to the anamorph of *O. clavigerum*; it has been isolated from many species of *Pinus* across North America and from a number of bark beetles, including *D. valens* LeConte, *D. terebrantis* (Oliv.) and *Hylurgops porosus* (LeConte), but it has not been found associated with beetle mycangia (Harrington 1988). Many *Ophiostoma* and *Leptographium* species exhibit a high degree of pleomorphism (Malloch and Blackwell 1993), and Tsuneda and Hiratsuka (1984) found a wide range in conidiophore and conidial morphology in *O. clavigerum*. This, and the high degree of similarity in isozyme phenotypes, led Zambino and Harrington (1992) to suggest that *O. clavigerum*, *L. pyrinum*, *L. terebrantis* and some other *Leptographium* species may be morphological variants of a single species.

Some uncertainty also exists whether *O. clavigerum* associated with *D. jeffreyi* and *O. clavigerum* associated with *D. ponderosae* constitute a single species or comprise a pair of cryptic species or physiologic races. No differences were seen in *O. clavigerum* isolates from the mycangia of the two beetles using morphology, isozymes or temperature tolerances (Six and Paine 1997). This fungus, however, when isolated from the two beetles, exhibits significant differences in tolerances for host tree-resin components, with greater tolerances for host resins than non-host resins when grown in artificial culture (Paine and Hanlon 1994). *Ophiostoma clavigerum* associated with the two beetles also exhibited differential growth in bolts of *Pinus contorta* Dougl. and *P. jeffreyi* Grev. & Balf., hosts of *D. ponderosae* and *D. jeffreyi*, respectively (Six and Paine 1998). *Dendroctonus jeffreyi* and *D. ponderosae* are sibling species that are morphologically and genetically very similar (Higby and Stock 1982, Wood 1982). However, there is no overlap in host tree species used by the two beetle species. *Dendroctonus jeffreyi* is monophagous and attacks only *P. jeffreyi* (Wood 1982). *Dendroctonus ponderosae* is polyphagous, attacking 13 species of *Pinus* but not *P. jeffreyi* (Wood 1982). The major resin components of the host trees of the two beetles differ considerably. n-Heptane is the major resin component of *P. jeffreyi*, while the main resin components of hosts of *D. ponderosae* are monoterpenes and resin acids (Mirov 1929, Smith 1967, Anderson et al 1969). Therefore, *O. clavigerum* associated with *D. jeffreyi* and *O. clavigerum* associated with *D. ponderosae* are isolated in different chemical environments, which ultimately

may result in divergence due to selection and random genetic drift. Divergence in morphologically simple fungi may result in genetic differentiation without concurrent morphological changes (Kemp 1977, Brasier 1986).

Our objectives were (1) to assess whether *L. pyrinum* is a species distinct from *O. clavigerum* or simply a morphologic variant, (2) to determine whether differentiation is occurring between *O. clavigerum* associated with *D. jeffreyi* and *O. clavigerum* associated with *D. ponderosae* and (3) to assess the relationship of the nonmycangial *L. terebrantis* to *O. clavigerum* and *L. pyrinum*. In addition to morphological comparisons, we used nuclear DNA (nDNA) fingerprinting and restriction fragment-length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA), techniques that have been used successfully to differentiate among species and strains of several fungi (Meyer et al 1991, DeScenzo and Harrington 1994).

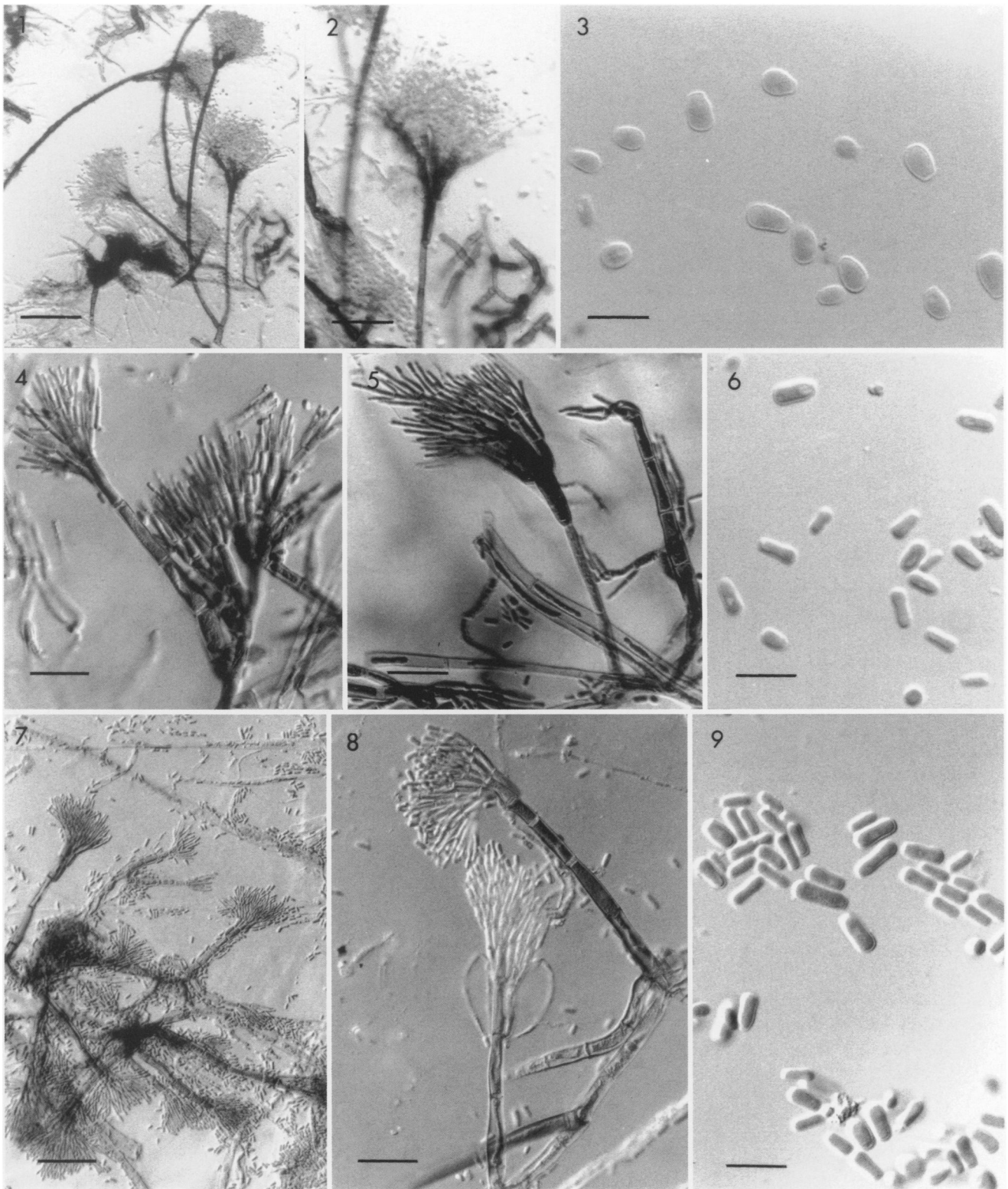
MATERIALS AND METHODS

Isolates.—Isolates used in this study are listed in TABLE I. Most isolates were obtained directly from mycangia of bark beetles. The procedure used for mycangial isolations is presented elsewhere (Six and Paine 1997). *Ophiostoma clavigerum* was isolated from *D. ponderosae* (DP) collected at three locations in California (isolates C836, C837, C838, C839 and C841) where *D. ponderosae* and *D. jeffreyi* are sympatric. *Ophiostoma clavigerum* also was isolated from *D. jeffreyi* (DJ) collected at 10 California sites that are representative of a majority of the geographic range of that beetle. In most, if not all, of these locations, *D. jeffreyi* and *D. ponderosae* are sympatric (Wood 1982). Fungi from allopatric populations of *D. ponderosae* also were included for comparison (C295, C293 and C86).

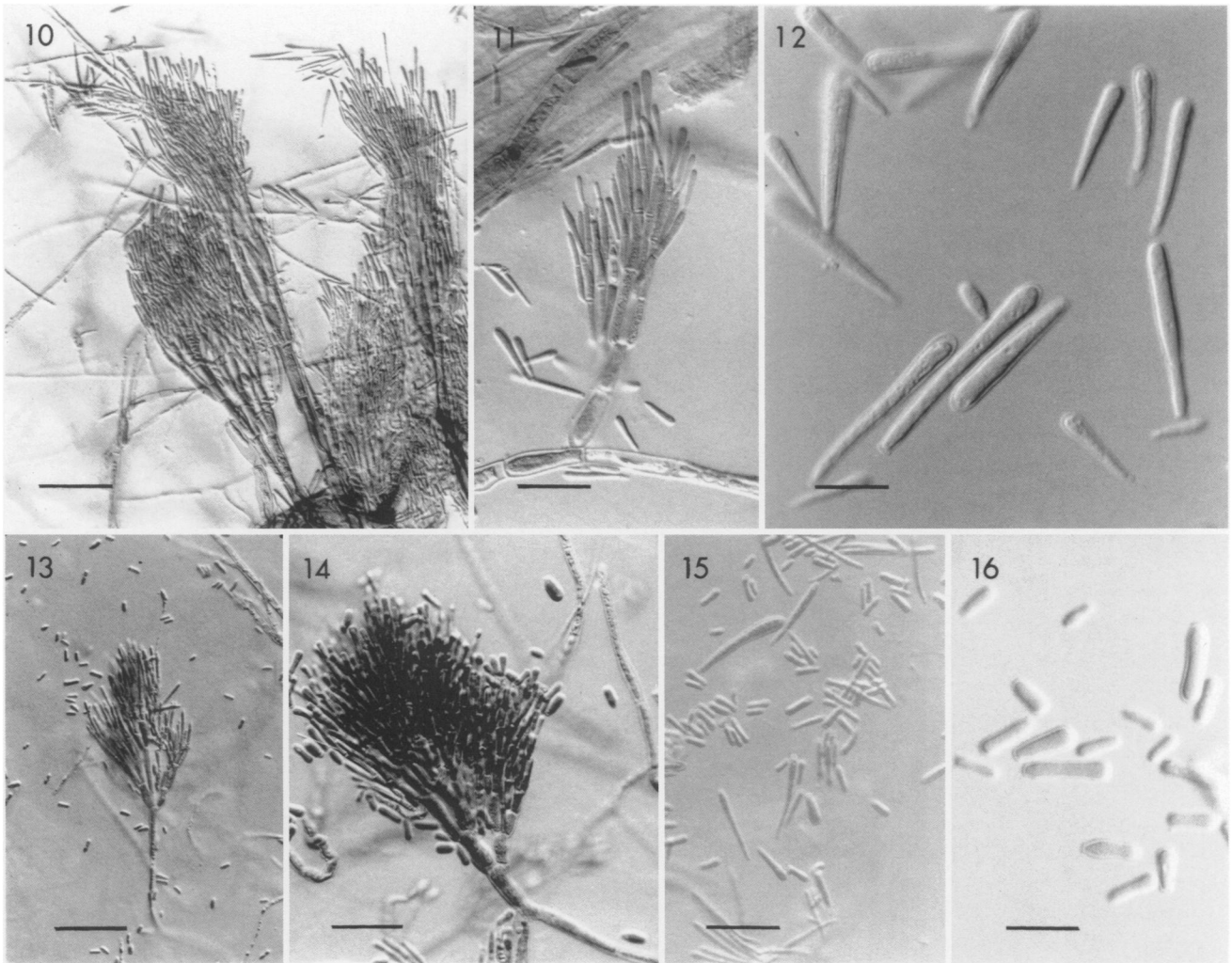
Morphology.—Cultures were grown in 95 mm plastic Petri dishes with MYEA (2% malt extract, 0.2% yeast extract and 1.5% agar) and autoclaved *Pinus strobus* twigs for at least 3 wk. Microscope slides were prepared of conidiophores and conidia produced near the twigs by mounting in cotton blue. For each isolate, 10 of the longest conidia were measured at 500× magnification and the number of septa was noted for each conidium.

Total genomic DNA extraction. Mycelia were grown in 25 mL liquid medium (2% malt extract, 0.2% yeast extract) in 125 mL Erlenmeyer flasks and held at room temperature (ca 21 C) in the light. After 14 d the mycelial mats were collected and dried with vacuum filtration through 1 mm Whatman No. 1 filter paper (Whatman International Ltd., Kent, England). The mats were placed between paper towels and dried for an additional 10 min. Immediately after drying, the mats were ground with mortar and pestle to a fine powder in liquid nitrogen.

Total genomic DNA extractions were carried out with the ground mycelia and a modification of the method devel-



FIGS. 1–9. *Leptographium pyrinum* and *L. terebrantis*. 1–3. *L. pyrinum*. 1, 2. Conidiophores. 3. Conidia. 1–3 from strain C833. 4–9. *L. terebrantis*. 4, 5. Conidiophores. 6. Conidia. 7, 8. Conidiophores. 9. Conidia. 4–6 from strain C680. 7–9 from strain C25. Scale bars: 1 = 100 μm ; 2, 7 = 50 μm ; 3, 6, 9 = 10 μm ; 4, 5, 8 = 25 μm .



FIGS. 10–16. *Ophiostoma clavigerum*. 10, 11. Conidiophores. 12. Clavate conidia. 13, 14. Conidiophores. 15. Clavate conidia. 16. Conidia. 10–12 from strain C187. 13–16 from strain C813. Scale bars: 10, 13 = 50 μm ; 11, 14, 15 = 25 μm ; 4, 5 = 25 μm .

oped by Dellaporté et al (1983) and detailed in DeScenzo and Harrington (1994).

mtDNA RFLPs.—RFLPs of mtDNA can be visualized directly from stained gels of total genomic DNA that has been digested with restriction enzymes possessing G-C, four-base recognition sites (Wingfield et al 1996). mtDNA exhibits a high degree of restriction fragment-length polymorphism at the intraspecific level, and length mutations have been shown to be the major cause of this variation (Sanders et al 1977, Taylor et al 1986, Bruns et al 1988). Total genomic DNA was digested with *Hae*III and *Msp*I restriction enzymes (Promega, Madison, Wisconsin), which recognize the base sequences GGCC and CCGG, respectively. These enzymes digest the majority of nuclear DNA to relatively short lengths, leaving relatively long pieces of AT-rich DNA, which are primarily from the mitochondrial genome. After electrophoresis, ethidium bromide-stained bands of uniform intensity are scored as mtDNA bands. Protocols used for electrophoresis and staining of RFLPs were developed by Wing-

field et al (1996). mtDNA RFLPs were not obtained for isolates C186, C847 and DLS568, and nDNA fingerprints were not obtained for isolate 608.

nDNA fingerprinting. nDNA fingerprints are produced by the hybridization of DNA probes (oligonucleotides) to restriction fragments of genomic DNA. These probes are homologous to hypervariable repetitive sequences often called “simple repetitive sequences” or “microsatellite DNAs”. These simple repetitive sequences often exhibit substantial variability in their repeat copy number for a given locus.

For nDNA fingerprinting, total genomic DNA was digested using the restriction enzyme *Pst*I (Promega, Madison, Wisconsin) and then hybridized with the synthetic oligonucleotide probe (CAC)₅. This probe has been shown to be useful in detecting variation in both basidiomycetes and ascomycetes (DeScenzo and Harrington 1994). Protocols for *Pst*I digestion, electrophoresis, radiolabeling, in-gel hybridization and autoradiography are described in DeScenzo and Harrington (1994).

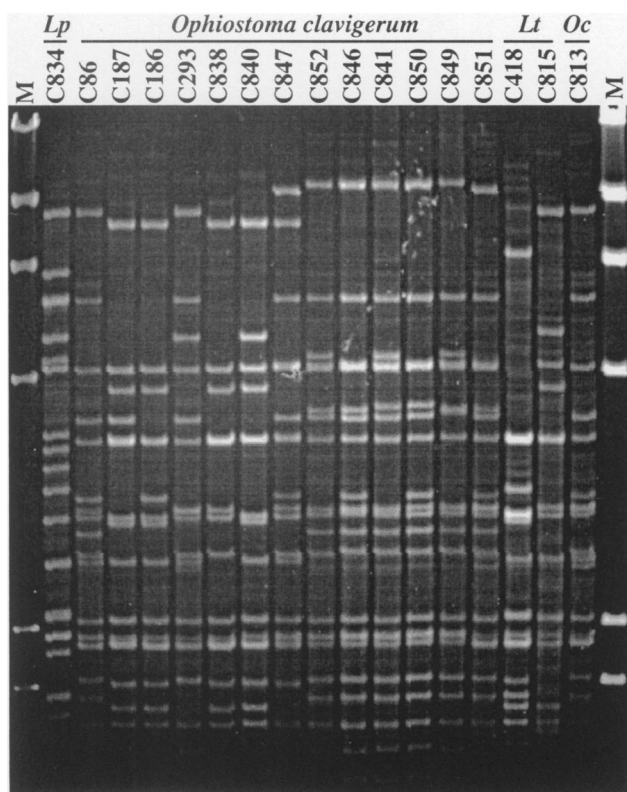


FIG. 17. Ethidium bromide-stained agarose gel with *Hae*III mtDNA patterns for *L. pyrinum* (lane 1), *O. clavigerum* (isolated from *D. ponderosae*), *O. clavigerum* (isolated from *D. jeffreyi*), and *L. terebrantis*. Lanes 1 and 20 are lambda DNA digested with *Hind*III, which was used to determine restriction fragment sizes.

Data analysis.—Band sizes for both mtDNA RFLPs and nDNA fingerprints were determined using Gelreader (version 2.0.5) (National Center for Supercomputing Applications 1991). Bands of the same molecular weight were scored as alleles possessing two character states (presence/absence). Gels used for DNA fingerprinting were run twice, and only bands distinct and scorable in both runs were analyzed.

Cluster analysis was performed using the GENDIST (Nei's genetic distance) and NEIGHBOR (UPGMA, unweighted pair-group method with arithmetic averaging) programs found within the PHYLIP package (version 3.5) (Felsenstein 1993). Trees were produced from PHYLIP files using TREEVIEW (version 1.6.6.) (Page 1996).

RESULTS

Morphology.—Three morphological species were recognized by conidial characteristics (TABLE I, FIGS. 1–16). Conidial masses of *L. terebrantis* were creamy yellow en masse when examined under a dissecting microscope, while all isolates of *L. pyrinum* and *O. clavigerum* had white conidial masses. The conidia of *L. pyrinum* were 4–7 μ m wide, had strongly flattened

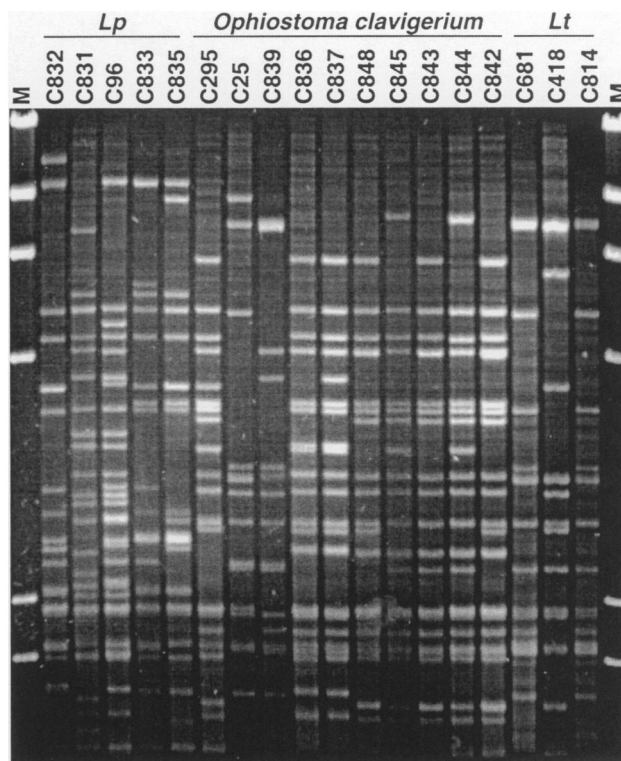


FIG. 18. Ethidium bromide-stained agarose gel with *Msp*I mtDNA patterns for *L. pyrinum*, *O. clavigerum* (isolated from *D. ponderosae*), *O. clavigerum* (isolated from *D. jeffreyi*), and *L. terebrantis*. Lanes 1 and 20 are lambda DNA digested with *Hind*III, which was used to determine restriction fragment sizes.

bases and tended to be pyriform to obovoid; while conidia of the other species were 2–4 μ m wide, had bases that are slightly truncate to rounded, and were cylindrical, to obovoid, to clavate. The length of *L. pyrinum* conidia was found to be 10–17 μ m, those of *L. terebrantis* to be 6–12 μ m and those of *O. clavigerum* to be highly variable. Most isolates of *O. clavigerum* formed clavate-shaped conidia that were large (40–70 μ m in length), with up to four septa, but smaller conidia, similar to those of *L. terebrantis* in size and shape, were common in all isolates of *O. clavigerum*. A number of isolates of *O. clavigerum* produced only small conidia of the *L. terebrantis* type in the examination reported in TABLE I but produced larger conidia in earlier examinations. Isolate C813 earlier produced clavate conidia from 10–35 μ m in length, and its conidial mass was white in color, consistent for *O. clavigerum*.

mtDNA RFLPs.—For *Hae*III and *Msp*I restrictions combined, the number of individual phenotypes was as great as the number of isolates investigated; that is each isolate had its own mitochondrial RFLP pattern. Examples of *Hae*III and *Msp*I restrictions are

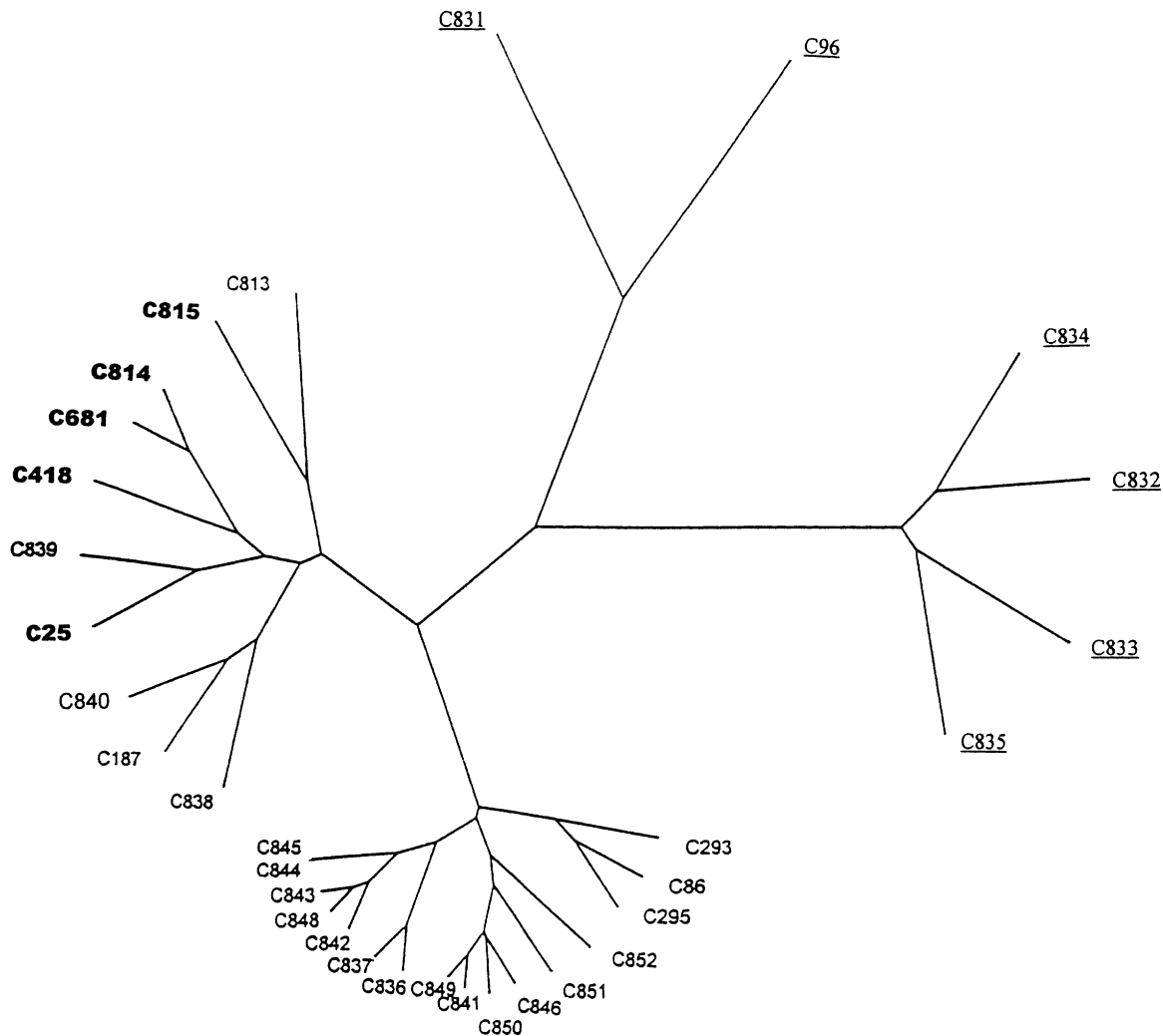


FIG. 19. UPGMA cluster analysis of mtDNA RFLP data. Isolates are designated as morpho-species: *L. pyrinum* (underline), *O. clavigerum* (plain), and *L. terebrantis* (bold).

presented in FIGS. 17 and 18, respectively. The UPGMA dendrogram developed from combined datasets for *Hae*III and *Msp*I restrictions resolved three major clusters (FIG. 19). The *L. pyrinum* isolates formed one cluster, while a second cluster included all *O. clavigerum* (DJ) isolates, as well as some isolates of *O. clavigerum* (DP). The third cluster included all isolates of *L. terebrantis*, as well as several isolates of *O. clavigerum* (DP).

When *Hae*III and *Msp*I restrictions were analyzed separately, isolates clustered into the same three groups, with the single exception of isolate C813. This isolate grouped with the main *O. clavigerum* cluster in the *Hae*III dendrogram, but with *L. terebrantis* in the *Msp*I dendrogram.

nDNA fingerprinting.—Fewer phenotypes were expressed in the nDNA fingerprints compared with the number of phenotypes observed with mtDNA RFLPs. The nDNA fingerprint of the four groups of fungi

probed with the $(CAC)_5$ oligonucleotide is shown in FIG. 20.

The UPGMA dendrogram developed from $(CAC)_5$ fingerprints resolved four clusters (FIG. 21). In this case, however, *L. pyrinum* and all isolates of *O. clavigerum* except C813 clearly were delineated in separate clusters. In this dendrogram, isolate C813 again resolved with *L. terebrantis*. C25, an isolate of *L. terebrantis*, resolved with *O. clavigerum*. Other *L. terebrantis* isolates grouped into two clusters separate from *L. pyrinum* and *O. clavigerum*.

DISCUSSION

Based on morphology, ecology, mtDNA RFLPs and nDNA fingerprints, *L. pyrinum*, *L. terebrantis*, and *O. clavigerum* appear to be very closely related and might have diverged only recently from a common ancestor. There was no evidence of cryptic species or

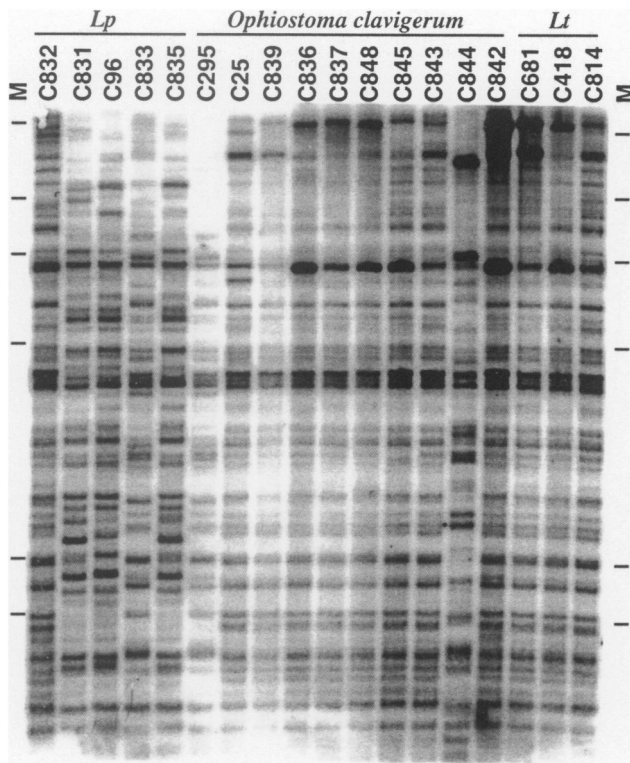


FIG. 20. DNA fingerprint patterns detected by $(CAC)_5$ in *L. pyrinum*, *O. clavigerum* (isolated from *D. ponderosae*), *O. clavigerum* (isolated from *D. jeffreyi*), and *L. terebrantis*.

physiologic race formation among isolates of *O. clavigerum* associated with *D. ponderosae* and *D. jeffreyi*.

The mtDNA RFLPs and nDNA fingerprints provided additional evidence that *L. pyrinum* is a good species and not a morphologic variant of *O. clavigerum*. However, evidence for the separation of *L. terebrantis* and *O. clavigerum* was not so strong. Most isolates of *L. terebrantis* and *O. clavigerum* clustered in agreement with the morphology consistent for their respective species in the dendrograms produced from mtDNA RFLPs and nDNA fingerprints. In each case, however, inconsistencies were seen. A possible explanation for these inconsistencies is that the two fungi actually comprise a single species that exhibits morphological variation linked to its ecology (mycangial versus nonmycangial). On the other hand, the two fungi might be distinct species that recently have diverged but, because of lineage sorting, are not clearly separable using these genetic markers.

Leptographium terebrantis is loosely associated with bark beetle species in several genera (Harrington 1988), while *O. clavigerum* has been found only with *D. ponderosae* (Whitney and Farris 1970) and the closely related *D. jeffreyi* (Six and Paine 1997) and *L. pyrinum* only with *D. adjunctus* (Six and Paine 1996). Furthermore, the unusually long septate and clavate-

shaped conidia and large spreading conidiophores of *O. clavigerum* might be derived characters related to ambrosial feeding by the associated beetles. Young adults are known to feed on dense sporogenous fungal growth in pupal chambers for several weeks before emergence from the natal host tree. In addition to the large conidia and spreading conidiophores, *O. clavigerum* produces smaller and less elaborately branched conidiophores and smaller conidia (Tsuneda and Hiratsuka 1984) that are morphologically very similar to those produced by *L. terebrantis*. Thus, while *O. clavigerum* may have retained the conidium and conidiophore morphology of *L. terebrantis*, it also produces a conidium and conidiophore type especially suitable for ambrosial feeding by young adult beetles. We can speculate that *L. terebrantis* might be the more primitive of the three species and that *O. clavigerum* and *L. pyrinum* might well have diverged from *L. terebrantis* when they developed close mycangial associations with specific bark beetles.

Microscopic examination of the conidia and conidiophores of the isolates studied for genetic markers showed three morphological groups that generally showed agreement with the clustering based on nDNA fingerprinting. That is those isolates that produced short, truncate conidia were in the *L. pyrinum* cluster; those that produced long, clavate conidia, often with one or two septations, tended to group in the *O. clavigerum* cluster. Further, the broad, spreading fascicles of conidiophores typical of *O. clavigerum* were seen in isolates of the *O. clavigerum* cluster but not in isolates in the *L. terebrantis* cluster. The two notable exceptions are isolates C25 and C813. The former isolate originally was identified as *L. terebrantis* in Harrington and Cobb (1983) but was found to produce clavate conidia in a later study (Zambino and Harrington 1992), in which it was referred to as *O. clavigerum*. Our more recent examinations of this strain revealed no clavate conidia, but the broad, spreading conidiophores typical of *O. clavigerum* were seen. The insect associate of C25 is not known, but C25 was isolated from *Pinus contorta*, which is a host of *L. terebrantis* and *O. clavigerum* and of the bark beetles, *D. valens* and *D. ponderosae*, that vector these fungi. Placement of C25 in the *O. clavigerum* cluster based on nDNA fingerprinting would suggest that C25 is *O. clavigerum*, but the culture has deteriorated and no longer produced the clavate conidial state, a deterioration that has been noted in other cultures of *O. clavigerum* (Tsuneda and Hiratsuka 1984). The other exceptional isolate is C813, which continues to produce large (55–65 μm), clavate conidia and broad, spreading conidiophores. Thus, C813 is morphologically *O. clavigerum*, though it was isolated from *D. valens* (rather than *D. ponderosae*)

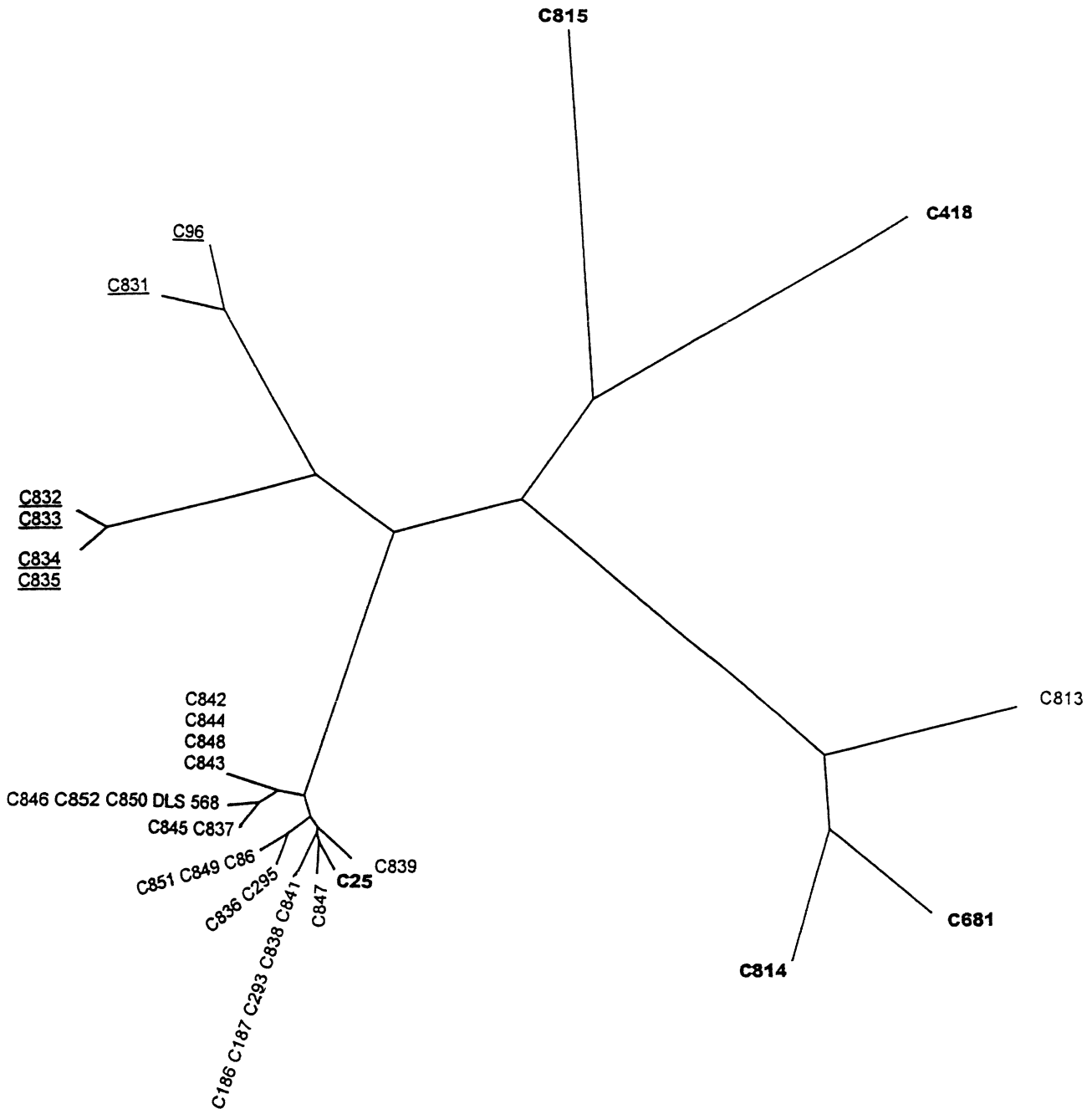


FIG. 21. UPGMA cluster analysis of DNA fingerprint data. Isolates are designated as morpho-species: *L. pyrinum* (underline), *O. clavigerum* (plain), and *L. terebrantis* (bold).

and it clusters with *L. terebrantis* based on nDNA fingerprints. Isolate C813 might be an intermediate in an ongoing process of speciation. It also might be that these two species are not genetically isolated, which also might be suggested by the mtDNA data.

A possible explanation for the resolution of five *O. clavigerum* (DP) associates in the *L. terebrantis* cluster in the dendrogram developed from the mtDNA RFLPs is that after the divergence of *L. terebrantis* and *O. clavigerum*, there has been horizontal transfer of mtDNA, but not nDNA, between the two species.

The horizontal transfer of mtDNA in fungi remains poorly understood, and the mechanisms involved are unknown. The occurrence of such transfers of mtDNA has been suggested in other fungi infecting trees (Brasier et al 1993, Harrington et al 1998). It is interesting to note that no isolates of *O. clavigerum* (DJ) showed evidence of possessing mtDNA polymorphisms typical of *L. terebrantis*. This might be due to a lack of contact between *O. clavigerum* (DJ) and *L. terebrantis* in *P. jeffreyi*. *D. valens*, a vector of *L. terebrantis*, can be found commonly in pines colo-

nized by *D. ponderosae* and *O. clavigerum* (DP), and *L. terebrantis* might interact in such trees. *D. valens* also attacks the bases of *P. jeffreyi* attacked by *D. jeffreyi*; however, it is not known how well *L. terebrantis* is able to colonize tissues of this tree. *P. jeffreyi* is quite different chemically than other pines and limits the growth of some ophiostomatoid fungi (Paine and Hanlon 1994, Six and Paine 1998). If growth of *L. terebrantis* is poor in *P. jeffreyi*, *Ophiostoma clavigerum* (DJ) might interface only rarely, or not at all, with this fungus.

On the other hand, the patterns we observed might suggest incomplete lineage sorting accompanied by limited morphological divergence, which can indicate recent or incomplete speciation events (Flowers and Folz 2001). However, this explanation still does not clarify the inconsistencies found using the nDNA probe (CAC)₅.

About half of the described *Leptographium* species have *Ophiostoma* teleomorphs, but the others, including *L. pyrinum* and *L. terebrantis*, are known only by their anamorphs (Harrington 1988, Jacobs and Wingfield 2001) and may be strictly asexual. The mode of reproduction (asexual or sexual) should not affect variation or polymorphism of mtDNA. However, variation and polymorphism of nDNA is predicted to be lower in asexual species than in sexual species. In this study, *Leptographium* species possessed as high or higher nuclear genetic variation than did the sexually reproducing *Ophiostoma clavigerum*. *Ophiostoma clavigerum* possessed the lowest genetic variation and was the least polymorphic of all fungi studied. These results concur with results of a study assaying genetic variation in *O. clavigerum* (DJ) using isozymes, which revealed little genetic variation and polymorphism in this fungus (Six and Paine 1999b).

The low genetic variability in *O. clavigerum* relative to the asexual *Leptographium* species may indicate that sexual reproduction in this species is uncommon. We have not observed the sexual state of *O. clavigerum* in nature, nor have we been able to produce it in artificial culture despite numerous pairings of isolates in the laboratory (Six and Paine 1997, T. C. Harrington unpubl, D. L. Six unpubl). The only known observations of ascomata for *O. clavigerum* were reported in a pair of related studies by Robinson (1962) and Robinson-Jeffrey and Davidson (1968) in which the authors observed neckless perithecia (unusual for *Ophiostoma* species) in sapwood and, more rarely, in culture.

The genus *Graphiocladiella* was erected for *Leptographium*-type species with individual conidiophores to those clustered into a synnema-like group (Upadhyay 1981). However, in all other respects these fungi appear to be *Leptographium* species (Harrington et

al 2001), and *O. clavigerum* is clearly closely related to other *Leptographium* species. Thus we propose to transfer this anamorph to *Leptographium*.

Ophiostoma clavigerum (Robinson-Jeff. & Davids.)
Harrington, Mycotaxon 28: 41. 1987.

Anamorph. *Leptographium clavigerum* (Upad.)
Harrington, Six et McNew, comb. nov. ≡ *Graphiocladiella clavigerum* Upad. Monogr. *Ceratocystis* and *Ceratocystiopsis*, p. 138, 1981.

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