Mycologia, 84(1), 1992, pp. 12–25.

CORRESPONDENCE OF ISOZYME CHARACTERIZATION WITH MORPHOLOGY IN THE ASEXUAL GENUS *LEPTOGRAPHIUM* AND TAXONOMIC IMPLICATIONS

PAUL J. ZAMBINO[1] AND T. C. HARRINGTON[2]

Department of Plant Biology, University of New Hampshire, Durham, New Hampshire 03824

ABSTRACT

The similarity of 88 isolates of 27 species of *Leptographium* was studied using enzyme electrophoresis. UGPMA cluster analysis of similarity matrices (Nei genetic identity, I) generated from data of 267 electrophoretic forms (electromorphs) of 15 enzymes showed a close correspondence between electrophoretic similarity and morphology. Isolates of a species had high similarity and clustered at $I \geq 0.60$, and in cases where different taxa clustered at $I > 0.60$, an examination of the morphology and history of the isolates suggested conspecificity. Isozyme characterization was also useful for identifying the extent of morphologic variation within versus between species and for comparing isolates with degenerate morphology after prolonged culture.

Key Words: *Ophiostoma, Ceratocystis,* Ascomycotina, taxonomy, stain fungi

Delimitation of species in anamorph genera remains a difficult problem. Without a sexual state, mating compatibility among strains cannot be determined, and the extent of acceptable morphologic variation within a species is ill-defined for most asexual genera. A number of genetic and phenotypic characters have been used to aid morphologic studies in delimiting species. As a source of non-morphologic characters, enzyme electrophoresis has been among the most widely used of available techniques (**3**, **4**, **13**, **25**, **26**, **30**), although its application to anamorph taxonomy has been somewhat limited (**8**, **39**). Herein, we examine the electrophoretic relatedness of species of *Leptographium* Lagerb. & Melin (= *Verticicladiella* Hughes), compare the morphology of the species, and evaluate the usefulness of electrophoresis in solving taxonomic problems.

Leptographium is a form genus that includes anamorphs of some species of *Ophiostoma* H. & P. Sydow (**18**). In *Leptographium,* the conidiophore stipe is pigmented and mononematous, the conidiogenous apparatus is penicilliately branched, and hyaline conidia are produced holoblastically by either sympodial or annellidic proliferation of conidiogenous cells (**18**). Conidia accumulate at the conidiophore apices in sticky droplets that facilitate dissemination by subcortical arthropods, such as bark beetles and their associated microfauna (**18**). Most species of *Leptographium* are lignicolous. Many species are agents of blue stain, and several species have been suggested to be at least weak root pathogens (**1**, **46**). The most pathogenic species, *L. wageneri* (Kendrick) Wingfield, causes black-stain root disease, a unique and destructive vascular wilt disease of conifers in the western United States and British Columbia, Canada (**6**).

For many species of *Leptographium,* morphologic descriptions are vague or poorly illustrated and type material inadequate or lacking (**18**). Cultures obtained from type material or other voucher specimens can be used to represent these taxa in contemporary comparisons among isolates, but comparisons based on such cultures are subject to error due to changes in culture morphology that may occur over time. The loss of the teleomorphic state is common in cultures of *Ophiostoma* (**12**, **42**), and anamorphs or one or more synanamorphs may also be lost when isolates are grown in continuous culture. For example, studies of the species *O. clavigerum* (Robins.-Jeffr. & Davids.) Harrington (**42**) and *O. araucariae* (Butin) de Hoog & Scheffer (**12**) indicated that fresh isolates of each species can initially produce five different imperfect states with morphologic complexity ranging from synnematal forms, to *Leptographium*-like conidiophores, to yeast-like forms. Cultures rapidly lost the ability

[1] Present address: U.S.D.A.-A.R.S. Cereal Rust Laboratory, 1551 Lindig St., University of Minnesota, St. Paul, Minnesota 55108.

[2] Present address: Department of Plant Pathology, 351 Bessey Hall, Iowa State University, Ames, Iowa 50011.

to produce synnemata, and the yeast-like budding form eventually predominated in some subcultures.

MATERIALS AND METHODS

Eighty-eight isolates representing 26 species of *Leptographium* were selected from the culture collection of the second author (TABLE I). Most of the species occur on conifers, principally the Pinaceae. The isolates represent about half of the described species of *Leptographium* (**18**), as well as unidentified and probably undescribed species. Where a number of isolates of a species was available, isolates were selected to represent the greatest possible geographic range.

Morphologic comparisons were made to confirm species identification. Isolates were grown on water agar continuing sterile sections of twigs of *Pinus resinosa* Ait. with bark removed and on malt extract agar (MEA; 1% malt extract and 1.5% agar). Cultures of *L. wageneri* were incubated at 18 C; cultures of other species were grown at room temperature. Cultures on twig medium were used for comparisons of conidiophores, conidia, and when present, ascocarps and ascospores. Cultures grown on MEA were used to compare growth rates, characteristics of the mycelia, and in some cases, conidiophore morphology.

To obtain fresh mycelium for enzyme extraction, plugs of mycelium grown on MEA were transferred to 30 ml of liquid medium (20 mg malt extract plus 1 mg yeast extract per ml) in 125 ml Erlenmyer flasks and incubated at 18 C or room temperature. The buffers and protocols by which enzymes were extracted from the mycelia of 14-day-old cultures were previously reported (**50**).

Preparation of 12% starch gels followed the method of Marty *et al.* (**23**). Gels were poured into gel forms of the design of Cardy *et al.* (**5**). Buffers, electrical requirements, and the amount of time required for electrophoretic separation of bands in each buffer system are listed in TABLE II.

Isolate NMD-2 of *L. wageneri* and isolate C297 of *L. serpens* (Goid.) Siem. were selected as reference isolates for each gel to test the evenness of electrophoretic migration and to calculate the relative mobility of each electrophoretic band. Samples of the two reference isolates were placed adjacent to each other at three locations on each gel. Up to 34 sample wicks were loaded per gel.

Following electrophoresis, horizontal slices of the starch gels were stained for enzyme activity (TABLE II). The distance between the electrophoretic origin and the electrophoretic bands was measured for each sample. Relative mobility (Rf) values were calculated as the ratio of the distances travelled by bands of the sample *vs* reference isolates and were used to determine the number of electrophoretically distinguishable forms (electromorphs) of each enzyme. Rf values from all buffer systems that gave well-resolved bands were used when determining electromorphs of an enzyme.

In earlier and preliminary studies of *L. wageneri* (**50**) and other species of *Leptographium* and *Ophiostoma* (unpubl. data), data from two or more independent extractions per isolate had been compared. Since banding patterns were consistant in different extractions, most data in this study are from a single extraction per isolate. Additional extractions were made when an isolate's banding pattern for an enzyme was weak on one or more gels. If an isolate continued to give poorly-resolved bands, the enzyme was not used for analysis.

Secondary isozyme patterns were used in some enzymes (e.g., IDH1, PGM1, and TPI1) for differentiating electromorphs of similar Rf values. Secondary isozymes or "shadow bands" may result when the electrophoretic mobility of a portion of the enzyme molecules is affected by changes in enzyme conformation, by binding to substrate or cofactor molecules, or by deamination. As the banding patterns that result from secondary isozymes are often highly characteristic for an allele (**20**), electromorphs of similar Rf value were considered distinct if secondary bands were apparent in different buffers and/or if banding patterns had differences in spacing among secondary bands.

Estimates were made of the putative number of genetic loci coding for each enzyme as required for calculation of genetic identity (**28**). Most enzymes had one set of bands, with or without shadow bands, and were considered the product of one genetic locus. If there were two sets of bands in different zones of migration in the gel, and variation in one set of bands was independent of the variation in the second set of bands (as occurred in malate dehydrogenase and NADH diaphorase), the bands of these enzymes were considered the products of two genetic loci. These estimates were checked against interpretations of genetic loci coding for enzymes in the related fungus *Ophiostoma nigrocarpum* (Davids.) deHoog (Zambino, unpubl.) and from studies of various unrelated fungi where the genetic basis of isozyme variation is known (**4**, **13**, **24**, **34**, **36**). Different electromorphs were assumed to represent the products of different alleles.

The routines SIMDIS and CLUSTER of the program BIOSYS-1 of Swofford and Selander (**40**, **41**) were used to calculate similarity matrices of Nei (**28**) genetic identity I (a measure of genetic relatedness) and to generate dendrograms reflecting similarity relationships and clustering of taxonomic units by the UGPMA method (unweighted group-pair method with arithmetic mean), respectively. A two step, hierarchical analysis after Swofford and Selander (**41**) was used to obtain the dendrograms presented in this study. In the first step, "electrophoretic types," i.e., groups of isolates that had identical electromorphs for each enzyme, were the taxonomic units analyzed in the calculation of a similarity matrix and dendrogram. In the second step, allelic frequencies from the electrophoretic types that comprised species were averaged before calculation of a similarity matrix and dendrogram. Species were groups of morphologically similar and identifiable isolates; in one cluster with morphologic plasticity in the anamorph, the "species" was arbitrarily defined as consisting of electrophoretic types related at $I > 0.60$. In the dendrogram presented in this study, clustering and branch lengths at $I > 0.60$ were obtained from the first step of the analysis, based on electrophoretic types; those at $I < 0.60$ were from the second analysis, based on morphologically similar isolates (species).

TABLE I

SPECIES AND ISOLATES OF *LEPTOGRAPHIUM* AND *OPHIOSTOMA* STUDIED USING ENZYME ELECTROPHORESIS

Species	Isolate numbers[a]	Geographic origin	Isolated from
L. abietinum	C10 (DAOM 37981A)	British Columbia	*Picea engelmanii*
	C11	Idaho	*Pseudotsuga menziesii*
	C18 (ATCC 58568, ORF-T)	Washington	*Abies grandis*
	C42	California	*Pinus ponderosa*
	C54 (ATCC 58567, NMA-103)	New Mexico	*Pinus ponderosa*
	C172 (Gregory 1211)	Scotland	*Picea sitchensis*
	C272	New York	*Picea rubens*
	C273	New York	*Picea rubens*
L. engelmannii	C29 (RWD 971, CO 456)	Colorado	*Picea engelmannii*
L. lundbergii	C23 (NFRI 69–168)	Norway	*Pinus sylvestris*
L. procerum	C17 (NFRI 59–84/2 as *L. phycomyces*)	Norway	*Pinus sylvestris*
	C83 (ATCC 58570, IDD-102)	Idaho	*Pseudotsuga menziesii*
	C124 (NFRI 80-53/7 as *Ceratocystis polonica*)	Norway	*Picea abies*
	C323 (CBS 145.41 as *Phialocephala phycomyces*)	unknown	wood pulp
L. pyrinum	C96 (CO 463)	unknown	unknown
L. serpens	C30 (CBS 141.36, from holotype)	Italy	unknown
	C56 (PREM 45442)	Rep. South Africa	*Pinus pinaster*
	C79 (ATCC 34322)	Italy	*Pinus pinea*
	C141 (CMW 90)	Rep. South Africa	*Pinus taeda*
	C153 (Horner VPI-173)	Mississippi	*Pinus taeda*
	C169 (Horner VPI-251)	Virginia	*Pinus strobus*
	C175 (Horner VPI-256)	Virginia	*Pinus strobus*
	C297 (ATCC 42810, from *V. alacris* holotype)	Rep. South Africa	*Pinus pinaster*
	C304 (CMW 304)	Rep. South Africa	*Orthomicus erosus*
	C305 (CMW 745)	Spain	*Pinus pinaster*
	C306 (CMW 310)	Rep. South Africa	*Pinus radiata*
	C307 (CMW 382)	Rep. South Africa	*Hylastes angustatus*
L. terebrantis	C63	Massachusetts	*Pinus resinosa*
L. truncatum	C8 (PREM 45699, from paratype)	New Zealand	*Pinus strobus*
	C59 (ATCC 22735 as *L. lundbergii*)	unknown	*Pinus sylvestris*
	C167 (Juzwik 8412Lr047)	Ontario	*Pinus resinosa*
L. wageneri			
var. *wageneri*	CAS-4 (ATCC 64194)	California	*Pinus monophylla*
	CAS-15 (ATCC 64195)	California	*Pinus monophylla*
var. *ponderosum*	BCL-3	British Columbia	*Pinus contorta*
	BCL-4	British Columbia	*Pinus contorta*
	CAP-36	California	*Pinus ponderosa*
	ORP-1	Oregon	*Pinus ponderosa*
var. *pseudotsugae*	BCH-1 (ATCC 42953)	British Columbia	*Tsuga heterophylla*
	CAD-55	California	*Pseudotsuga menziesii*
	COD-2 (ATCC 64191)	Colorado	*Pseudotsuga menziesii*
	IDD-2 (James 80-1)	Idaho	*Pseudotsuga menziesii*
	MOD-1 (ATCC 58578)	Montana	*Pseudotsuga menziesii*
	NMD-1	New Mexico	*Pseudotsuga menziesii*
	NMD-2 (Mielke 800509)	New Mexico	*Pseudotsuga menziesii*
	ORD-Q	Oregon	*Pseudotsuga menziesii*
Leptographium sp. A	C19	Idaho	*Pseudotsuga menziesii*
	C39	Idaho	*Pseudotsuga menziesii*
Leptographium sp. E	C32	New Mexico	*Pinus ponderosa*
	C41	New Mexico	*Pinus ponderosa*
	C46 (ATCC 58571, NMP-106)	New Mexico	*Pinus ponderosa*
Leptographium sp. F	C6	California	*Pseudotsuga menziesii*
	C21 (ORD-O)	Oregon	*Pseudotsuga menziesii*
	C33 (UI 791010 as *L. abietinum*)	Idaho	*Pseudotsuga menziesii*

TABLE I
CONTINUED

Species	Isolate numbers[a]	Geographic origin	Isolated from
	C36 (Mielke 800514)	New Mexico	*Pseudotsuga menziesii*
	C40 (ATCC 58572, COD-101)	Colorado	*Pseudotsuga menziesii*
	C47	California	*Pseudotsuga menziesii*
Leptographium sp. H	C22 (ATCC 58573, IDD-101)	Idaho	*Pseudotsuga menziesii*
Leptographium sp. I	C154 (CMW 41)	Virginia	*Pinus strobus*
	C155 (CO 83-74)	Colorado	*Pinus edulis*
	C156 (CO 83-97)	Colorado	*Pinus edulis*
	C157 (CO 83-96)	Colorado	*Pinus edulis*
	C182	North Carolina	*Pinus strobus*
	C183	North Carolina	*Pinus strobus*
	C184	North Carolina	*Pinus strobus*
Leptographium sp. J	C289 (NFRC C840 as *Ophiostoma huntii*)	Alberta	*Pinus contorta*
Leptographium sp. K	C173 (J. Hoffman "A")	Idaho	*Pinus edulis*
	C174 (J. Hoffman "B")	Idaho	*Pinus edulis*
Leptographium sp. L	C15 (ATCC 58566 as *L. terebrantis*, IDL-101)	Idaho	*Pinus contorta*
O. abiocarpum	C135 (RWD 494 from paratype)	unknown	*Picea engelmannii*
O. adjuncti	C119 (ATCC 34942 from holotype)	unknown	*Pinus ponderosa*
O. aureum	C88 (ATCC 16936 from holotype)	British Columbia	*Pinus contorta*
O. clavigerum	C25 (ATCC 58565 as *L. terebrantis*, BCL-101)	British Columbia	*Pinus contorta*
	C86 (CO 453)	Wyoming	*Pinus contorta*
	C187 [D. Owen 84EC (B)]	California	*Pinus ponderosa*
	C291 (NFRC C837)	Alberta	*Pinus contorta*
	C295 (NFRC C1215)	British Columbia	*Pinus contorta*
O. crassivaginatum	C95 (CO 498)	unknown	*Populus tremuloides*
O. europhioides	C129	Idaho	*Pseudotsuga menziesii*
	C274	New York	*Picea rubens*
	C290 (ATCC 16059)	Ontario	*Picea mariana*
O. huntii	C12	California	*Pinus ponderosa*
	C113 (CO 468, RWD 776)	unknown	unknown
	C139	California	*Pinus ponderosa*
O. penicillatum	C5 (NFRI 1731/3)	Norway	*Picea abies*
	C7 (NFRI 1716/2)	Norway	*Picea abies*
O. robustum	C109 (CO 452)	unknown	unknown
Ophiostoma sp. M	C158	New Hampshire	*Picea rubens*
	C160	New Hampshire	*Picea rubens*

[a] Isolate numbers are those used in the collection of T. C. Harrington. Abbreviations in parentheses indicate alternate isolate numbers found in culture collections as follows: American Type Culture Collection (ATCC); Centraalbureau voor Schimmelcultures, Baarn, Netherlands (CBS); Plant Research Institute, Dept. of Agriculture, Mycology, Ottawa, Canada (DAOM); Norwegian Forest Research Institute, As, Norway (NFRI); Northern Forestry Research Centre, Edmonton, Canada (NFRC); Plant Protection Research Institute, Pretoria, South Africa (PREM); and the collections of R. W. Davidson (RWD), T. E. Hinds, U.S. Forest Service, Rocky Mountain Forest and Range Experiment Station, Fort Collins, Colorado (CO), A. D. Partridge, University of Idaho, Moscow (UI), and M. J. Wingfield, University of the Orange Free State, Bloemfontein, Republic of South Africa (CMW).

RESULTS

Fifteen putative genetic loci coding for the enzymes listed in TABLE II were used for the analysis. Isolates had also been screened for differences in β-glucosidase (EC 3.2.1.21) and esterase (EC 3.1.1.1) using the fluorescent stain methods of Marty *et al.* (**23**), but these enzymes and the less anodal (slower migrating) form of NADH diaphorase (DIA2) were not used in the analysis due to one or more of the following reasons: poor resolution in some isolates, complex, multibanded patterns, unequal number of bands, and changes in banding patterns when tests were replicated.

TABLE II

ENZYMES USED IN STARCH GEL ELECTROPHORESIS OF *LEPTOGRAPHIUM* SPP., THE NUMBER OF ELECTROMORPHS DETERMINED PER ENZYME, AND BUFFERS AND STAINING PROCEDURES FAVORING RESOLUTION

Enzyme (EC number)[a]	Enzyme abbreviation[b]	Number of electromorphs	Buffer systems[c]	Stain reference[d]
Aconitase (4.2.1.3)	ACO1	20	A, D, HC7	1
Aspartate aminotransferase (2.6.1.1)	AAT1	15	B2, D	1
NADH diaphorase (1.8.1.4)	DIA1	10	A, D	1
Fumarase (4.2.1.2)	FUM1	12	A	1
Glucose-6-phosphate dehydrogenase (1.1.1.49)	G6PD1	10	B2	1
Glucosephosphate isomerase (5.3.1.9)	GPI1	24	B, D, E	1
Glutamate dehydrogenase (NADP) (1.4.1.3)	GDH1	12	B2	1
Isocitrate dehydrogenase (1.1.1.42)	IDH1	22	D, E	1
Leucine aminopeptidase (3.4.11.1)	LAP1	23	M	2
Malate dehydrogenase (1.1.1.37)	MDH1	18	D, E	1
	MDH2	20	D, E	1
Mannitol dehydrogenase (1.1.1.67)	MAN1	29	A, D, E, HC7	3
Phosphoglucomutase (5.4.2.2)	PGM1	19	A, E, HC7, M	1
Superoxide dismutase (1.15.1.1)	SOD1	8	A, B2, E, HC7	4
Triose-phosphate isomerase (5.3.1.1)	TPI1	26	A, HC7	1

[a] Nomenclature Committee of the International Union of Biochemistry (**29**).

[b] Multiple enzyme forms are designated in order of decreasing anodal migration.

[c] Buffer systems, electrical requirements, and references: A: pH 8.5/8.1 discontinuous TRIS citrate/lithium borate system (RW) using 50 mA constant current until wave front reaches 8 cm; Marty *et al.* (**23**). B: pH 5.7 continuous histidine citrate system using 250 V constant voltage for 4.5 h; Shields *et al.* (**37**). B2: pH 8.8/8.0 discontinuous TRIS citrate/sodium borate system (B) using 50 mA constant current until wave front reaches 8 cm; Conkle *et al.* (**7**). D: pH 6.1 continuous morpholine citrate system using 250 V constant voltage for 5.0 h; Conkle *et al.* (**7**). E: buffer D with pH adjusted to 8.1 using morpholine citrate, with same voltage and run time as D. HC7: pH 7.0/7.0 histidine/citrate system (HC) using 250 V constant voltage for 5.0 h; Marty *et al.* (**23**). M: pH 8.9 continuous TRIS borate EDTA system using 275 V constant voltage for 4.5 h; Micales *et al.* (**25**).

[d] 1, Marty *et al.* (**23**); 2, Conkle *et al.* (**7**); 3, Micales *et al.* (**25**); 4, Vallejos (**45**).

The number of electromorphs for the selected enzyme loci ranged from 8 to 29 (TABLE II). For the less variable enzymes, species or groups of several related species were often found to be monomorphic, i.e., to have the same electromorph in all isolates (TABLE III). In contrast, each electromorph of the most variable enzymes was found in only one species, and many species were polymorphic at these enzyme loci.

Cophenetic correlation (the correlation between values of relatedness in a similarity matrix *versus* a dendrogram) was 0.951 for the dendrogram from the first step in the hierarchical analysis, in which electrophoretic types were the taxonomic units for comparison, and 0.824 for the dendrogram from the second step of the analysis, in which allelic averages from species were compared. A single difference in branching patterns was noted between the dendrograms of the two steps of the analysis: the branch containing *O.*

abiocarpum (Davids.) Harrington and *O. penicillatum* (Grosm.) Siem. clustered with *O. crassivaginatum* (Griffin) Harrington at a genetic identity of I = 0.13 in the analysis using electrophoretic types, but clustered at I = 0.08 with the branch containing *L. abietinum* (Peck) Wingfield in the analysis using species averages. Also, the value of I was up to 0.08 higher for some branches of the dendrogram when species were used as taxonomic units than when electrophoretic types were used.

Electrophoretic relatedness among isolates and species generally corresponded to their morphologic similarity. For each morphological species, there were from one to nine electrophoretic types that clustered at values of genetic identity of $I \geq 0.62$ (FIG. 1). Many pairs of species that had minor morphological differences clustered at values of I between 0.25 and 0.60. Species with the greatest differences in anamorph and teleomorph morphology clustered only at $I < 0.10$.

At the top of FIG. 1 is shown the electrophoretic relatedness among isolates denoted here as the "*L. serpens* cluster": *L. procerum* (Kendr.) Wingfield, *L. serpens,* three varieties of *L. wageneri,* and four groups of isolates listed as *Leptographium* species E, F, H, and I that have been recognized as distinct (**18**, **19**) but have not been described as taxa. All species of this cluster are from roots of conifers. In our cultures, all produced conidia that were held in slime droplets, masses of young conidia that were generally white, and conidiophores that arose individually from hyphae without branching of the conidiophore stipe, even on older conidiophores. These species could be considered asexual, although there have been unconfirmed reports of sexual states occurring in two species of this cluster, i.e., *Ophiostoma wageneri* (Goheen & Cobb) Harrington (**14**, **18**) and *Ophiostoma serpens* (Goid.) von Arx (**15**, **18**).

Three groups of isolates were evident within *L. wageneri* and corresponded to the three described, host-specialized varieties (**18**). The three groups were related at I = 0.66 and 0.72. An isolate (C22) designated *Leptographium* sp. H, isolated from roots of *Pseudotsuga menziesii* (Mirb.) Franco., was closely related to *L. wageneri* (I = 0.53). *Leptographium* sp. H is weakly pathogenic to conifers (**19**) but does not cause the symptoms of black stain root disease caused by *L. wageneri.* Although conidiophores of the two species were similar, the isolate of *Leptographium* sp. H had a growth rate much slower than that of *L. wageneri.*

In addition to *L. wageneri* and *Leptographium* sp. H, there were seven other cases where clusters of isolates were similar at an intermediate level (I between 0.25 and 0.60). In each case, isolates of these clusters were morphologically distinguishable but similar. *Leptographium* sp. E [a fungus found in roots of *Pinus ponderosa* Laws. (**19**)] and *Leptographium* F [a fungus frequently isolated from roots of *Pseudotsuga menziesii* and an associate of the root-feeding bark beetle *Hylastes nigrinus* Mannerheim (**19**)] clustered with each other at I = 0.46 and were related to *L. wageneri* and *Leptographium* sp. H at I = 0.34. Species E and F are reported to be weak pathogens (**19**).

Isolates of *L. procerum* from the United States and Norway were uniform for the tested enzymes and clustered with the aforementioned species at I = 0.20. This species has a wide range of reported coniferous hosts and has been reported as a root pathogen of pines (**1**).

Isolates of *L. serpens,* another reported root pathogen of pines (**46**), were of three electrophoretic types. One electrophoretic type consisted of two isolates from Italy, including isolate C79 from the holotype. A second type consisted of a mixture of isolates from the southern United States and the Republic of South Africa, including isolate C297 from the holotype of *Verticicladiella alacris* Wingfield & Marasas [later synonymized with *L. serpens* by Wingfield and Marasas (**47**)]. The third type consisted of isolate C141 from South Africa and isolate C305 from Spain. The three electrophoretic types clustered at $I \geq 0.80$.

Isolate C289, listed as *Leptographium* sp. J, was originally identified as *O. huntii* (Robins.-Jeffr.) deHoog & Scheffer, but we found it to lack the serpentine hyphae typical of *O. huntii* (**33**), and conidia were more rounded than those of the three examined isolates of the latter species. No ascocarps were produced in our cultures. In electrophoretic comparisons, this fungus was somewhat related to *L. serpens* but was distinct from *O. huntii.*

A second cluster, of 14 species, included seven species with *Ophiostoma* teleomorphs and seven species that apparently lack a sexual state, including the type species for the genus *Leptographium, L. lundbergii* Lagerb. & Melin. This cluster of species, denoted here as the "*L. lundbergii* cluster," was heterogeneous in morphol-

TABLE III

ENZYME ELECTROMORPHS OF SPECIES AND ISOLATES OF *LEPTOGRAPHIUM* AND *OPHIOSTOMA* DETERMINED USING STARCH GEL ELECTROPHORESIS

Species	Number of isolates tested	Enzymes					
		ACO1[a]	AAT1	DIA1	FUM1	G6PD1	GPI1
L. abietinum	7	J[b]	B, E	F	A, D	J	D, E, J
L. abietinum? (C172)	1	G	E	F	D	F	J
L. engelmannii	1	J	B	F	D	J	D
L. lundbergii	1	J	J	H	E	D	V
L. procerum	4	H	I	C	C	B	I
L. pyrinum	1	O	A	H	H	B	R
L. serpens	12	A	I	F	C, G	B	B, J
L. terebrantis	1	S	C	J	F	A	Q
L. truncatum	3	T	F	C, F	H	D	S
L. wageneri							
var. *wageneri*	2	F	L	C	F	B	K
var. *ponderosum*	4	H	M	C	F	B	K, P
var. *pseudotsugae*	8	H	L, M	C	F	B	K
Leptographium sp. A	2	M	C	H	G	C	X
Leptographium sp. E	3	D	D	C	G	B	B
Leptographium sp. F	6	C, E	D	C, G	G	B	H
Leptographium sp. H	1	H	O	C	G	B	K
Leptographium sp. I	7	L	F	C	B	D	C
Leptographium sp. J	1	K	F	F	F	E	M
Leptographium sp. K	2	M	F	C	B	D	A
Leptographium sp. L	1	I	E	H	K	K	T
O. abiocarpum	1	P	H	E	G	B	F
O. adjuncti	1	B	N	D	L	G	G
O. aureum	1	M	C	H	F	D	X
O. clavigerum	5	N, O	A	H, I, J	H	B	N, R
O. crassivaginatum	1	Q	G	A	J	H	W
O. europhioides	3	I	I	J	F	D	S
O. huntii	3	K	I	H	G	D	O
O. penicillatum	2	P	G	B	J	I	L
O. robustum	1	N	A	J	H	B	N
Ophiostoma sp. M	2	R	K	J	I	D	U

[a] Enzyme abbreviations from TABLE II.

[b] Electromorphs are designated alphabetically with electromorph A having the greatest anodal migration. In MAN1, electromorphs AA, BB, and CC have less anodal migration than Z.

ogy. Conidia produced by different species were held in slime droplets or as semi-dry masses that ranged in color from white, yellow, light tan, to grey. Conidiophore stipes varied from single to caespitose, and some species had branching stipes. Of the species that produce *Ophiostoma* teleomorphs, some form ascocarps that are ostiolate and necked, whereas others produce non-ostiolate ascocarps. Species were isolated from stems or roots of Pinaceae.

Isolates listed as *Leptographium* sp. I have conidiophores with morphology similar to those of *L. serpens,* but the two fungi are apparently of low relatedness. Harrington (**18**) has previously reported the width of the conidiogenous cells of Lackner and Alexander's (**21**) isolate C154 of species I to be narrower than in isolates of *L. serpens.* Additionally, in the current study, the highly serpentine hyphae in isolates of *L. serpens* was seen to differ from the slightly undulating but curved hyphae of species I. Lackner and Alexander (**21**) originally identified isolate C154 as *L. serpens* and noted its association with darkly stained roots of *Pinus strobus* L. Other isolates of *Leptographium* sp. I were from *Pinus strobus* and *Pinus edulis* Engelm. Isolates of *Leptographium* sp. K, which clustered with *Leptographium* sp. I at I = 0.43, were obtained from roots of *Pinus edulis.* Thus, all isolates of species I and K were from members of the white or "soft pine" group.

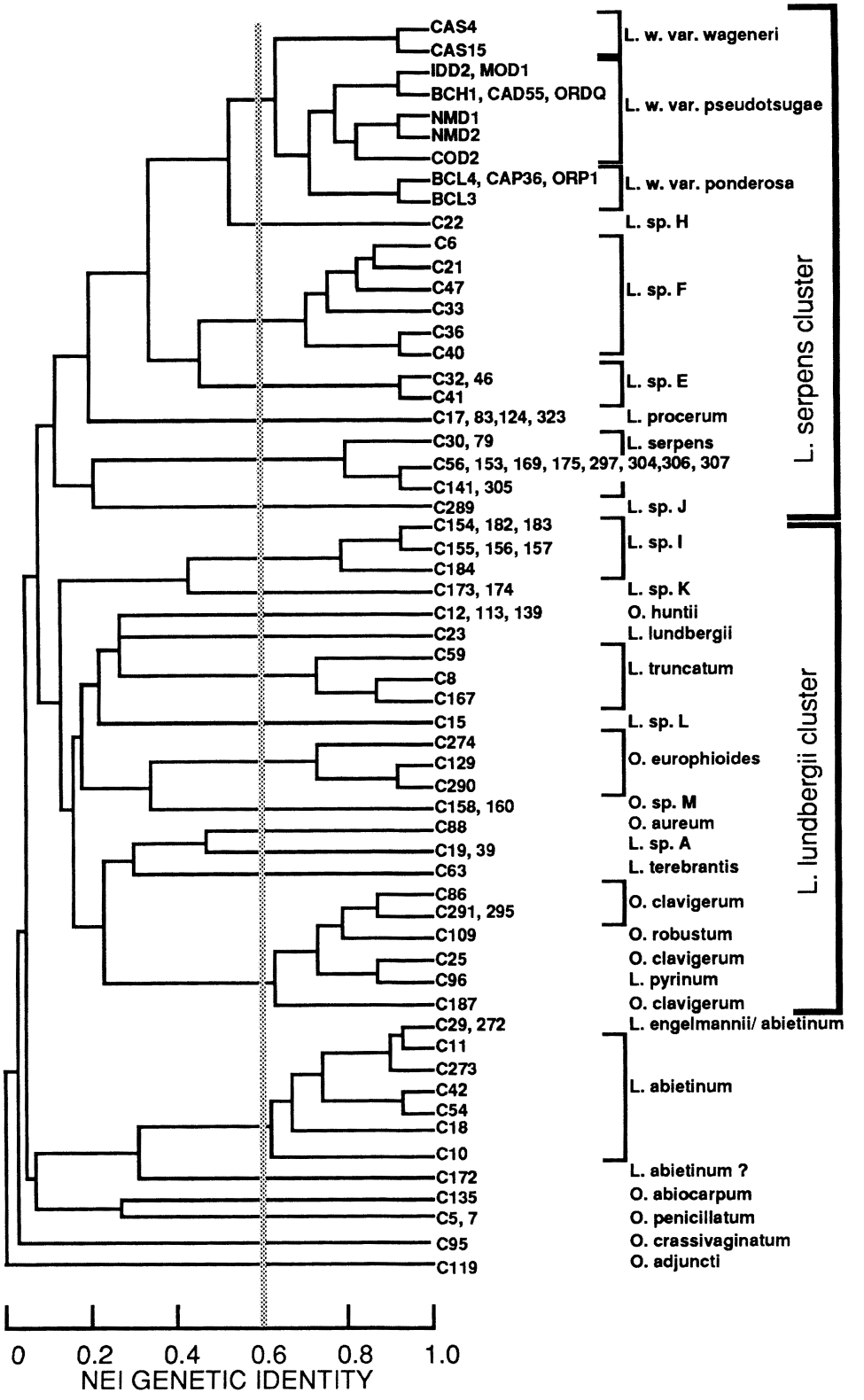
Ophiostoma huntii was described as an asso-

TABLE III
EXTENDED

Enzymes								
GDH1	IDH1	LAP1	MAN1	MDH1	MDH2	PGM1	SOD1	TPI1
D	K, L, R	C, F, M	K, U	G, O	C, H	I, N	B	I
D	V	S	N	N	K	Q	B	A
E	L	M	U	O	H	N	B	I
L	M	O	BB	Q	P	O	A	L
A	C	V	D	R	F	D	F	D
D	M	H	V	J	F	O	D	S
H	B	K	C	B	G	F, N	A	F, V, Y
B	P	E	T	J	P	K	A	T
G, H	E	I	P, S	A, M	F	O	A	G, L
D	S	R	G	F	O	B, E	F	M
D	G	R	G	F	M	E	G	H
D	G	R	G	F	M, O	B, E	F	H, O
B	M	G	J	J	N	O	A	T
D	A, B	W	B	O	F	E	A	F
D	A, B, G, Q	L, Q	B, C, E	F	A	A, C	E	F
D	G	P	F	F	O	C	A	U
E	N	D	O, R, X	E, S	Q, T	O	B	D
C	N	K	W	K	F	K	A	C
D	M	D	L	C	P	O	C	J
F	H	J	Q	A	F	O	A	X
I	D	K	I	J	D	R	B	B
G	T	U	H	I	R	H	H	E
B	M	H	S	J	J	P	B	W
D	M	H, O	V	J	F, N	M O	A, D	P, R, S
K	W	N	CC	H	E	G	A	K
I	G, H	B	Y	D, J	B, I, F	S	A	N, T
G	I	B	M	L	S	O	A	N
I	U	T	AA	P	D	J	B	D
D	M	H	V	J	F	O	A	P
J	J	A	Z	J	L	S	A	Q

ciate of *Dendroctonus monticola* Hopk. on bark and sapwood of *Pinus contorta* Dougl. (**33**). It produces ostiolate, necked ascocarps and hyphae that are highly serpentine. This fungus clustered with the asexual *L. lundbergii* and *L. truncatum* (Wingf. & Marasas) Wingf. at I = 0.27. *Leptographium lundbergii* is one of the common blue stain fungi on stems of *Picea* and *Pinus* species in Europe (**18, 22**), whereas *L. truncatum* has a wider distribution and is associated with dying roots (**18, 46, 48**). We observed hyphae to be moderately serpentine in *L. lundbergii* but only undulating to curved in *L. truncatum.* Conidia of the two species are similar, but basal flanges distinguish the conidia of *L. truncatum* from other species (**48**). Although the flanges were pronounced in conidia of isolate C8 (from a paratype specimen), they were difficult to discern in isolate C59. Both isolates C59 and C23 had been received as *L. lundbergii,* but culture morphology of C23 more closely matched the description of *L. lundbergii* by Lagerberg *et al.* (**22**).

Isolates C15, C25, and C63 had been chosen to represent variation within *L. terebrantis* Barras & Perry. After the electrophoretic analysis showed little similarity among the isolates, further morphologic comparisons were made among 21 isolates of *L. terebrantis* from the collection of T. C. Harrington. Isolate C63 and most of the other isolates were typical of Barras and Perry's (**2**) description of *L. terebrantis,* whereas isolate C25 was found to have conidiophores that fit within the range of morphologies observed in *O. clavigerum.* Isolate C15 differed in conidiophore and mycelial characteristics from the description of *L. terebrantis, O. penicillatum,* and all other


CAS4
CAS15
IDD2, MOD1
BCH1, CAD55, ORDQ
NMD1
NMD2
COD2
BCL4, CAP36, ORP1
BCL3
C22
C6
C21
C47
C33
C36
C40
C32, 46
C41
C17, 83,124, 323
C30, 79
C56, 153, 169, 175, 297, 304,306, 307
C141, 305
C289
C154, 182, 183
C155, 156, 157
C184
C173, 174
C12, 113, 139
C23
C59
C8
C167
C15
C274
C129
C290
C158, 160
C88
C19, 39
C63
C86
C291, 295
C109
C25
C96
C187
C29, 272
C11
C273
C42
C54
C18
C10
C172
C135
C5, 7
C95
C119
L. w. var. wageneri
L. w. var. pseudotsugae
L. w. var. ponderosa
L. sp. H
L. sp. F
L. sp. E
L. procerum
L. serpens
L. sp. J
L. sp. I
L. sp. K
O. huntii
L. lundbergii
L. truncatum
L. sp. L
O. europhioides
O. sp. M
O. aureum
L. sp. A
L. terebrantis
O. clavigerum
O. robustum
O. clavigerum
L. pyrinum
O. clavigerum
L. engelmannii/ abietinum
L. abietinum
L. abietinum ?
O. abiocarpum
O. penicillatum
O. crassivaginatum
O. adjuncti
L. serpens cluster
L. lundbergii cluster
0
0.2
0.4
0.6
0.8
1.0
NEI GENETIC IDENTITY

examined species. It was isolated from *Pinus contorta* and may be of the same taxon reported by Mielke (**27**) as *L. penicillatum* [see Harrington (**18**)]. Harrington and Cobb (**19**) reported the pathogenicity of C15 and C25 under the name *L. terebrantis.* Isolate C63 of *L. terebrantis* had conidia, conidiophores, and mycelia that differed greatly from *O. aureum,* with which it clustered at I = 0.30.

Isolates identified as *O. europhioides* (Wright & Cain) H. Solheim and as *Ophiostoma* sp. M were related at I = 0.34 and produced ostiolate perithecia and ascospores of similar dimensions. Clustered, branched conidiophores typical of the species (**49**) were abundant in isolates of *O. europhioides,* but the isolates of *Ophiostoma* sp. M lacked a *Leptographium* anamorph. *Ophiostoma* sp. M also lacked the branched ascocarp necks found with isolates of *O. europhioides* (**49**). The isolates of *Ophiostoma* sp. M were also compared with the description of *O. piceaperdum* (Rumb.) von Arx, a putative synonym of *O. europhioides* (**43**). *Ophiostoma piceaperdum* reportedly produces abundant conidiophores (**43**), but Rumbold (**35**) did not specifically mention neck branching in *O. piceaperdum.*

A culture (C88) from the holotype of *O. aureum* (Robins.-Jeffr. & Davids.) Harrington, a species associated with the stems of beetle-infested pines (**32**), clustered at I = 0.47 with the two isolates labelled as *Leptographium* sp. A from *Pseudotsuga menziesii* attacked by *Dendroctonus pseudotsugae* Hopk. (**18**). In culture, isolates of both fungi produced conidiophores that were similar in size, shape, and the arrangement of metulae. Masses of conidia were yellow in both species, but conidium size was up to twice as long in *O. aureum* as in *Leptographium* sp. A. Although ascocarps of *O. aureum* were not produced in the current study, they reportedly (**32**) lack necks and are non-ostiolate.

Ophiostoma clavigerum, O. robustum (Robins.-Jeffr. & Davids.) Harrington, and *L. pyrinum* Davids. clustered with one another at I ≥ 0.63, indicating close relatedness. These three species are found in stems of pines attacked by bark beetles (**11, 32**). Anamorph morphologies of the isolates were consistent with their respective species descriptions (**11, 32**). *Ophiostoma clavigerum* produced clavate conidia of two sizes, some extremely long and multicellular, others small and unicellular; *Ophiostoma robustum* produced rounded to oval conidia of several sizes; and in both species many conidia had thick cell walls. *Leptographium pyrinum* produced pear-shaped conidia with unthickened cell walls. Some isolates of *O. clavigerum* produced synnematal structures in addition to mononematous conidiophores typical of *Leptographium,* a salient feature of Upadhyay and Kendrick's genus *Graphiocladiella* (**43**). The production of the larger, synnematal structures was not found in all isolates of *O. clavigerum,* however. Only mononematous conidiophores were found in *O. robustum* and *L. pyrinum.* There was some similarity between *O. aureum, Leptographium* sp. A, and *O. clavigerum* in the branching patterns of the metulae of the conidiophores. The teleomorphs of *O. aureum, O. robustum,* and *O. clavigerum* have been reported to be similar if not identical (**32**).

FIGURE 1 also shows the relatedness among the *L. serpens* cluster, the *L. lundbergii* cluster, and the remaining species used in the study. Isolates of *L. abietinum* (Peck) Wingf. were from various genera of Pinaceae and geographic origins. Isolate C29, received from Davidson's collection as *L. engelmannii* Davids. and isolated from *Picea engelmannii* Parry in Colorado, was similar in morphology to the other isolates of *L. abietinum* and was electrophoretically identical to isolate C272 from *Picea rubens* Sarg. in New York. Isolate C172, from diseased roots of *Picea sitchensis* (Bong.) Carr. in Scotland (**16**), had been previously identified as *L. abietinum* by Harrington (**18**), but this isolate had several morphologic differences that distinguished it from *L. abietinum.* Conidia and conidiophores of C172 were similar to *L. abietinum,* but thin branches that resembled hyphae in width and pigmentation originated near the base of the conidiophores and ended with a single conidiogenous

←

FIG. 1. UGPMA cluster analysis of isozyme data from 15 putative enzyme loci showing relatedness [Nei's (**28**) genetic identity I] among isolates of *Leptographium* and *Ophiostoma.* Within each group clustering to the right of the gray line (arbitrarily placed at I = 0.60), isolates showed limited morphologic variation and were considered conspecific.

cell. All other isolates of *L. abietinum* lacked such branches. Growth of C172 was slower than isolates of *L. abietinum* on MEA and was markedly zonate on twig medium. This isolate was related to typical isolates of *L. abietinum* at I = 0.31.

Isolates representing the species *O. penicillatum* and *O. abiocarpum* were related at I = 0.27. The conidiophores and conidia of isolates C5 and C7 were typical of the anamorph of *O. penicillatum,* but isolate C135 of *O. abiocarpum* did not produce conidiophores in culture. Ascocarps were not produced by *O. penicillatum* or *O. abiocarpum.* Davidson (**10**) reported the lack of a *Leptographium* state to be typical in *O. abiocarpum* and questioned whether *Leptographium*-like conidiophores found near ascocarps of this species were produced by the same fungus, but Upadhyay (**43**) confirmed the anamorph-teleomorph connection.

The species used in this study represent four distinct ascospore morphologies. In the *L. lundbergii* cluster, ascospores, when present, have gelatinous sheaths and appear hat-shaped in side view. Ascospores of *O. penicillatum* and *O. abiocarpum* are allantoid (**10**, **38**), and the gelatinous ascospore sheaths are variously reported as lacking (**10**) or of variable thickness (**38**). *Ophiostoma adjuncti* (Davids.) Harrington, a species associated with the bark beetle *Dendroctonus adjunctus* Blandford attacking *Pinus ponderosa,* produces sheathed ascospores that appear rectangular or pillow-shaped (**11**). *Ophiostoma crassivaginatum* has falcate ascospores and occurs primarily on hardwoods (**18**, **43**). Relatedness was very low ($I \leq 0.07$, FIG. 1) among the four branches that represent these ascospore morphologies, with *O. adjuncti* having the least relatedness to the other branches.

DISCUSSION

Isozyme variation generally corresponded with morphological variation and proved useful in showing relationships among groups of isolates, infraspecific taxa, species, and species clusters of the genus *Leptographium.* With the enzymes and isolates examined in this study, the arbitrary value of genetic identity of I = 0.60 appeared to delimit species. Clustering at greater levels of relatedness may indicate the need for taxa to be synonymized (e.g., *L. abietinum* and *L. engelmannii*), although some clusters within species at $I > 0.60$ could represent subspecies or varieties. In *L. wageneri,* for example, the three primary electrophoretic groups corresponded to host-specialized varieties having minor but consistent morphologic characteristics. Conversely, relatedness at less than I = 0.60 may indicate the need for isolates differing slightly in morphology to be recognized as distinct taxa (e.g., *Leptographium* sp. I from *L. serpens*).

To the extent that isozyme studies of different genera can be compared, the value of I = 0.60 as the lower limit for relatedness of isolates within species of *Leptographium* is in general agreement with the minimum relatedness of approximately I = 0.50 for isolates of the ascomycete *Cryphonectria cubenses* (Bruner) Hodges (**26**). The results of the latter study also support the idea that in at least some fungal genera, isozyme comparisons among numerous isolates and species can be used to determine a level of electrophoretic similarity that can serve as a "genetic yardstick" for assessing species limits.

Other genera differ from *Leptographium* in the amount and distribution of variation within and between taxa. In the genus *Phytophthora,* Nygaard *et al.* (**30**) reported that isolates of *Phytophthora megasperma* specialized to different hosts and isolates of three other *Phytophthora* species each form groups with distinct isozyme patterns; isozyme variation within groups is minimal or lacking, and differences among *formae speciales* are in some cases of the same magnitude as differences among species (**30**). Cruikshank and Pitt (**8**) also found little variation in isozyme patterns within most of the *Penicillium* species they examined. In phylogenetic (parsimony) and phenetic analyses of electrophoretic data in the genus *Trichoderma,* Stasz *et al.* (**39**) reported poor correspondence between species delineation and relatedness. In some species, isolates composed more than one distinct cluster, and Stasz *et al.* (**39**) have suggested that the recognized species of *Trichoderma* may contain morphologically cryptic species. Values of genetic identity were not reported for these latter three studies.

A number of specific taxonomic problems were addressed in our study, and our results demonstrate the usefulness of enzyme electrophoresis in delimiting species and in the study of old, pleomorphic cultures. Isolates of *O. clavigerum, O. rubustum,* and *L. pyrinum* were closely related and formed a distinct cluster. In light of the inherent variability in anamorph morphology not-

ed in *O. clavigerum* (**32**, **42**) and the lack of differences in teleomorph characteristics between *O. clavigerum* and *O. robustum* (**32**), it is likely that the three taxa are morphologic variants of the same species and should probably be synonymized. However, more isolates and holotype material should be examined. It is noteworthy that the isolate from the holotype of *O. aureum* is morphologically similar but did not cluster closely with *O. clavigerum.*

A suggestion by Harrington (**18**) that *L. engelmannii* and *L. abietinum* may be conspecific is supported by close clustering. Davidson's (**9**) description of *L. engelmannii* closely resembles *L. abietinum,* and since the diagnosis of *L. engelmannii* did not include a comparison with *L. abietinum,* Davidson may have been unaware of the resemblance between his fungus and the earlier-described *L. abietinum.* Isolate C29 from the collection of Davidson is probably more than 35 years old and is the only known isolate of *L. engelmannii* (**18**), but its relationship to the type specimen is uncertain.

Electophoretic results of this study support the decision by Wingfield and Marasas (**47**) to synonymize *Verticicladiella alacris* with *L. serpens* and demonstrate the utility of this technique in identifying older or otherwise atypical cultures. *Leptographium serpens* was described over 50 years ago from an isolate in pine roots in Italy. The original material used to describe this species was lost, but isolate C30 from the type specimen is available. Morphology of this culture differs from recent cultures of *L. serpens* in having shorter conidia, slower growth, less serpentine hyphae, and unusual side branches along the main stipe of the conidiophore (**18**, **47**). In contrast to culture morphology, this study has shown that electrophoretic characters of this culture were apparently unaffected by its age.

Taxa that were morphologically similar but distinguishable and that had relatedness values of I between 0.25 and 0.60 can be interpreted as distinct but closely related species, i.e., sib-species. Davidson (**10**) first recognized *O. abiocarpum* as distinct from its European sib-species *O. penicillatum* by its lack or rarity of *Leptographium* conidiophores, and these species had relatedness of I = 0.27. Isolates of *Ophiostoma* sp. M differed from those of its sib-species *O. europhioides* by the lack of conidiophores and the unbranched ascocarp necks in *Ophiostoma* sp. M (**49**). Similarly, isolate C172 was distinct from all other isolates of *L. abietinum* by its production of thin conidiogenous branches from the base of the conidiophore stipe, was related to the other isolates at I = 0.31, and may be a sib-species to the latter fungus.

Although dendrogram branching may be considered as increasingly subject to error as relatedness nears its lower extreme, the low relatedness among species representing the four ascospore morphologies of Olchowecki and Reid (**31**), i.e., *O. adjuncti, O. crassivaginatum, O. penicillatum/O. abiocarpum,* and the *L. lundbergii* cluster, provided some justification for the subdivision of the genus *Ophiostoma* into sections or groups along these lines. The fact that *O. adjuncti* of the *Ips* group (**31**) had lower relatedness than *O. crassivaginatum* to other *Ophiostoma* species also supports suggestions by de Hoog and Scheffer (**12**) and Harrington (**17**, **18**) that many of the species of Upadhyay's (**43**, **44**) genus *Ceratocystiopsis* can be accomodated in *Ophiostoma.*

In conclusion, enzyme electrophoresis has been shown to be a valuable tool for clarifying the taxonomy of *Leptographium* species. Further, the results suggest the further use of this method for determining the extent of variation in asexual fungi at the infra-specific, species, and higher ranks.

ACKNOWLEDGMENTS

The authors thank Dr. Robert Eckert of the Department of Forest Resources at the University of New Hampshire for use of his facilities. Our thanks also go to the many individuals that have supplied us with *Leptographium* isolates used in this study. This work was supported in part by a graduate research grant from the Central University Research Fund of the University of New Hampshire awarded to PJZ. Scientific Publication number 1695 of the New Hampshire Agricultural Experiment Station.

LITERATURE CITED

1. **Alexander, S. A., W. E. Horner, and K. J. Lewis.** 1988. *Leptographium procerum* as a pathogen of pines. Pp. 97–112. *In*: Leptographium *root diseases on conifers.* Eds., T. C. Harrington and F. W. Cobb, Jr. APS Press, St. Paul, Minnesota.
2. **Barras, S. J., and T. Perry.** 1971. *Leptographium terebrantis* sp. nov. associated with *Dendroctonus terebrans* in loblolly pine. *Mycopathol. Mycol. Appl.* **43**: 1–10.
3. **Bonde, M. R., G. L. Peterson, W. M. Dowler, and B. May.** 1984. Isozyme analysis to differentiate species of *Peronosclerospora* causing downy

mildews of maize. *Phytopathology* **74**: 1278–1283.

4. **Burdon, J. J., A. P. Roelfs, and A. H. D. Brown.** 1986. The genetic basis of isozyme variation in the wheat stem rust fungus (*Puccinia graminis tritici*). *Canad. J. Genet. Cytol.* **28**: 171–175.
5. **Cardy, B. J., C. W. Stuber, and M. M. Goodman.** 1983. *Techniques for starch gel electrophoresis of enzymes from maize* (Zea mays *L.*). Institute of Statistics Mimeo Series No. 1317R. North Carolina State University, Raleigh, North Carolina. 35 p.
6. **Cobb, F. W., Jr.** 1988. *Leptographium wageneri,* cause of black-stain root disease: a review of its discovery, occurrence and biology with emphasis on pinyon and ponderosa pine. Pp. 41–62. *In*: Leptographium *root diseases of conifers.* Eds., T. C. Harrington and F. W. Cobb, Jr. APS Press, St. Paul, Minnesota.
7. **Conkle, M. T., P. D. Hodgekiss, L. B. Nunnally, and S. C. Hunter.** 1982. *Starch gel electrophoresis of conifer seeds: a laboratory manual.* U.S. Forest Service General Technical Report PSW-64. 18 p.
8. **Cruikshank, R. H., and J. I. Pitt.** 1987. Identification of species in *Penicillium* subgenus *Penicillium* by enzyme electrophoresis. *Mycologia* **79**: 614–620.
9. **Davidson, R. W.** 1955. Wood-staining fungi associated with bark beetles in Engelmann spruce in Colorado. *Mycologia* **47**: 58–67.
10. ———. 1966. New species of *Ceratocystis* from conifers. *Mycopathol. Mycol. Appl.* **28**: 273–286.
11. ———. 1978. Staining fungi associated with *Dendroctonus adjunctus* in pines. *Mycologia* **70**: 35–40.
12. **deHoog, G. S., and R. J. Scheffer.** 1984. *Ceratocystis* versus *Ophiostoma*: a reappraisal. *Mycologia* **76**: 292–299.
13. **Gessner, R. V., M. A. Romano, and R. W. Schultz.** 1987. Allelic variation and segregation in *Morchella deliciosa* and *M. esculenta. Mycologia* **79**: 683–687.
14. **Goheen, D. J., and F. W. Cobb, Jr.** 1978. Occurrence of *Verticicladiella wageneri* and its perfect state *Ceratocystis wagenerii* sp. nov. in insect galleries. *Phytopathology* **68**: 1192–1195.
15. **Goidanich, G.** 1936. Il genere di Ascomiceti *Grossmania* G. Goid. *Boll. Staz. Patol. Veg. Roma.* **16**: 26–60.
16. **Gregory, S. C., and D. B. Redfern.** 1987. The pathology of Sitka spruce in northern Britain. *Proc. Roy. Soc. Edinburgh,* Sect. B., **93**: 145–156.
17. **Harrington, T. C.** 1987. New combinations in *Ophiostoma* of *Ceratocystis* species with *Leptographium* anamorphs. *Mycotaxon* **28**: 39–43.
18. ———. 1988. *Leptographium* species, their distributions, hosts, and insect vectors. Pp. 1–39. *In*: Leptographium *root diseases on conifers.* Eds., T. C. Harrington and F. W. Cobb, Jr. APS Press, St. Paul, Minnesota.
19. ———, **and F. W. Cobb, Jr.** 1983. Pathogenicity of *Leptographium* and *Verticicladiella* spp. isolated from roots of western North American conifers. *Phytopathology* **73**: 596–599.
20. **Harris, H., and D. A. Hopkinson.** 1976. *Handbook of enzyme electrophoresis in human genetics.* Oxford American Elsevier Publishing Co., New York, New York.
21. **Lackner, A. L., and S. A. Alexander.** 1983. Root disease and insect infestations on air-pollution-sensitive *Pinus strobus* and studies of pathogenicy of *Verticicladiella procera. Pl. Dis.* **67**: 679–681.
22. **Lagerberg, T., G. Lundberg, and E. Melin.** 1927. Biological and practical researches into blueing in pine and spruce. *Sven. Skogsvårdsfören. Tidskr.* **25**: 145–272.
23. **Marty, T. L., D. M. O'Malley, and R. P. Guries.** 1984. *A manual for starch gel electrophoresis: new microwave edition.* Staff Paper #20. Department of Forestry, University of Wisconsin, Madison. 24 p.
24. **May, B., and D. L. Royse.** 1982. Confirmation of crosses between lines of *Agaricus brunnescens* by isozyme analysis. *Exp. Mycol.* **6**: 283–292.
25. **Micales, J. A., M. R. Bonde, and G. L. Peterson.** 1986. The use of isozyme analysis in fungal taxonomy and genetics. *Mycotaxon* **27**: 405–449.
26. ———, **R. J. Stipes, and M. R. Bonde.** 1987. On the conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis. Mycologia* **79**: 707–720.
27. **Mielke, M. E.** 1981. Pathogenicity of *Verticicladiella penicillata* (Grosm.) Kendrick to northern Idaho conifers. *Forest Sci.* **27**: 103–110.
28. **Nei, M.** 1972. Genetic distance between populations. *Amer. Naturalist* **106**: 283–292.
29. **Nomenclature Committee of the International Union of Biochemistry.** 1984. *Enzyme nomenclature 1984.* Academic Press Inc., Orlando, Florida. 646 p.
30. **Nygaard, S. L., C. K. Elliott, S. J. Cannon, and D. P. Maxwell.** 1989. Isozyme variability among isolates of *Phytophthora megasperma. Phytopathology* **79**: 773–780.
31. **Olchowecki, A., and J. Reid.** 1974. Taxonomy of the genus *Ceratocystis* in Manitoba. *Canad. J. Bot.* **52**: 1675–1711.
32. **Robinson-Jeffrey, R. C., and R. W. Davison.** 1968. Three new *Europhium* species with *Verticicladiella* imperfect states on blue-stained pine. *Canad. J. Bot.* **46**: 1523–1527.
33. ———, **and A. H. H. Grinchenko.** 1964. A new fungus in the genus *Ceratocystis* occurring on lodgepole pine attacked by bark beetles. *Canad. J. Bot.* **42**: 527–532.
34. **Royse, D. J., M. C. Spear, and B. May.** 1983. Single and joint segregation of marker loci in the shiitake mushroom, *Lentinus edodes. J. Gen. Appl. Microbiol.* **29**: 217–222.
35. **Rumbold, C. T.** 1936. Three blue-staining fungi, including two new species, associated with bark beetles. *J. Agric. Res.* **52**: 419–437.
36. **Shattock, R. C., P. W. Tooley, and W. E. Fry.** 1986. Genetics of *Phytophthora infestans*: de-

termination of recombination, segregation, and selfing by isozyme analysis. *Phytopathology* **76**: 410–413.

37. **Shields, C. R., T. J. Orton, and C. W. Stuber.** 1983. An outline of general resource needs and procedures for the electrophoretic separation of active enzymes from plant tissue. Pp. 443–483. *In*: *Isozymes in plant genetics and breeding, part A*. Eds., S. D. Tanksley and T. J. Orton. Elsevier Science Publishers, Amsterdam, Netherlands.
38. **Solheim, H.** 1986. Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*. *Nordic J. Bot.* **6**: 199–207.
39. **Stasz, T. E., K. Nixon, G. E. Harmon, N. F. Weedon, and G. A. Kuter.** 1989. Evaluation of phenetic species and phylogenetic relationships in the genus *Trichoderma* by cladistic analysis of isozyme polymorphism. *Mycologia* **81**: 391–403.
40. **Swofford, D. L., and R. B. Selander.** 1981. Biosys-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Heredity* **72**: 281–283.
41. ———, **and** ———. 1989. *Biosys-1. A computer program for the analysis of allelic variation in population genetics and biochemical systematics*. Release 1.7. Illinois Natural History Survey, Urbana, Illinois. 43 p.
42. **Tsuneda, A., and Y. Hiratsuka.** 1984. Sympodial and annellidic conidiation in *Ceratocystis clavigera*. *Canad. J. Bot.* **62**: 2618–2624.
43. **Upadhyay, H. P.** 1981. *A monograph of* Ceratocystis *and* Ceratocystiopsis. University of Georgia Press, Athens. 176 p.
44. ———, **and W. B. Kendrick.** 1975. Prodromus for a revision of *Ceratocystis*. *Antonie van Leeuwenhoek Ned. Tijdschr. Hyg.* **41**: 353–360.
45. **Vallejos, C. E.** 1983. Enzyme activity staining. Pp. 469–516. *In*: *Isozymes in plant genetics and breeding*, part A. Eds., S. D. Tanksley and T. J. Orton. Elsevier Science Publishers B. V., Amsterdam, Netherlands.
46. **Wingfield, M. J., P. Capretti, and M. MacKenzie.** 1988. *Leptographium* spp. as root pathogens of conifers. An international perspective. Pp. 113–128. *In*: *Leptographium root diseases on conifers*. Eds., T. C. Harrington and F. W. Cobb, Jr. APS Press, St. Paul, Minnesota.
47. ———, **and W. F. O. Marasas.** 1981. *Verticicladiella alacris*, a synonym of *V. serpens*. *Trans. Brit. Mycol. Soc.* **76**: 508–510.
48. ———, **and** ———. 1983. Some *Verticicladiella* species, including *V. truncata* sp. nov., associated with root diseases of pine in New Zealand and South Africa. *Trans. Brit. Mycol. Soc.* **80**: 231–236.
49. **Wright, E. F., and R. F. Cain.** 1961. New species of the genus *Ceratocystis*. *Canad. J. Bot.* **39**: 1215–1230.
50. **Zambino, P. J., and T. C. Harrington.** 1989. Isozyme variation within and among host-specialized varieties of *Leptographium wageneri*. *Mycologia* **81**: 122–133.

Accepted for publication August 5, 1991