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J. Perez de la Rosa · S. A. Harris · A. Farjon

Noncoding chloroplast DNA variation in Mexican pines

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Abstract Universal primers were used for PCR amplification of three noncoding regions of chloroplast DNA in order to study restriction site variation in 12 Mexican pine species. Two length mutations were identified that are of diagnostic value for two subgenera or sections of the genus. Phylogenetic analysis of the restriction site and length variation showed patterns of variation largely consistent with previous arrangements of these pines, except for the position of *Pinus nelsonii*, indicating that *Pinus* section *Parraya* Mayr, as circumscribed by Little and Critchfield (1969) and later authors, is not a monophyletic group.

Key words *Pinus* · Noncoding cpDNA · Genetic variation · Phylogeny · Conservation

Introduction

The genus *Pinus* is the largest of 11 genera in the family Pinaceae with approximately 120 species distributed across the Northern Hemisphere from boreal to subtropical zones. Unlike other coniferous genera, the region of greatest species diversity is situated along the western fringe of the North American continent, with a concentration of 43 species and ten infraspecific taxa in Mexico (Farjon and Styles 1995). This region is both one of recent evolution and speciation (Styles 1993) and of palaeo-endemics, some of which may represent very old lineages going back to the early evolution of the genus in the Mesozoic. In both categories taxonomic problems occur; those with the relict pertain to their place in a phylogenetic system of the genus, whilst species delimitation is the most important issue of debate in the recently evolved or still evolving 'species complexes'. Classical morphological approaches, as well as inventories of terpenes, have led to often very different results and a profusion of nomenclature. Since the rather conservative approach by Shaw (1909), the present monograph for Flora Neotropica (Farjon and Styles 1995) is the first critical, comprehensive treatment of Latin American pines. Yet some questions remain and require new approaches.

Restriction fragment analysis of the chloroplast genome has been used to study the systematics of pines from Europe (e.g. Szmidt and Wang 1993), North America (e.g. Wagner et al. 1992) and Asia (e.g. Wang and Szmidt 1993). However, this approach requires large amounts of high quality leaf or seed material. These materials may not be available for many interesting, but poorly known, subtropical pine species. Noncoding regions of chloroplast DNA (cpDNA) are expected to evolve more rapidly than coding regions (Gielly and Taberlet 1994) and therefore be of greater utility for assessing variation at the infrageneric level. Taberlet et al. (1991) designed primer sets for the amplification of three noncoding cpDNA sequences by means of the polymerase chain reaction (PCR). The primers were based on the consensus sequences of highly conserved, adjacent tRNA genes that flank the short, noncoding regions. These, presumably variable noncoding regions, are thought to be of particular interest to the study of genetic variation of cpDNA within and among closely related species. Restriction analysis of the PCR-amplified cpDNA regions has been shown to be of value in the phylogenetic analysis of genera such as *Astragalus* and *Datisca* (Liston 1992; Liston et al. 1992).

In the study presented here we investigate the cpDNA of dried Mexican pine species using PCR amplification of noncoding regions and subsequent restriction fragment analysis for phylogenetic analysis in the genus *Pinus*.

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J. Perez de la Rosa
Universidad de Guadalajara, Departmento de Botanica y Zoologia, Km 15.5 carreterra Guadalajara-Nogales, Apartado Postal 139, 45110 Zapopan, Jalisco, Mexico

S. A. Harris (✉) · A. Farjon
Oxford Forestry Institute, Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, U.K.

Material and methods

Young leaves, leaf buds or branches were collected in the field from 12 Mexican pine species and 1 *Picea* species (Table 1). The material was collected into plastic bags where it was dried with silica gel (Chase and Hills 1991), kept at ambient temperature until it was returned to the laboratory and stored at −20 °C.

Intact, total DNA was extracted from 200 mg of dried material according to the method of Doyle and Doyle (1987), but with the following modifications: 1% PVP-40T was added to the 2 × CTAB extraction buffer and the mixture incubated for 30 min at 65 °C. Synthetic primers (labelled a–f) designed by Taberlet et al. (1991) were used for amplification of the *trn*T-*trn*L-*trn*F region (Fig. 1). Amplification reaction (100 µl) contained: 42 µl water; 10 µl 1 m*M* dATP; 10 µl 1 m*M* dCTP; 10 µl 1 m*M* dGTP; 10 µl 1 m*M* dTTP; 10 µl 10 × Dynazyme™ buffer (100 m*M* Tris-HCl, pH 8.8; 15 m*M* $MgCl_2$; 500 m*M* KCl; 1% Triton-X-100); 2 µl 100 µ*M* forward primer; 2 µl 100 µ*M* reverse primer; 2 µl DNA solution; 1 µl (1 unit) Dynazyme™ (Finnzymes OY; Flowgen Laboratories). The reaction mixture was subjected to amplification in a Techne PHC-3 thermocycler for 40 cycles consisting of 1 min at 94 °C, 1 min at 54 °C and 2 min at 72 °C. A final cycle of 8 min at 72 °C was used to complete extension of any remaining products. Twenty-six restriction enzymes (*Alu*I, *Bam*HI, *Bcl*I, *Bgl*II, *Cfo*I, *Cla*I, *Dde*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hin*dIII, *Hinf*I, *Hpa*II, *Mse*I, *Nci*I, *Nru*I, *Nsi*II, *Pvu*II, *Rsa*I, *Sau*3AI, *Sst*I, *Stu*I, *Taq*I, *Xba*I, *Xho*I) were used to screen amplified products (5 µl) of *Picea chihuahuana* and *Pinus nelsonii* for the presence of restriction sites. Ten of these enzymes (*Alu*I, *Dde*I, *Hinf*I, *Hpa*II, *Mse*I, *Nci*I, *Rsa*I, *Sau*3AI, *Taq*I, *Xba*I) were used to screen the 13 taxa used in the study. Restriction fragments were resolved on 2% agarose gels, in Tris-acetate buffer containing 0.5 µg/ml ethidium bromide. Banding patterns were visualised under UV light , recorded on Polaroid 667 film and scored. Restriction fragment sizes were calculated by comparison with a 123-bp ladder (Gibco-BRL) and fitting a least-squares regression (Weir 1990).

The distribution of restriction fragments in the *trn*T-*trn*L-*trn*F region was determined by comparison with the known cpDNA sequence of *Pinus thunbergii* (Wakasugi et al. 1994) and from these data the presence or absence of restriction sites and length mutations were determined. These data were scored as characters for a parsimony-based analysis using the heuristic option [tree bisection and reconnection branch swapping (TBR); random addition; 1000 replicates] of the computer package PAUP 3.1.1 (Swofford 1993), with *Picea chihuahuana* as a functional outgroup.

Results

Three pairs of primers (Fig. 1) were used to study restriction site variation in noncoding regions of the chloroplast genome from 12 species of Mexican *Pinus* and 1 species of *Picea* (Fig. 2). Primers a and b were used to amplify the intergenic spacer between the *trn*T (UGU) gene and the *trn*L (UAA) 5′ exon (Fig. 2A). Two fragments were identified in the taxa analysed, one of approximately 465 bp in *Pinus nelsonii*, *P. pinceana*, *P. maximartinezii*, *P. ayacahuite* var 'veitchii', *P. strobus* var 'chiapensis', *P. cembroides*, *P. rzedowskii* and *Picea chihuahuana* and the other of approximately 505 bp in *Pinus hartwegii*, *P. patula*, *P. pseudostrobus*, *P. devoniana* and *P. greggii*. In *Pinus thunbergii* (Wakasugi et al. 1994) this region is 426 bp in length, based on sequence analysis, compared to estimates, based on restriction analysis, of between 480 bp to 600 bp in other pine species (Boscherini et al. 1994; Taberlet et al. 1991). Primers c and d were used to amplify the intron of the *trn*L (UAA) gene (Fig. 2B). A single fragment of 570 bp was identified in all 13 species studied. In *Pinus thunbergii* (Wakasugi et al. 1994) this region is 523 bp in length, compared to estimates of approximately 600 bp from other conifers (Taberlet et al. 1991). Primers e and f were used to amplify the intergenic spacer between the *trn*L (UAA) 3' exon and the *trn*F (GAA) gene (Fig. 2C). Two fragments were identified in the taxa analysed, one of approximately 460 bp in *Picea chihuahuana*, *Pinus hartwegii*, *P. patula*, *P. pseudostrobus*, *P. devoniana* and *P. greggii* and the other of approximately 420 bp in *Pinus nelsonii*, *P. pinceana*, *P. maximartinezii*, *P. ayacahuite* var 'veitchii', *P. strobus* var 'chiapensis', *P. cembroides* and *P. rzedowskii* . In *Pinus thunbergii* (Wakasugi et al. 1994) this region is 426 bp in length, compared to estimates of 480 bp and 500 bp in other pine species (Boscherini et al. 1994).

Two length mutations and 19 restriction site mutations explained all of the restriction fragment patterns identified (Table 2). One length mutation, approximately 40 bp, was located in the *trn*T–*trn*L 5′ exon intergenic spacer, whilst a second length mutation, approximately 40 bp, was located in the *trn*F–*trn*L 3′ exon intergenic spacer. Restriction sites were distributed across the three noncoding regions analysed in Mexican pines (Table 3).

Table 1 Taxa and localities of species used in the unconding cpDNA analysis. All vouchers are Farjon and Mejía, except *Picea chihuahuana*, which is Nimsch

Voucher	Taxon	Locality	Lat. N	Long. W
335	*Pinus nelsonii* Shaw	Tula, Tamaulipas	23° 15′	99° 40′
373	*P. pinceana* Gordon	Aguaje, Guadalcazar, San Luis Potosí	22° 38′	100° 30′
317	*P. hartwegii* Lindl.	Sierra de Juárez, Oaxaca	17° 35′	96° 25′
330	*P. greggii* Engelm.	Zimapán, Hidalgo	20° 50′	99° 17′
326	*P. ayacahuite* var 'veitchii' Shaw	Tlaxco, Puebla	19°,24′	98° 05′
312	*P. strobus* var chiapensis Mtz.	Chalchiuitán, Chiapas	16° 55′	92° 38′
331	*P. patula* Schl. et Cham.	Zimapán, Hidalgo	20° 51′	99° 17′
329	*P. pseudostrobus* Lindl.	Zimapán, Hidalgo	20° 51′	99° 17′
314	*P. devoniana* Lindl.	Oaxaca, Oaxaca	17° 14′	96° 33′
328	*P. cembroides* Zucc.	Zimapán, Hidalgo	20° 46′	99° 17′
338	*P. maximartinezii* Rzedowski	Pueblo Viejo, Juchipila, Zacatecas	21° 23′	103° 15′
353	*P. rzedowskii* Mad. et Cab.	Dos Aguas, Coalcomán, Michoacán	18° 49′	102° 56′
356a	*Picea chihuahuana* Mtz.	Nuevo León[a]	–	–

[a] Collected from *Pinetum Blijdenstein* (The Netherlands)

Fig. 1 Location of the three noncoding regions on the complete cpDNA sequence of *Pinus thunbergii* (Wakasugi et al. 1994). *Numbers* refer to nucleotide positions on the complete sequence. Primers (Taberlet et al. 1991) used for hybridisation are indicated as *arrows*: *a* $^{5'}$CATTACAAATGCGATGCTCT$^{3'}$, *b* $^{5'}$TCTACCGATTTCGCCATATC$^{3'}$, *c* $^{5'}$CGAAATCGGTAGACGCTACG$^{3'}$, *d* $^{5'}$GGGGATAGGGACTTGAAC$^{3'}$, *e* $^{5'}$GGTTCAAGTCCCTCTATCCC$^{3'}$, *f* $^{5'}$ATTTGAACTGGTGACACGAG$^{3'}$

Between 6 and 12 restriction sites were found in the *trn*T–*trn*L 5′ exon intergenic spacer, of which 5 were variable. 8–10 restriction sites were found in the *trn*L intron, of which 2 were variable, and 9–13 restriction sites were found in the *trn*F–*trn*L 3′ exon intergenic spacer, of which 6 were variable.

Parsimony analysis of the total data matrix resulted in 6 shortest trees, each 25 steps long (CI = 0.875; RI = 0.840), whilst analysis of only the 19 restriction site characters resulted in 5 shortest trees each 22 steps long (CI = 0.864; RI = 0.870). In both analyses the strict consensus trees were identical (Fig. 3a).

Six site mutations (characters 1, 6, 9, 10, 13, 15) were shared only by the *Pinus* ingroup, whilst 2 (characters 5, 11) were unique to taxa within the ingroup (*P. strobus* var 'chiapensis' and *P. nelsonii*, respectively). Two major clades were identified within the *Pinus* ingroup. One clade (*P. hartwegii*, *P. greggii*, *P. patula*, *P. pseudostrobus*, *P. devoniana*) was defined by the 40-bp length mutation in the *trn*T–*trn*L 5′ exon intergenic spacer (character 20). The second clade (*P. pinceana*, *P. ayacahuite* var 'veitchii', *P. strobus* var 'chiapensis', *P. maximartinezii*, *P. rzedowskii*) was defined by the 40-bp length mutation in the *trn*F–*trn*L 3′ exon intergenic spacer (character 21). *Pinus nelsonii* possessed neither of the length mutations (Fig. 3). Three characters (3, 16, 17) showed parallelisms, whilst 1 character (8) showed a reversal.

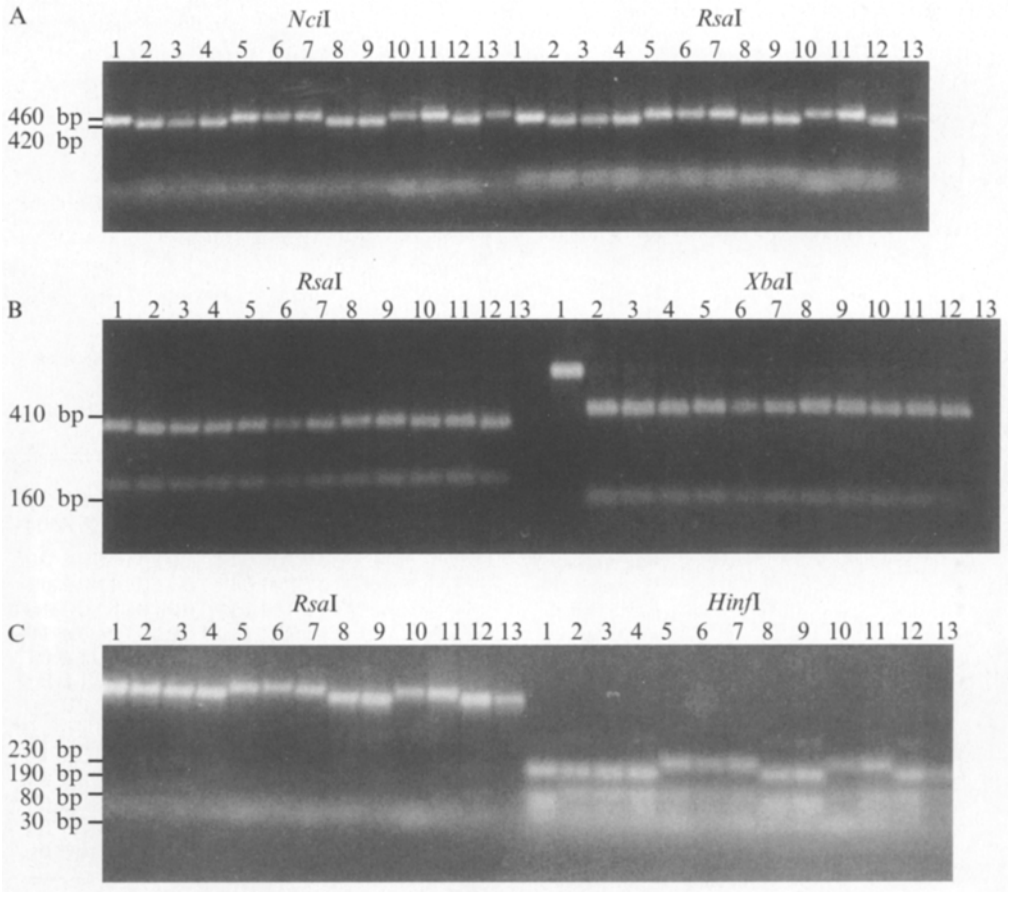

Fig. 2A–C Electrophoretic patterns of PCR products from 13 Mexican conifer species after amplification of three noncoding cpDNA regions and subsequent restriction digestion. **A** Products obtained using primers "a" and "b" and digested with *Nci*I and *Rsa*I. **B** Products obtained using primers "c" and "d" and digested with *Rsa*I and *Xba*I. **C** Products obtained using primers "e" and "f" and digested with *Rsa*I and *Hinf*I. *Lane numbers*: *1 Picea chihuahuana*, *2 Pinus rzedowskii*, *3 P. maximartinezii*, *4 P. cembroides*, *5 P. devoniana*, *6 P. pseudostrobus*, *7 P. patula*, *8 P. strobus* var 'chiapensis', *9 P. ayacahuite* var 'veitchii', *10 P. greggii*, *11 P. hartwegii*, *12 P. pinceana*, *13 P. nelsonii*

Table 2 Number of restriction fragments identified by analysis of the *trn*T-*trn*L-*trn*F noncoding cpDNA of 12 *Pinus* species with ten restriction enzymes. The number in parentheses indicates the number of sites inferred from the *P. thunbergii* cpDNA sequence (Wakasugi et al. 1994) (*n.d.* not determined, since a large number of fragments were not resolved)

Enzyme	Sequence 5′ – 3′	Number of sites		
		*trn*T-*trn*L 5′	*trn*L intron	*trn*F-*trn*L 3′
*Alu*I	AG/CT	1(1)	2(2)	1(2)
*Dde*I	C/TNAG	0–1(2)	1(2)	1–2(1)
*Hinf*I	G/ANTC	2–3(6)	1–2(4)[a]	1–2(3)
*Hpa*II	C/CGG	0(0)	1(1)	1(1)
*Mse*I	T/TAA	0–2(0)	1–2(2)	2–3(2)
*Nci*I	CC/(C or G)GG	0(0)	0(0)	1(1)
*Rsa*I	GT/AC	0(0)	1(1)	1(1)
*Sau*3AI	/GATC	2(4)	1(3)	n.d. (9)
*Taq*I	T/CGA	1–3(5)	0–1(2)	1–2(3)
*Xba*I	T/CTAGA	0(0)	1(1)	0(0)
	Total	6–12(18)	8–10(14)	9–13(23)

[a] *Hinf*I sites were not scored as characters in the analysis since they were difficult to compare with the *Pinus thunbergii* sequence

Table 3 Characters and character state distributions amongst *Picea chihuahuana* and 12 Mexican *Pinus* species. + indicates present, – indicates absent. The number in parentheses indicates fragment not resolved on gel

Character number	Region	Enzyme	Mutation (bp	
			0	1
Site mutations				
1	*trn*L	*Xba*I	570	410 + 160
2	*trn*L	*Taq*I	335 + 195	530
3	*trn*L	*Mse*I	200	160 + 40
4	*trn*T	*Mse*I	465	400 + 65
5	*trn*T	*Mse*I	465	265 + 200
6	*trn*T	*Taq*I	240	190 + 50
7	*trn*T	*Taq*I	145 + 70	215
8	*trn*T	*Hinf*I	70 + (10)	80
9	*trn*T	*Sau*3AI	160 + (20)	180
10	*trn*T	*Alu*I	310 + 85	395
11	*trn*T	*Dde*I	460	230 + 230
12	*trn*F	*Dde*I	425	310 + 115
13	*trn*F	*Taq*I	380	220 + 160
14	*trn*F	*Taq*I	145 + 145	290
15	*trn*F	*Mse*I	460	400 + 60
16	*trn*F	*Mse*I	160 + (20)	180
17	*trn*F	*Mse*I	90 + (30)	120
18	*trn*F	*Hinf*I	110 + (40)	150
19	*trn*F	*Hinf*I	370	330 + (40)
Length mutation				
20	*trn*T	40 bp	–	+
21	*trn*F	40 bp	+	–

	Character number 0000000001111111111 22 1234567890123456789 01
Pinus nelsonii	10000101111010100 00
P. pinceana	10110101110010110 00 01
P. hartwegii	11100111110010101 00 10
P. greggii	11100111110010100 00 10
P. ayacahuite var 'veitchii'	10100100110111110 11 01
P. strobus var 'chiapensis'	10101100110111101 11 01
P. patula	11100111110010101 00 10
P. pseudostrobus	11100111110010101 00 10
P. devoniana	11100111110010101 00 10
P. cembroides	10000101110010100 00 01
P. maximartinezii	10110101110010100 00 01
P. rzedowskii	10000101110010100 00 01
Picea chihuahuana	00000000000000000 00

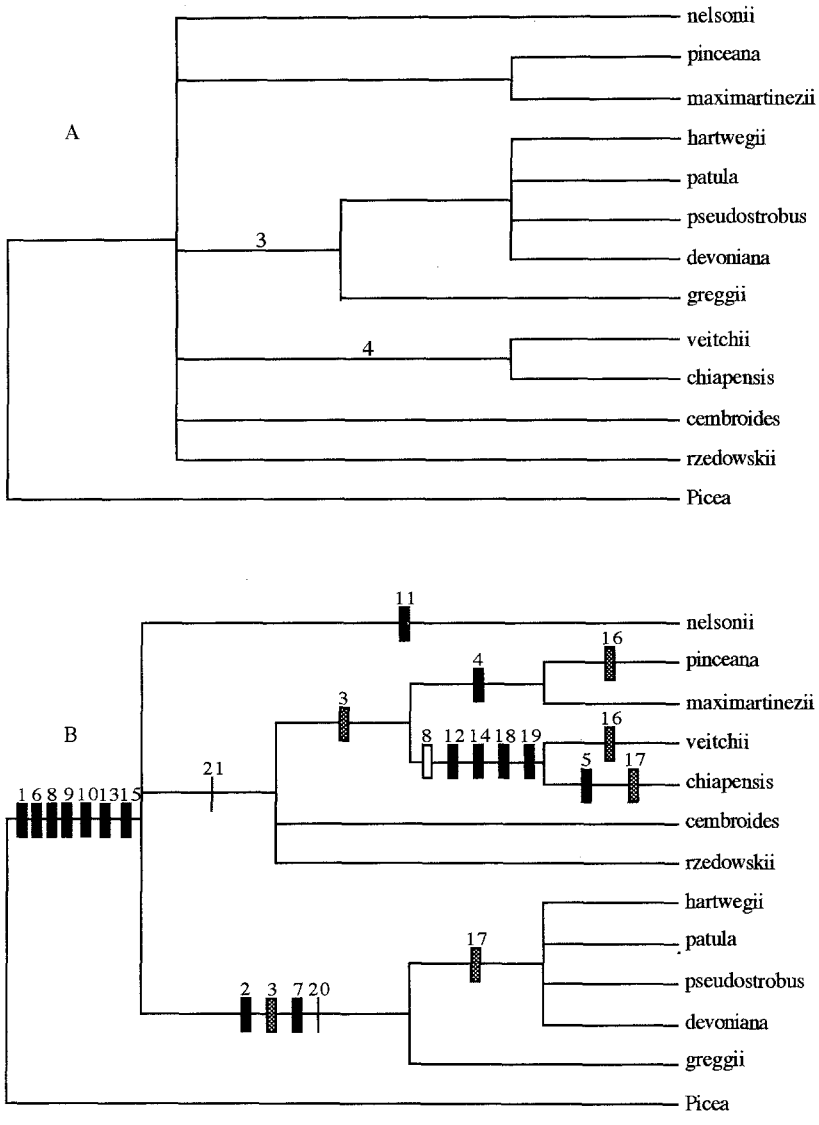

Fig. 3A, B Phylogenetic trees of 12 Mexican *Pinus* species, based on restriction analysis of the *trn*T-*trn*L-*trn*F cpDNA region, using 19 site mutations and 2 length mutations. **A** Strict consensus of the 6 shortest trees (steps = 25; C.I. = 0.875; RI = 0.840). *Number above* the *branches* indicates the number of additional steps required to collapse the clade to a polytomy. **B** One of the 6 shortest trees showing character state distributions. *Number above* the *vertical boxes* refer to characters (Table 3). *Solid boxes* indicate a 0 → 1 change, *stippled boxes* indicate a 0 → 1 parallelism and the *hollow box* indicates a 1 → 0 character state reversal. *Single vertical lines* are length mutations

Discussion

Restriction enzyme analysis of the *trn*T-*trn*L-*trn*F region showed that cpDNA intergenic spacers and introns can be used as a source of characters for the phylogenetic analysis of Mexican *Pinus* species. Comparison of these data with information available from the total sequence of the *P. thunbergii* chloroplast genome (Wakasugi et al. 1994) showed that the number of sites for some enzymes (e.g. *Sau*3AI) were apparently underestimated (Table 3). This is likely to be the result of sites differences between *P. thunbergii* and the Mexican pines and the relatively poor resolving power of agarose gels when analysing small restriction fragments. However, the results reported here are comparable with the number of sites identified in *P. leucodermis* and, for the enzymes *Taq*I and *Mse*I, in *P. brutia*, *P. halepensis*, *P. nigra* and *P. pinaster* (Boscherini et al. 1994). Studies of other species (e.g. *Quercus robur and Q. petraea*; Ferris et al. 1993) indicate that intraspecific variation in the region is minimal. However, this cannot be ruled out in the present study, particularly for those species, e.g. *P. cembroides*, *P. hartwegii* and *P. pseudostrobus*, that have a wide geographic range.

DNA sequence analysis has been used to study variation in the *trn*T-*trn*L-*trn*F region (e.g. Gielly and Taberlet 1994; van Ham et al. 1994). However, the value of this region appears to depend on the group of taxa being studied; for example no sequence divergence was found between the *trn*L introns of the genera *Fraxinus* and *Alnus*, but 6% sequence divergence was found within the genus *Gentiana* (Gielly and Taberlet 1994). Short-length mutations appear to be frequent in the *trn*T-*trn*L-*trn*F region. An analysis of the Crassulaceae and some related families showed 50 independent length mutations were responsible for the observed size variation of the *trn*F-*trn*L 3′ exon intergenic spacer (von Ham et al. 1994). All of the *Pinus* species used in this study showed little evidence of length variation, except for the presence of the 40-bp length mutations in the two intergenic spacers. Comparison of the restriction fragments with those of *P.*

thunbergii located the length mutations to single regions of their respective intergenic spacers. It therefore seems highly likely that the observed length variants are evolutionarily homologous. At the subgeneric or sectional level these length mutations are particularly valuable characters, although restriction site variation differentiates closely related pine species.

The systematic interpretation of the observed variation can only be tentative given the limited number of taxa studied. However, the lack of intraspecific sampling may not be a serious problem since intraspecific sampling in *Pinus leucodermis* (Boscherini et al. 1994) did not reveal any variation in either the *trn*T-*trn*L 5′ exon intergenic spacer or the *trn*F-*trn*L 3′ exon intergenic spacer. As in other studies that have looked at cpDNA of more than a few species of *Pinus* traditionally classified in various major groupings (e.g. Wang and Szmidt 1993), the resulting cladograms confirm both the monophyly of the genus and its division into major groups, now increasingly recognised as subgenera. However, *P. nelsonii*, traditionally classified in subgenus *Strobus*, section *Parraya* (Farjon 1984; Little and Critchfield 1969) separates on a level with the subgenera, as it possess neither of the observed length mutations (characters 20 and 21). These data indicate that its traditional placement in subsection *Cembroides* of section *Parraya* (Little and Critchfield 1969) cannot be upheld, and it may be that its separate classification from the 'Pinyon pines' (Farjon 1984) is correct and that a change of taxonomic rank is required.

Furthermore, other members of subsection *Cembroides*, *P. pinceana* and *P. maximartinezii* on the one hand and *P. cembroides* and *P. rzedowskii* on the other, do not group well, but why they form two separate 'pairs' is more difficult to explain given the substantial morphological differences between the members of these 'pairs'. This analysis casts some doubt on the monphyly of section *Parraya* and subsection *Cembroides*, although the absence of additional putatively related taxa is a limitation. Finally, 4 species in the subgenus *Pinus* sharing character 17 are members of 'species complexes', to a greater or lesser extent related amongst each other, although by all accounts distinct species. Interestingly, *P. greggii* separates from them; this 'closed-cone' pine is perhaps more closely related to Californian 'closed-cone' pines such as *P. attenuata*, *P. muricata* and *P. radiata* (Farjon and Styles 1995). Resolution of some of these problems may be possible by sequence analysis of the cpDNA intergenic regions studied here or a combination of sequence and restriction analyses of other cpDNA intergenic regions (Aldrich et al. 1988; Golenberg et al. 1993; Manen et al. 1994; Savolainen et al. 1994; Spichger et al. 1993; vom Stein and Hachtel 1988).

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