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Relationships among the Spruces (*Picea*, Pinaceae) of Southwestern North America

F. Thomas Ledig,[1,4] Paul D. Hodgskiss,[1] Konstantin V. Krutovskii,[1] David B. Neale,[1] and Teobaldo Eguiluz-Piedra[2,3]

[1]Institute of Forest Genetics, Pacific Southwest Research Station, USDA Forest Service, and Department of Environmental Horticulture, University of California, One Shields Avenue, Davis, California 95616;
[2]Centro de Genética Forestal, Universidad Autónoma Chapingo, Apartado Postal No. 37, Chapingo, México, C.P. 56230, México;
[3]Present address: Grupo Genfor, Calle Huautla No. 109, San Luis Huexotla, Texcoco, México, C.P. 56230, México;
[4]Author for correspondence (e-mail: tledig@ucdavis.edu)

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Abstract. Numerous populations from six spruce taxa, including four relict endemics, *Picea chihuahuana* (Chihuahua spruce), *P. martinezii* (Martínez spruce), *P. mexicana* (Mexican spruce), and *P. breweriana* (Brewer spruce), and two widespread species, *P. engelmannii* (Engelmann spruce) and *P. pungens* (blue spruce), were compared at homologous isozyme loci to test various hypotheses about their affinities and origins. Each of the species was clearly separated, and Neighbor-Joining and Unweighted Pair Group analyses of Nei's genetic distance grouped all populations within a taxon into their own clusters. Spruces from Flys Peak, Chiricahua Mountains, Arizona, joined a *P. engelmannii* cluster and were not a bridge to *P. mexicana* as previously believed. Spruces from Cerro Mohinora, Chihuahua, were clearly *P. mexicana*, not phantom hybrids of *P. chihuahuana* and *P. pungens*. Nuclear random amplified polymorphic DNA and chloroplast simple sequence repeat and cleaved amplified polymorphic genetic markers were compared in a smaller sample of populations, using distance and parsimony approaches. DNA markers, like isozymes, clearly identified spruces from Cerro Mohinora as *P. mexicana*. In contradiction to the most recent taxonomic treatment, *P. chihuahuana* and *P. martinezii* were separated as distinct species by both isozyme and DNA markers, and formed a sister-species group. *Picea engelmannii* and *P. mexicana* formed a separate cluster, and the genetic distance between them was similar to values associated with closely related species but greater than distances typical of subspecies or varieties in conifers. *Picea pungens*, which is so similar to *P. engelmannii* that the two are frequently misidentified, was clearly distinguished from it, sometimes joining a *P. chihuahuana-martinezii* group and sometimes a *P. engelmannii-mexicana* group, depending on analysis. *Picea breweriana* was well isolated from all other taxa. Both DNA and isozyme phylogenies agreed with results from crossability studies and contradicted intrageneric relationships constructed largely on cone morphology.

Spruce (*Picea* A. Dietr.) is a taxonomically difficult genus because the species encompass a relatively narrow range in morphology and ecological preference (Wright 1955; Taylor and Patterson 1980; Rehfeldt 1994). Taxonomists have encountered problems in delimiting species and constructing phylogenies, and early attempts at dividing the genus into sections and series were based on comparisons of few species (as reviewed by Aldén 1987). Intrageneric classifications based on morphology (Bobrov 1970; Schmidt 1989) made implausible groupings when compared to the results of controlled hybridization, which are assumed to reflect genetic similarities and differences. The situation is complicated by hybridization in areas of sympatry (Roche 1969; Bobrov 1970; Daubenmire 1974; Taylor et al. 1975; Gordon 1976b; Krutovskii and Bergmann 1995; Rajora and Dancik 1999).

Spruces belong to the Pinaceae, and include 28 to 50 species, depending on the taxonomic authority (Wright 1955; Everett 1981; Schmidt 1989). Most taxonomists have accepted about 36 to 37 species (Bobrov 1970; Schmidt-Vogt 1977; Rushforth 1987; Schmidt 1989), to which we would add *Picea martinezii* T.F. Patterson (Martínez spruce), described in 1988 (Patterson 1988). The recent conifer checklist (Farjon 2001) recognized 34 species, three subspecies, and 15 varieties, but considered *P. martinezii* conspecific with *P. chihuahuana* Martínez (Chihuahua spruce). Most species are Asian, and found predominantly in boreal and cool temperate or montane biomes. In Taiwan and México, montane species extend south of the Tropic of Cancer.

Of a total ten taxa in North America, six occur in the southwestern United States and México (Fig. 1). Four of the six are relicts, based on fossil evidence or biogeography, and are now rare endemics (Wolfe 1964; Lozano-García 1993; Ledig et al. 2000b); i.e., *P. martinezii*, *P. chihuahuana*, *P. mexicana* Martínez (Mexican spruce), and *P. breweriana* S. Wats. (Brewer spruce). In all of the three Mexican species, trees number only in the hundreds to thousands (Ledig et al. 2000b). Because of their rarity, few studies of any kind have been published on *P. breweriana*, *P. martinezii*, *P. chihuahuana*, or *P. mexicana*.

Picea pungens Engelm. (blue spruce) is more widespread than the endemic relicts. It occurs in the Rocky Mountains, primarily in Wyoming, Utah, Colorado, and New Mexico, with disjunct outliers as far north as northern Montana, as far west as the Great Basin, and

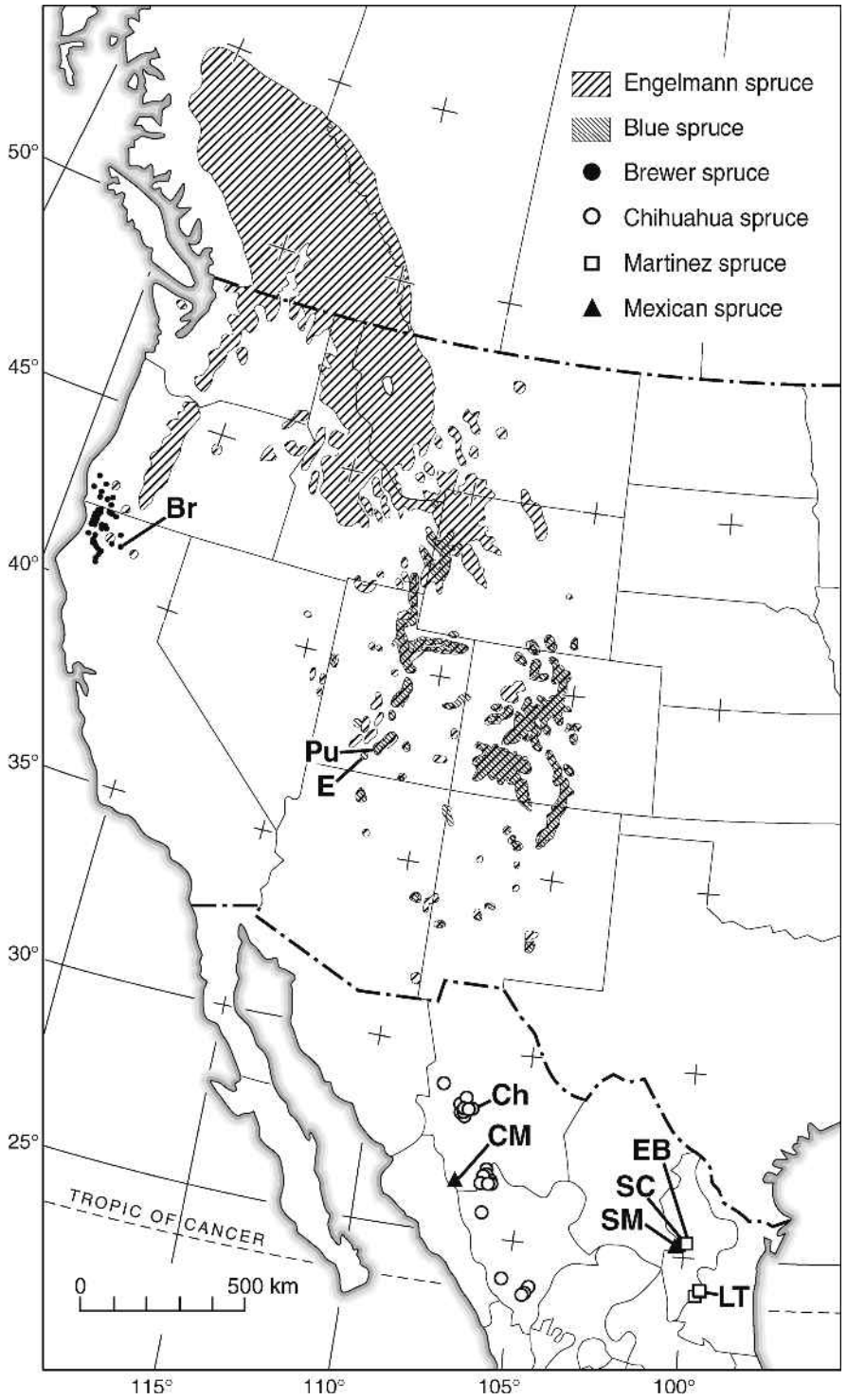

FIG. 1. The natural distributions of *P. engelmannii*, *P. pungens*, *P. breweriana*, *P. chihuahuana*, *P. martinezii*, and *P. mexicana* in western North America (after Little 1971, Griffin and Critchfield 1976, and Ledig et al. 2000b), with location of sampling sites used for DNA markers: *P. chihuahuana* (**Ch**), *P. martinezii*—Cañon el Butano (**EB**) and La Tinaja (**LT**); *P. mexicana*—Sierra el Coahuilón (**SC**), Sierra la Marta (**SM**), and Cerro Mohinora (**CM**); *Picea engelmannii* (**E**); *P. pungens* (**Pu**); *P. breweriana* (**Br**).

as far south as southern Arizona (Little 1971). *Picea engelmannii* Parry ex Engelm. (Engelmann spruce) has the widest range of any species in the group, extending from the southernmost United States to northern British Columbia, Canada. *Picea engelmannii* is predominantly a species of the Rocky Mountains, but is also found on the east slope of the Coast and Cascade Ranges through British Columbia, Washington, and Oregon, southward to an outlier in the Siskiyou Mountains of California, and on montane sky islands of the Great Basin (Little 1971).

Based on its distribution, *P. mexicana* appears to be a relict stranded on the highest peaks of the Sierra Madre Oriental and the Sierra Madre Occidental by warming temperatures. It occurs at only three confirmed locations, on Sierra la Marta (3,500 m) and Sierra el Coahuilón (3,470 m) in the Sierra Madre Oriental and 676 km away on Cerro Mohinora (3,185 m) in the Sierra Madre Occidental. *Picea mexicana* has been called a variety of *P. engelmannii* (var. *mexicana* (Martínez) Silba) by Taylor and Patterson (1980). However, others (A. G. Gordon, pers. comm. 1988) felt that the differences between *P. mexicana* and *P. engelmannii* were sufficient to warrant their recognition as distinct species. The spruces on Cerro Mohinora remained problematic for several years after they were first observed by Correll (1960). They were popularly known as *P.* ''indeterminada'' (J. Sánchez-Cordova pers. comm. 1988) and *P.* ''hybrida'' (Taylor and Patterson 1980), alluding to the confusion over whether they were phantom hybrids of *P. engelmannii* with *P. pungens* and/or the local *P. chihuahuana*. *Picea chihuahuana* occurs only 57 km away from the spruces on Cerro Mohinora, but the nearest populations of *P. pungens* are about 780 km distant in southern Arizona. Initially, Taylor and Patterson (1980) leaned toward a hybrid origin, but later considered the spruces from Cerro Mohinora conspecific with *P. mexicana* (Taylor et al. 1994). Spruce from the Chiricahua Mountains of southern Arizona (e.g., Flys Peak) could be a link between *P. engelmannii* and *P. mexicana*, or could even be *P. mexicana*, because they have greater morphological affinity to *P. mexicana* than to *P. engelmannii* from central Arizona (Taylor and Patterson 1980).

Picea chihuahuana has a north-south range of 687 km in the Sierra Madre Occidental of México. Within this range, however, it occurs only in scattered populations that range in size from 11 to 2,342 trees $\geq$ 10 cm diam-breast-high (Ledig et al. 2000b). Populations differ extensively in allele frequency as a result of random genetic drift, suggesting their relictual status (Ledig et al. 1997). Based on pollen evidence, *Picea* spp. occurred at least 500 km further south than the present distributions of *P. chihuahuana* and *P. martinezii* as recently as 8,000 years ago (Lozano-García et al. 1993), and apparently retreated northward during Holocene warming.

Botanists discovered *P. martinezii* in two locations in the Sierra Madre Oriental in 1984, but reported the discovery as new records of *P. chihuahuana* (Müller-Using and Alanís-Flores 1984; Müller-Using and Lässig 1986). After further observation, Patterson (1988) decided that *P. martinezii* was sufficiently different from *P. chihuahuana* in needles, pulvini, and margins of the cone scales to name it a new species. More recently, we have recorded a few new populations of *P. martinezii* (Ledig et al. 2000b) in addition to the original discoveries by Müller-Using and Alanís-Flores (1984).

Picea breweriana is a relict of the widespread Arcto-Tertiary flora, now confined to scattered ridges and north slopes in the western Klamath Geological Province (Wolfe 1964; Griffin and Critchfield 1976; Waring 1969; Waring et al. 1975). It has a north-south range of about 220 km and outliers may occur as far as 150 km from the Pacific coast. It is easily recognized by its large cones and weeping habit.

We believe that morphology is not a reliable guide to relationships within *Picea*. For example, because of the difficulty in identifying *P. pungens* and *P. engelmannii* when the two occur in sympatry, widespread hybridization was long invoked, suggesting a close relationship (Habeck and Weaver 1969; Daubenmire 1972; Taylor et al. 1975). However, the cross proved difficult to make, and molecular data has revealed no, or few, natural hybrids (Ernst et al. 1990). Because of the difficulty in making controlled crosses between *P. engelmannii* and *P. pungens*, a high rate of abnormalities in the hybrid progeny, and a paucity of natural hybrids (Ernst et al. 1990), *P. engelmannii* and *P. pungens* seem to be good biological species in the sense of Mayr (1963).

Picea breweriana and *P. pungens* have cones in the 6–12 cm range, and *P. chihuahuana* and *P. martinezii* in the range 8.5–16 cm, while those of *P. engelmannii* and *P. mexicana* are smaller, about 3–7 cm (Martínez 1953, 1961; Taylor 1993). *P. breweriana* is distinguished by its needles that are flattened, blunt at the apex and have stomata restricted to the adaxial surface, while all the other species in this study have quadrangular needles in cross-section, apices that are acuminate to sharply acuminate, and stomata on all surfaces (Weng and Jackson 2000). *Picea breweriana*, *P. chihuahuana*, and *P. martinezii* all have two continuous resin ducts, which distinguishes them from the other species, which have discontinuous resin ducts (mostly 3–4 in *P. pungens* and mostly 2, or occasionally none, in *P. engelmannii* and *P. mexicana*; Weng and Jackson 2000).

Because morphology results in questionable groupings, and to develop a more robust phylogeny, it is critical that the characteristics analyzed directly reflect the genome. Although molecular markers have been

used in several attempts to clarify spruce phylogeny, none of these attempts have included *P. martinezii* (e.g., Wellendorf and Simonsen 1979; Sigurgeirsson 1992; Nkongolo 1999). Furthermore, few have truly taken intraspecific variation among natural populations into account, and intraspecific differences may be important in relict species whose genetic structure has been shaped by random genetic drift (e.g., Ledig et al. 1997). As part of a long-term study of the endemic spruces of southwestern North America, we accumulated comparable data on allele frequencies at isozyme loci for all four endemics, and for *P. pungens* and *P. engelmannii*.

Isozymes are a class of polypeptide markers that can be separated by electrophoresis and are fairly directly related to differences at the DNA level. However, in theory only ca. 30% of the possible amino acid substitutions result in a difference in charge (Shaw 1970). Therefore, some genetic variation among taxa might go undetected. Furthermore, isozymes provide only a small number of markers and represent a biased set of gene loci, predominantly those involved in the glycolitic pathway or intermediary metabolism. By contrast, the number of DNA markers is almost unlimited, except by constraints of cost, and DNA markers are probably distributed randomly throughout the genome. While isozymes provide no information on the number of evolutionary steps separating one allele from another, DNA markers may reflect differences at a single nucleotide pair. Therefore, we also used restriction fragment length polymorphisms of PCR amplified chloroplast DNA (cpRFLP) or chloroplast cleaved amplified polymorphisms (cpCAP), chloroplast microsatellites or simple sequence repeats (cpSSR), and nuclear RAPDs to compare samples and bolster conclusions based on isozymes.

Our specific objectives were: to determine whether the genetic differences between *P. engelmannii* and *P. mexicana* were typical of the varietal level or the species level; to examine the genetic relationship of spruces from Cerro Mohinora to other populations of *P. mexicana*; to determine whether spruces from the Chiricahua Mountains were aligned with *P. mexicana* or with *P. engelmannii*; to determine the genetic relationship between *P. martinezii* and *P. chihuahuana*; to compare the relictual *P. breweriana* with the Mexican relicts; and to confirm or reject the genetic differences reported between *P. engelmannii* and *P. pungens* using a broad sample of populations, because previous studies on molecular genetic differences between the two were concentrated in a single drainage, the Dolores River, Colorado (Ernst et al. 1990). We also wished to compare our results on genetics of natural populations to the classifications of spruce based on morphological criteria, usually measured on herbarium specimens or on arboretum specimens planted outside their native ranges, which might lead to unknown biases.

Comparisons using isozymes allowed us to address whether the six taxa were genetically distinct entities and answered questions about relationships among populations. DNA markers provided additional support with regard to relationships among species.

We are using the biological species concept and our criterion to delimit species is reproductive isolation, identified by a gap (e.g., quantified by Nei's genetic distance) that suggests reduced gene flow. Our techniques are equally applicable to a phylogenetic species concept (Luckow 1995).

Materials and Methods

Isozymes. Plant Materials. We compared nine populations of *P. breweriana*, four of *P. pungens*, ten of *P. chihuahuana*, ten of *P. engelmannii*, two of *P. martinezii*, and three of *P. mexicana* (Table 1). Samples for *P. martinezii* and *P. mexicana* represented all the populations known at the time. Further details on the locations of *P. chihuahuana*, *P. martinezii*, and *P. mexicana* populations were provided in Ledig et al. (1997, 2000a, b, 2002). Papers are in preparation on genetic diversity in *P. breweriana*, *P. pungens*, and *P. engelmannii*, and detailed coordinates for these samples will be reported there.

Electrophoresis. Cones were collected, seeds extracted, germinated, and extracts of soluble proteins prepared for electrophoresis over a period of 20 yr. We used starch gel electrophoresis to assay isozymes (Conkle et al. 1982).

Seeds were germinated in petri dishes, and when radicles emerged, megagametophytes and embryos were dissected, separated, and extracted. For *P. breweriana*, *P. chihuahuana*, *P. martinezii*, and *P. mexicana*, seeds were kept separate by seed tree, and at least six megagametophytes from each seed tree were used in electrophoresis to infer parental tree genotype. The number of genomes (twice the number of trees) varied from 16 to 76 per population, but was generally around 50 (Table 1). Genotypic frequencies of seed trees were used to calculate allele frequencies for the populations. For *P. engelmannii* and *P. pungens*, except for *P. engelmannii* from Flys Peak, Chiricahua Mountains, Arizona, we assayed 50 to 60 haploid megagametophytes per population from bulked seed collections of many trees without knowledge of parentage; allele frequencies were recorded. For Flys Peak, seeds were available from only seven trees, so we recorded pollen allele frequencies by assaying megagametophytes and embryos side by side.

In spruce, the nutritive tissue of the seed, or megagametophyte, consists of haploid cells that have the same haplotype as the egg. Therefore, the genotype of a seed parent can be determined by sampling multiple megagametophytes. When two different alleles at a locus segregate, the seed parent is unequivocally a heterozygote. When only one allele is detected, the tree is classified as a homozygote, although the possibility remains that it is a heterozygote and by chance the sampled seeds included only one allele. The probability of misclassification decreases with increase in sample size. With a sample of six megagametophytes from the same tree, there is a probability of 0.03125 of misclassifying a heterozygote as a homozygote. That is, the probability that all six megagametophytes in a sample from a heterozygous tree carry the same allele is $2(½)^6 = 0.03125$, assuming random 1:1 segregation of alleles. Knowing the contribution of the egg (the haploid genotype of the megagametophyte) to the embryo, the pollen contribution can be deduced by subtraction. At Flys Peak, allele frequencies were very similar for the pollen (85 to 175 genomes per locus) and the seven seed trees (14 genomes), except that more low-frequency alleles were detected in the pollen than in the megagametophytes because of the larger sample size for the pollen.

Methods and reagents changed subtly during the course of the spruce investigations, and enzymes that were well resolved in one species were not always scorable in another. To reconcile results

TABLE 1. Spruce populations included in isozyme studies (mean number of genomes sampled per locus in parentheses).

Picea pungens. 1. **Rudd Knoll**, Apache-Sitgreaves National Forest, Arizona (49.3) 2. **Scotch Creek**, San Juan National Forest, Colorado (50.0) 3. **Wildcat** Guard Station, Dixie National Forest, Utah (39.4) 4. **West Fork** Little Colorado River, Apache-Sitgreaves National Forest, Arizona (49.3)

P. breweriana. 1. **Little Grayback**, Klamath National Forest, California (39.4) 2. **Doolittle Creek**, Klamath National Forest, California (39.2) 3. **Poker Flat**, Klamath National Forest, California (38.6) 4. **Prescott Cabin**, Six Rivers National Forest, California (40.2) 5. **Russian Peak**, Klamath National Forest, California (40.8) 6. **Rock Creek Butte**, Klamath National Forest, California (39.8) 7. **Baldy Mountain**, Klamath National Forest, California (40.0) 8. **Flattop**, Siskiyou National Forest, Oregon (38.0) 9. **Iron Mountain**, Siskiyou National Forest, Oregon (76.2)

P. chihuahuana. 1. **Arroyo de la Pista**, Durango (46.0) 2. **Arroyo del Infierno**, Durango (30.0) 3. **Faldeo de Cebollitas**, Durango (50.0) 4. **Arroyo del Indio Ignacio**, Durango (36.2) 5. **Rio Vinihueachi**, Chihuahua (37.4) 6. **Talayotes**, Chihuahua (16.0) 7. **Cerro de la Cruz**, Chihuahua (23.6) 8. **El Realito**, Chihuahua (28.6) 9. **La Tinaja**, Chihuahua (28.2) 10. **Arroyo Ancho**, Chihuahua (42.6)

P. engelmannii. 1. **Panther Creek**, Salmon National Forest, Idaho (53.0) 2. **Summit Lake**, Payette National Forest, Idaho (48.3) 3. **Beartooth Pass**, Shoshone National Forest, Wyoming (48.6) 4. **Six Bit Spring**, Cache National Forest, Utah (48.3) 5. **Navajo Lake**, Dixie National Forest, Utah (54.0) 6. **East Gavilan Canyon**, Carson National Forest, New Mexico (49.3) 7. **Sierra Blanca**, Lincoln National Forest, New Mexico (54.4) 8. **Barlow Lake**, San Juan National Forest, Colorado (48.4) 9. **San Francisco Mountain**, Coconino National Forest, Arizona (48.4) 10. **Flys Peak**, Coronado National Forest, Arizona (154.0)

P. martinezii. 1. **La Encantada**, Nuevo León (48.4) 2. **Cañon el Butano**, Nuevo León (48.4)

P. mexicana. 1. **Sierra la Marta**, Nuevo León (72.0) 2. **Sierra el Coahuilón**, Coahuila (44.0) 3. **Cerro Mohinora**, Chihuahua (48.0)

of studies that took place over so many years, we performed co-electrophoresis. That is, we ran extracts from the various species together on the same gel. Bands that migrated to the same position on the gel were assumed to represent the same allele for comparisons among species.

We realize that isozymes with the same charge need not necessarily be identical in their amino acid sequence and, therefore, the underlying DNA sequences. However, within a putatively monophyletic genus such as the spruces, identity in migration is almost certain to reflect orthology, and bootstrap analysis accounts for failures in the assumption. The argument for homology is further strengthened by the consistency in number of banding zones among all species in this study, and by structural genomics, which indicates a highly conserved map for isozyme loci even among genera in Pinaceae (Krutovskii, unpubl. data).

Nine loci could be reconciled for all six species: *Gdh, Got1, Idh1, Mdh2, 6Pg2, Pgi2, Pgm, Skd1,* and *Skd2*. However, 13 loci were common to five species, excluding *P. breweriana* and the *P. engelmannii* from Flys Peak. The additional loci were *Got3, Mdh1, Mdh3,* and *Mdh5.*

GENETIC AND PHYLOGENETIC ANALYSIS. BIOSYS-2 (Swofford et al. 1981) was used to calculate Nei's (1978) unbiased genetic distance and modified Rogers' distance among populations, hierarchic F-statistics among populations within species and among species, and chi-square contingency table analyses of heterogeneity among populations within species, and to generate bootstrap data to produce 1,000 matrices of Nei's (1972) genetic distances to perform unweighted pair group (UPGMA) and Neighbor-Joining (NJ) cluster analyses using PHYLIP (Felsenstein 1995).

TABLE 2. Location and elevation of sampling sites for six spruce taxa included in DNA marker studies.

Picea chihuahuana: Rio Vinihueachi, Chihuahua, México, 2160 m. ***P. martinezii***: Cañon el Butano, Nuevo León, México, 2180 m; La Tinaja, Nuevo León, México, 2515 m. ***P. mexicana***: Sierra el Coahuilón, Coahuila, México, 3470 m; Sierra la Marta, Nuevo León, México, 3500 m; Cerro Mohinora, Chihuahua, México, 3185 m. ***P. engelmannii***: Cedar City Ranger District, Dixie National Forest, Utah, USA, 2995 m. ***P. pungens***: Powell Ranger District, Dixie National Forest, Utah, USA, 2415 m. ***P. breweriana***: Castle Crags Wilderness, Shasta-Trinity National Forest, California, USA, 1705 m.

Since TreeBASE does not accommodate raw genotypes, allele frequencies, or distance matrices, the data sets will be provided in electronic format upon request to the senior author.

DNA Markers. PLANT MATERIALS. Three trees from each of nine populations (27 trees in total) were sampled in six spruce taxa. This included *P. chihuahuana* from one of the largest known populations, Rio Vinihueachi, Chihuahua; two populations of *P. martinezii*, representing the northern and southern extremes of its distribution; three of *P. mexicana*, including spruces from Cerro Mohinora, Chihuahua; and one population each of *P. engelmannii*, *P. pungens*, and *P. breweriana*. Location of sampling sites is listed in Table 2 and shown in Fig. 1. Twigs with needles were collected from *P. chihuahuana* on November 21 and 22, 1995; from *P. martinezii* and *P. mexicana* on Sierra la Marta and Sierra el Coahuilón on May 18 to 21, 1996; and from Cerro Mohinora on May 6, 1997. *Picea pungens*, *P. engelmannii*, and *P. breweriana* were collected in July 1997. In the field, samples were placed in plastic bags and put on wet ice in coolers for transport to the laboratory. In the laboratory, the samples were frozen in liquid nitrogen within seven days of collection.

DNA EXTRACTION. Needles were stripped and ground in liquid nitrogen. DNA was isolated from the needles using the FastPrep FP120 instrument for fast and efficient cell/tissue disruption with the FastDNA Kit S (Qbiogene Inc., Carlsbad, California, USA).

CHLOROPLAST CLEAVED AMPLIFIED POLYMORPHISMS. We studied six genes *rbcL, psbA, psbD, frxC, trnK,* and *16S* used previously in the phylogenetic study of conifers by Tsumura et al. (1995). Five of these genes (excepting *psbD*) were amplified in all spruce species in our study using polymerase chain reaction (PCR) primers and conditions described in Tsumura et al. (1995). The amplified PCR product was digested with restriction endonucleases and subjected to electrophoresis in 2% agarose gel in the tris-acetate-EDTA (TAE) buffer to reveal restriction fragment length polymorphisms (RFLPs). We chose restriction endonucleases that revealed polymorphisms in two spruce species sampled in Tsumura et al. (1995).

CHLOROPLAST SIMPLE SEQUENCE REPEATS (CPSSR). We tested our spruce samples with 20 primer pairs, Pt1254, Pt9383, Pt15169, Pt26081, Pt30204, Pt36480, Pt41093, Pt45002, Pt48210, Pt51873, Pt63718, Pt71936, Pt79951, Pt87268, Pt100783, Pt102584, Pt107148, Pt107517, Pt109567, and Pt110048, that are based on the complete chloroplast genome sequence of *Pinus thunbergii* Franco (Japanese black pine; GenBank accession #D17510) and which amplified specific simple sequence repeat (SSR) regions in the chloroplast DNA (cpSSRs) of many different conifer species (Powell et al. 1995; Ven-

dramin et al. 1996). The locus name corresponds to the position of the forward primer in *Pinus thunbergii* cpDNA (Vendramin et al. 1996). All primer pairs flank mononucleotide repeats, but we were able to resolve alleles using high resolution 2.5–3% MetaPhor agarose gels (BioWhittaker Molecular Application (BMA), Rockland, Maine, USA #50180) in the TBE buffer. All primers have a melting temperature (T_m) close to 58°C (see primer sequences in Vendramin et al. 1996 or at http://dendrome.ucdavis.edu/Data/chloroplast.html). Results included in the data set were obtained from only eight primer pairs, Pt15169, Pt30204, Pt36480, Pt63718, Pt71936, Pt87268, Pt107148, and Pt107517, that amplified cpSSR fragments of expected or close to expected size in all spruce species.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD). We tested 64 RAPD Operon primers (Operon Technologies, Alameda, California, USA): A01-A05, A07-A17, A19, A20, B03-B05, B07, B09, B11-B20, C20, K01-K09, K11-K20, L10, L12, L13, L19, M08, N05, N19, P03, Q11, S09, and U02. The DNAs of three individuals per population were pooled together for PCR to maximize detection of species-specific or population-specific markers. Based on PCR performance, 26 RAPD primers, A04, A05, A07, A08, A09, A12, A14, A15, A16, A19, B05, B09, B11, B17, B18, B19, K02, K04, K05, K06, K08, K13, K14, K15, K16, and K18, that yielded 61 markers in total, were included in the final analysis.

PHYLOGENETIC ANALYSIS USING BINARY DATA. The limited sample size did not allow us to infer allele or haplotype frequencies from RAPD and cpDNA data. Therefore, a phenetic approach based on presence/absence of amplified RAPD or cpDNA microsatellite fragments and cpDNA RFLP bands of the same size were used to study genetic similarity among species. This approach is well justified considering that the major objective was to analyze phylogenetic relationships rather than population structure, and also considering the relatively low within-population variation in Mexican spruce species (Ledig 1997, 2000a, 2002).

The Wagner parsimony analysis was performed using binary data, the MIX program, and 1,000 data sets generated by the SEQBOOT program in the PHYLIP package (Felsenstein 1995). The parsimony analysis uses only informative traits that are shared by at least two individuals but not all. To take into account all traits, we also applied distance-based phylogenetic analysis. The similarity indices between species were calculated using the Nei and Li (1985) measure of similarity: $S = 2N_{AB}/(N_A + N_B)$, where N_{AB} is the number of bands that individuals A and B share in common, N_A is the number of bands in individual A, and N_B is the number of bands in individual B. This measure is recommended when comparing different species. It does not take into account the potentially false similarity based on the shared absence of a band, because the assumption that the absence of a band in two individuals arose from the identical ancestral mutation (i.e., recessive alleles are identical in state) is likely violated when individuals from different species are compared. There are potentially many different point mutations at or around the primer annealing sites that could interrupt annealing and lead to an ''absence'' phenotype in PCR amplification. Furthermore, different inversions that involve the annealing sites would also prevent amplification, and large inserts between the annealing sites would make amplification impossible under regular PCR conditions. The assumption that recessive alleles are identical in state is not valid in all these cases, and scoring of a shared recessive phenotype may overestimate relatedness among individuals.

A species-by-species dissimilarity distance (d) matrix was calculated from S, as d = 1—S. All similarity and distance indices were obtained using the RAPDPLOT program version 3 (Black 1997). This program also allowed us to test the statistical support for individual branches using a bootstrap analysis. Bootstrapping was performed via random resampling of our dataset. The goal of bootstrapping was to test the consistency with which our dataset supported the phenetic relationships among taxa. High bootstrap scores (> 90%) suggest strong support for a particular cluster, whereas lower levels of support suggest a lower order of differentiation and the need for additional data to either support a particular branch or collapse that branch. A set of 1,000 dissimilarity distance matrices, based on 1,000 datasets generated by bootstrapping over loci, was produced, and the distance matrices were then used to produce phylogenetic trees using the UPGMA and the NJ methods in the NEIGHBOR clustering program of the PHYLIP package (Felsenstein 1995).

For both isozymes and DNA markers, the majority-rule consensus trees were generated from bootstrap trees in both distance-based and parsimony analyses using the program CONSENSE in the PHYLIP package. The phylogenetic trees were viewed and drawn using the TREEVIEW program (Page 1996).

Multivariate analysis was performed employing principal coordinate analysis (PCA) based on the dissimilarity distance matrix and using the NTSYS computer program (Rohlf 1997).

RESULTS

Isozyme Polymorphism within Species. Differences between taxa were consistent to the degree that all six spruce species could be identified unambiguously by their allelic profiles (Table 3). Cluster analysis using nine loci, Nei's (1972) genetic distance (D), and the NJ method cleanly separated all taxa, and no population from any taxon clustered within another group (Fig. 2). Trees constructed with the distance Wagner procedure and Rogers'; modified genetic distance were very similar, as were results using the UPGMA method (not shown). However, with the UPGMA procedure and only nine loci, one of the *P. mexicana* populations, Cerro Mohinora, was close to *P. engelmannii* and was supported within the *P. mexicana* cluster by a bootstrap value of only about 49%.

Although taxa were distinct, considerable differentiation was evident between populations, especially in *P. breweriana*, *P. engelmannii*, and *P. chihuahuana* (Figs. 2, 3). In *P. engelmannii*, a division into two groups was evident based on 13 loci; one group included populations from the northern Rocky Mountains in Idaho, and the other included populations from Colorado, Utah, Arizona, and New Mexico (Fig. 3). It is notable that Flys Peak, Chiricahua Mountains, Arizona, fell solidly within the *P. engelmannii* group, and not with the *P. mexicana* populations (Fig. 2).

D between populations within species varied from 0.011 to 0.147 in *P. engelmannii* and 0.000 to 0.091 in *P. breweriana* based on nine loci (Table 4). These ranges are much greater than those in the other four species, none of which exceeded 0.032. Results for 13 loci were very similar (Table 4).

Contingency chi-square tests also demonstrated the greater divergence among populations within *P. engelmannii* and *P. breweriana* than among populations within the other four species (Table 5). The tests were significant for all nine polymorphic loci in *P. engelmannii*, and for five of six polymorphic loci in *P. breweriana*. For comparison, contingency chi-square was significant for four of five polymorphic loci in *P. pungens*, two of three in *P. chihuahuana*, one of three in *P. martinezii*, and two of eight in *P. mexicana*. Overall, the variation among populations relative to species (F_{PS}) was 0.127 (Table 6).

Isozyme Phylogeny. *Picea engelmannii* and *P. mexi-*

TABLE 3. Distribution of alleles for nine isozyme loci in *P. engelmannii* (En), *P. mexicana* (Mx), *P. pungens* (Pu), *P. martinezii* (Ma), *P. chihuahuana* (Ch), and *P. breweriana* (Br); 0 = allele not present, 1 = allele fixed in nearly all populations sampled, p = polymorphic allele with a frequency > 0.05 but ≤ 0.95 in at least some populations, + = rare allele present in at least some populations but frequency < 0.05 unless noted; bold type indicates species-specific alleles. Notes: [1] frequency = 0.053 in one of ten populations, absent in remaining nine; [2] frequency = 0.060 in one of four populations, absent in remaining three; [3] frequency = 0.060 in one of eight populations, present in frequency < 0.050 in two, and absent in five; [4] frequency = 0.075 in one of four populations, present in frequency < 0.050 in two, and absent in one; [5] frequency = 0.077 in one of ten populations, absent in remaining nine; [6] frequency = 0.067 in one of eight populations, present in frequency < 0.050 in one, and absent in eight; [7] frequency = 0.065 in one of eight populations, present in frequency < 0.050 in one, and absent in eight.

		Species					
Locus	Allele	En	Mx	Pu	Ma	Ch	Br
Gdh	1	p	1	+	1	1	p
	2	p	0	1	0	0	0
	3	0	0	0	0	0	**p**
Got1	1	1	1	1	1	1	+[1]
	2	+	0	0	0	0	1
	3	**+**	0	0	0	0	0
	4	**+**	0	0	0	0	0
Idh1	1	p	1	1	0	1	1
	2	**+**	0	0	0	0	0
	3	**p**	0	0	0	0	0
	4	**p**	0	0	0	0	0
	5	0	0	0	**1**	0	0
Mdh2	1	p	1	1	1	1	p
	2	**p**	0	0	0	0	0
	3	0	0	0	0	0	**p**
6Pg2	1	p	1	p	1	1	p
	2	+	0	p	0	0	p
	4	**p**	0	0	0	0	0
	5	p	+	0	0	0	0
	6	**+**	0	0	0	0	0
Pgi2	1	**p**	0	0	0	0	0
	2	0	0	0	0	**+**	0
	3	0	0	0	**p**	0	0
	4	+	0	1	p	1	+
	5	p	0	0	0	0	p
	6	p	1	+[2]	0	0	p
	7	**+**	0	0	0	0	0
	8	**+**[3]	0	0	0	0	0
	9	**p**	0	0	0	0	0
Pgm	1	+	0	p	0	p	0
	2	0	p	0	0	0	p
	3	p	p	p	0	0	p
	4	p	0	0	1	0	0
	5	0	0	0	0	**p**	0
Skd1	1	+	0	+	0	p	0
	2	p	0	p	0	0	0
	3	p	p	+[4]	1	0	0
	4	p	0	p	0	0	0
	5	p	p	p	0	+[5]	1
	6	**p**	0	0	0	0	0
	7	**p**	0	0	0	0	0
	8	**+**[6]	0	0	0	0	0
	9	p	0	+	0	p	0
Skd2	1	p	1	1	p	0	0
	2	**p**	0	0	0	0	0
	3	0	0	0	**p**	0	0
	4	0	0	0	0	0	**1**
	5	0	0	0	0	**1**	0
	6	**+**[7]	0	0	0	0	0

cana were the most closely related pair of taxa among the spruces of southwestern North America, especially based on genetic distances for the subset of nine loci (Table 4). Between *P. mexicana* and *P. engelmannii*, D averaged 0.147, with a range from 0.100 to 0.241 for nine loci, depending on which pairs of populations were compared. The mean of 0.147 is half or less the distance associated with any other pair of species (Table 4). The range among the other 15 pairwise combinations of species was 0.336 to 1.457. Results for 13 loci were similar to those for nine (Table 4).

Picea chihuahuana and *P. martinezii* formed another group, but were surprisingly divergent from each other (especially based on 13 loci as in Fig. 3), considering that *P. martinezii* was originally confused with *P. chihuahuana* (Müller-Using and Alanís-Flores 1984; Müller-Using and Lässig 1986).

Picea breweriana was well isolated from the other species (Fig. 2). The position of *P. pungens*, a species often confused with *P. engelmannii* in the field, varied—it joined the *P. engelmannii-mexicana* group in NJ trees based on 13 loci (Fig. 3), but showed more affinity to the *P. chihuahuana-martinezii* group when based on only nine loci (Fig. 2) or in UPGMA trees (not shown) with either 13 or nine loci.

Variation among species relative to the total genetic variation (F_{ST}) was 0.661; i.e., 66.1% of the total genetic diversity was among species (Table 6). For comparison, among 16 populations within the *Pinus ayacahuite* complex, covering a range from Honduras (14°07′ N) to northern Mexico (25°14′) and variously assigned to two species and one variety or to three varieties, F_{ST} was 0.087 and F_{PS} was 0.121, based on 21 isozyme loci (Ledig et al. unpublished data). Thus, although spruce species may lack the morphological distinctions that characterize species in other genera (e.g., among species of *Pinus*), differences in allele frequency at these nine loci were pronounced among the spruce taxa of southwestern North America.

Chloroplast DNA Polymorphism. All species except *P. breweriana* had the same chloroplast haplotype based on 21 cpCAP markers representing 21 gene-enzyme combinations or 40 restriction sites (binary presence-absence traits). *Picea breweriana* differed from all other spruces by two restriction enzyme sites, for *Taq*I in *rbcL* and for *Eco*RV in *trnK*.

The cpSSR markers also were not highly polymor-

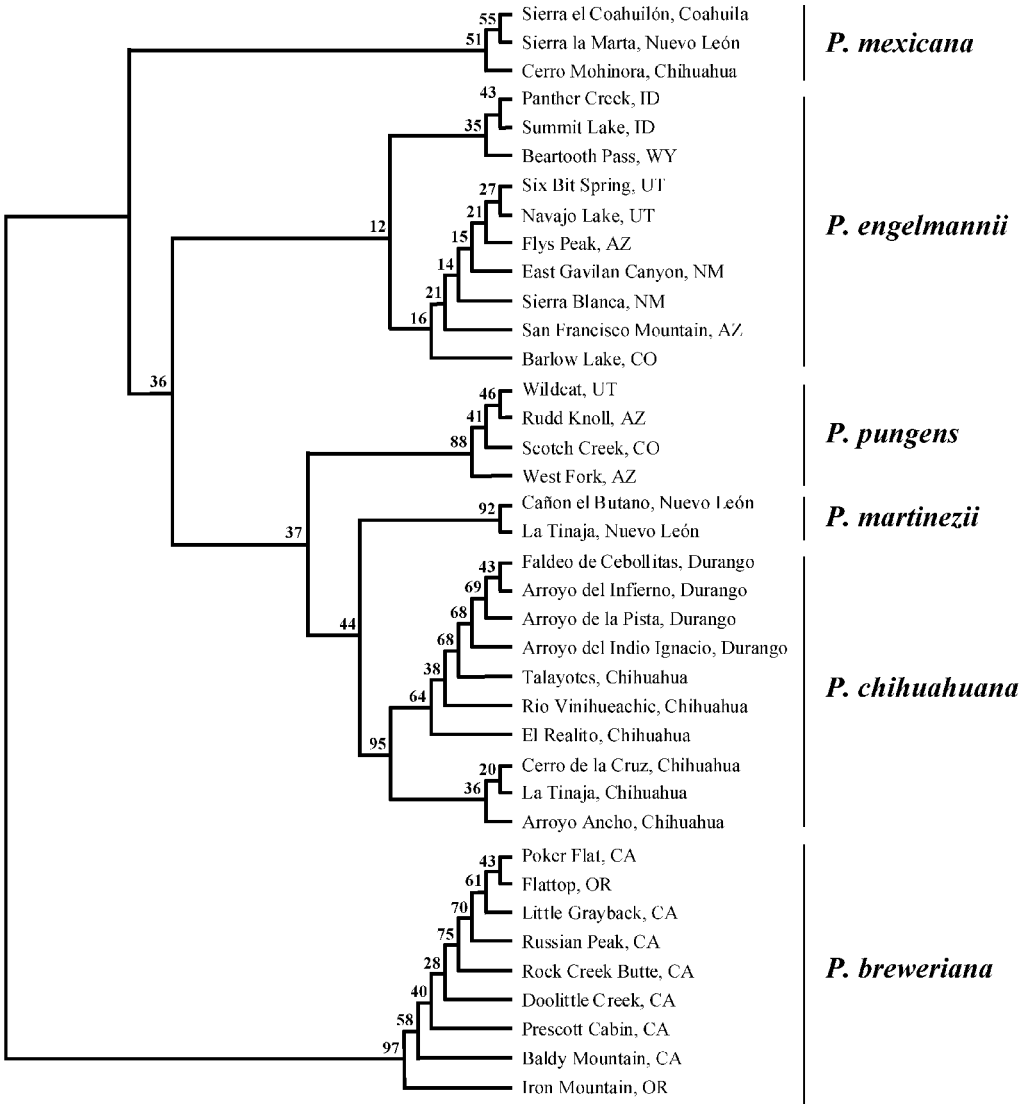

FIG. 2. Neighbor-Joining phylogenetic tree based on Nei's (1972) genetic distance calculated for nine isozyme loci in *P. engelmannii*, *P. pungens*, *P. breweriana*, *P. chihuahuana*, *P. martinezii*, and *P. mexicana*, and rooted with *P. breweriana* as an outgroup. Numbers near nodes represent percentage of occurrences in 1,000 bootstrap-generated data sets.

phic, but one species-specific cpDNA 150bp fragment was observed with primer Pt71926 in *P. martinezii*; one 96bp fragment with primer Pt63718 in *P. pungens*; and two 151bp fragments each with primers Pt30204 and Pt36480 in *P. breweriana*. None of the other four primers yielded species-specific fragments.

All individuals within a species had the same chloroplast haplotype, except in *P. chihuahuana* and *P. martinezii* at Pt30204. Among three individuals in *P. chihuahuana*, one had allele *148* (which is more characteristic of *P. martinezii* and *P. pungens*) while the other two had allele *145* (common in all other Mexican spruces and in *P. engelmannii*). One among six individuals of *P. martinezii* had allele *145*, while the other five had allele *148*. In total, based on both cpCAP and cpSSR markers, six chloroplast haplotypes (**A** to **F**) were found: **A** in *P. chihuahuana*, *P. mexicana* (including spruces from Cerro Mohinora), and *P. engelmannii*, **B** in *P. chihuahuana*, **C** in *P. martinezii* from both La Tinaja and Cañon el Butano, **D** in *P. martinezii* from La Tinaja, **E** in *P. pungens*, and **F** in *P. breweriana*.

Nuclear DNA Polymorphism. RAPD markers were more polymorphic and revealed more species-specific markers than chloroplast DNA, and even population-specific markers (for *P. mexicana* and *P. martinezii*, which were sampled from more than one population; Table 7). Although chloroplast markers revealed no species-specific alleles in *P. engelmannii*, *P. mexicana*, or *P. chi-*

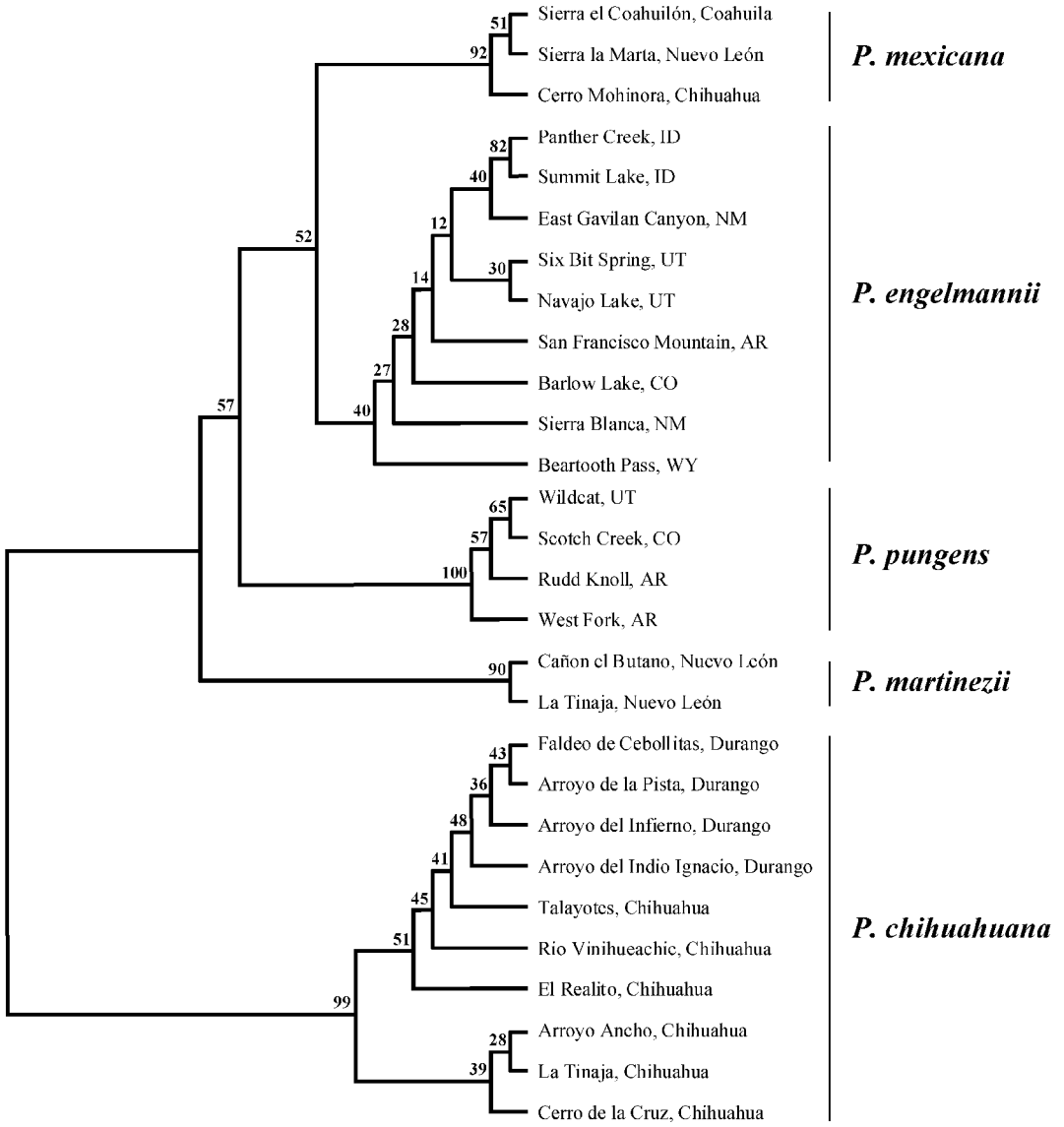

FIG. 3. Neighbor-Joining phylogenetic tree based on Nei's (1972) genetic distance calculated for 13 isozyme loci in *P. engelmannii*, *P. pungens*, *P. chihuahuana*, *P. martinezii*, and *P. mexicana*, and rooted with *P. chihuahuana* as an outgroup. Numbers near nodes represent percentage of occurrences in 1,000 bootstrap-generated data sets.

huahuana, seven species-specific RAPD fragments were amplified in *P. engelmannii*, two in *P. mexicana*, and two in *P. chihuahuana*, and another four RAPD fragments were specific to the *P. engelmannii-mexicana* complex (Tables 7, 8). The total number of species-specific markers observed for chloroplast (cpCAP and cpSSR) and nuclear (RAPD) DNA are given in Table 8. The presence of some RAPD markers across all species is also interesting, and attests to the conservative nature of the spruce genome.

Phylogenetic Analysis Using DNA Markers. In total, 11 unique multi-locus genotypes were found and used in phylogenetic analysis (one in each of *P. mexicana* from Cerro Mohinora, *P. engelmannii*, *P. pungens*, and *P. breweriana*, two in *P. mexicana* from Sierra la Marta and Sierra el Coahuilón and in *P. chihuahuana*, and three in *P. martinezii*, based on 114 binary traits that represented both chloroplast and nuclear markers).

Due to highly monomorphic cpDNA markers, only 64 of 114 binary traits were variable, and 40 of them were informative and used in parsimony analysis. In the original data set, there were three equally parsimonious trees, practically identical except for the position of Cerro Mohinora within the *P. mexicana* cluster. Consistency and retention indices were 0.79 and 0.85, respectively.

TABLE 4. Means of Nei's (1978) unbiased genetic distances ± one standard deviation between populations of *Picea engelmannii* (En), *P. mexicana* (Mx), *P. pungens* (Pu), *P. martinezii* (Ma), *P. chihuahuana* (Ch), and *P. breweriana* (Br) based on nine isozyme loci (below diagonal) and 13 loci (above diagonal), the range of genetic distances between pairs of populations (in parentheses), and number of populations (N).

Species	N (9 loci)	Species						N (13 loci)	Species
		En	Mx	Pu	Ma	Ch	Br		
		0.045 ± 0.026	0.157 ± 0.032	0.383 ± 0.060	0.331 ± 0.030	0.525 ± 0.043		9	En
		(0.013–0.103)	(0.108–0.233)	(0.267–0.503)	(0.289–0.386)	(0.429–0.591)			
			0.016 ± 0.014	0.395 ± 0.026	0.346 ± 0.020	0.485 ± 0.010		3	Mx
			(0.000–0.026)	(0.364–0.443)	(0.323–0.374)	(0.464–0.505)			
En	10	0.055 ± 0.035		0.019 ± 0.011	0.606 ± 0.006	0.571 ± 0.018		4	Pu
		(0.011–0.147)		(0.003–0.034)	(0.599–0.618)	(0.530–0.605)			
Mx	3	0.147 ± 0.035	0.020 ± 0.018		0.000	0.460 ± 0.009		2	Ma
		(0.100–0.241)	(0.000–0.032)			(0.446–0.477)			
Pu	4	0.336 ± 0.069	0.395 ± 0.028	0.006 ± 0.002		0.007 ± 0.007		10	Ch
		(0.214–0.445)	(0.359–0.436)	(0.004–0.009)		(0.000–0.018)			
Ma	2	0.417 ± 0.039	0.406 ± 0.008	0.581 ± 0.011	0.000				
		(0.348–0.494)	(0.396–0.418)	(0.565–0.599)					
Ch	10	0.514 ± 0.032	0.546 ± 0.018	0.530 ± 0.026	0.557 ± 0.014	0.010 ± 0.010			
		(0.447–0.575)	(0.512–0.571)	(0.474–0.560)	(0.536–0.577)	(0.000–0.025)			
Br	9	0.811 ± 0.116	0.700 ± 0.076	1.023 ± 0.115	1.457 ± 0.160	1.026 ± 0.100	0.020 ± 0.025		
		(0.586–1.032)	(0.604–0.827)	(0.816–1.199)	(1.164–1.694)	(0.813–1.184)	(0.000–0.091)		

The NJ tree (Fig. 4) revealed two major, well-separated groups—*P. engelmannii-mexicana* and *P. chihuahuana-martinezii*. *Picea pungens* was closest to the *P. engelmannii-mexicana* group, and *P. breweriana* stayed well apart from both groups. *Picea mexicana* from Cerro Mohinora (the problematic *P.* hybrida), which is isolated from the populations on Sierra la Marta and Sierra el Coahuilón by ca. 675 km, was not differentiated from the latter two populations. Maximum parsimony (MP) and UPGMA, not presented here, gave trees practically identical to the NJ tree. PCA (results not shown) confirmed the parsimony- and distance-based analyses. All spruce taxa in this study can be considered distinct species.

DISCUSSION

Identity of Species and Variation among Populations. Isozyme loci were useful in identifying affinities among spruce populations of southwestern North America; species were marked by clear differences in allele frequencies, often fixation of alternative alleles (Table 3).

Picea pungens and *P. engelmannii* are often difficult to identify in the field, and putative hybrids have been suspected (Taylor et al. 1975). However, isozymes separate *P. pungens* and *P. engelmannii* very cleanly. The seedlot from the West Fork of the Little Colorado River, Apache-Sitgreaves National Forest, Arizona, which we received as *P. engelmannii*, fell squarely within the *P. pungens* cluster. The seedlot's isozyme profile identified it as *P. pungens* without doubt. Likewise, a common garden test and monoterpene composition unequivocally established this seedlot as *P. pungens* (Rehfeldt 1994). If hybridization between *P. engelmannii* and *P. pungens* occurs in sympatric populations, it has not been detected with molecular markers or common garden studies (Ernst et al. 1990; Rehfeldt 1994) and must be an infrequent event.

Large differences in allele frequencies between *P. engelmannii* and *P. pungens* were found by Ernst et al. (1990); the species were fixed for alternate alleles at some isozyme loci. Four of the loci used by Ernst et al. (1990), their *Gdh, Got3, Pgi2,* and *Pgm,* are common to our study. In their study and in ours, *Got3* and *Pgi2* were nearly fixed for alternate alleles in *P. pungens* and *P. engelmannii*. And in both studies, *P. pungens* was nearly fixed at *Gdh* and *Pgm,* while *P. engelmannii* was highly polymorphic at these two loci.

The division between northern and southern populations of *P. engelmannii* is intriguing (e.g., Fig. 2). Even for the subset of only nine loci, the mean D between the northern and southern clusters is 0.083 (± 0.007, one standard error of the mean), which is twice as great as the mean distances within clusters, and approaches the level characterizing different varieties or subspecies. The mean genetic distance within the northern cluster of populations from Idaho and Wyo-

TABLE 5. Chi-square contingency table analyses of heterogeneity among populations in *P. engelmannii, P. mexicana, P. pungens, P. martinezii, P. chihuahuana,* and *P. breweriana.*

	P. engelmannii		*P. mexicana*		*P. pungens*		*P. martinezii*		*P. chihuahuana*		*P. breweriana*	
Locus	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Gdh	163.20	0.00	0.00	1.00	6.02	0.11	—	—	—	—	23.27	0.00
Got1	75.93	0.00	—	—	—	—	—	—	—	—	18.75	0.02
Idh1	275.63	0.00	0.00	1.00	—	—	—	—	—	—	—	—
Mdh2	186.93	0.00	0.00	1.00	—	—	0.00	1.00	—	—	110.04	0.00
6Pg2	227.05	0.00	0.87	0.65	27.17	0.00	—	—	—	—	18.47	0.02
Pgi2	600.59	0.00	0.00	1.00	17.07	0.00	6.35	0.01	8.65	0.47	48.35	0.00
Pgm	195.17	0.00	133.57	0.00	40.70	0.00	—	—	100.12	0.00	9.49	0.30
Skd1	968.31	0.00	25.69	0.00	89.70	0.00	—	—	45.21	0.00	—	—
Skd2	258.48	0.00	0.00	1.00	—	—	0.00	1.00	—	—	—	—

ming was 0.037 ± 0.004, and within the southern cluster of populations from Utah, Colorado, Arizona, and New Mexico, it was only 0.030 ± 0.003. The difference between *P. engelmannii* from the northern and southern Rocky Mountains was noted in common garden tests (Rehfeldt 1994). However, Rehfeldt (1994) placed the dividing line on the northern borders of Arizona and New Mexico; he considered Utah and Colorado part of the range of northern *P. engelmannii.*

Perhaps, the differences between *P. engelmannii* in the southern Rocky Mountains and the populations in Idaho and Wyoming (Fig. 2), particularly the population from Panther Creek, reflect introgression from *P. glauca* (Moench) Voss (white spruce) in the north. Hybridization and introgression between *P. engelmannii* and *P. glauca* is common in Alberta and interior British Columbia, Canada, and *P. engelmannii* was considered a subspecies of *P. glauca* by Taylor (1959), i.e., subsp. *engelmannii* (Parry) T. M. C. Taylor. S. T. Jackson (pers. comm. 2002) has observed hybrids between *P. glauca* and *P. engelmannii* in the Big Horn Mountains, Wyoming. More intensive sampling of *P. engelmannii* and the use of a variety of markers, including chloroplast and mitochondrial DNA, might shed light on the apparent break between northern and southern *P. engelmannii* populations.

TABLE 6. Hierarchical F-statistics for nine polymorphic loci in *P. engelmannii, P. mexicana, P. pungens, P. martinezii, P. chihuahuana,* and *P. breweriana*; $\mathbf{F_{PS}}$ = population diversity relative to diversity among species; $\mathbf{F_{PT}}$ = population diversity relative to total diversity; $\mathbf{F_{ST}}$ = species diversity relative to total diversity.

Locus	F_{PS}	F_{PT}	F_{ST}
Gdh	0.082	0.618	0.584
Got1	0.024	0.979	0.978
Idh1	0.126	0.564	0.501
Mdh2	0.200	0.605	0.506
6pg2	0.040	0.318	0.289
Pgi2	0.082	0.744	0.721
Pgm	0.154	0.593	0.520
Skd1	0.165	0.676	0.612
Skd2	0.087	0.924	0.917
Combined	0.127	0.704	0.661

Genetic distance gives no indication that *P. engelmannii* from Arizona is conspecific with *P. mexicana.* Rehfeldt (1994) had speculated that southwestern *P. engelmannii* might be synonymous with *P. mexicana,* and suggested that additional studies were needed. Spruce from Flys Peak in the Chiricahua Mountains had allele frequencies that were definitely not typical of *P. mexicana*, and fell clearly within the range of variation expected in *P. engelmannii.* We found that the smallest D value between *P. engelmannii* and *P. mexicana* was 0.100 between *P. mexicana* from Sierra el Coahuilón, Coahuila, and a *P. engelmannii* population from Barlow Lake, San Juan National Forest, Colorado, and not with *P. engelmannii* from the Chiricahua Mountains, Arizona. Genetic distance between *P. mexicana* populations and *P. engelmannii* from Flys Peak in the Chiricahua Mountains varied from 0.153 to 0.164. No evidence from this study suggests that the Chiricahua Mountains are a bridge between *P. engelmannii* and *P. mexicana.* On the basis of common garden tests, G. E. Rehfeldt (pers. comm. 2002) now believes that spruces from the Chiricahua Mountains belong to a cline that extends throughout the range of *P. engelmannii.*

Isozyme Phylogeny. No comparisons of *P. chihuahuana* and *P. martinezii* using molecular markers have been reported prior to the present study, undoubtedly because *P. martinezii* was so recently discovered and because its habitat was so remote and difficult of access. Although *P. chihuahuana* and *P. martinezii* form a cluster in the NJ tree based on nine loci, this group is weakly supported and the two species are nearly as divergent as any other pair of spruces in this study (Fig. 2). For example, the mean D (nine loci) between *P. chihuahuana* and *P. martinezii* was 0.557 compared to 0.581 between *P. martinezii* and *P. pungens* and only 0.417 between *P. martinezii* and *P. engelmannii* (Table 4). Genetic distance between *P. chihuahuana* and *P. martinezii* was not as great when based on 13 loci, but comparisons were similar (Table 4). Our data suggest that *P. chihuahuana* and *P. martinezii* have been separated for a long time, and contradicts Farjon's (2001) treatment

TABLE 7. RAPD fragments observed in six spruce taxa. DNA fragments are presented as number of nucleotide base pairs (bp) corresponding to amplified fragment lengths measured using a DNA size standard. Markers specific for the *P. engelmannii-mexicana* spruce complex are marked by bold italic and for the *P. chihuahuana-martinezii* group by bold font alone; all species-specific markers are marked by bold, underlined font.

RAPD Primer	*P. chihuahuana*	*P. martinezii*	*P. mexicana*	*P. mexicana* from Cerro Mohinora	*P. engelmannii*	*P. pungens*	*P. breweriana*
A04	1200	1200	950, 1200	950, 1200	950, 1200	—	**810**, 950, 1200
A05	1180	1180	*600*, 1180	***600***, 1180	***600***, 1180	1180	1180
A07	620	620	550, 620	550, 620	620	620	**400**, 550, 620
A08	**410, 490**	**410, 490**	—	—	—	—	—
A09	410, **470**, 750	410, **470**, 750	**250**, 410, 750	**250**, 410, 750	410, 750	410, 750	410, 750
A12	650	**250**, 650	***350, 510***, 650	***350, 510***, 650	***350, 510***, 650	650	—
A14	620	620	620	620	620	620	—
A15	**380**, 1500	**380**	500, 1500	500, 1500	500, 1400	500, 1400, 1500	**330**
A16	**490**	**490, 600**	—	—	—	—	—
A19	**410**	310, **500**	—	***760***	310, ***760***	—	—
B05	450	450	450/0	450	450	450	—
B09	—	**590**	—	—	—	—	—
B11	550	550	550, 700	550, 700	550, 700	550, 700	550, 700
B17	—	175/0	175	175	175	175	—
B18	—	—	410	410	410	410	—
B19	—	—	980	980	980	—	980
K02	1350	1350	1350/0	1350	1350	1350	1350
K04	—	—	900	900	900	900	—
K05	—	—	380	380	380	380	—
K06	—	410/0	410	410	410	—	410
K08	450	450	450, ***1200***	450, ***1200***	450, ***1200***	**400**, 450	**490**
K13	**380**	**380**	—	—	—	—	—
K14	550, 575	550, 575	550, 575	550, 575	550, 575	550, 575	550
K15	—	510, 750, 910	475, 510, **620**, 750, 910	475, 510, **620**, 750, 910	475, 510, 750, 910	475, 510, 750, 910	510, 750, **890**
K16	**410**, 810	810	810	810	810	**700**, 810	**470, 790**
K18	510	510	—	—	—	510	—

TABLE 8. Number of species-specific markers observed in spruce taxa for chloroplast (cpCAP and cpSSR) and nuclear (RAPD) markers (*P. engelmannii* had no species-specific markers, although the *P. engelmannii-mexicana* complex had specific markers).

Species	cpCAP	cpSSR	RAPD	Total
P. chihuahuana			2	2
P. martinezii		1	4	5
P. pungens		1	2	3
P. breweriana	2	2	7	11
P. mexicana			2	2
P. engelmannii-mexicana complex			5	5

of the two species as synonymous. *Picea martinezii* deserves recognition as a species.

The status of *P. mexicana* as an independent species or as a variety of *P. engelmannii* depends on interpretation. *Picea mexicana* and *P. engelmannii* were well separated based on 13 loci, although the distinction was not as great based on only nine (compare Figs. 2, 3). Genetic distance between the two taxa for either nine or for 13 loci was less than half the distance between any other pair of species (Table 4). Moreover, genetic distance between some populations within *P. engelmannii* was as great as the mean genetic distance between *P. engelmannii* and *P. mexicana*. For comparison, we (Ledig et al. 2001) found that D averaged 0.218 between *Pinus pinceana* Gordon (weeping piñon) and *Pinus maximartinezii* Rzedowski (maxipiñon), two morphologically distinct pine species that belong to the same subsection of *Pinus* (section *Strobus*, subsection *Cembroides*; Gernandt et al. 2001). *Picea mexicana* populations approach this level of differentiation from *P. engelmannii* only when compared to *P. engelmannii* from Panther Creek, Salmon National Forest, Idaho, the most geographically distant population from *P. mexicana* in our sample; the mean genetic distance (nine loci) between the Panther Creek population of *P. engelmannii* and *P. mexicana* was 0.232, which is significantly different

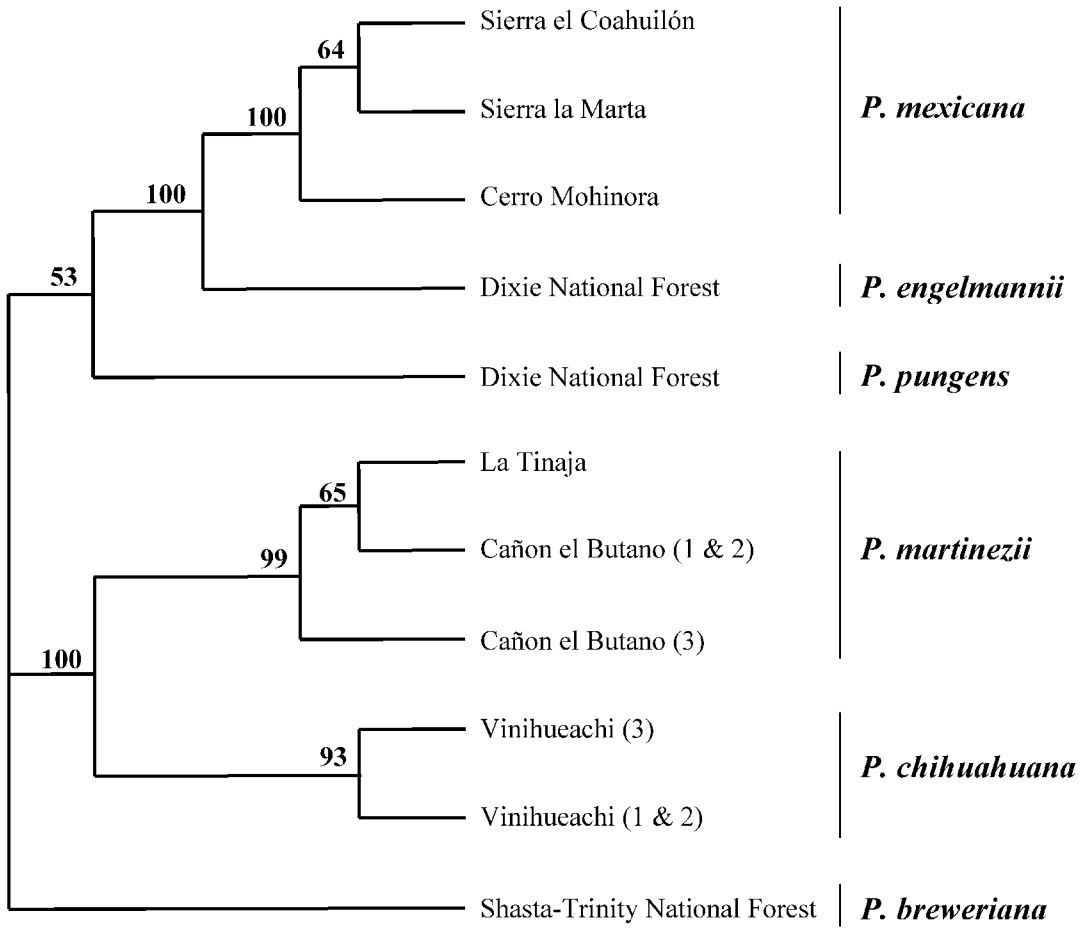

FIG. 4. Neighbor-Joining phylogenetic tree based on dissimilarity distance (Nei and Li 1985) estimated from 114 binary traits representing 21 cpCAP, 8 cpSSR, and 61 RAPD markers. Numbers near relevant nodes are percentage of 1,000 bootstrap replicates. Numbers at the ends of the branches refer to individual trees within species or populations.

from the mean genetic distance of *P. mexicana* from all other populations of *P. engelmannii*, 0.137 (t = 7.48, df = 28). In a review of the use of isozymes in systematics, Gottlieb (1977) found average genetic distance between conspecific plant populations was 0.05, while genetic distance between congeneric species averaged 0.33. The distance between *P. mexicana* and *P. engelmannii* is much greater than the distance expected for conspecific populations but only half as large as that suggested by Gottlieb (1977) for distinct congeneric species.

Nevertheless, *P. mexicana* and *P. engelmannii* are as divergent as several other conifer species pairs. For example, genetic distance between *P. sitchensis* (Bong.) Carr. (Sitka spruce) and *P. glauca* was only 0.121 (Yeh and Arnott 1986), and these are considered good morphological species, although they hybridize in coastal Alaska. The genetic distance between *Picea abies* (L.) Karst. (Norway spruce) and *Picea obovata* Ledeb. (Siberian spruce), taxa that introgress over a broad hybrid zone, was only 0.072, about half the genetic distance between *P. mexicana* and *P. engelmannii* (Krutovskii and Bergmann 1995). The two Eurasian taxa were considered species. Genetic distances among pairs of five stone pine taxa (genus *Pinus*, section *Strobus*, subsection *Cembrae*), spanning three continents, ranged from 0.105 to 0.256, except for the distance between *Pinus sibirica* Du Tour (Siberian stone pine) and *Pinus cembra* L. (Swiss stone pine; Krutovskii et al. 1995), which was only 0.065. The genetic distance of 0.065 was still about ten times greater than genetic distances between populations within species. Thus, *P. mexicana* and *P. engelmannii* differ genetically to a degree greater than that seen in some other well-accepted conifer species pairs. The genetic distance between *P. mexicana* and *P. engelmannii* would suggest that each deserves species rank.

While most authorities have considered the relationship between *P. mexicana* and *P. engelmannii* a close one (Schmidt 1989; Taylor et al. 1994), one cluster analysis based on isozymes separated the two taxa by a wide gap (Wellendorf and Simonsen 1979). In that study, genetic distance measures were not used; the analysis was based on a crude statistic reflecting presence or absence of alleles, making the results difficult to interpret.

Although the bootstrap support for *P. engelmannii* appears low (Figs. 2, 3), that may be misleading. *Picea engelmannii* is a wide-ranging species with great genetic diversity. Its high polymorphism means that it shares alleles with several other species and, therefore, genetic distance between *P. engelmannii* and other spruces are relatively low (Table 4). In the phylogeny based on 13 isozyme loci, only *P. engelmannii* shows low bootstrap values, but this does not mean that populations of *P. engelmannii* join other groups in bootstrap replicates. This would be rare because the *P. pungens* cluster is supported in 100% of the bootstrap trees, the *P. chihuahuana* cluster in 99%, the *P. martinezii* in 90%, and even the node connecting the three populations of *P. mexicana* is supported in 92% of the replicates, so that there are zero or few opportunities for populations of *P. engelmannii* to join those groups. Rather, in individual bootstrap replicates, single populations of *P. engelmannii* may join other clusters because of the high heterogeneity within the species. Even though the *P. engelmannii* cluster has low support from bootstrapping (Figs. 2, 3), its interspecific distinction is consistent with the phylogeny based on RAPD and other molecular markers (Fig. 4), suggesting that the relationships revealed by isozymes are meaningful.

Picea pungens joined the *P. chihuahuana-martinezii* cluster in the UPGMA tree based on 13 loci and in the NJ tree based on nine loci (Fig. 2). We have little confidence in this relationship because we have included only a few species of the genus and because *P. pungens* joins the *P. engelmannii-mexicana* group in our NJ tree based on 13 loci (Fig. 3). However, *P. pungens* also joined a *P. chihuahuana* clade in Sigurgeirsson's (1992) UPGMA tree that was based on cpDNA restriction fragment polymorphisms. *Picea pungens* and *P. engelmannii* were also reported to be members of different clades based on nuclear ribosomal 18s sequences and an internal transcribed spacer (Smith and Klein 1994), but this work was shown to be in error (Smith and Klein 1996; Camacho et al. 1997). The ribosomal 18s sequences were actually from an endophytic fungus, *Hormonema dematioides* (Camacho et al. 1997). *Picea pungens* did cluster with *P. engelmannii* in an investigation based on random amplified polymorphic DNA (Nkongolo 1999), but neither *P. chihuahuana* nor *P. martinezii* were included in that study. Whether *P. pungens* has closer affinity to the *P. engelmannii-mexicana* or to the *P. chihuahuana-martinezii* clusters, it is distinctly different from all of the other spruces in southwestern North America.

DNA Phylogeny. Because of the very limited sample size, the species-specific markers in Tables 7 and 8 must be viewed with caution. However, in phylogeny the number of markers is more important than the number of individuals, especially in the Mexican spruces because of their relatively low level of within-species polymorphism (Ledig et al. 1997, 2000a, 2002).

The phylogeny (Fig. 4) is also strictly limited by the number of species included. Nevertheless, it answers specific questions about relationships among species and about problematic populations in the spruces of southwestern North America, and we believe the result is fairly robust. The trees produced from similarities and differences in cpDNA and RAPDs were almost identical to the results based on nine isozyme loci. Both isozyme and DNA markers indicated that spruce from Cerro Mohinora was conspecific with *P. mexicana*

and showed no evidence of being a hybrid derivative that included *P. chihuahuana* or *P. pungens* in its ancestry. The *P. mexicana* population from Cerro Mohinora was marginally closer to *P. engelmannii* than were *P. mexicana* populations from the Sierra Madre Oriental, as suggested by Taylor et al. (1994), but both isozyme and DNA markers leave no doubt that the spruces on Cerro Mohinora are conspecific with those from Sierra la Marta and Sierra el Coahuilón.

Both isozymes and DNA showed that *P. chihuahuana* and *P. martinezii* were related but distinct species, verifying Patterson's (1988) decision to publish *P. martinezii*. *Picea martinezii* was not included in the more global phylogenies of Sigurgeirsson and Szmidt (1993) or of Wellendorf and Simonsen (1979).

Both our sets of markers indicated that *P. mexicana* was more similar to *P. engelmannii* than would be expected for a well-differentiated species, which may suggest that it should be considered a variety, as preferred by Taylor and Patterson (1980). Among 31 taxa, *P. engelmannii* and *P. mexicana* were among the most closely related pairs, and formed a clade with *P. glauca* (Sigurgeirsson and Szmidt 1993). We conclude, however, that the differences between *P. engelmannii* and *P. mexicana*, when combined with information on their low crossability (Gordon 1980, 1984; see below), are great enough to consider them distinct species.

Based on either isozymes or DNA, *P. breweriana* was the most isolated taxon. In other studies that used molecular markers (Wellendorf and Simonsen 1979; Sigurgeirsson 1992), *P. breweriana* also stood out as an isolated taxon. In Wellendorf and Simonsen's (1979) cluster analysis of 20 species, *P. breweriana* was nearly as isolated as their outgroup, *Pinus contorta* Dougl. ex Loud. (lodgepole pine). In Sigurgeirsson and Szmidt's (1993) phylogeny based on restriction fragment length polymorphisms of cpDNA, *P. breweriana* was highly differentiated on either an UPGMA phylogenetic tree or a strict consensus tree based on the most parsimonious Wagner trees. The weight of evidence from isozymes and other molecular markers suggests that *P. breweriana* is an ancient and divergent species.

Although isozymes were conclusive in addressing relationships among populations, bootstraps did not provide overwhelming evidence for a species phylogeny. However, the DNA markers provided strong support for the sister group relationships between *P. chihuahuana* and *P. martinezii* and between *P. engelmannii* and *P. mexicana* that were suggested by isozymes.

The placement of *P. pungens* in the DNA study does not resolve the conflict between the results based on nine isozyme loci (Fig. 2) and those based on 13 isozyme loci (Fig. 3). Whether *P. pungens* is more closely related to the *P. engelmannii-mexicana* complex or to the *P. chihuahuana-martinezii* complex or not closely related to either, was not clear, and studies with additional markers and additional species are necessary to determine its affinities. The weight of the present analyses indicated that *P. pungens* is slightly closer to the *P. engelmannii-mexicana* group than to the *P. chihuahuana-martinezii* group, but not as close as suggested by *P. pungens'* morphological similarity to *P. engelmannii*.

Genetic Isolation and Crossability. Any classification that ignores crossability abandons the biological species concept, whether the classification is based on morphological and biochemical phenotype, or on more direct genetic markers such as isozyme and DNA polymorphisms. *Picea engelmannii* and *P. glauca* are highly crossable and *P. mexicana* also crossed readily with *P. glauca* in some attempts (Gordon 1982). It is curious, therefore, that *P. mexicana* and *P. engelmannii* exhibit only ''low crossability'' (Gordon 1980, 1984). *Picea glauca*, *P. engelmannii*, and *P. mexicana* all cross with *P. sitchensis* (Gordon 1986). In fact, introgression between *P. glauca* and *P. sitchensis* is common in parts of coastal Alaska and British Columbia (Roche 1969; Sigurgeirsson et al. 1991). Thus, evolution of species within the *P. glauca-engelmannii-mexicana-sitchensis* complex is not independent.

However, neither *P. breweriana*, *P. pungens*, nor *P. chihuahuana* are part of the *P. glauca* complex. *Picea pungens* crosses with *P. engelmannii* with difficulty, and the cross can only be made with *P. engelmannii* as the seed parent (Kossuth and Fechner 1973; Ernst et al. 1990). *Picea pungens* failed to cross with *P. mexicana* or *P. sitchensis*, and success in crosses with *P. glauca* was very low (Wright 1955; Gordon 1980). Few crosses have been attempted with *P. chihuahuana* and none with *P. martinezii*. Hybrids from crosses of *P. glauca* and *P. chihuahuana* were unconfirmed (Gordon 1980).

Picea breweriana is so isolated in the genus that even hybrids and tri-hybrids of other species ''would not accept *P. breweriana* pollen'' (Gordon 1986). The results of controlled hybridization (Fig. 5) are consistent with the isolation of *P. breweriana* as shown in Figs. 2 and 4. *Picea breweriana* was distinctly different from all other spruces in this study, as in other investigations using other markers (Sigurgeirsson and Szmidt 1993; Weng and Jackson 2000; C. S. Campbell pers. comm. 2002). Its failure to cross with other species supports its isolation. *Picea breweriana* has no close relatives among the spruces of southwestern North America, and probably not among the extant spruces of boreal and eastern North America either.

The results of controlled hybridization also support the separation of *P. pungens* and *P. chihuahuana* from the *P. engelmannii-mexicana* group as shown in Figs. 2 and 3.

Attempted crosses of *P. chihuahuana*, *P. pungens*, and *P. breweriana* with members of the *P. omorika* (Panc.) Purk. (Serbian spruce), *P. rubens* Sarg. (red spruce), and *P. mariana* (Mill.) Britt. (black spruce) complex have

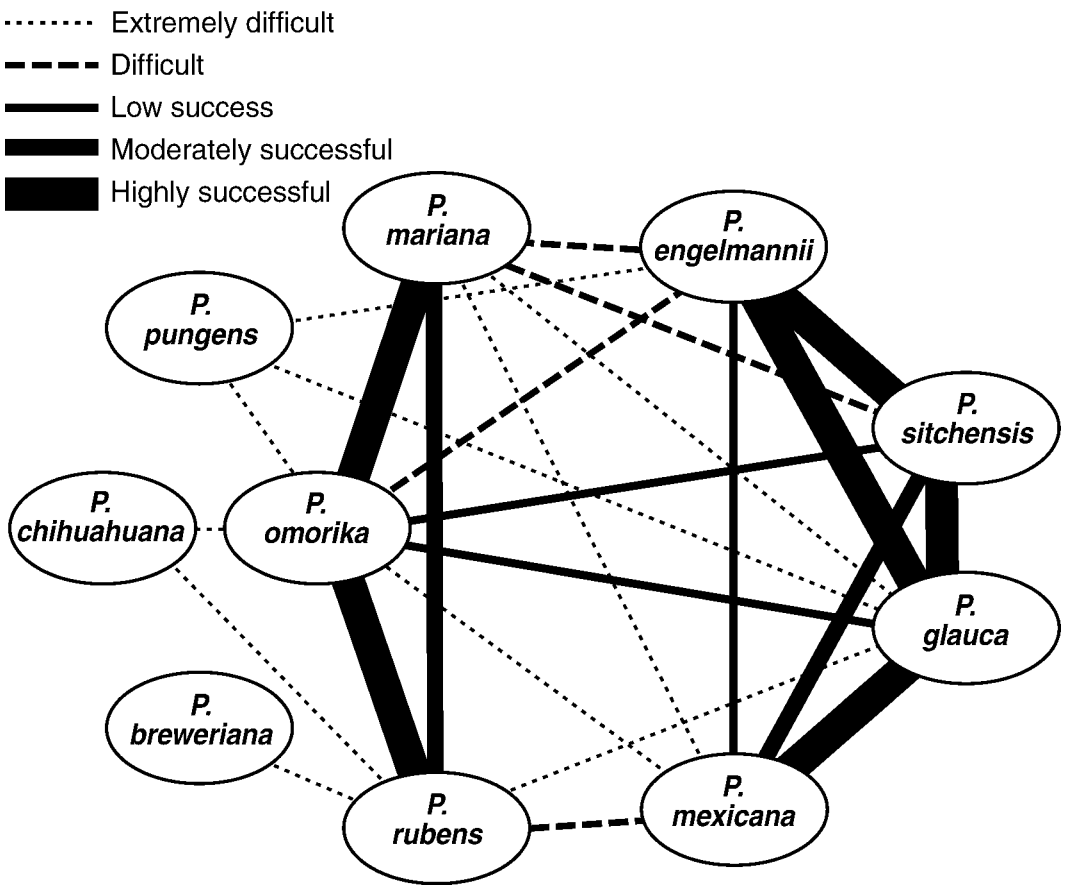

FIG. 5. Crossability among spruces of North America and *P. omorika*. Weight of the line is roughly proportional to the ease of the cross, from very difficult (light dash) to highly successful (broad bar). Crosses that have been attempted and have consistently failed are (not necessarily with the maternal parent listed first): *P. breweriana* with *P. mariana*, *P. rubens*, *P. omorika*, and *P. mexicana*; *P. pungens* with *P. mariana*, *P. rubens*, *P. sitchensis*, and *P. mexicana*; *P. chihuahuana* with *P. mariana*.

failed, or produced unconfirmed hybrids with low success (Wright 1955; Gordon 1976a, 1978, 1980, 1982, 1984, 1986, 1989). However, *P. omorika*, a European species, provides a connection between the two North American spruce complexes (the *P. rubens-mariana* complex and the *P. glauca-engelmannii-sitchensis-mexicana* complex), even though Europe and North America have been separated since the destruction of the North Atlantic land bridges near the Eocene/Oligocene boundary, a date soon after the deposition of the earliest dated spruce fossils in the Canadian Arctic (LePage 2001).

We acknowledge that relationships based on crossability may not be an accurate measure of total genetic similarity or difference. Small genetic changes can result in reproductive isolation. Furthermore, crossability is not the sole determinant of reproductive isolation. Nevertheless, the erection of crossability barriers marks the point at which further evolutionary divergence can occur.

Systematics. Some intrageneric classifications of spruce based on morphology have made implausible groupings (Bobrov 1970; Schmidt 1989) when compared to molecular phylogenies or the results of controlled hybridization. Following earlier authors, Bobrov (1970) recognized three sections within the genus; i.e., *Casicta* Mayr, *Picea* (sect. *Eupicea* M. Willk.), and *Omorika* M. Willk., but closely related species were assigned to different sections and unrelated species grouped together. For example, *P. engelmannii* was placed in sect. *Casicta* Mayr ser. *Pungentes* Bobr., while *P. glauca* was placed in sect. *Omorika* ser. *Glaucae* Bobr., even though the two naturally hybridize to such an extent that introgression between the taxa occurs in Alberta, Canada (Taylor 1959; Rajora and Dancik 1999). *Picea sitchensis*, which also forms a hybrid zone with *P. glauca* (Roche 1969; Sigurgeirsson et al. 1991), was placed in still a third series, in sect. *Casicta*, ser. *Ajanenses* Bobr.

Schmidt's (1989) classification also separated *P. glauca* and *P. engelmannii*, this time into two different subgenera, subgenus *Picea* sect. *Picea* and subg. *Casicta* sect. *Pungentes*, respectively. *Picea sitchensis* was separated

into still a third section, subg. *Casicta* sect. *Sitcha* P. A. Schmidt.

Picea chihuahuana was grouped with *P. glauca* by both Schmidt (1989) and Bobrov (1970), but separated from *P. glauca* and placed in a section with *P. engelmannii* by Aldén (1987). *Picea pungens* was placed in the same section as *P. engelmannii* in both Bobrov's (1970) and Schmidt's (1989) classifications. *P. martinezii* was published too late to be included in either treatment of the genus.

Bobrov's (1970) or Schmidt's (1989) classifications are not supported by crossability studies, by our study, or by other studies that used molecular markers. All studies based on DNA markers group *P. engelmannii* with *P. glauca* (Sigurgeirsson 1992; Karvonen et al. 1994; Nkongolo 1999), and based on crossability, they are closely related (Wright 1955). Our phylogeny is in strong contrast to the sectional divisions established by either Bobrov (1970) or Schmidt (1989), which relied heavily on ovulate cone morphology. By comparison, our phylogeny is in close agreement with relationships based on needle anatomy (Weng and Jackson 2000) and on crossability patterns (Gordon 1976a, b, 1978, 1982, 1984, 1986, 1988, 1989; Fowler 1983). Thus, ovulate cone morphology seems to be a poor indicator of genetic relationships in spruce. Perhaps, Wright (1955) was correct when he stated: ''There is no natural break in the genus sufficient to warrant the erection of section lines.'' All previous classifications that divided spruces into subgenera and/or sections and series based solely on morphology (Bobrov 1970, Schmidt 1989) are flawed and unacceptable. They have shed little light on a complex genus.

In contrast to systematic arrangements that relied heavily on cone morphology, needle anatomy produced relationships largely in agreement with those based on isozymes and crossability (Weng and Jackson 2000). Needle anatomy indicates that *P. breweriana* is distinct from other North American spruces; places *P. engelmannii*, *P. glauca*, *P. sitchensis*, and *P. mexicana* in a clade cleanly separated from *P. pungens*; and places *P. chihuahuana* and *P. martinezii* in another clade (Weng and Jackson 2000).

Evolution of Spruce. Spruce probably originated in boreal North America, then spread southward in North America and westward to Asia, and from Asia to Europe, based on the fossil record. The oldest spruce fossils occur in middle Eocene deposits (ca. 45 Myr B.P.) on Axel Heiberg Island in the Canadian Arctic (ca. 80°N lat.), and the presence of three distinct species indicates that the genus had already diversified (LePage 2001). Therefore, the origin of the genus was probably in the early Tertiary or Late Cretaceous. Many taxonomists believed that spruce evolved in Asia because so many species were native to Japan and China (e.g., Wright 1955). More recently, a North American origin was suggested based on molecular evidence (Sigurgeirsson 1992; Sigurgeirsson and Szmidt 1993). We favor a North American origin because no fossils as old as 45 Myr B.P. have been found in Asia or Europe, and fossils are progressively younger moving westward from North America and southward from the Bering Straits. Several spruce fossils have been dated to the late Eocene in North America, but the only late-Eocene fossils reported from Asia are from the Kamchatka Peninsula and Honshu Island on the Pacific Rim (cited in LePage 2001), which may suggest migration from North America through Beringia. Most reports of spruce in Asia are from the Oligocene, Miocene, and Pliocene (LePage 2001). In North America, spruce had spread as far south as the Rio Grande Depression (ca. 33 to 34°N lat.) by the late Eocene (35 Myr B.P.; Axelrod and Bailey 1976).

All spruce fossils in Europe are rather recent, from the Pliocene, with the exception of one report of pollen from the late Eocene (cited in LePage 2001). The DeGeer Route and the Thulian Route connected North America and Europe until about the Eocene-Oligocene boundary, and might have provided a land bridge from the Canadian Arctic to Europe. However, while the bridge was open, the climate of Europe was tropical to subtropical, and LePage and Basinger (1991) concluded that the difference in paleolatitude would have presented a barrier to the dispersal of boreal taxa. Thus, the likely scenario is that European spruces descended from Asian progenitors.

Although temperatures in the middle Eocene had already declined from their early Eocene peak, the Canadian Arctic at 45 Myr B.P. was much warmer than today. As a result, spruce fossils on Axel Heiberg Island are associated with macrofossils of several other coniferous genera, such as *Metasequoia* Miki ex Hu et Cheng and *Glyptostrobus* Endlicher, and with angiosperms, such as *Juglans* L. (walnut) and *Alnus* Miller (alder), of temperate affinities (LePage 2001). Temperature cooled gradually during the Eocene and then plummeted abruptly at the beginning of the Oligocene. However, even prior to the Eocene-Oligocene transition, spruce had moved south; *P. lahontense* MacGinitie (Lahontan spruce), similar to modern *P. engelmannii*, was described from the late Eocene flora of Copper Basin, Nevada (Axelrod 1966). The fossil species, *P. coloradensis* Axelrod (Colorado spruce), which resembled *P. pungens*, was already in southern Colorado (ca. 38°N lat.) by the Oligocene (Axelrod 1987). Therefore, possible precursors of *P. engelmannii* and *P. pungens* had diverged in North America before 26.5 Myr B.P. Fossils attributed to *P. breweriana* appeared in the Stewart Spring, Nevada, flora in the very late Miocene (ca. 5.3 Myr B.P.; Wolfe 1964). Because all or most of the fossil occurrences of *P. breweriana* were of seeds and seed wings, they must be viewed with caution. Neverthe-

less, spruces had probably spread into the Southwest as a result of the uplift of the North American Cordillera during the Miocene, because the Cordillera provided cool, montane habitat for the first time (Axelrod 1990).

The principal upheaval of the Cordillera was coincident with the uplift of the Trans-Mexican Volcanic Belt during the Miocene (Graham 1993). Mountain building continued into the Pliocene and was accompanied by dramatic cooling, undoubtedly increasing habitat for montane spruces, and providing a route south into México via the Sierra Madre Oriental and Sierra Madre Occidental. Spruce may have reached ca. 18°N lat. during this period, as evidenced by spruce pollen in middle Pliocene deposits of the Paraje Solo flora on the Isthmus of Tehuantepec, México (Graham 1993).

Spruce persisted in the Trans-Mexican Volcanic Belt at ca.19°N lat. at least from the last glacial maximum until 8,000 yr B.P. (Lozano-García et al. 1993), and on desert ranges of Coahuila in northern México at ca. 27°N lat., where it is not now known, from at least 32,000 to 12,000 yr B.P. (Meyer 1973). During several interglacial periods in the Quaternary, climate became inhospitable for spruce in México, the last event being the late Pleistocene to the present. In the early Holocene, spruce was driven up in elevation and populations were fragmented for the last time (Ledig et al. 1997, 2000a, 2002). *Picea mexicana* may have evolved during the Pleistocene as a relict population of *P. engelmannii*, isolated from it during one of the interglacials in the middle Quaternary, or at least prior to the current interglacial, judging from the genetic distance between the two taxa.

An extinct spruce, *P. critchfieldii* Jackson and Weng (Critchfield spruce), had cones at least 10 cm long and may have been related to *P. chihuahuana* or *P. martinezii* (Jackson and Weng 1999). The association of *P. critchfieldii* with temperate hardwoods in the Tunica Hills of Louisiana and Mississippi during the glacial maximum (Jackson et al. 2000), is suggestive of the present habitat of *P. martinezii*, but the two species differ in a number of morphological and anatomical characteristics (Jackson and Weng 1999).

Time since Isolation and Divergence between Taxa. How does the genetic evidence on divergence correlate with the fossil record of evolution in spruce? Under the neutral mutation theory, time (T) since divergence is given by:

$$T = D/2\alpha$$

where D is Nei's genetic distance and α is mutation rate (Nei 1975). Mutation rates are usually assumed to be on the order of 10^{-7} (Nei 1975), although some evidence suggests that they are higher in long-lived woody trees (Klekowski and Godfrey 1989; Lowenfeld and Klekowski 1992). Neutral mutation theory may not apply well to the spruces of southwestern North America because of the pronounced effects of recent bottlenecks (Ledig et al. 1997, 2002) or if selection has operated on these loci, as well may be the case. Nevertheless, it is of interest to compare estimated times since divergence with the paleoclimatic and fossil records.

Based on Nei's genetic distance, it is likely that *P. mexicana* separated from *P. engelmannii* during a previous interglacial period in the middle Pleistocene (ca. 725,000 yr B.P.) and they have had limited contact since. The clearly related species pair, *P. chihuahuana* and *P. martinezii*, have been separated much longer, perhaps being isolated in separate mountain ranges 2–3 Myr B.P., although bottlenecks experienced by these species (Ledig et al. 1997, 2000a, 2002) might be a source of bias and the actual time to separation could be less. It would be very interesting to know to what degree the cross between *P. chihuahuana* and *P. martinezii* was compatible.

The 2–3 Myr estimate for divergence between *P. chihuahuana* and *P. martinezii* is especially surprising since the data suggest that the more divergent species pair, *P. pungens* and *P. engelmannii*, separated only 1–2 Myr B.P. The fossil record, however, indicates that possible precursors of *P. engelmannii* and *P. pungens* (i.e., the fossil species *P. lahontense* and *P. coloradensis*), were distinct at least as long ago as the Oligocene, about 32 Myr B.P. (Axelrod 1987). Estimates based on genetic distance suggest that *P. chihuahuana* and *P. martinezii* have been isolated from the *P. engelmannii-mexicana* complex for ca. 2 Myr, placing the date near the end of the Pliocene.

Picea breweriana is the most divergent of the spruces of southwestern North America, as was expected from other studies on molecular phylogeny of spruce (Wellendorf and Simonsen 1979; Sigurgeirsson and Szmidt 1993; C. S. Campbell pers. comm. 2002) and needle anatomy (Weng and Jackson 2000). Based on genetic distance, *P. breweriana* may have diverged from the other species 4 to 7 Myr ago, at the Miocene-Pliocene boundary, a time of very rapid climate change. In fact, fossils credited to *P. breweriana* appear ca 5.3 Myr B.P. in the very late Miocene flora of Nevada (Wolfe 1964). However, Wolfe (1964) considered the fossil *P. sonomensis* Axelrod (Sonoma spruce) similar or identical to *P. breweriana*, and *P. sonomensis* appeared over much of the western United States even earlier, in the Oligocene and Miocene. The Oligocene is inconsistent with the estimate from D, and if the fossil *P. sonomensis* and modern *P. breweriana* are synonymous, all values calculated here must be underestimates. Alternatively, the fossil evidence may be considered inconclusive because it is based only on seeds and seed wings, not cones or foliage.

Taxonomy and Distribution. Although spruces

may be taxonomically difficult, the six spruces of southwestern North America are clearly distinct based on isozymes and other molecular markers. Perhaps, this is because three of the species are allopatric and have no overlap with any congeners (Fig. 1). Among the remaining three potential species-pairs, two combinations are sympatric, and the other combination, *P. breweriana* and *P. pungens*, is allopatric. One of the two sympatric pairs, *P. breweriana* and *P. engelmannii*, occur together only near Russian Peak in the Klamath Mountains, California, but remain distinct in their isozyme profile. The ranges of only a single pair, *P. engelmannii* and *P. pungens*, overlap extensively, and although they are sometimes difficult to tell apart in the field, they are easily separated by their isozyme profiles and by chloroplast and nuclear DNA markers.

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