An electrophoretic study of representatives of subgenus *Diploxylon* of *Pinus*

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Relationships among 14 taxa in subgenus *Diploxylon* of *Pinus* from Mexico and the United States were analyzed using 15 isozyme loci obtained from both frozen and fresh needle tissue. The phenogram obtained from cluster analysis of genetic distance values and phylogenetic trees implementing the distance Wagner procedure were in general agreement with classifications based on morphological features. *Pinus leiophylla* is genetically distinct from other species. Two groupings, one comprising *P. oocarpa* and *P. pringlei* and another comprising *P. lawsonii* and *P. teocote*, correspond to sections *Serotinos* and *Teocote* (sensu Martínez), respectively. Classification of the remaining eight taxa has varied, ranging from their placement in three, two, or only one section. Isozyme analysis resulted in a group that included *P. cooperi*, *P. douglasiana*, *P. durangensis*, *P. michoacana*, and *P. montezumae*, which suggests genetic affinity between Sections *Pseudostrobus* and *Montezumae* (sensu Martínez). This result corresponds more closely to the placement of these species in subsection *Pseudostrobi*. The distant relationship between *P. engelmannii* and the ponderosa pines may be anomalous (an artifact of small sample size) or may indicate greater genetic divergence than previously recognized.

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À partir d'aiguilles fraîches et congelées et en observant les positions de 15 isoenzymes, les auteurs ont analysé les relations qui existent entre les 14 taxons du sous-genre *Diploxylon* et *Pinus* qu'on retrouve au Mexique et aux États-Unis. Les phénogrammes obtenus par l'analyse du regroupement des valeurs des distances génétiques et les dendrographes phylogénétiques mettant en application la méthode des distances de Wagner montrent une concordance générale avec les classifications basées sur les caractéristiques morphologiques. Le *Pinus leiophylla* s'avère génétiquement distinct des autres espèces. Deux regroupements, un comprenant le *P. oocarpa* et le *P. pringlei* et un autre comprenant le *P. lawsonii* et le *P. teocote*, correspondent aux sections *Serotinos* et *Teocote* (sensu Martínez), respectivement. La classification des huit autres taxons a varié; on les retrouvent dans trois, deux ou une seule section. L'analyse des isoenzymes conduit à un groupe qui inclut *P. cooperii*, *P. douglasiana*, *P. durangensis*, *P. michoacana* et *P. montezumae*, ce qui suggère une affinité génétique entre les sections *Pseudostrobus* et *Montezumae* (sensu Martínez). Ce résultat se rapproche de l'attribution de ces trois espèces à la subsection *Pseudostrobi*. La faible relation entre le *P. engelmannii* et les pins du type ponderosa pourrait être une anomalie (un artefact venant d'un faible échantillonnage) ou pourrait indiquer une plus grande divergence génétique que celle reconnue jusqu'ici.

[Traduit par la revue]

Introduction

The genus *Pinus* consists of more than 100 species, which constitutes about half the number of species in the family Pinaceae (Mirov 1967; Farjon 1984). Several classifications have been proposed for the genus *Pinus* including the work by Shaw (1914), Pilger (1926), and Critchfield and Little (1966). A summary of the subgeneric classifications of pines prior to 1967 is given in Mirov (1967). A recent classification based upon the wood anatomy and morphological features of 78 species was proposed by Van der Burgh (1973). Farjon (1984) conducted a cladistic analysis of the pines following the classification by Van der Burgh (1973). In both studies, eight sections of *Pinus* were recognized. Two sections, *Strobus* and Parya, include the soft pines with only one vascular bundle per needle, equivalent to subgenus *Haploxylon* of Mirov (1967). Six sections, *Leiophylla*, *Sula*, *Lumholtzii*, *Pinea*, *Pinus*, and *Pinaster*, include the hard pines with two vascular bundles per needle, which were placed in subgenus *Diploxylon* by Mirov (1967).

Mexico harbors a high diversity of pine species, many of which are considered evolutionarily recent (Martínez 1948; Mirov 1967; Farjon 1984). The pines of this area have received considerable taxonomic attention, especially after the monographic works by Shaw (1909, 1914). The most comprehensive and detailed study of the Mexican pines was by Martínez (1948) who recognized 38 species, 21 varieties, and 8 forms among 9 sections. Although this work clarified many taxonomic problems, evolution and hybridization still complicate classification and subgeneric affinities remain obscure (Looke 1950). Taxonomic investigation of Mexican pines is an active area of research as shown by the discovery of at least 12 new species or subspecies since 1967 (e.g., Robert 1978; Perry 1987).

For this study, mature needles from 14 taxa of *Pinus* subgenus *Diploxylon* were used for electrophoretic analysis. A comparison of classifications for these pines based upon the studies of Martínez (1948), Critchfield and Little (1966), and Farjon (1984) is given in Table 1. These classifications are similar in terms of species delimitation; however, sectional and subsectional concepts vary. The major difference in the grouping of species involves subsection *Oocarpae* (Farjon 1984), which includes species assigned to sections *Teocote* and *Serotinos* according to Martínez (1948) and subsections *Oocarpae* and *Ponderosae* according to Critchfield and Little (1966). Besides *P. leiophylla* (Section *Ternatae*) and *P. oocarpa* and *P. pringlei* (Section *Pinus*, Subsection *Oocarpae*), all the remaining species examined in this work were placed in subsection *Ponderosae* by Critchfield and Little (1966).

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TABLE 1. Comparison of classifications for the pines used in this study

Martínez 1948	Critchfield and Little 1966	Farjon 1984
Section *Leiophylla*	Section *Ternatae*	Section *Leiophylla*
P. leiophylla Schiede & Deppe	*P. leiophylla*	*P. leiophylla*
Section *Teocote*	Section *Pinus*	Section *Pinaster*
P. lawsonii Riezl.	Subsection *Oocarpae*	Subsection *Oocarpae*
P. teocote Schiede & Deppe	*P. oocarpa*	*P. lawsonii*
Section *Serotinos*	*P. pringlei*	*P. oocarpa*
P. oocarpa Schiede	Subsection *Ponderosae*	*P. pringlei*
P. pringlei Shaw	*P. cooperi*	*P. teocote*
Section Pseudostrobus	*P. douglasiana*	Subsection *Pseudostrobi*
P. douglasiana Martínez	P. durangensis	P. cooperi
Section *Montezumae*	*P. engelmannii*	*P. douglasiana*
Group *Rudis*	*P. lawsonii*	*P. durangensis*
P. cooperi C. E. Blanco	P. michoacana	P. michoacana
Group *Montezumae*	*P. montezumae*	*P. montezumae*
P. durangensis Martínez	*P. ponderosa* var. *ponderosa*	Subsection *Ponderosae*
P. montezumae Lamb.	P. ponderosa var. *scopulorum*	*P. engelmannii*
Group *Michoacana*	*P. teocote*	*P. ponderosa* var. *scopulorum*
P. michoacana Martínez		P. ponderosa var. *ponderosa*
Section Ponderosa		
P. engelmannii Carr.		
P. ponderosa var. *ponderosa* Lam.		
P. ponderosa var. *scopulorum* Engelm.		

Enzyme electrophoresis is useful for the study of phylogenetic relationships among organisms as it provides reliable estimates of genetic distances between taxa (Nei 1975). Genetic variation in natural populations of plants can be practically surveyed using isozymes and this method has been extensively utilized for the pines. Genotypes can be determined from electrophoretic phenotypes because of the overall similarity of enzyme substructure (Gottlieb 1981) and the general conservation of isozyme number among higher plants (Gottlieb 1982). During the past decade, enzyme electrophoresis has played a major role in conifer systematics and population genetics (Wheeler and Guries 1982; Wheeler et al. 1983; Jacobs et al. 1984; and Miller et al. 1988), hence a large amount of information exists on isozyme number and levels of genetic variation within and between populations.

The objective of this study was to compare estimates of relationships among 14 *Diploxylon* pines obtained from this electrophoretic anlaysis with those based mainly upon morphological characters.

Materials and methods

Pine needle collection was done in two different ways. First, needle samples were collected while in Mexico and were immediately frozen over dry ice. The samples were stored at −100°C after returning to Illinois. The second method involved using fresh needles from pines grown from seed under greenhouse conditions. The source of this seed material and other collection information is given in Table 2. The locations of these pine populations in Mexico is illustrated in Fig. 1. Seedlings were allowed to reach at least 15 cm in height before adult foliage was harvested for enzyme extraction. Only mature needles were used to circumvent possible developmental variation in enzyme banding patterns.

Enzyme extraction

Pine-specific electrophoretic conditions were determined by a series of scans conducted with several extraction buffers, gel buffers, and enzyme systems. The effects of freezing upon enzyme activity were also determined. Extraction buffer No. 12 of Pitel and Cheliak (1984) was found to be most suitable. This pH 7.2 buffer is composed of 0.1 M Tris, 0.5 M sucrose, 0.06 M sodium tetraborate, 0.03 M sodium metabisulfite, 0.02 M diethyldithiocarbamic acid, 6 mM ascorbic acid (Na salt), 6 mM cysteine-HCl, 1 mM dithiothreitol, 0.5 mM EDTA (Na_2), 0.4 mM NAD, 0.2 mM pyridoxal-5′-phosphate, 10% dimethyl sulfoxide, 10% (w/v) polyvinylpyrrolidone (PVP-40), 1% Tween, 1% 2-phenoxyethanol, 1% polyethylene glycol, 1% 2-mercaptoethanol, and 0.1% bovine serum albumin.

Pine needles were cut into ca. 4-cm long pieces (ca. 5 g wet weight) and ground to a powder in a mortar under liquid nitrogen. The powder was then quickly transferred to a precooled (50 mL, 2.5 cm diameter) test tube and 8 mL of extraction buffer was added. The mixture was homogenized for ca. 30 s at 0°C using a Brinkman Polytron with a 2-cm diameter generator. The homogenate was then centrifuged for 15 min at 10 000 × *g*. The supernatant was either applied immediately to filter paper wicks and subjected to electrophoresis or was stored in 1.5-mL microfuge tubes at −100°C.

Rapid extraction with a Polytron homogenizer after grinding with nitrogen appears to greatly improve the quality of isozyme extracts. Apparently protein degradation is arrested as the buffer components are quickly brought into contact with the disrupted cells. When extracts from both frozen and fresh samples were immediately subjected to electrophoresis, clear banding patterns were obtained. Although frozen (−100°C) protein extracts retained enzyme activity for as long as a month, fresh homogenates resulted in greater enzyme activity and sharper banding patterns. These results indicate that, for long term storage (weeks to months), frozen intact needles are preferable to frozen homogenates. If protein extracts must be frozen prior to electrophoresis, storage for less than 1 week is preferable.

Electrophoresis and data analysis

The samples were absorbed onto filter paper wicks and subjected to electrophoresis on 14% starch gels (Sigma Chemical Company) using standard techniques. Eleven enzyme systems coding for a total of 15 gene loci were used in this study (Table 3). The number of loci was inferred by band number, intensity, and position. A review of the relevant literature provided valuable insight into the number of loci per enzyme system and the amount and type of variation expected per locus (El-Kassaby 1981; Wheeler and Guries 1982). For enzyme systems with two or more loci, the most anodal locus was designated as No. 1 and the others were numbered accordingly. The most

TABLE 2. Collection Information

Popul. No.	Pine species	Collector, No., and year	Locality	Method of needle collection[a] and sample size
1	*P. cooperi*	Nickrent 2060 1985	Mexico, Estado Durango, km 122 along hwy. 40 near Las Adjuntas, 23°45′N, 105°31′W	fn, 11
2	*P. douglasiana*	Nickrent 2050 1985	Mexico, Estado Durango, 1.6 km W of El Madroño along hwy. 40, 23°38′N, 105°47′W	fn, 48
3	*P. durangensis*	Guries Lot 472[b] 1975	Mexico, Estado Michoacana, Municipio Paracho, 19°39′N, 102°1′W	s, 20
4	*P. engelmannii*	Nickrent 2066 1985	Mexico, Estado Durango, along hwy. 40 at Los Mimbres Canyon, 42 km W of Durango, 23°56′N, 104°55′W	fn, 10
5	*P. lawsonii*	Nickrent 2042 1985	Mexico, Estado Oaxaca, km 121 on hwy. 175, 40 km N of Ixtlan, 17°35′N, 96°26′W	fn, 26
6	*P. leiophylla*-1	Nickrent 2068 1985	Mexico, Estado Durango, Los Mimbres Canyon, 48 km W of Durango on hwy. 40, 23°55′N, 104°57′W	fn, 10
7	*P. leiophylla*-2	Guries s.n. 1978	Mexico, Estado Durango, 1.6 km W of El Salto 23°46′N, 105°23′W	s, 10
8	*P. michoacana*-1	Nickrent 2028 1985	Mexico, Estado Oaxaca, 6 km south of San Miguel Suchixtepec along hwy. 175, 16°4′N, 96°29′W	fn, 7
9	*P. michoacana*-2 var. *cornuta*	Guries Lot 699 1978	Mexico, Estado Chiapas, La Laguna, Ocasingo, 16°49′N, 92°8′W	s, 22
10	*P. montezumae*-1	Guries Lot 655 1977	Mexico, Estado Chiapas, Rancho Nuevo, Mitzinton, near San Cristóbal de las Casas, 16°43′N, 92°33′W	s, 18
11	*P. montezumae*-2	Nickrent 2017 1985	Mexico, Estado Veracruz, 4 km S of Sierra de Aqua off hwy. 140, 19°34′N, 97°9′W	fn, 8
12	*P. oocarpa*	Guries Lot 692 1978	Mexico, Estado Chiapas, Aserradero, San Martin, Jitotol, 17°0′N, 92°51′W	s, 26
13	*P. ponderosa* var. *ponderosa*	DeLucia s.n. 1987	U.S.A., Nevada, Washoe Co., SW of Peavine Mountain, 39°39′N, 119°59′W	s, 13
14	*P. ponderosa* var. *scopulorum*	Hawksworth s.n. 1986	U.S.A., Larimer Co., State Forest Nursery, Ft. Collins, CO, 40°35′N, 105°6′W	s, 25
15	*P. pringlei*	Guries Lot 87 1964	Mexico, Estado Mexico, Valle de Bravo, 19°14′N, 100°07′W	s, 24
16	*P. teocote*	Nickrent 2021 1985	Mexico, Estado Veracruz, 4 km S of Sierra de Aqua off hwy. 140, 19°34′N, 97°9′W	fn, 17

[a]fn, field collected frozen needles; s, needles from plants grown from seeds.
[b]Seed lot number from Instituto Nacional de Investigaciones Forestales, Departamento de Mejoramiento Genetico, Centro de Germoplasma Forestal. Seeds were obtained through R. P. Guries.

common allele (allozyme) for each enzyme locus was designated as 100 and other allelic variants were assigned mobility numbers relative to this standard. Statistical analyses of the isozyme data were performed using the computer program BIOSYS-1 by Swofford and Selander (1981).

Results

All loci included in this study were polymorphic, some with major differences in allele frequencies among populations. The allele frequencies for the 15 loci are shown in Table 4. Fixation for different alleles at a locus was observed among several species. Levels of heterozygosity and percent polymorphic loci varied widely among the species tested (0 to 0.2 and 6.7 to 60, respectively). Possible explanations for this are low sample sizes within populations, few populations sampled, and the frequency of homozygote and heterozygote genotypes may have been biased owing to seed derived from one or few cones. Average values of percent polymorphic loci and heterozygosity for the group of pines listed in Hamrick et al. (1981) were

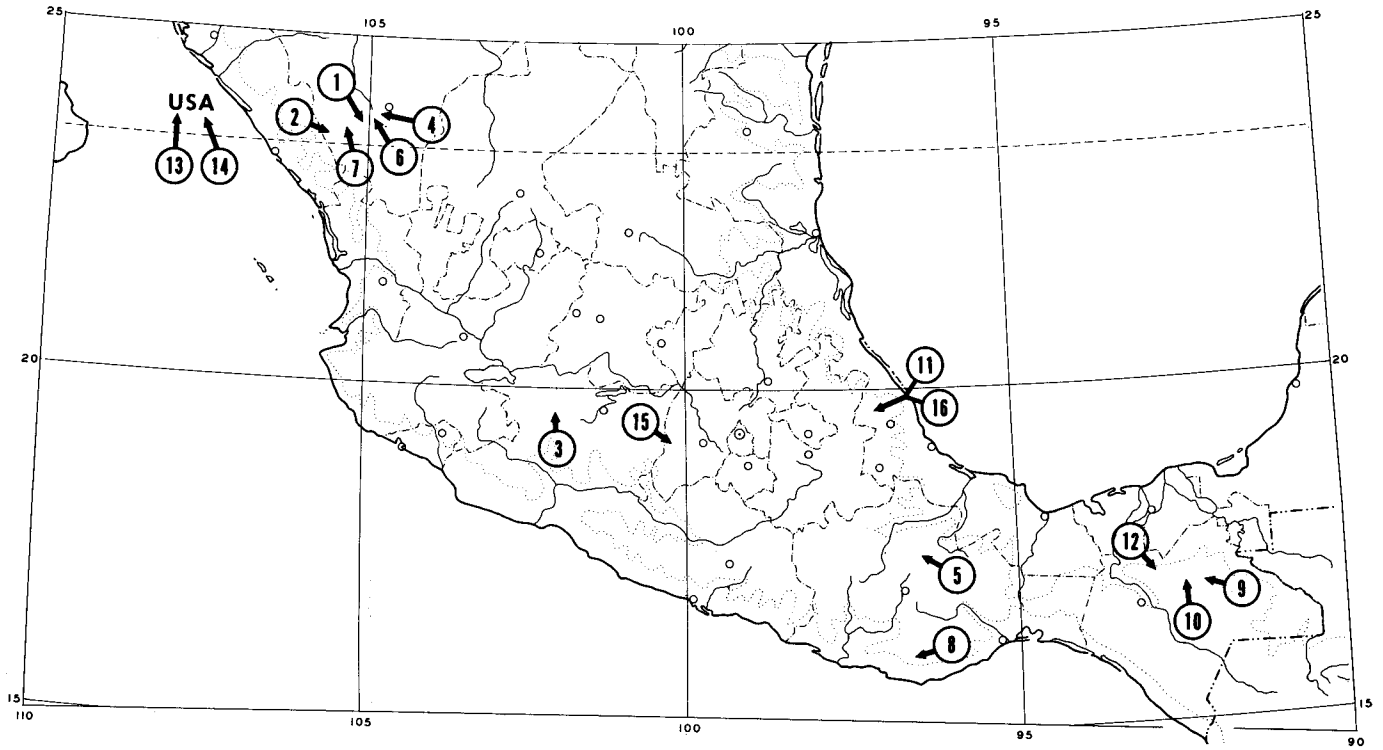

FIG. 1. Locations of the 14 Mexican pine taxa used in this study. See Table 2 for complete collection information for all 16 populations.

TABLE 3. Enzyme systems and buffers

Enzyme	Abbreviation	E.C. No.	Buffer system[a]	No. of loci used
Aspartate amino transferase	AAT	2.6.1.1	A	2
Glucose-6-phosphate dehydrogenase	G-6-PDH	1.1.1.49	B	1
Isocitrate dehydrogenase	IDH	1.1.1.42	A	1
Leucine amino peptidase	LAP	3.4.11.1	A	1
Malate dehydrogenase	MDH	1.1.1.37	C	2
Malic enzyme	ME-2	1.1.1.40	C	1
Menadione reductase	MDR	1.6.99.2	C	1
6-Phosphogluconate dehydrogenase	6-PGDH	1.1.1.44	B	2
Phosphoglucoisomerase	PGI-2	5.3.1.9	B	1
Phosphoglucomutase	PGM	2.7.5.1	A	2
Shikimate dehydrogenase	SKDH	1.1.1.25	C	1

[a]A, lithium hydroxide, pH 8.3 (Selander et al. 1971); B, citrate-morpholine, pH 6.5 (Nickrent 1986); and C, Tris–citrate, pH 7.5 (Soltis et al. 1983).

ca. 60% and 0.2, respectively. Although levels of heterozygosity and polymorphism determined in this study may not be representative of the species, the effect of low sample size upon genetic distance estimates is less acute (Gorman and Renzi 1979).

Several genetic distance and genetic identity measures were calculated and used to generate phenograms and cladograms. The chord distance (Cavalli-Sforza and Edwards 1967) was chosen for UPGMA (Sneath and Sokal 1973) phenogram construction. The distance Wagner method, using the Prevosti distance (Wright 1978), was used to generate cladograms since it is free of the assumption implicit in UPGMA methods that evolutionary rates in all taxa are equal. These genetic distance values are shown in Table 5. The UPGMA phenogram shown in Fig. 2 had a cophenetic correlation of 0.944 and a percent standard deviation of 8.57. Cophenetic correlation values from other genetic similarity and distance measures ranged from 0.88 to 0.935, with percent standard deviation values ranging from 10.6 to 28.7.

Phenetic analyses of isozyme data

The UPGMA cluster analysis of pine genetic distances (Fig. 2) reflects species relationships in general agreement with one or more of the classification systems shown in Table 1. The segregation of *Pinus leiophylla* by isozyme characters corresponds to the recognition of sections *Leiophylla* (Martínez 1948; Farjon 1984) and *Ternatae* (Critchfield and Little 1966). The grouping of *P. oocarpa* with *P. pringlei* is supported by the placement of these taxa in section *Serotinos* (Martínez 1948) and subsection *Oocarpae* (Critchfield and Little 1966). The affinity of *P. lawsonii* and *P. teocote* (as proposed by Farjon 1984) with *P. oocarpa* and *P. pringlei* is not

TABLE 4. Allele frequencies (%) for 15 loci and 16 populations of *Pinus*

Locus	Rel. mob.	Populations															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AAT-1	75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	95.8	0
	87	0	0	0	25.0	0	0	0	0	0	0	0	0	0	0	0	0
	100	100.0	100.0	100.0	75.0	100.0	100.0	100.0	100.0	90.9	93.3	100.0	100.0	100.0	100.0	4.2	100.0
	110	0	0	0	0	0	0	0	0	0	6.7	0	0	0	0	0	0
	116	0	0	0	0	0	0	0	0	9.1	0	0	0	0	0	0	0
AAT-2	50	0	0	0	0	0	0	0	0	0	0	50.0	0	0	0	0	0
	60	0	0	0	0	0	0	0	0	25.0	0	0	0	0	0	0	0
	78	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.2	0
	100	100.0	100.0	100.0	40.0	100.0	0	0	100.0	70.5	100.0	50.0	0	100.0	94.0	2.1	100.0
	125	0	0	0	0	0	0	0	0	0	0	0	0	0	6.0	14.6	0
	136	0	0	0	0	0	0	0	0	0	0	0	100.0	0	0	79.2	0
	143	0	0	0	60.0	0	100.0	100.0	0	0	0	0	0	0	0	0	0
	165	0	0	0	0	0	0	0	0	4.5	0	0	0	0	0	0	0
G-6PDH	100	100.0	100.0	100.0	100.0	100.0	0	0	100.0	100.0	94.3	100.0	100.0	100.0	100.0	100.0	100.0
	113	0	0	0	0	0	100.0	100.0	0	0	0	0	0	0	0	0	0
	130	0	0	0	0	0	0	0	0	0	5.7	0	0	0	0	0	0
IDH	80	0	0	0	0	0	0	0	0	0	0	0	0	3.8	0	0	0
	90	0	0	0	15.0	0	0	0	0	0	0	0	0	0	0	0	0
	100	100.0	100.0	100.0	85.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	92.3	100.0	50.0	100.0
	113	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50.0	0
	124	0	0	0	0	0	0	0	0	0	0	0	0	3.8	0	0	0
LAP	87	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.2	0
	100	100.0	100.0	100.0	95.0	0	0	0	57.1	93.2	100.0	50.0	0	50.0	50.0	0	0
	105	0	0	0	0	0	0	0	42.9	6.8	0	50.0	0	50.0	50.0	0	0
	111	0	0	0	0	0	100.0	100.0	0	0	0	0	100.0	0	0	95.8	0
	117	0	0	0	5.0	100.0	0	0	0	0	0	0	0	0	0	0	100.0
MDH-1	67	0	8.3	0	0	16.7	0	0	28.6	0	7.1	6.3	0	0	0	0	0
	73	0	0	0	0	0	0	0	0	0	0	0	0	3.8	2.0	0	0
	100	100.0	91.7	65.0	100.0	83.3	100.0	100.0	71.4	84.1	92.9	93.8	100.0	96.2	98.0	100.0	100.0
	108	0	0	35.0	0	0	0	0	0	0	0	0	0	0	0	0	0
	117	0	0	0	0	0	0	0	0	15.9	0	0	0	0	0	0	0
MDH-2	56	0	2.8	0	0	0	0	0	0	15.9	0	6.3	0	0	0	0	0
	72	100.0	88.9	100.0	20.0	0	0	0	0	15.9	100.0	93.8	0	34.6	50.0	0	0
	81	0	5.6	0	20.0	0	0	0	0	0	0	0	0	0	0	4.2	0
	87	0	2.8	0	30.0	0	0	0	0	0	0	0	0	0	0	0	0
	100	0	0	0	0	34.6	100.0	100.0	100.0	68.2	0	0	100.0	61.5	50.0	95.8	35.3

TABLE 4 (*continued*)

Locus	Rel. mob.	Populations															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	108	0	0	0	30.0	0	0	0	0	0	0	0	0	0	0	0	0
	138	0	0	0	0	0	0	0	0	0	0	0	0	3.8	0	0	0
	151	0	0	0	0	65.4	0	0	0	0	0	0	0	0	0	0	64.7
MDR	90	0	22.2	0	0	0	0	0	0	0	0	0	7.7	0	0	2.1	0
	100	100.0	77.8	65.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	86.5	100.0	98.0	97.9	100.0
	113	0	0	35.0	0	0	0	0	0	0	0	0	0	0	2.0	0	0
	123	0	0	0	0	0	0	0	0	0	0	0	5.8	0	0	0	0
ME-2	64	0	20.0	0	0	0	0	0	0	0	0	0	5.8	0	0	0	0
	77	0	0	0	0	0	0	0	0	0	11.1	0	0	0	0	0	0
	83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.2	0
	88	0	0	0	0	0	0	0	0	4.5	0	0	0	0	0	0	0
	93	0	0	0	0	0	0	0	0	0	4.4	0	0	0	0	0	0
	100	100.0	80.0	100.0	100.0	100.0	100.0	100.0	100.0	95.5	84.4	100.0	94.2	100.0	100.0	95.8	100.0
6-PGDH-1	81	0	20.0	10.0	0	0	0	0	0	13.6	0	0	0	0	0	0	0
	84	0	0	0	0	0	0	0	0	0	6.3	100.0	0	100.0	100.0	0	0
	100	100.0	80.0	85.0	100.0	100.0	0	0	100.0	86.4	81.3	0	0	0	0	0	100.0
	106	0	0	5.0	0	0	0	0	0	0	4.2	0	0	0	0	0	0
	108	0	0	0	0	0	100.0	100.0	0	0	8.3	0	100.0	0	0	100.0	0
6-PGDH-2	86	0	0	0	15.0	0	0	0	0	0	0	0	0	0	0	0	0
	100	100.0	100.0	100.0	85.0	100.0	60.0	0	100.0	100.0	100.0	100.0	0	100.0	100.0	0	100.0
	107	0	0	0	0	0	40.0	100.0	0	0	0	0	100.0	0	0	100.0	0
PGI-2	32	0	0	0	0	0	0	6.3	0	0	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.2	0
	78	0	5.0	0	65.0	0	0	0	0	0	3.1	0	0	0	0	0	0
	84	0	5.0	0	0	0	0	0	0	0	0	0	0	0	0	25.0	0
	100	100.0	90.0	100.0	20.0	0	0	0	100.0	100.0	96.9	100.0	0	100.0	100.0	2.1	0
	116	0	0	0	0	100.0	0	0	0	0	0	0	0	0	0	0	100.0
	125	0	0	0	15.0	0	100.0	93.8	0	0	0	0	100.0	0	0	68.8	0
PGM-1	94	18.2	0	0	80.0	0	0	0	57.1	0	0	0	0	0	0	18.8	0
	100	72.7	97.2	2.5	0	94.7	80.0	100.0	0	100.0	90.0	100.0	100.0	0	0	77.1	100.0
	102	0	2.8	0	0	0	0	0	0	0	8.9	0	0	0	14.0	0	0
	105	0	0	97.5	10.0	5.3	20.0	0	21.4	0	0	0	0	100.0	86.0	4.2	0
	108	9.1	0	0	10.0	0	0	0	0	0	1.1	0	0	0	0	0	0
	114	0	0	0	0	0	0	0	21.4	0	0	0	0	0	0	0	0

TABLE 4 (*concluded*)

Locus	Rel. mob.	Populations 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
PGM-2	74	100.0	100.0	100.0	0	100.0	0	0	0	0	100.0	0	0	0	0	0	0
	100	0	0	0	0	0	100.0	100.0	100.0	80.0	0	100.0	100.0	0	100.0	100.0	100.0
	106	0	0	0	100.0	0	0	0	0	20.0	0	0	0	100.0	0	0	0
SKDH	86	86.4	0	100.0	15.0	0	0	0	0	29.5	0	0	1.9	38.5	0	0	100.0
	93	0	0	0	0	0	0	0	0	0	0	0	1.9	3.8	0	0	0
	100	13.6	100.0	0	85.0	100.0	100.0	100.0	100.0	70.5	97.8	100.0	76.9	57.7	100.0	100.0	0
	106	0	0	0	0	0	0	0	0	0	1.1	0	0	0	0	0	0
	115	0	0	0	0	0	0	0	0	0	1.1	0	1.9	0	0	0	0
	142	0	0	0	0	0	0	0	0	0	0	0	17.3	0	0	0	0

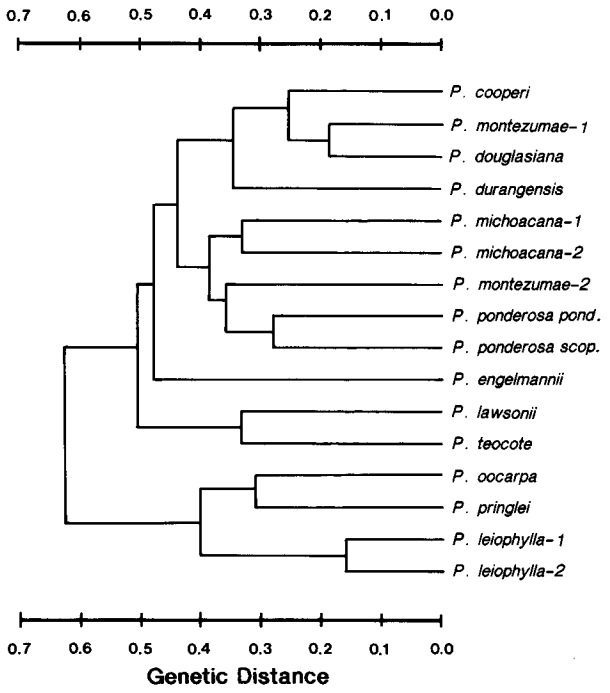

FIG. 2. UPGMA phenogram of 14 pine taxa in Subgenus *Diploxylon* using the Cavalli-Sforza and Edwards (1967) chord distance.

supported by isozyme analysis. The former two taxa do not appear related to section *Serotinos* (Martínez 1948) but to a group of pines placed in subsection *Ponderosae* (Critchfield and Little 1966). The cluster composed of *P. oocarpa* and *P. pringlei* joins the *P. leiophylla* branch at a genetic distance value of 0.4. Although distantly related, this analysis may reflect an affinity between these groups (sections) that has to date not been recognized.

The sectional placement of the remaining eight pine taxa (*P. cooperi*, *P. douglasiana*, *P. durangensis*, *P. engelmannii*, *P. michoacana*, *P. montezumae*, *P. ponderosa* var. *ponderosa*, and *P. ponderosa* var. *scopulorum*) varies among the treatments shown in Table 1. These taxa are placed in three sections by Martínez (1948), two sections by Farjon (1984), and one section by Critchfield and Little (1966). Farjon (1984) and Martínez (1948) recognized the relationships between *P. ponderosa* (two varieties) and *P. engelmannii* and placed these taxa (and others) within subsection *Ponderosae* and section *Ponderosa*, respectively. Unlike these treatments, the isozyme analysis showed a distant relationship between this accession of *P. engelmannii* and the ponderosa pines. The cluster composed of *P. cooperi*, *P. douglasiana*, *P. durangensis*, and *P. montezumae*-1 (with the exception of *P. michoacana*) corresponds to subsection *Pseudostrobi* (Farjon 1984). The two populations of *P. michoacana* do not cluster with Section *Montezumae*; instead they join the ponderosa pines at a genetic distance of 0.38. The two populations of *P. montezumae* also do not cluster together. *Pinus montezumae*-2 shows greater affinity for the ponderosa pines than to the cluster including *P. montezumae*-1 (see Discussion).

Cladistic analyses of isozyme data

The distance Wagner procedure (Farris 1972) was employed using the Prevosti genetic distance measures (Wright 1978) given in Table 5. The shortest tree obtained (1.938) is shown in Fig. 3. The topology of this tree has many features in

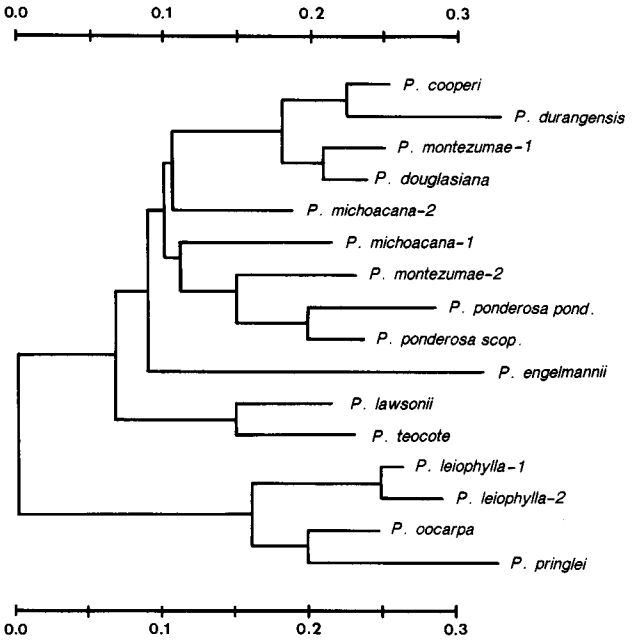

FIG. 3. Distance Wagner tree generated from the Prevosti (Wright 1978) genetic distance matrix. OTUs were added according to the multiple addition criterion (Swofford 1981); the goodness-of-fit criterion used the *F* value of Prager and Wilson (1976); the maximum number of partial networks saved during distance Wagner operation was 20; and the tree was rooted using the midpoint method of Farris (1972). The total tree length was 1.938 (shortest found) with a cophenetic correlation of 0.969.

common with the UPGMA phenogram, i.e., the relationships among *P. lawsonii* and *P. teocote*, *P, oocarpa* and *P. pringlei*, and the two populations of *P. leiophylla*. Both methods succeed in placing the two varieties of *P. ponderosa* as sister taxa. Differences include the closer relationship between *P. cooperi* and *P. durangensis* than between the former taxon and the cluster composed of *P. douglasiana* and *P. montezumae*-1. None of the cladograms obtained using the Prevosti distance matrix (or any other genetic distance matrix available through BIOSYS) represented the two populations of *P. michoacana* as sister taxa. Unlike the UPGMA phenogram, *P. michoacana*-2 cluster with *P. cooperi*, *P. douglasiana*, *P. durangensis*, and *P. montezumae*-1. This grouping corresponds to subsection *Pseudostrobi* of Farjon (1984). Although *P. engelmannii* occupies a similar relative position in Figs. 2 and 3, several trees with high cophenetic correlations and nearly equal length were found that placed this pine basal to the clade corresponding to subsection *Pseudostrobi* (Farjon 1984).

Discussion

Few of the current classifications of *Pinus* provide information on intersectional relationships (or equivalent levels). Farjon (1984), using morphological features of 92 pine species, depicted phylogenies by means of "cladograms." Unfortunately, the cladistic methodology used was not clearly stated. The hierarchical relationships derived from these analyses are represented in Table 1. The cladograms (Farjon 1984, p. 202), section *Leiophylla* is shown as a discrete group diverging from the majority of the genus during the Mesozoic. The clade representing section (*Pinaster*) is shown diversifying into eight subsections during the Tertiary. Within *Pinaster*,

TABLE 5. Matrix of genetic distance coefficients for 16 *Pinus* populations[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 *P. cooperi*		0.262	0.275	0.442	0.458	0.661	0.689	0.448	0.356	0.243	0.430	0.649	0.459	0.480	0.679	0.477
2 *P. douglasiana*	0.137		0.212	0.368	0.251	0.540	0.569	0.290	0.222	0.189	0.397	0.626	0.485	0.461	0.658	0.539
3 *P. durangensis*	0.131	0.375		0.496	0.528	0.699	0.743	0.486	0.444	0.371	0.513	0.704	0.437	0.466	0.731	0.536
4 *P. engelmannii*	0.349	0.460	0.427		0.546	0.627	0.663	0.458	0.464	0.464	0.539	0.660	0.468	0.521	0.668	0.572
5 *P. lawsonii*	0.287	0.427	0.385	0.424		0.604	0.638	0.483	0.474	0.425	0.537	0.604	0.555	0.546	0.640	0.338
6 *P. leiophylla*-1	0.569	0.641	0.658	0.540	0.491		0.165	0.578	0.566	0.626	0.586	0.382	0.628	0.575	0.455	0.603
7 *P. leiophylla*-2	0.609	0.669	0.712	0.587	0.525	0.058		0.622	0.597	0.654	0.616	0.349	0.678	0.629	0.434	0.633
8 *P. michoacana*-1	0.293	0.445	0.338	0.343	0.315	0.432	0.486		0.334	0.438	0.425	0.587	0.416	0.334	0.597	0.496
9 *P. michoacana*-2	0.232	0.360	0.318	0.343	0.316	0.447	0.474	0.202		0.369	0.368	0.556	0.416	0.395	0.595	0.432
10 *P. montezumae*-1	0.113	0.069	0.209	0.359	0.246	0.522	0.556	0.278	0.219		0.374	0.622	0.462	0.434	0.652	0.537
11 *P. montezumae*-2	0.284	0.244	0.383	0.431	0.377	0.444	0.471	0.253	0.233	0.229		0.432	0.406	0.301	0.616	0.536
12 *P. oocarpa*	0.554	0.509	0.648	0.577	0.486	0.215	0.166	0.447	0.416	0.515	0.581		0.641	0.594	0.307	0.587
13 *P. pond* var. *pond.*	0.317	0.351	0.305	0.364	0.418	0.542	0.595	0.268	0.284	0.332	0.248	0.538		0.277	0.664	0.568
14 *P. pond* var. *scop.*	0.331	0.309	0.325	0.423	0.390	0.449	0.503	0.182	0.259	0.289	0.139	0.463	0.126		0.610	0.552
15 *P. pringlei*	0.629	0.603	0.739	0.614	0.567	0.314	0.276	0.505	0.512	0.593	0.518	0.180	0.619	0.526		0.637
16 *P. teocote*	0.294	0.382	0.388	0.463	0.148	0.483	0.510	0.329	0.285	0.376	0.371	0.455	0.425	0.383	0.558	

[a]Above diagonal: Cavalli-Sforza and Edwards (1967) chord distance; below diagonal: Prevosti distance (Wright 1978).

subsections of interest in this study are *Oocarpae*, *Pseudostrobi*, and *Ponderosae*. Unfortunately, these subsections are shown arising from a polychotomy; hence, it is impossible to determine their mutal affinities. Critchfield and Little (1966) placed all but three taxa (*P. leiophylla*, *P. oocarpa*, and *P. pringlei*) into section *Ponderosae*, which provides no further information on interspecific and intersectional relationships.

The genetic distances and phylogenetic relationships shown in the phenogram (Fig. 2) and the cladogram (Fig. 3) can be used to address intersectional affinities. Section *Leiophylla*, treated in all classifications as a distinct group (Table 1), is also shown to be distinct using isozymes. Phenetic and cladistic analysis of electrophoretic data indicate that *P. oocarpa* and *P. pringlei* are a distinct group, separate from *P. lawsonii* and *P. teocote*. This classification is in accord with Martínez (1948), who placed these pines within section *Serotinos*. In addition, isozyme evidence indicates an affinity between sections *Serotinos* and *Leiophylla*. The latter section has traditionally been separated from others based upon the deciduous (vs. persistent) fascicle sheath. How well this character reflects sectional or phylogenetic differences is not known.

The isolation of *P. engelmannii* from the ponderosa pines is surprising given their morphological similarities and ability to form natural hybrids (Peloquin 1971). The affinities of this pine to members of the *Montezumae* complex such as *P. durangensis* and *P. cooperi* have been noted by Martínez (1948, Fig. 169, p. 211), Mirov (1967, p. 557), and Farjon (1984, p. 206). Unlike *P. ponderosa* and other members of the section, *P. engelmannii* lacks Δ^3-carene (Mirov 1967, p. 539). These data suggest that *P. engelmannii* may have undergone significant genetic differentiation relative to members of the sections *Montezumae* and *Ponderosa*. Alternatively, the small number (10) of individuals examined may not provide a representative sample of genetic variation within the species as a whole. Further systematic studies are needed to determine the relationships among *P. engelmannii* and other Mexican pines.

The separation of *P. michoacana* from section *Montezumae* in the phenetic analysis may be anomalous or may reflect actual genetic divergence. In the cladistic analysis, a relationship to subsection *Pseudostrobi* (Farjon 1984) or section *Montezumae* (Martínez 1948) is more evident for *P. michoacana*-2 than for *P. michoacana*-1 (Fig. 3). It is notable that *P. michoacana*-2 (from Chiapas) represents variety *cornuta* whereas the Oaxacan population is the typical variety. Within the *Montezumae* complex, Martínez (1948) recognized three groups (Table 1). Group *Michoacana* includes the typical variety, var. *cornuta*, and var. *quevedoi*. Within these varieties, several forms have also been named, further indicating a high degree of morphological variation in this taxon. It is possible that the taxa included within *P. michoacana* as a group merit independent sectional rank and that more than one species is present within the complex itself.

The pine collected in Veracruz, Mexico (Nickrent 2017) is tentatively identified as *P. montezumae*-2, but it may have affinity with *P. pseudostrobus*. The site, approximately 15 km west of Jalapa, harbors a rich assemblage of pine species. Among others, Perry (1987) lists the following as occurring in this vicinity: *Pinus montezumae*, *P. nubicola*, *P. oaxacana*, *P. pseudostrobus*, and *P. rudis*. In mixed stands where these species occur together, morphologically intermediate individuals can be seen, indicating hybridization and backcrossing (Perry 1987). The ability of various members of sections *Montezumae* and *Pseudostrobus* (Martínez 1948) to form natural hybrids obscures species boundaries and may partially account for the position of *P. montezumae*-2 shown in Figs. 2 and 3. Until more detailed populational work can be conducted, the concept of Critchfield and Little (1966) that includes a large number of the Mexican five-needled pines within one subsection (*Ponderosae*) may more accurately depict genetic relationships among these closely related, recently evolved species.

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