

Phylogenetics of *Pinus* Subsections *Cembroides* and *Nelsoniae* Inferred from cpDNA Sequences

DAVID S. GERNANDT,^{1,4} AARON LISTON,² and DANIEL PIÑERO³

¹Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Hidalgo, Apartado Postal 1-69
Plaza Juárez, Pachuca, Hidalgo, Codigo Postal 42001, México;

²Department of Botany and Plant Pathology, 2082 Cordley Hall, Oregon State University,
Corvallis, Oregon 97331-2902;

³Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México,
Apartado Postal 70-275, Ciudad Universitaria, Codigo Postal 04510, México;

⁴Author for Correspondence (gernandt@miranda.ecologia.unam.mx)

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ABSTRACT. We sequenced chloroplast DNA from the *matK*, *rbcl*, and *rpl16* regions to infer interrelationships within the pinyon pines (*Pinus* subsections *Cembroides* and *Nelsoniae*). Pinyons, together with subsections *Balfourianae*, *Gerardianae*, and *Krempfianae* have been classified in section *Parrya*, characterized by a dorsal umbo (raised area) on the ovulate cone scale. All three cpDNA regions support the separation of pinyon pines into subsection *Cembroides* and a monotypic subsection *Nelsoniae* and indicate that section *Parrya* is paraphyletic. We propose restricting section *Parrya* to the North American clade (subsection *Cembroides*, *Nelsoniae*, and *Balfourianae*) and transferring the Asian subsections *Gerardianae* and *Krempfianae* to section *Quinquefolius* (generally known as section *Strobos*). The data moderately support a sister relationship between subsections *Nelsoniae* and *Balfourianae*, rendering the pinyons paraphyletic. Several monophyletic groups can be identified within subsection *Cembroides*, including a sister relationship between *P. maximartinezii* and *P. pinceana*, which is at variance with morphological cladistic analyses. In general, relationships inferred from cpDNA are less consistent with morphological evidence than with internal transcribed spacer region data, despite paralogy in the latter marker.

Pinus is composed of approximately 110 species distributed widely throughout the Northern Hemisphere (Price et al. 1998). The genus is divided into two subgenera, differentiated by the presence of either one fibrovascular bundle in the needle (subgenus *Strobos*, or soft pines), or two (subgenus *Pinus*, or hard pines). Subgenus *Strobos* is further divided into two sections: sect. *Parrya* with a dorsal umbo (a raised area that is exposed in the second year of cone maturation) on the apophysis of the female cone scale, and sect. *Quinquefolius* (sect. *Strobos* is a synonym, see below) with a terminal umbo. Recent molecular systematic studies of the nuclear ribosomal (nr) DNA internal transcribed spacer (ITS) region (Liston et al. 1999) and four chloroplast (cp) DNA regions (Wang et al. 1999; Geada López et al. 2002) have corroborated the monophyly of both subgenera. Within subgenus *Strobos*, molecular studies sampling both Eurasian and North American representatives of sect. *Parrya* have suggested to varying degrees that this section is paraphyletic to sect. *Quinquefolius* (Strauss and Doerksen 1990; Liston et al. 1999; Wang et al. 1999; Gernandt et al. 2001).

Relationships among the members of sect. *Parrya* are unclear. Using restriction fragment length analysis, Strauss and Doerksen (1990) inferred that the North American subsection *Balfourianae* is the sister group to all other soft pines, with subsections *Cembroides* and *Gerardianae* successively paraphyletic to sect. *Quinquefolius*. Using cpDNA sequences, Wang et al. (1999) recovered a paraphyletic *Parrya* with subsection *Balfourianae* at the base, and with subsections *Gerardianae* and *Krempfi-*

anae successively paraphyletic to sect. *Quinquefolius*. Although sampling differed between the two studies, the results are consistent. Section *Parrya* was paraphyletic in the partial ITS region analysis of Liston et al. (1999) but subsectional interrelationships, particularly the placement of subsections *Nelsoniae* and *Balfourianae*, were poorly supported and varied depending on the sequence alignment. Section *Parrya* was also inferred to be paraphyletic in a recent phylogeny of almost-complete ITS region sequences (Gernandt et al. 2001), in which North American subsections *Balfourianae*, *Cembroides*, and *Nelsoniae* were monophyletic, and Eurasian subsection *Gerardianae* was the sister group to sect. *Quinquefolius*. Despite apparent agreement of subsectional relationships with previous studies, ITS region paralogy was found to affect phylogenetic inference among closely related species.

Morphology-based views of pinyon phylogeny have tended to recognize two lineages, though the circumscription of the two lineages has varied. Cladistic analyses of mainly morphological characters (Malusa 1992; Farjon and Styles 1997) have recovered a paraphyletic group of pinyons divided into two lineages, a monophyletic group of "typical" pinyons and a paraphyletic grade of four putatively relictual species occurring in a sister relationship to other members of subgenus *Strobos*. The typical pinyons have subglobose ovulate cones with a flat base (Malusa 1992); the cones are shorter than those of their close relatives and have fewer (≤ 60) seed scales per cone (Farjon and Styles 1997). The relictual pinyons are confined to one or a

few small populations in Mexico and share character states with other soft pines, including ovulate cones with a conical base, longer seed cone peduncles, longer and wider ovulate cones, more (60–110) seed scales per cone, and longer needles (Farjon and Styles 1997). The dichotomy of pinyons into typical and relictual pines is reflected in the classification of Perry (1991). Putative relicts, *P. nelsonii* and *P. pinceana*, occur as sister species in morphological cladistic studies and both are included in subsect. *Nelsoniae* by Farjon (1996) and Farjon and Styles (1997), although subsect. *Nelsoniae* originally only included *P. nelsonii* (van der Burgh 1973). Farjon and Styles chose not to classify *P. rzedowskii* and *P. maximartinezii* to subsection, pending “more phylogenetic work”. Finally, Price et al. (1998) divided pinyons into subsect. *Cembroides* and a monotypic subsect. *Rzedowskianae* Carvajal.

Pine phylogenies based on molecular data have offered limited insights into pinyon relationships. Studies of cpDNA restriction sites (Perez de la Rosa et al. 1995) and nrDNA ITS sequences (Liston et al. 1999, Gernandt et al. 2001) have been unable to determine whether pinyons are monophyletic, paraphyletic, or polyphyletic, although they have all separated *P. nelsonii*, but not *P. pinceana*, from other pinyons. CpDNA and ITS indicate that *P. pinceana* is the sister species of *P. maximartinezii* (Perez de la Rosa et al. 1995; Gernandt et al. 2001). Taken together, molecular studies suggest that pinyons are biphyetic, composed of sect. *Cembroides* and a monotypic subsect. *Nelsoniae*. A large basal polytomy in the study of Perez de la Rosa et al. (1995) and ITS paralogy in the study of Gernandt et al. (2001) limit further insights into pinyon relationships.

In this study we use sequences from three cpDNA regions, the maturase K (*matK*) and ribulose 1,5-bisphosphate carboxylase large subunit (*rbcl*) genes and the ribosomal protein L16 (*rpl16*) intron, to infer interrelationships among pinyon pines and to test the monophyly of sect. *Parrya*. We also compare these results with previous morphology-based cladistic analyses (Malusa 1992; Farjon 1996; Farjon and Styles 1997). Wang et al. (1999) have published *matK* and *rbcl* sequences for many Eurasian pines and for the North American subsect. *Balfourianae*, but none for pinyons. This study also integrates pinyon pine sequences, plus other new sect. *Parrya* sequences, into the molecular data sets of Wang et al. (1999).

MATERIALS AND METHODS

Sampling. All species of sect. *Parrya* recognized by Price et al. (1998) were sampled (Table 1). Also included were *P. catarinae*, possibly a synonym of *P. remota*; *P. quadrifolia*, possibly a hybrid between *P. juarezensis* and *P. monophylla*; and infraspecific taxa of *P. cembroides*. Price et al. recognized two subspecies of *P. monophylla*, *P. monophylla* subsp. *californiarum* (D.K. Bailey) Zavarin and *P. monophylla* subsp. *fallax* (Little) Zavarin, that were not included in the present study. Whenever possible, material was collected from

wild populations. Two exemplars were taken from the type location of *P. juarezensis*, but only one of the two (DSG01099) had a high percentage (approximately 80%) of fascicles with five needles; the second may better correspond to *P. quadrifolia*, proposed by Lanner (1974) to be a hybrid between *P. juarezensis* and *P. monophylla*, although this conclusion is controversial (Farjon and Styles 1997).

All analyses included the corresponding region or regions from the chloroplast sequence of *P. thunbergii*, NC_001631.1 (Wakasugi et al. 1994). Additional *matK* and *rbcl* sequences (Wang et al. 1999) were downloaded from GenBank (accession numbers AB019795-AB019867). More recently deposited sequences of hard pines (Gada López et al. 2002) had not yet been published at the time the analyses were performed.

DNA Extraction, Amplification, and Sequencing. Total genomic DNA was extracted from needles of single trees using a modified CTAB method (Doyle and Doyle 1997). Final concentrations of polymerase chain reactions (PCR) were 1X *Tfl* PCR buffer, 1.5 mM MgCl₂, 200 μM each dNTP, 1 μM of each primer, 2X PCR enhancer with betaine, 1.0 U *Tfl* DNA polymerase (Epicentre Technologies), and approximately 1 ng/μL of DNA. Primer information is given in Table 2. PCR cycles involved an initial denaturing step at 94°C for three min, then 30 cycles of 94°C for one min, 50°C for 50 s, and 72°C for 80 sec. At the end of the cycles, tubes were held at 72°C for an additional five min, and then cooled to 4°C. PCR reactions were checked on 1% agarose gels and successful amplifications were purified using a GeneClean Bio101 DNA purification kit. PCR products were sequenced using Perkin-Elmer BigDye terminator kits on either an ABI Model 310, 373 or 377 automated sequencer (PE Applied Biosystems, Inc.).

Analysis. Sequence reads were assembled and edited in BioEdit Sequence Alignment Editor (Hall 1999). The 34 taxon *rpl16* data set (Table 1 plus *P. thunbergii*) included 825–832 bp from the *rpl16* intron and 285 bp from the 5' end of exon 1 (98.4% complete), was 1,134 bp in length, and had four cells scored as missing (0.010%). Three gaps in the *rpl16* sequence alignment were phylogenetically informative within sect. *Parrya*, a four bp gap in all sequences except *P. bungeana* and *P. gerardiana* (position 610 in *P. cembroides*), a seven bp gap in *P. cembroides* subsp. *cembroides*, *orizabensis*, and *lagunae* (position 712 in *P. cembroides*), and a five bp gap in subsect. *Balfourianae* (position 803 in *P. cembroides*); these were scored as “0” or “1” and appended to the sequence matrix (Steele and Vilgalys 1994). All other gaps were uninformative for sect. *Parrya* and were treated as missing characters. The 28 taxon *rbcl* data set included 1398 bp of the *rbcl* gene (97.9% complete, missing 30 bp from the 5' end) plus 20 bp of the 3' flanking region, was 1418 bp in length (no gaps), and had ten cells scored as missing (0.025%). The 39 taxon *matK* data set included 1431 bp of the *matK* gene (92.4% complete, missing approximately 117 bp from the 5' end) plus 170 bp of the 3' flanking region, was 1601 bp in length, and had ten cells scored as missing (0.016%). No missing cells were at positions known to be variable. The *matK* and *rbcl* sequences from more distantly related pines in GenBank were truncated relative to the study group, and no *rpl16* sequences were available. As a result, in the combined 56 taxon matrix, 50,445 cells were scored as missing (21.62%). Of these, 585 cells were missing from subsects. *Balfourianae*, *Cembroides*, *Nelsoniae*, *Gerardiana*, and *Krempfianae*, 568 of which were from the 5' *matK* GenBank sequence of *P. krempfii*. The morphological matrix of Malusa (1992) had 33 cells scored as missing (8.6%) and an additional 11 cells scored as polymorphic (2.9%). The data set is available on TreeBASE (study accession number = S953; matrix accession numbers = M1579, M1580, M1581, and M1582).

Phylogenetic analyses were run in PAUP* 4.0B8 for Windows (Swofford 2002). Heuristic searches used equally weighted parsimony as the selection criterion and tree-bisection and reconnection (TBR) branch swapping. Random addition sequence with 250 replicates was used with no limit to the number of trees saved. Branch support was measured with bootstrap values (Felsenstein 1985) and decay indices (Bremer 1994). Bootstraps were performed with 500 replicates using simple taxon addition and TBR swapping, with 100 trees saved per replicate. Decay indices were calculated

TABLE 1. *Pinus* taxa collection information, with voucher information and Genbank accession numbers.

Subsection *Cembroides*. *P. catarinae*—Pinetum M. Martínez, Chapingo 66, Mexico, *Gernandt 00800* (MEXU) (*matK* AY115772, *rpl16* AY115811, *rbcL* →). *P. catarinae* 2—E. of Santa Catarina, Nuevo León, Mexico, *Gernandt 01301* (MEXU) (*matK* AY115773, *rpl16* AY115812, *rbcL* AY115749). *P. cembroides*—Pinalito, Hidalgo, Mexico, *Gernandt 00398* (MEXU) (*matK* AY115782, *rpl16* AY115803, *rbcL* AY115751). *P. cembroides* 2—Royal Botanic Gardens, Kew 1977.6986 (K) (*matK* AY115781, *rpl16* →, *rbcL* →). *P. cembroides* subsp. *lagunae*—Pinetum M. Martínez, Chapingo 159, Mexico, *Gernandt 01600* (MEXU) (*matK* AY115783, *rpl16* AY115805, *rbcL* AY115752). *P. cembroides* subsp. *orizabensis*—San Salvador El Seco, Puebla, Mexico, *Gernandt 07399* (MEXU) (*matK* AY115784, *rpl16* AY115804, *rbcL* AY115753). *P. cembroides* subsp. *orizabensis* 2—Royal Botanic Gardens, Kew 1910.67001 (K) (*matK* AY115785, *rpl16* →, *rbcL* →). *P. culminicola*—Cerro Potosí, Nuevo León, Mexico, *Gernandt 24298* (MEXU) (*matK* AY115776, *rpl16* AY115806, *rbcL* AY115748). *P. discolor*—S. of San Luis Potosí, San Luis Potosí, Mexico, *Gernandt 2101* (MEXU) (*matK* AY115780, *rpl16* →, *rbcL* →). *P. discolor* 2—Pinetum M. Martínez, Chapingo 66, Mexico, *Gernandt 6699* (MEXU) (*matK* AY115777, *rpl16* AY115747). *P. edulis*—Eagle, CO, USA, *Gernandt 03399* (OSC) (*matK* AY115765, *rpl16* AY115807, *rbcL* AY115738). *P. edulis* 2—Royal Botanic Gardens, Kew 1995.3959 (K) (*matK* AY115766, *rpl16* AY115808, *rbcL* AY115739). *P. johannis*—Concepción del Oro, Zacatecas, Mexico, *Gernandt 08199* (MEXU) (*matK* AY115778, *rpl16* AY115813, *rbcL* AY115746). *P. johannis* 2—N. of Charcas, San Luis Potosí, Mexico, *Gernandt 02001* (MEXU) (*matK* AY115779, *rpl16* AY115814, *rbcL* AY115747). *P. juarezensis*—La Rumorosa, Baja California Norte, Mexico, *Gernandt 01099* (MEXU) (*matK* AY115770, *rpl16* AY115819, *rbcL* AY115742). *P. juarezensis*—La Rumorosa, Baja California Norte, Mexico, *Gernandt 01499* (OSC) (*matK* AY115769, *rpl16* AY115818, *rbcL* AY115743). *P. monophylla*—White Mountains, CA, USA, *Gernandt 02399* (OSC) (*matK* AY115768, *rpl16* AY115817, *rbcL* AY115740). *P. monophylla* 2—Royal Botanic Gardens, Kew 1987.5767 (K) (*matK* AY115767, *rpl16* AY115816, *rbcL* AY115741). *P. maximartinezii*—Pueblo Viejo, Zacatecas, Mexico, *Gernandt 07699* (MEXU) (*matK* AY115790, *rpl16* AY115821, *rbcL* AY115755). *P. maximartinezii* 2—Pinetum M. Martínez, Chapingo 67, Mexico, *Gernandt 01200* (MEXU) (*matK* AY115789, *rpl16* →, *rbcL* →). *P. pinceana*—Barranca de Tolantongo, Hidalgo, Mexico, *Gernandt 03998* (MEXU) (*matK* AY115787, *rpl16* AY115822, *rbcL* →). *P. pinceana* 2—E. of Parras, Coahuila, Mexico, *Gernandt 08899* (MEXU) (*matK* AY115788, *rpl16* AY115823, *rbcL* AY115754). *P. pinceana* 3—Pinetum M. Martínez, Chapingo 162, Mexico, *Gernandt 07199* (MEXU) (*matK* AY115786, *rpl16*, *rbcL*). *P. quadrifolia*—Riverside Co., CA, USA, *Gernandt 01999* (OSC) (*matK* AY115771, *rpl16* AY115820, *rbcL* AY115744). *P. remota*—El Oso, Coahuila, Mexico, *Gernandt 19298* (MEXU) (*matK* AY115775, *rpl16* AY115809, *rbcL* AY115750). *P. remota* 2—E. of La Cuesta, Coahuila, Mexico, *Gernandt 00801* (MEXU) (*matK* AY115774, *rpl16* AY115810, *rbcL* →). *P. rzedowskii*—El Varaloso, Michoacán, Mexico, *Quijada 9008* (no voucher) (*matK* AY115791, *rpl16* AY115824, *rbcL* AY115756).

Subsection *Nelsoniae*. *P. nelsonii*—Miquihuana, Tamaulipas, Mexico, *Gernandt 30798* (MEXU) (*matK* AY115793, *rpl16* AY115825, *rbcL* AY115757). *P. nelsonii* 2—San Antonio Peña Nevada, Nuevo León, Mexico, *Gernandt 10398* (MEXU) (*matK* AY115792, *rpl16* →, *rbcL* →).

Subsection *Balfouriana*. *P. aristata*—Echo Lake, CO, USA, *Gernandt 03299* (OSC) (*matK* AY115795, *rpl16* AY115826, *rbcL* AY115758). *P. aristata* 2—Royal Botanic Gardens, Kew 1992.747 (K) (*matK* AY115794, *rpl16* AY115827, *rbcL* →). *P. balfouriana*—Loblolly Mountains, CA, USA, *Oline CA 11* (OSC) (*matK* AY115799, *rpl16* AY115830, *rbcL* AY115760). *P. balfouriana* 2—Royal Botanic Gardens, Kew 1969.10666 (K) (*matK* AY115798, *rpl16* AY115831, *rbcL* →). *P. longaeva*—White Mountains, CA, USA, *Gernandt 03099* (OSC) (*matK* AY115796, *rpl16* AY115828, *rbcL* AY115759). *P. longaeva* 2—Royal Botanic Gardens, Kew 1972.6349 (K) (*matK* AY115797, *rpl16* AY115829, *rbcL* →).

Subsection *Gerardiana*. *P. bungeana*—Royal Botanic Gardens, Kew 1999.226 (K) (*matK* AY115800, *rpl16* AY115832, *rbcL* AY115761). *P. gerardiana*—Royal Botanic Gardens, Kew 1991.1245 (K) (*matK* AY115801, *rpl16* AY115833, *rbcL* AY115762). *P. squamata*—Qiaojia County, Yunnan Province, China, RMP0412 (OSC) (*matK* AY115802, *rpl16* AY115834, *rbcL* AY115763).

Subsection *Krempfiana*. *P. krempfii*—Royal Botanic Gardens, Edinburgh RMP0411 (E) (*matK* →, *rpl16* AY115835, *rbcL* AY115764).

TABLE 2. Source and position of chloroplast primers used in this study. 3' position given is for *P. thunbergii*.

matK:

orf515-900F: TACGCAATTTCTCATGATCA (3' position 3159); Gadek et al. (2000).
 2496R: TTTTCAGTGAATTCGACGAG (3' position 2496); this study.
 2R: TAAACGATCCTCTCATTACGCA (3' position 2567); Wang et al. (1999).
 orf505-2000F: TCAGGRCGGCCCAATTAGTAA (3' position 2100); Gadek et al. (2000).
 1F: GAACTCGTCGGATGGAGTG (3' position 1548); Wang et al. (1999).

rbcL:

1: ATGTCACCACAAACAGARACTAAAGC (3' position 44448); Olmstead et al. (1992).
 3F: ACCCAATTTTGGTTTGATAG (3' position 43956); Wang et al. (1999).
 2F: GGACATACGCAATGCTTTAG (3' position 43530); Wang et al. (1999).
 1R: ACAATGGCCTACTCTTTCAC (3' position 43598); Wang et al. (1999).
 12: CTTTTAGTAAAAGATTGGGCCGAG (3' position 43017); Olmstead et al. (1992).

rpl16:

F62274: TTTGGAACCGTGCTATGCTT (3' position 62274); this study.
 R1516: CCCTTCATTCTCTCTATGTTG (3' position 61418); Kelchner and Wendel (1996).
 R61126: TCTAGCGACGGTTCCGGATA (3' position 61126); this study.

by using the KEEP command in PAUP* and evaluating the strict consensus for trees up to five steps longer than the most parsimonious trees. Decay indices for branches with higher decay indices were calculated using the CONVERSE and CONSTRAINTS commands. To test whether the character state distributions underlying the morphological and cpDNA phylogenies were significantly different, we applied the Templeton test (Templeton 1983).

RESULTS

Individual Data Sets. The G+C content was 38.1% (S.D. = 0.14%) in the 34 taxon *rpl16* matrix, 38.1% (S.D. = 0.14%) in the 28 taxon *rbcL* matrix, and 37.0% (S.D. = 0.11%) in the 39 taxon *matK* matrix. The *rpl16* matrix had 14 informative sites including three gaps (45 variable sites, 42 in the intron), the *rbcL* matrix had 23 informative sites (54 variable, all in the 1398 bp gene-coding region), and the *matK* matrix had 38 informative sites (125 variable, only one in the 3' flanking region, a synapomorphy for subsect. *Gerardiana*). Informative sites occurred in the *rbcL* gene as follows: six (12 variable) at the first codon position, four (five variable) at the second, and 13 (37 variable) at the third. Fourteen amino acid changes were inferred, ten of which occurred in more than one sequence. Informative sites occurred in the *matK* gene as follows: 13 (36 variable) at the first codon position, seven (41 variable) at the second, and 17 (47 variable) at the third. Seventy-two amino acid changes were inferred, 25 of which occurred in more than one sequence.

The *rpl16* search recovered six equally most parsimonious trees (MPTs; not shown) with a length (L) of 49 steps, a consistency index (CI) of 0.92, a CI excluding uninformative characters (CI_{exc}) of 0.78, a retention index (RI) of 0.94, and a rescaled consistency index (RC) of 0.86. The *rbcL* search recovered one hundred and fifty-six MPTs (not shown; L = 75 steps, CI = 0.73, CI_{exc} = 0.55, RI = 0.81, and RC = 0.60). The *matK* search recovered four MPTs (L = 136 steps, CI = 0.93, CI_{exc} = 0.81, RI = 0.93, RC = 0.86; Fig. 1). Resolution was highest in the *matK* strict consensus tree, intermediate in the *rbcL* tree, and lowest in the *rpl16* tree.

Combined CpDNA Matrix. As expected, no topological conflict was observed among the three cpDNA trees, so a combined matrix was assembled for 26 representatives of subsects. *Balfouriana*, *Cembroides*, *Nelsonia*, *Gerardiana*, and *Krempfiana*. As a result of difficulty in amplification, only two regions, *rbcL* and *rpl16*, were sequenced for *P. krempfii*; a shorter *matK* sequence was taken from Wang et al. (1999). Sequences for these 27 representatives were combined with the three corresponding regions in *P. thunbergii*, plus *matK* and *rbcL* sequences from ten species of subsects. *Cembrae* and *Strobi*, fourteen species of subgenus *Pinus*, and four outgroups (Wang et al. 1999). The *rbcL* sequences from Wang et al. were 1322 bp in length compared to 1418 bp for the sect. *Parrya* and *P. thunbergii* sequences and the *matK* sequences were 1019–1032 bp in length

compared to 1601 bp for the sect. *Parrya* and *P. thunbergii* sequences. Wang et al. did not sequence *rpl16*. Thus, a total of 1802 bp from 28 sequences were scored as missing in the 4163 bp matrix.

Phylogenetically informative sites for the 27 subsect. *Balfouriana*, *Cembroides*, *Nelsonia*, *Gerardiana*, and *Krempfiana* sequences were distributed among the three cp regions as follows: 12 (23 variable) in *rpl16*, 23 (37 variable) in *rbcL*, and 27 (42 variable) in *matK*. To calculate (uncorrected) pairwise divergence between species, 19 complete sequences were chosen, while *P. krempfii* plus seven pinyon sequences (*P. edulis* 2, *P. monophylla* 2, *P. juarezensis*, *P. juarezensis* 2, *P. catarinae*, *P. cembroides* subsp. *orizabensis*, and *P. cembroides* subsp. *lagunae*) were excluded. The mean number of substitutions per site was 0.0036 (S.D. = 0.0025) for *rpl16*, 0.0065 (S.D. = 0.0032) for *rbcL*, and 0.0054 (S.D. = 0.0035) for *matK*.

The 56 taxon combined matrix had 169 informative sites (433 variable). A heuristic search recovered 936 MPTs of length = 552 steps (Fig. 2). Despite the large number of trees found, the number of subsect. *Balfouriana*, *Cembroides*, *Nelsonia*, *Gerardiana*, and *Krempfiana* branches supported by bootstrap values of 90% or greater increased from between zero to three in the separate analyses to six in the combined. A seventh bootstrap value above 90% supported the monophyly of subsects. *Gerardiana*, and *Krempfiana* with sect. *Quinquefolius*. Constraining *P. nelsonii* to monophyly with subsect. *Cembroides* (rather than paraphyly) cost one additional step.

Morphological Vs. CpDNA Phylogenies. A comparison between morphological (Malusa 1992) and cpDNA phylogenies for subsects. *Balfouriana*, *Cembroides*, *Nelsonia*, and *Gerardiana* is given in Fig. 3. The morphological tree is based on 20 unordered "morphological" characters (17 external, one chemical, one anatomical, and one phenological; Table 3), with multistate characters treated as polymorphic rather than missing. For cone and seed length characters, residuals from linear correlations of log-transformed values were used rather than uncorrected values because cone and seed characters were positively correlated with cone and seed length (see Malusa 1992 for details). In a preliminary analysis, we recovered trees of identical topology, but one step longer than reported by Malusa (1992). We determined that this is due to a typographical error in the published matrix for character 17, growth habit, where *P. rzedowskii* is incorrectly scored as a shrub (also, the binary coding of this character and character 20, timing of pollen release, are reversed in the data matrix and appendix of character descriptions). Correcting this results in the 90 step trees reported by Malusa. When rooted using subsect. *Gerardiana*, the pinyon pines are rendered paraphyletic by the position of subsect. *Balfouriana* within the pinyons. Constraining

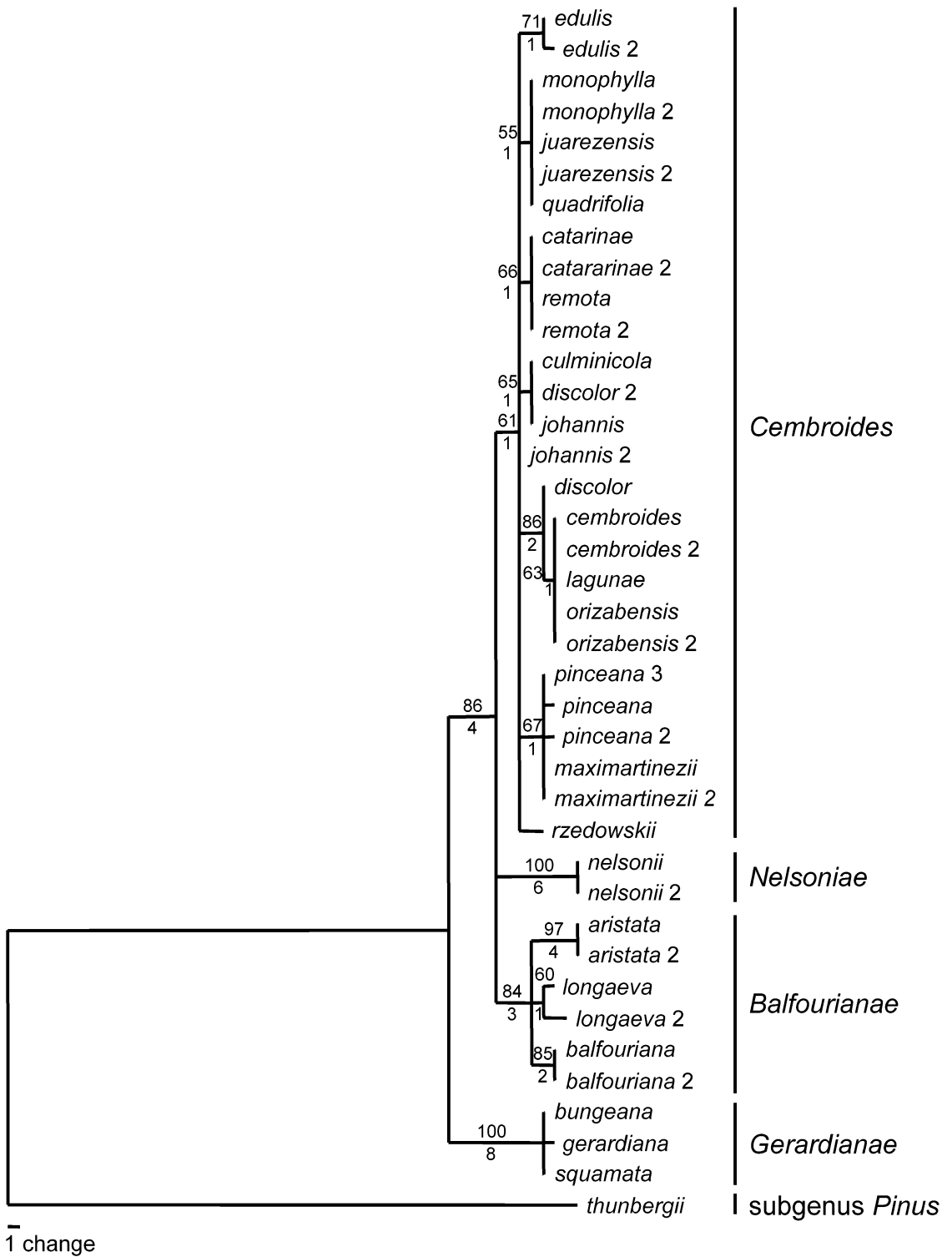


FIG. 1. One of four equally most parsimonious *matK* trees (38 informative characters, L = 136 steps, CI = 0.93, CI_{exc} = 0.81, RI = 0.93, RC = 0.86). Bootstrap values over 50% are shown above branches, and decay indices are shown below branches. Branch lengths are proportional to the number of nucleotide changes.

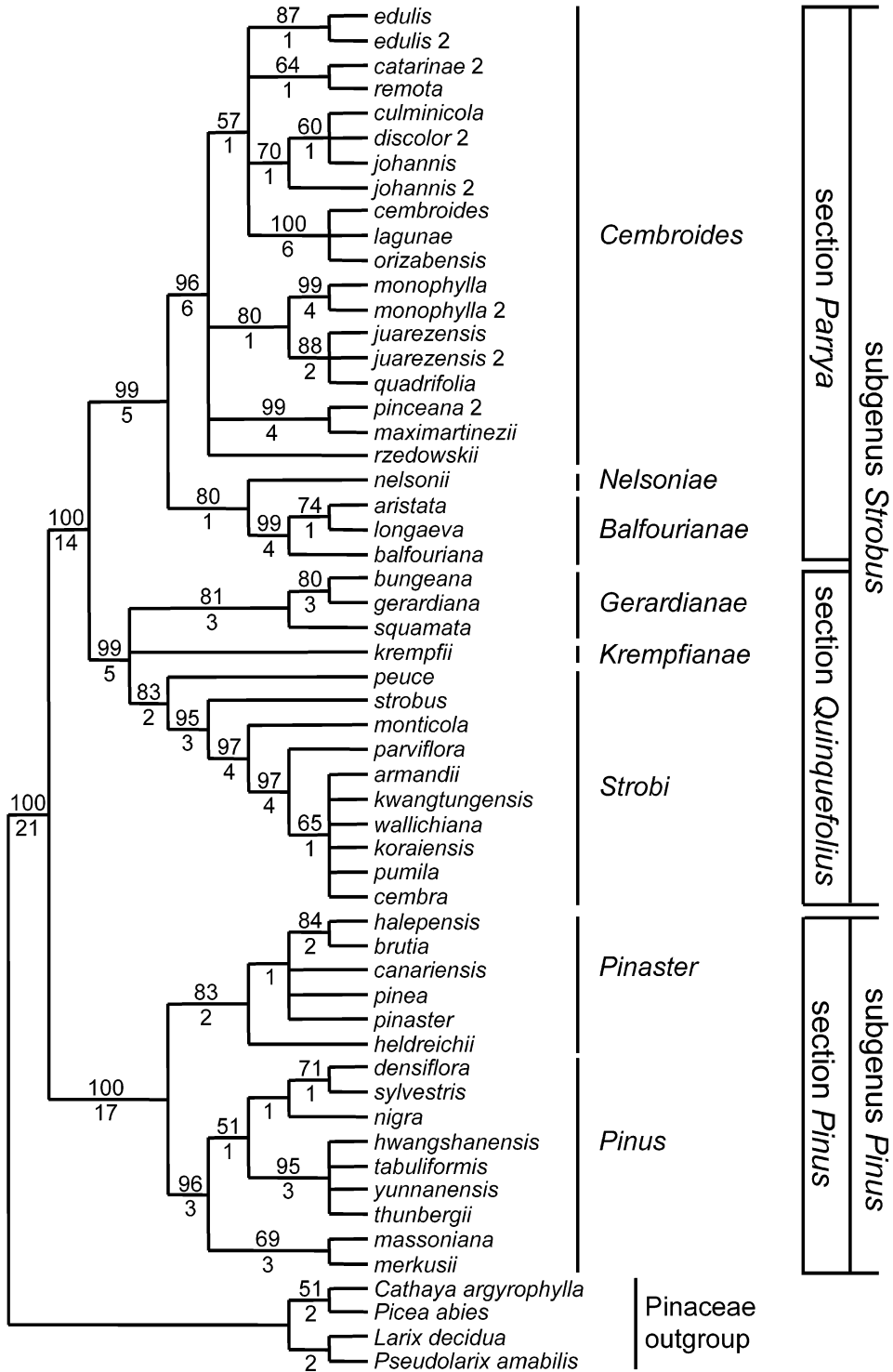


FIG. 2. Strict consensus of 936 trees for the combined *rp116*, *rbcL*, and *matK* matrix (4163 bp, 169 informative characters, L = 552 steps, CI = 0.821, CI_{exc} = 0.649, RI = 0.920, RC = 0.755). Sequences from Wakasugi et al. (1994) and Wang et al. (1998) are also included (see text for details). Bootstrap values over 50% are shown above branches, and decay indices are shown below branches. Infrageneric groups as modified in this study are shown on the right. Subsectional classification of subgenus *Pinus* is based on Liston et al. (1999).

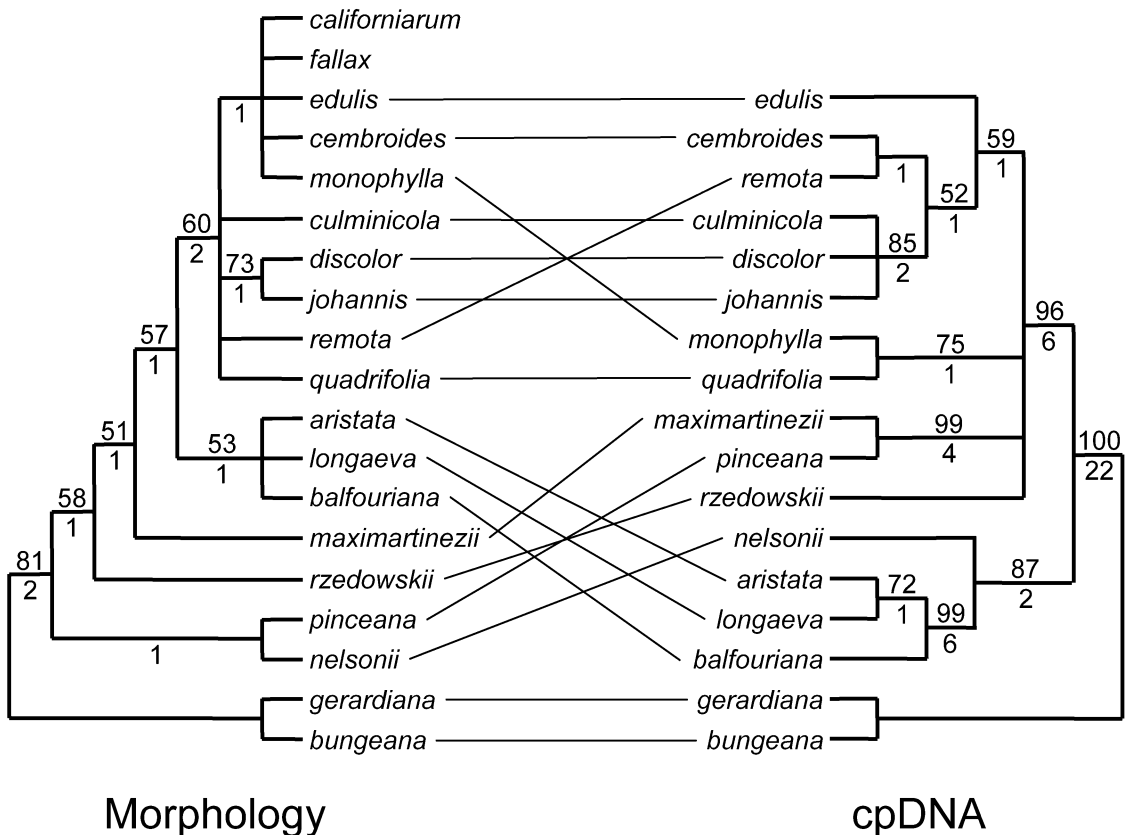


FIG. 3. Topological incongruence between the morphology/ecology data of Malusa (1992) and cpDNA sequences (this study). The morphology cladogram is a strict consensus of eight trees (19 informative characters, $L = 90$ steps, $CI = 0.67$, $CI_{exc} = 0.66$, $RI = 0.70$, $RC = 0.47$). The cpDNA cladogram is a strict consensus of six most parsimonious trees (56 informative characters, $L = 116$ steps, $CI = 0.83$, $CI_{exc} = 0.74$, $RI = 0.84$, $RC = 0.70$). Bootstrap values over 50% are shown above branches, and decay indices are shown below branches.

ing subjects. *Cembroides* and *Nelsoniae* to monophyly in the morphology tree costs six additional steps.

The cpDNA phylogeny (Fig. 3) is based on the combined matrix with all taxa not found in the morphological tree removed. The wild-collected exemplar is used when a species was represented by more than one sequence, or in the case of *P. johannis*, the collection from the type locality (Concepción del Oro, Zacatecas) was used. The cpDNA tree is based on 56 informative characters and has a length of 116 steps. Bootstrap values and decay indices are higher in the cpDNA tree than in the morphological tree. The topologies of the two trees vary both within subsect. *Cembroides*, and relative to subsects. *Gerardianae* and *Balfourianae*. Other trees from Malusa (1992) were based on ordering eight characters; these trees recovered another clade found in the cpDNA tree: *P. culminicola* in a sister relationship to *P. discolor* and *P. johannis*.

To make the morphological and molecular data sets compatible for congruence tests, we treated polymorphic morphological character states as uncertain and deleted two taxa from the morphological tree (*P. cali-*

forniarum subsp. *californiarum* and *P. californiarum* subsp. *fallax*) that are usually considered subspecies of *P. monophylla* (Zavarin et al. 1990; Price et al. 1998) and were not sampled in our cpDNA study. The changes resulted in 31 most parsimonious morphology trees with a length of 75 steps. Character distributions underlying the morphological and cpDNA trees were significantly incongruent. Using the Templeton test, the 31 morphology trees were 28 to 34 steps less parsimonious than the six cpDNA trees when tested on the cpDNA data (maximum $P = 0.0001$). In the converse test, the six cpDNA trees were 17 to 18 steps less parsimonious than the 31 morphology trees (maximum $P = 0.008$).

To investigate whether morphological and molecular data conflict was the result of adaptive convergence in particular suites of characters used by Malusa (1992), such as foliar adaptations for drought tolerance or general adaptations for seed dispersal by birds, we mapped the morphological characters onto the cpDNA tree (McCracken et al. 1999). This approach also allowed us to evaluate the amount of homoplasy in the

TABLE 3. Step lengths for individual morphological characters from Malusa (1992) compared on morphology and cpDNA trees. Trees are from Fig. 3 except that *P. monophylla* subsp. *californarium* and *P. monophylla* subsp. *fallax* are deleted from the morphology analysis.

Character	RI on cpDNA tree	No. states	No. cells missing or polymorphic	No. of steps		Difference
				Morphology trees 1-8	CpDNA trees 1-6	
Seed shell thickness	0.50	6	3	6-7	7	0-1
Seed shell length	0.43	6	0	8-9	9	0-1
Seed shell width	0	3	6	3	3	0
Cone scale thickness	0	4	2	4-6	7	1-3
Cone peduncle length	0	6	1	7-9	11	2-4
Cone length	0.63	6	1	7-8	8	0-1
Cone width	0.86	3	0	2	3	1
% fat in gametophyte	0	2	10	1-2	2	0-1
Cone base	0.71	3	0	3	4	1
Cone resin	0.40	3	5	4-5	5	0-1
Needles per fascicle	0.33	5	2	4-5	7	2-3
Needle margin	0.67	2	0	2	2-3	0-1
Fascicle sheath	0.50	3	1	2	3	1
Needle length	0.60	2	0	1	3	2
Stomate position	0.33	2	1	2-3	5	2-3
Resin canals	0.2	4	2	5-6	6	0-1
Habit	undefined	2	2	1	1	0
Bark of mature trees	1.0	3	0	2	2	0
Seed wing	0.67	3	0	3	3	0
Pollen release	1.0	2	7	1-2	1	0-1

continuous quantitative traits (Table 3; characters 1-7) and other cone and seed traits (characters 8-10 and 19) associated with the method of seed dispersal. The increase in steps on the cpDNA tree for each of the 20 morphological characters ranged from zero to four (Table 3), with several characters varying in length among equal length trees, particularly on the 31 morphology trees. The four characters with the most missing data (seed integument width, percent fat in the gametophyte, cone resin, and timing of pollen release) did not show any consistent increases in character length, although two were one step longer in a subset of the six cpDNA trees. Characters at least two steps longer in the cpDNA trees were cone peduncle length, needles per fascicle, needle length, and stomate position. Four other characters were always a minimum of one step longer on the cpDNA trees: cone scale thickness, cone width, cone base, and fascicle sheath. In other words, four of the eleven cone and seed characters (including three of the eight continuous quantitative characters) were always at least one step longer on the cpDNA trees and four of the six foliar and fascicular characters were at least one step longer. Altogether, the six foliar and fascicular characters accounted for the greatest increase in length, adding a minimum of seven steps, while the eight continuous characters accounted for a minimum increase of four steps.

One possible explanation for homoplasy in foliar characters is parallel adaptation to drought, which could have occurred simultaneously in several lineages. To evaluate this possibility we examined character state changes on cpDNA trees. Under both accelerated

and decelerated character state transformation (ACCTRAN and DELTRAN, respectively) the branch leading to subsect. *Balfourianae* involves character changes in all three homoplastic foliar characters. This branch undergoes a decrease in needle length and a loss of dorsal stomata, but contrary to the pattern expected for drought tolerance, an increase in needle number. Under ACCTRAN optimization all three foliar characters also undergo a simultaneous change in the pinyons, on the terminal branch leading to *P. edulis*. In this case, the number of leaves per fascicle drops from three to two and leaves become shorter, but dorsal stomata are gained. Needle length only changes three times, twice with both other homoplastic character changes (but only once under DELTRAN). Two homoplastic foliar characters change together twice under ACCTRAN: the common ancestor of *P. remota* and *P. cembroides* (from three to two needles per fascicle and dorsal stomata are gained) and *P. monophylla* (from three to one needles per fascicle and dorsal stomata are gained). Under DELTRAN, *P. quadrifolia* undergoes an increase from three to four (or five) needles per fascicle and dorsal stomata are lost, and *P. maximartinezii* undergoes an increase from three to five needles per fascicle and dorsal stomata are lost. Needle length and needle number changed simultaneously three out of three times under ACCTRAN and one out of three times under DELTRAN; needle number and stomate position changed simultaneously three out of five times under both ACCTRAN and DELTRAN, and needle length and stomate position change simultaneously two out of three times under ACCTRAN and one out of three

times under DELTRAN. Despite this level of simultaneous character change, the polarity of the three changes expected from adaptations to drought (decrease in needle number, decrease in needle length, loss of dorsal stomata) never occur together. Of the six simultaneous changes in two characters, such a polarity occurs twice (as mentioned above): in the common ancestor of subsect. *Balfourianae* (decrease in needle length and loss of dorsal stomata) and in *P. edulis* (decrease in needle number and length).

Homoplasy can have a positive effect on the information content of data sets when reversals or parallelisms identify additional monophyletic groups; the retention index (RI) is a better measure of information content or phylogenetic signal than the consistency index (Farris 1989, Savolainen et al. 2000). Morphological characters with RI values of 0.5 or greater when mapped on the cpDNA tree were seed shell thickness, cone length, cone width, shape of cone base, needle margin, fascicle sheath, needle length, bark of mature trees, seed wing, and pollen release (Table 3). Three continuous quantitative characters (seed shell width, cone scale thickness, and cone peduncle length) had RI values of 0. The foliar characters needles per fascicle, stomata position, and number of resin canals had values of 0.33, 0.33 and 0.2, respectively. RI values remained the same regardless of whether polymorphic characters were treated as missing or polymorphic.

DISCUSSION

Relative Phylogenetic Utility of CpDNA Regions.

In the present study, 92.4% of *matK* was used, plus 170 bp of the 3' flanking region. Forty-two variable sites were found (41 from the exon) in the 27 subsect. *Balfourianae*, *Cembroides*, *Nelsoniae*, *Gerardianae*, and *Krempfianae* *matK* sequences included in the combined analysis (Fig. 2), and 37 variable sites were found in the *rbcl* sequences (97.9% complete). Thus, when nearly complete sequences are used for subgenus *Strobis*, *matK* yields slightly more variable (and informative) sites than *rbcl*, but nucleotide divergence is 13% lower in *matK* (0.0054) than in the shorter *rbcl* region (0.0062). These results are mainly consistent with those from Wang et al. (1999) based on 32 pine species, in which nucleotide divergence in 15 species from subgenus *Strobis* was 0.0061 and 0.0063 for *matK* and *rbcl*, respectively. Fewer variable sites were found in *matK* (23) than in *rbcl* (30) for that study, presumably because their *matK* sequences were only approximately 50% complete compared to their 93.2% complete *rbcl* sequences.

Wang et al. (1999) also sequenced the *trnV* intron (542–543 bp, 100% complete plus six bp of exon) and the *rpl20-rps18* spacer (256 bp in *P. thumbergii*, 100% complete plus 186 bp of *rpl20* and 134 bp of *rps18*). Across both subgenera there were 91 variable sites in

matK, 57 in *rbcl*, 32 in the *rpl20-rps18* region, and 16 in the *trnV* intron. Nucleotide divergence for *matK* in subgenus *Pinus* was 1.8 times higher than in subgenus *Strobis* (0.0108 versus 0.0061), while *rbcl* divergence was 34% lower (0.0041 vs. 0.0063). In subgenus *Pinus*, *matK* evolved 2.6 times faster than *rbcl*, a result comparable to the 2–4 times higher rate found in Cupressaceae and in angiosperms (Soltis and Soltis 1998), but in subgenus *Strobis*, rates were almost equal in *matK* and *rbcl* (.0061 versus 0.0062, respectively).

This study reports the first phylogenetic data from the *rpl16* region (1110–1117 bp) in Pinaceae. With 23 variable and 12 informative sites for 27 subsect. *Balfourianae*, *Cembroides*, *Nelsoniae*, *Gerardianae*, and *Krempfianae* sequences in the combined analysis (Fig. 2), this region has little more than half the variable sites found in either *matK* or *rbcl*. The ingroup had only one variable site and no phylogenetically informative sites in the exon between R1516 and R61126 (259 bp), although two more variable sites occur there relative to *P. thumbergii*. Thus, phylogenetic information could have been obtained more economically by sequencing only the 850–857 bp intron region between F62274 and R1516. Although taxonomic sampling is not equivalent, the 23 variable sites from 27 subgenus *Strobis* *rpl16* sequences (representing approximately 19 species) is 15 more than in *trnV* and 11 more than in the *rpl20-rps18* region for 15 subgenus *Strobis* sequences by Wang et al. (1999). Like the *rpl20-rps18* region, *rpl16* also includes indels that can also be scored as characters. Accounting for differences in lengths among the three markers, nucleotide divergence was only slightly higher in the *rpl16* sequences (0.0036) than in *trnV* (0.0030) and lower than that encountered in the *rpl20-rps18* region (0.0043).

Sectional and Subsectional Taxonomy. A revised classification for *Pinus* subgenus *Strobis* is given in Table 4 and a key to the sections and subsections is given in Table 5. Phylogenetic analyses of nrDNA (Liston et al. 1999; Gernandt et al. 2001) and cpDNA (Wang et al. 1999; Wang et al. 2000; this study) support morphological evidence that *Pinus* is biphyletic. The well-supported dichotomy between subgenera *Pinus* and *Strobis* does not justify the recognition of a third, monotypic subgenus *Ducampopinus* (A. Cheval.) de Ferré. *Pinus krempfii*, the sole member of this subgenus as recognized by Little and Critchfield (1969), is a basal member of subgenus *Strobis* in molecular phylogenies. Members of subgenus *Strobis*, including *P. krempfii*, have a single vascular bundle per needle, while members of subgenus *Pinus* have two.

Van der Burgh (1973) and Price et al. (1998) include members of subsections. *Gerardianae* and *Krempfianae* together with subsections. *Cembroides*, *Nelsoniae*, and *Balfourianae*, but these groups are paraphyletic in this study and in prior DNA sequence phylogenies (Liston

TABLE 4. Reclassification of *Pinus* subgenus *Strobos* based on sequences from the ITS region and from cpDNA. The status of *P. monophylla* subsp. *californiarum* (D.K. Bailey) Zavarin and *P. monophylla* subsp. *fallax* (Little) Zavarin remains under investigation.

Subgenus <i>Strobos</i> Lemmon (Haploxyton or soft pines)
Section <i>Parrya</i> Mayr
Subsection <i>Balfourianae</i> Engelm.
1 <i>P. aristata</i> Engelm.
2 <i>P. balfouriana</i> Balf.
3 <i>P. longaeva</i> D.K. Bailey
Subsection <i>Cembroides</i> Engelm.
4 <i>P. cembroides</i> Zucc.
subsp. <i>cembroides</i>
subsp. <i>lagunae</i> (Rob.-Pass.) D.K. Bailey
subsp. <i>orizabensis</i> D.K. Bailey
5 <i>P. culminicola</i> Andresen & Beaman
6 <i>P. discolor</i> D.K. Bailey & Hawksw.
7 <i>P. edulis</i> Engelm.
8 <i>P. johannis</i> Rob.-Pass.
9 <i>P. maximartinezii</i> Rzed.
10 <i>P. monophylla</i> Torr. & Frém.
11 <i>P. pinceana</i> Gordon
12 <i>P. quadrifolia</i> Parl. ex Sudw. (syn. <i>P. juarezensis</i> Lanner)
13 <i>P. remota</i> (Little) D.K. Bailey & Hawksw. (syn. <i>P. catarinae</i> Rob.-Pass.)
14 <i>P. rzedowskii</i> Madrigal & Caballero
Subsection <i>Nelsoniae</i> van der Burgh
15 <i>P. nelsonii</i> Shaw
Section <i>Quinquefolius</i> Duhamel
Subsection <i>Gerardianae</i> Loudon
16 <i>P. bungeana</i> Zucc.
17 <i>P. gerardiana</i> Wallich ex D. Don
18 <i>P. squamata</i> X.W. Li
Subsection <i>Krempfianae</i> Little & Critchfield
19 <i>P. krempfii</i> Leconte
Subsection <i>Strobi</i> Loudon (including subsection <i>Cembrae</i> Loudon) 21 species

et al. 1999; Wang et al. 1999; Gernandt et al. 2001), which support a different dichotomy in subgenus *Strobos*. DNA-based phylogenies indicate that subsects. *Cembroides*, *Nelsoniae*, and *Balfourianae* form a separate monophyletic group from subsects. *Krempfianae*, *Gerardianae*, and *Strobi*. Koehne (1893) grouped subsects. *Balfourianae*, *Cembroides*, and *Gerardianae* together based on the shared presence of a dorsal umbo on the female cone, mucronate or aristate conelet scales, similar leaf epiderm and hypoderm appearing as a single tissue, external resin ducts, ray tracheids with smooth walls and ray cells with thick walls and small pits (Shaw 1914). These character states are present in one or more hard pines, indicating that sect. *Parrya* is defined either by the retention of symplesiomorphies or by homoplastic characters rather than synapomorphies that would define it as a natural group. To recognize the two clades composing subgenus *Strobos*, subsects. *Gerardianae* and *Krempfianae* should be separated from subsects. *Balfourianae*, *Cembroides*, and *Nelsoniae*. The holotype for sect. *Parrya* is *P. parryana* Engelm., a synonym for *P. quadrifolia* of subsect. *Cembroides* (Little and Critchfield 1969). Therefore we have retained the North American subsections in sect. *Parrya* and transferred the Eurasian subsections (Table 4).

The correct name for the monophyletic group composed of subsects. *Gerardianae*, *Krempfianae*, and *Strobi* is sect. *Quinquefolius* Duhamel (1755). Recent classifications (Farjon and Styles 1997; Price et al. 1998) have followed Little and Critchfield (1969) in using the name sect. *Strobos* Lemm. In adopting sect. *Strobos*, Little and Critchfield (1969) cited ICBN article 22, which recommends that the type for a subgenus should suggest the epithet for sectional names in the absence of any obstacles. This choice disregards the priority of Duhamel's (1755) sect. *Quinquefolius* (which we have modified to the ablative form, *Quinquefolius*).

TABLE 5. Key to sections and subsections of *Pinus* subgenus *Strobos*.

1. Ovulate cones with dorsal umbos, fascicles sheaths persistent or curling back to form a rosette and then deciduous (W USA, Mexico)	Sect. <i>Parrya</i>
2. Seeds with long functional wings, fascicle sheaths curling back to form a rosette, five needles per fascicle, needle length < 6 cm (W USA)	Subsect. <i>Balfourianae</i>
2. Seeds enlarged and functionally wingless, or with long functional wings but with 3–5 needles per fascicle and needle length 6–10 cm.	
3. Seeds enlarged and functionally wingless, connate needles, persistent fascicle sheaths, three needles per fascicle (Mexico)	Subsect. <i>Nelsoniae</i>
3. Seeds enlarged and functionally wingless or rarely winged, fascicle sheaths reflexing, usually curling back to form a rosette, one to five needles per fascicle (W USA, Mexico)	Subsect. <i>Cembroides</i>
1. Ovulate cones with either dorsal or terminal umbos, needle fascicles deciduous, not forming a rosette (N America and Eurasia)	Sect. <i>Quinquefolius</i>
4. Ovulate cones with a dorsal umbo.	
5. Seed wings reduced, needles in fascicles of three or seed wings functional and needles in fascicles of four to five (E Asia, Himalayas)	Subsect. <i>Gerardianae</i>
5. Seeds with functional wings, needles flattened, in fascicles of two (Vietnam).	Subsect. <i>Krempfianae</i>
4. Ovulate cones with a terminal umbo, seed wings absent, rudimentary or functional, needles in fascicles of five: (North America, Eurasia)	Subsect. <i>Strobi</i>

The ICBN now provides for the conservation of families, genera, and species "to avoid disadvantageous nomenclatural changes entailed by the strict application of the rules" (Greuter et al. 2000), but this principle has not been applied to the rank of sections. In the present situation, the name change has the potential benefit of alerting users to the recircumscription of the section proposed here.

Section *Parrya* can be circumscribed as North American soft pines (one vascular bundle per needle) with dorsal cone scale umbos and with fascicle sheaths that usually curl back to form a rosette and fall before the needles (subjects. *Balfourianae* and *Cembroides*), or are persistent (subject. *Nelsoniae*). Section *Quinquefolius* can be circumscribed as North American or Eurasian soft pines with either a dorsal cone scale umbo (subjects. *Gerardianae* and *Krempfianae*) or a terminal cone scale umbo (subject. *Strobi*), and deciduous fascicle sheaths that do not form rosettes. Price et al. (1998) further point out that Duhamel's (1755) work provides the earliest name corresponding to the "New World Diploxylon Pines", sect. *Trifolius* (ablative form of Duhamel's sect. *Trifoliis*). This section is monophyletic in Liston et al. (1999) and Gada López et al. (2002).

Molecular evidence does not support the separation of subjects. *Strobi* and *Cembrae* into mutually monophyletic groups. Genomic restriction fragment length polymorphism analysis (Strauss and Doerksen 1990) could not resolve two species of subject. *Strobi* from two species of subject *Cembrae*. The cpDNA sequences of Wang et al. (1999) for *P. cembra*, *P. koraiensis*, and *P. pumila* are derived from within subject *Strobi* (Fig. 2 of this study) and unresolved together with species classified in subject. *Strobi* (*P. armandii*, *P. kwangtungensis*, and *P. wallichiana*). ITS region sequences for *P. pumila*, *P. cembra* and *P. albicaulis* have been polyphyletic and poorly resolved relative to subject. *Strobi* (Liston et al. 1999, in press). Members of subject. *Cembrae* have indehiscent female cones and wingless seeds compared to dehiscent cones and either winged or wingless seeds in subject. *Strobi*. Molecular phylogenies have been inconclusive as to whether indehiscent cones are a synapomorphy uniting the five species commonly classified in subject. *Cembrae*. Considering the limited cpDNA sequence divergence among some members of the two subsections, and more importantly, the paraphyly of subject. *Strobi*, we do not recognize subject. *Cembrae*.

Pinus squamata is a recently described species from Yunnan, China (Li 1992). It is known from only 32 individuals in the wild, and is considered extremely endangered (Zhang et al. 2003). It was provisionally placed in subject. *Gerardianae* by Price et al. (1998). Our study confirms the placement of *P. squamata* in subject. *Gerardianae* (Zhang et al. 2003).

In this study, *P. nelsonii* is assigned to the monotypic

subject. *Nelsoniae* as proposed by van der Burgh (1973). Recent classifications are in disagreement. Perry (1991) placed *P. nelsonii* and the three other putatively relictual pinyon pines in subject. *Pinceana*. Morphological cladistic analyses (Malusa 1992; Farjon and Styles 1997) have recovered a sister relationship between *P. nelsonii* and *P. pinceana*, leading Farjon (1996) and Farjon and Styles (1997) to include *P. pinceana* with *P. nelsonii* in subject. *Nelsoniae*. Styles (1993) and Price et al. (1998) grouped *P. nelsonii* with subject. *Cembroides*. Despite its functionally wingless seeds, *P. nelsonii* has a number of characters that separate it from all other pinyons (Shaw 1909, 1914). Its female cones are attached by a persistent, recurved peduncle; its connate needles have serrate edges on the dorsal margins and persistent fascicles sheaths; and its bark is smooth and somewhat thin. Shoots and female cones do not have a period of summer dormancy. As a result of this uninterrupted growth, the female cones have an indefinite dorsal umbo. Hudson (1960) noted that the degree of dentition on the inner walls of the ray tracheids of this species is much higher than other soft pines and more similar to that in hard pines. *Pinus nelsonii* was separated from other pinyons in the cpDNA restriction site study of Perez de la Rosa et al. (1995), but overall resolution in that study was low. ITS region studies (Liston et al. 1999; Gernandt et al. 2001) also have separated *P. nelsonii* from other pinyons, but ITS paralogy rendered phylogenetic inferences uncertain, at least among closely related species in subsection *Cembroides*. This study provides the strongest molecular evidence to date of the phylogenetic distinctness of *P. nelsonii*. The only other species in subgenus *Strobis* that forms a monotypic subsection is *P. krempfii* (sect. *Quinquefolius*).

Species Relationships in Subsect. *Cembroides*. Although three other pinyons, *P. maximartinezii*, *P. pinceana*, and *P. rzedowskii*, have often been either classified with *P. nelsonii* (e.g., Perry 1991; Malusa 1992), or at least apart from other pinyons (Farjon and Styles 1997; Price et al. 1998), ITS region (Gernandt et al. 2001) and cpDNA sequences (this study) indicate that these species form a well-supported monophyletic group with other pinyons. Their poorly resolved position at the base of subject. *Cembroides* in this study and their sister position to typical pinyons inferred from ITS sequences (Gernandt et al. 2001) is consistent with their proposed relictual nature, although they are not closely related to the other relictual species, *P. nelsonii*. The present study corroborates the sister relationship between *P. maximartinezii* and *P. pinceana*. The two are sister species here and in previous cpDNA (Perez de la Rosa et al. 1995) and ITS (Gernandt et al. 2001) studies. The monoterpene compositions in these two species are highly similar; limonene is the primary mono-

terpene compared to α - and β -pinenes in *P. nelsonii* (Zavarin and Snajberk 1987).

The remaining pinyons of subsect. *Cembroides* exhibit less interspecific morphological variation, and as a consequence, taxonomic difficulties in this group often relate to species delineation. For example, Perry (1991) recognized ten species in this group, while Farjon and Styles (1997) recognized six (including *P. edulis* with a range outside Mexico), and Price et al. (1998) recognized eight (excluding *P. maximartinezii*, *P. nelsonii*, and *P. pinceana*). Non-recombining DNA sequences have been proposed to be useful in delineating phylogenetic species (Brower 1999). Despite low resolution, this approach can be applied in a preliminary way by determining whether groups in the cpDNA trees (Figs. 1 and 2) corroborate a priori hypotheses of species boundaries. Within subsect. *Cembroides*, two populations each from most potential species are represented in the *matK* tree (Fig. 1), and several species are represented twice in the combined tree (Fig. 2).

The most strongly supported monophyletic group unites *P. cembroides* subsp. *cembroides*, *P. cembroides* subsp. *orizabensis*, and *P. cembroides* subsp. *lagunae*. All cpDNA sequences in this clade are identical. *Pinus cembroides* subsp. *lagunae*, endemic to southern Baja California, was elevated to *P. lagunae* by Passini (1987). Perry (1991) adopted this new ranking but Farjon and Styles (1997) and Price et al. (1998) considered it a subspecies of *P. cembroides*. Our single exemplar of this species is from a pinetum in central Mexico and not collected in the wild by the authors. We confirmed the identification by observing the foliar characters and marked apical dominance that distinguish this subspecies from other subspecies of *P. cembroides*. Of the two exemplars of *P. cembroides* subsp. *orizabensis*, one was collected from the wild, and the other corresponds to the same individual at the Royal Botanic Gardens Kew that was noticed by Bailey (1983), prompting the taxonomic investigations that led to the description of this subspecies. Finally, the RBG Kew exemplar of *P. cembroides* subsp. *cembroides* was collected from Texas, compared to south-central Mexico for the other *P. cembroides* exemplars, confirming that this haplotype lineage is latitudinally widespread.

Pinus cembroides var. *bicolor* was described based on a small pinyon in northern Mexico and the southern U.S. with a dark green dorsal leaf surface lacking stomata and a bright white ventral surface, and smaller seeds and cones than *P. cembroides* (Little 1968). Robert-Passini (1978) described *P. johannis* from the mountains above Concepción del Oro, Zacatecas, a population considered by Little (1968) as *P. cembroides* var. *bicolor*. Bailey and Hawksworth (1979) elevated the rank of *P. cembroides* var. *bicolor* to *P. discolor*. Morphological differences between these taxa are unclear; the above names have been considered to be synonyms regard-

less of rank by some (Farjon and Styles 1997; Passini 1994), and two separate species, *P. discolor* and *P. johannis*, by others (Perry 1991; Price et al. 1998). The cpDNA sequence from *P. johannis* collected at its type locality forms an unresolved trichotomy with a *P. discolor* (arboretum tree of unknown wild origin) and *P. culminicola* sequence and apart from *P. cembroides* (Figs. 2 and 3), suggesting it is not a subspecific taxon of *P. cembroides* as suggested by Farjon and Styles (1997). A second *P. johannis* sequence from San Luis Potosí is in a sister position to the other three sequences, rendering *P. johannis* at best paraphyletic. Further complicating the matter, a second *P. discolor* exemplar from San Luis Potosí has a *matK* sequence in a sister group to *P. cembroides* (Fig. 1). In a phenetic morphological analysis, Romero et al. (2000) found that San Luis Potosí populations of what they called *P. johannis*, corresponding to the *P. johannis* and *P. discolor* populations sampled here, were easily distinguished from *P. johannis* in Zacatecas. In view of the cpDNA and morphological diversity observed in this group, and the absence of sampling of *P. discolor* from the Sierra Madre Occidental or from its type locality in Arizona, more extensive molecular and morphological studies of *P. johannis*-*P. discolor* populations are needed. *Pinus culminicola*, *P. discolor*, and *P. johannis* were also monophyletic in the Malusa (1992) study when eight characters were ordered. He identified two synapomorphies for this group: small cone size and summer pollen release. *Pinus culminicola* apparently has the same cpDNA lineage as *P. johannis* in Zacatecas, and together with *P. cembroides* should be included in future studies of species limits in *P. johannis* and *P. discolor*.

Pinus remota differs from *P. cembroides* primarily in its thin seed integument, though it also tends to have two needles per fascicle rather than three (Farjon and Styles 1997). Robert-Passini (1981) proposed *P. catarinae* for a shrub-like population in Nuevo León that was originally included in *P. remota*. This division was accepted by Perry (1991) but not by Farjon and Styles (1997) or Price et al. (1998). Two sequences for each of these proposed species form a monophyletic group (Figs. 1 and 2) and all four have identical *rpl16* and *matK* sequences although the *rbcL* sequences differ by a single base pair. The type locality of *P. remota* was not sampled, but these results are consistent with morphological evidence that suggests *P. catarinae* is a synonym of *P. remota*. Little (1966) and Malusa (1992) have suggested that *P. remota* could be a hybrid, or could have introgressed with *P. cembroides*, *P. edulis*, or *P. monophylla* subsp. *fallax*. The cpDNA sequences for *P. remota* are distinct from all known pinyons, suggesting that this species is not a hybrid of contemporary taxa, although historical introgression would probably not be detected with cpDNA markers.

Pinus quadrifolia typically has four needles per fas-

cicle but exhibits much variation in needle number from three to five and rarely two or six (Farjon and Styles 1997). Focusing primarily on needle counts and number of resin canals per needle, Lanner (1974) concluded that *P. quadrifolia* is a hybrid between *P. monophylla*, with predominantly one needle per fascicle, and *P. juarezensis*, a species with a very limited distribution in Baja California Norte with predominantly five needles per fascicle. Lanner further speculated that pollen flow from *P. monophylla* was effectively introgressing *P. juarezensis* out of existence. *Pinus juarezensis* was accepted tentatively by Perry (1991) and Price et al. (1998) but rejected by Farjon and Styles (1997). These authors, while accepting the possibility of limited hybridization between *P. monophylla* and *P. quadrifolia*, pointed out that needle number varies throughout the range of *P. quadrifolia* and can also vary within an individual from year to year. Based on similarities in cone morphology, seed shell thickness, and the early deciduous fascicle sheaths of both species, Farjon and Styles (1997) predicted that *P. monophylla* and *P. quadrifolia* were closely related. Although this prediction was not supported by previous morphology based cladistic analysis (Malusa 1992 and Fig. 3), it is by cpDNA sequences, in which *P. quadrifolia* (and *P. juarezensis*) form a sister group to *P. monophylla* (Figs. 1–3). Although *matK* sequences are identical for *P. quadrifolia* and *P. monophylla* (Fig. 1), *rbcl* and *rpl16* sequences each show differences at three nucleotide positions. Chloroplast DNA is paternally inherited in pines (Neale and Sederoff 1989), thus the relatively clear cpDNA differentiation between *P. monophylla* and *P. quadrifolia* suggests that the individuals of *P. quadrifolia* (or *P. juarezensis*) that we sampled had not been introgressed with pollen from *P. monophylla*. Data from the ITS region are inconclusive (Gernandt et al. 2001). ITS clones from *P. monophylla* show intra-individual polymorphism, but one clone was in a weakly supported sister group with the representative clone of *P. juarezensis*. This could be argued to favor the hypothesis that our *P. juarezensis* sample is actually introgressed with *P. monophylla* ITS (and could be considered a misidentified "*P. quadrifolia*"), but substantial ITS polymorphism does not allow for reliable phylogenetic conclusions among closely related species. More specifically, the placement of two other *P. quadrifolia* clones away from *P. juarezensis* and either *P. monophylla* clone indicates there are three ITS lineages; only two would be expected in the absence of gene paralogy, regardless of whether *P. quadrifolia* is a hybrid or whether *P. juarezensis* is a synonym of *P. quadrifolia*. Based on the interpretation of morphological evidence by Farjon and Styles (1997) and on the cpDNA sequences reported here, we conclude that *P. juarezensis* is a synonym of *P. quadrifolia*. Nevertheless, it would be useful to include cpDNA sequences of *P. monophylla* from populations

closer to *P. quadrifolia*, and to include nuclear and mitochondrial data.

Conflict Between Morphological and Molecular Data. We attribute the character conflict between the cpDNA tree and the morphological tree of Malusa (1992) to three properties of the morphological data: 1) the small number of morphological characters (20) included, 2) the lack of a subgenus *Pinus* outgroup, and 3) somewhat high levels of homoplasy in foliar characters. The morphology tree had fewer branches with bootstrap support of 50% or more (seven compared to ten) and only two were over 70%, compared to eight over 70% in the cpDNA tree (Fig. 3). The two relationships receiving bootstrap support over 70% in the morphology tree are the sister relationship between *P. gerardiana* and *P. bungeana* (81%) and the sister relationship between *P. discolor* and *P. johannis* (73%). Both are consistent with the cpDNA tree. Therefore, no conflict was found between the morphology and cpDNA tree at moderately or well-supported branches.

Conflict between morphological and molecular data is often probably a spurious result arising from the undersampling of taxa or characters (Hillis and Wiens 2000). In Malusa's morphology matrix, inclusion of more distant outgroups would have increased the number of informative characters and allowed for better optimization. For example, presence of persistent fascicle sheaths (*P. nelsonii*) was scored as missing data, but this character would have been a symplesiomorphy if a representative of subgenus *Pinus* had been included. Although fewer species of pinyon pines are represented, Farjon (1996) includes both subgenera of *Pinus* plus *Picea* in a morphological analysis in which *P. nelsonii* is the sister group to remaining soft pines. Farjon (1996) makes no distinction between strongly and weakly curling rosettes, and instead distinguishes between early deciduous sheaths (as in *P. monophylla* and *P. quadrifolia*) and fascicle sheaths forming a weak rosette. This changes the distribution of character states. For example *P. pinceana* was the only pinyon scored as stramineous (a state shared with subsect. *Gerardianae*) by Malusa, but as early deciduous (a shared plesiomorphy in pinyons) by Farjon and Styles (1997). Terminal versus dorsal umbo are informative characters in the analyses of Farjon (1996) and Farjon and Styles (1997) and furthermore, Farjon (1996) used a number of additional characters informative within pinyons not used by Malusa (1992) such as female cone opening and persistence, presence or absence of a dorsal spine on the umbo, cataphyll bases virtually non-decurrent or decurrent, and number of scales on the female cone. Despite probable improvements in the character matrix by Farjon (1996) and Farjon and Styles (1997), taxonomic sampling was limited to Mexican pines; subsects. *Balfourianae* and *Gerardianae* were not included.

Ortiz-García (1999) analyzed 70 morphological characters from 46 pine species. This study had good subsectional representation for the genus, but only included seven pinyons: six members of subsect. *Cembroides*, and *P. nelsonii*. Subgenus *Strobus* was monophyletic in the strict consensus of 24 morphology trees (bootstrap support below 50%), with *P. nelsonii*, then subsect. *Gerardianae* (*P. bungeana* and *P. gerardianae*) successively paraphyletic to a clade of subsect. *Cembroides*. Within the pinyon clade, *P. maximartinezii*, *P. pinceana*, and *P. cembroides* formed a paraphyletic grade leading to *P. culminicola* and *P. johannis*. Except for the paraphyly rather than monophyly of *P. maximartinezii* and *P. pinceana*, this topology is consistent with molecular results and shows that *P. nelsonii* and *P. pinceana* do not form a sister group when more taxa and characters are sampled (although some corrections are needed in this matrix). Nevertheless, *P. rzedowskii* is separated from the remaining members of subsect. *Cembroides* by subsect. *Balfourianae* (*P. aristata*), and in a position basal to subsect. *Strobi*. None of these basal relationships receive bootstrap support of 50%.

Suites of adaptive characters can exhibit parallel (homoplastic) changes in response to a common ecological pressure (e.g., McCracken et al. 1999). Comparison of cpDNA and morphological data in pinyon pines and their close relatives reveals that foliar characters show low congruence with the cpDNA tree (Table 3). For example, several sister species of pinyon pines differ by more than one needle per fascicle (e.g. *P. maximartinezii* and *P. pinceana*, *P. monophylla* and *P. quadrifolia*, and *P. culminicola* and *P. johannis/P. discolor*). In pines, parallel reduction in needle number and needle length (thus decreasing surface area for transpiration), and reduction in the distribution of stomata might be adaptations for summer drought tolerance, but Lanner (1974) and subsequently Malusa (1992) have pointed out that while *P. monophylla*, with usually one needle per fascicle, is found in one of the driest pinyon habitats, *P. quadrifolia*, with needles in fascicles usually of four to five, is found in similar if not drier ones. Furthermore, homoplastic foliar characters did not show what could be considered simultaneous adaptations for drought (reduction in needles per fascicle, shorter needles, and strictly adaxial stomata). Such adaptations might be expected for pinyons from low rainfall habitats, but only a single co-occurrence of two such adaptations was found in the inferred reduction of needle number and needle length in *P. edulis*. Regarding phylogenetic reconstruction, the ordering of homoplastic characters such as needle number would have the undesirable effect of giving them additional weight; for example, grouping *P. monophylla* with *P. quadrifolia*, as occurs in the cpDNA tree, would require three transformations in needle number, from one to four. Comparison with molecular phylogenies suggests that rather

than ordering foliar characters, it may be better to downweight them in morphological cladistic analyses of pines.

The eleven seed and cone characters (1–10 and 19) used by Malusa (1992) did not show large increases in steps on the cpDNA tree. Only one cone character, cone peduncle length, cost a minimum of two additional steps on the cpDNA tree. Cone and seed characters conceivably could show a correlated and homoplastic pattern of character transformation if more distantly related pine subsections with vertebrate-dispersed seeds are compared, such as some members of subsections. *Strobi* and *Pinaster*. This prediction requires more thoroughly sampled molecular and morphological phylogenies of *Pinus*.

Malusa's seed and cone characters (Table 3) were quantitative. He used the Sheffe F-test to search for significant differences between mean taxon values (successful in characters 1 and 7) and when that failed to distinguish five characters, used a controversial method, generalized gap coding (Archie 1985) to code characters 2 to 6. Character means were ranked, and a standard deviation of 2.0 was chosen as his criterion to delineate groups. Inclusion of the five gap-coded characters resulted in fewer minimum length trees and better resolution. In the present study, two quantitative characters (cone scale thickness and cone width) increased a minimum of one step on the cpDNA tree, and one (cone peduncle length) increased by a minimum of two steps. All three characters had an RI value of 0 on the cpDNA tree, indicating a lack of information content, but values for the other quantitative characters were higher. The seven quantitative characters accounted for a minimum length increase of four steps on the cpDNA tree while six foliar and fascicular characters accounted for seven additional steps. For the foliar characters, RI values were low for the number of needles per fascicle, position of stomata, and number of resin canals, but higher for needle margin, fascicle sheath and needle length. Generally, the eleven seed and cone characters, even when quantitative and scored in a controversial manner (Pimentel and Riggins 1987), performed better than most foliar characters when judged on the cpDNA tree (five steps versus seven steps). Although the phenological character, timing of pollen release, cost an additional step on a subset of the six cpDNA trees despite being scored as missing data in seven taxa, two other characters, habit (uninformative) and bark of mature trees, did not increase in length on the cpDNA tree. These observations on the relative performance of data sources might be useful in choosing characters for future morphological cladistic analyses of pines.

Biogeography of Pinyon Pines. Based on wood, needles, pollen, and anatomically verified cones, Millar (1998) recognized approximately 25 species of Creta-

ceous pines thought to represent both subgenera and seven subsections. These fossils are from eight middle or upper latitude regions of Eurasia and North America. Meijer (2000) reported fossil wood assigned to sect. *Parrya* from Late Cretaceous deposits (85 million years ago) in Europe. In addition to the apparently widespread Cretaceous fossil record of pines, five of the six extant subsections of subgenus *Strobis* are concentrated around the Pacific Ocean (subsects. *Balfouriana*, *Cembroides*, and *Nelsoniae* in western North America and subsects. *Gerardiana* and *Krempfiana* in eastern Asia), and the sixth subsection (*Strobi*), though more widespread, is also represented around the Pacific Rim. Although taxonomic assignment of some Cretaceous fossils attributed to *Pinus* may require reinterpretation, past and present distributions suggest that the common ancestor of extant soft pines could have been distributed across the Bering Land Bridge, which was probably available for migration during the Eocene (reviewed by Tiffney and Manchester 2001). Subsection *Cembroides* has a minimum age of 27.2 million years (late Oligocene; Axelrod 1986; Wolf and Schorn 1990). Axelrod (1986) hypothesized that pinyons occupied refugia in Mexico during the Eocene and radiated subsequently, but this estimate may have been too early as it was based on subsequently invalidated Eocene fossils (Wolfe and Schorn 1990). The first Pinaceae pollen from Mexico is found in Chiapas in early Miocene deposits (about 23 million years ago; Graham 1999). Lanner (1981) argued that pinyons radiated in Mexico based on the presence of summer shoots, which are probably adaptations for summer rains, in northern species where summers are dry. He also linked the radiation of pinyons to dispersal by corvids, whose fossil record begins in the late Miocene. Most pinyon species have disjunct distributions and have seeds that are dispersed by corvids. Nevertheless, the cpDNA tree indicates that two clades of sister species have adjacent or near adjacent distributions; *P. johannis*, *P. discolor*, and *P. culminicola* haplotypes from the Sierra Madre Oriental are monophyletic (*P. discolor* populations also occur in the Sierra Madre Occidental), and *P. monophylla* and *P. quadrifolia* from the western U.S. are monophyletic. This suggests that vicariance may have been more important than long distance dispersal in the evolution of these groups, which has some relevance for certain theories of pinyon evolution. For example, Lanner (1981) and Malusa (1992) hypothesized that tolerance to summer drought is a recently derived character in pinyons. Did drying climates stimulate speciation in pinyons, or was geographic separation the primary cause of speciation, with interspecific differences subsequently reinforced by adaptation to summer drought? The extent of summer drought is apparently correlated to proximity to the southwest coast of the U.S (Malusa 1992). *Pinus monophylla* and *P.*

quadrifolia are both found in California and Baja California Norte, and *P. edulis* reaches eastern Nevada. If *P. edulis* is found to be the sister group to *P. monophylla* and *P. quadrifolia*, it could be argued that this group evolved in response to summer drought, but the same relationship could be explained by vicariance even if some factor other than drought was the cause. Malusa also studied the origin of resistance to calcareous soils, but acknowledged that the Sierra Madre Oriental is predominantly limestone while the Sierra Madre Occidental is predominantly metamorphic. These characters are also confounded with geography, and therefore the monophyly of *P. culminicola*, *P. johannis*, (and *P. discolor*) in the Sierra Madre Oriental could equally be attributed to limestone tolerance or to vicariance.

Pinyon pines have been a subject of much systematic study, both from a morphological and a molecular perspective. Their nomenclatural history, geographic distribution, and most recently, phylogenetic position within *Pinus* are reasonably well understood. The separation of *P. nelsonii* from other pinyons, first proposed based on morphology, is now corroborated by nuclear and chloroplast DNA. Nevertheless, relationships within subsection *Cembroides* are only partially understood, and species concepts, particularly for *P. johannis* and its possible synonym *P. discolor*, remain unresolved. Future studies are needed to provide an improved morphological cladistic analysis of pinyons and *Pinus* in general. Molecular phylogenetic studies must sample more populations, particularly remote populations and type localities. Inclusion of additional chloroplast, nuclear, and mitochondrial regions are needed both to increase phylogenetic resolution and explore issues of hybridization and introgression in this fascinating group of pines.

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