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Chloroplast DNA-based phylogeny of Asian *Pinus* species (*Pinaceae*)

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Abstract: The genus *Pinus* includes over 90 species with approximately 24 species native to Asia. We have analyzed the chloroplast (cp) DNA variation of 18 *Pinus* species, including 15 Asian, two Eurasian, and one European species using seven restriction enzymes and ten non-overlapping probes and inferred their phylogenetic relationships. Results of phenetic and cladistic approaches to phylogeny reconstruction were largely in agreement, suggesting two major lineages within the genus and confirmed the ancient character of haploxylon and diploxylon subgenera. Species from section *Parrya* appear to have diverged earliest from the hypothesized phylogenetic centre for the haploxylon pines, with *P. bungeana* and *P. gerardiana* forming two basal, monotypic lineages. The range of estimated pairwise nucleotide substitutions per site (d) was higher among haploxylon pines than among diploxylon species. CpDNA divergence was found to be low within the section *Sylvestres*, relative to the divergence among haploxylon species, suggesting that the radiation of this group of taxa from its common ancestor occurred after the diversification of other groups. The low cpDNA divergence in this subsection corroborated earlier evidence for its phylogenetic cohesiveness and existence as a monophyletic group.

The genus *Pinus* includes nearly one-hundred species and is one of the most widely distributed genera of trees in the Northern Hemisphere. The genus is usually divided into two subgenera: *Strobus* (hereafter referred to as haploxylon) and *Pinus* (hereafter referred to as diploxylon) (CRITCHFIELD & LITTLE 1966, MIROV 1967, LITTLE & CRITCHFIELD 1969, FARJON 1984). The subdivision into haploxylon and diploxylon subgenera appears to be well established (CRITCHFIELD & LITTLE 1966, MIROV 1967, LITTLE & CRITCHFIELD 1969, FARJON 1984). However, the taxonomic assignment of individual species to lower taxonomic ranks is still subject to debate (CRITCHFIELD & LITTLE 1966, MIROV 1967, LITTLE & CRITCHFIELD 1969, FARJON 1984, MALUSA 1992). The difficulties in genetic delineation are especially evident in the case of species occurring in Asia which are among the most poorly known taxa of the genus *Pinus* (MIROV 1967, FARJON 1984).

Eurasian diploxylon species appear to be less differentiated than their counterparts from North America. Notably, all diploxylon species native to this region belong to one subsection *Sylvestres*. In contrast, North American species from this

subgenus show considerable diversity and are subdivided into several different subsections (CRITCHFIELD & LITTLE 1966, LITTLE & CRITCHFIELD 1969, FARJON 1984). A similar, although less vivid pattern can be discerned in the haploxylon subgenus. For instance, the section *Parrya* includes only two Asian species (*P. bungeana* and *P. gerardiana*), forming a separate subsect. *Gerardianae*. The North American species from this section are divided into two subsections (*Cembroides* and *Balfourianae*) (FARJON 1984). On the other hand, species belonging to the other section *Strobus* of the haploxylon subgenus have intercontinental distribution (FARJON 1984).

Attempts were made to apply different methods such as analysis of morphological and/or anatomical characters, crossability, karyotype, and allozyme polymorphism, to assess the genetic relationships among *Pinus* species (DUFFIELD 1952; SAYLOR 1972, 1983; WHEELER & al. 1983; BURGH 1984; PRICE & al. 1987; FRANKIS 1988; MILLAR & al. 1988; KARALAMANGALA & NICKRENT 1989; KLAUS 1989; MALUSA 1992; SHURKHAL & al. 1992). Comparative studies of chloroplast (cp) DNA variation may shed new light on the phylogenetic structure of this systematically neglected geographic group of pines. The genome's uniparental inheritance, compact size, and slow rate of evolution are among the features eliciting its high resolving power for systematic comparisons (SZMIDT 1991, SZMIDT & WANG 1992 a and references therein). For these reasons, there has been a surge of studies carried out on cpDNA in recent years, aimed at inferring phylogenetic relationships at intrageneric or higher taxonomic levels (BREMER 1991, RIESEBERG & BRUNSFELD 1992 and references therein). Earlier phylogenetic studies of *Pinus* employing this approach included very few Asian species (SZMIDT & al. 1988, STRAUSS & DOERKSEN 1990, GOVINDARAJU & al. 1992). To date, only one preliminary analysis attempted to reconstruct cpDNA-based phylogeny of Asian *Pinus* species (WANG & al. 1991 b). In the present study, we evaluate phylogenetic relationships among *Pinus* species from Asia and Europe derived through the use of phenetic and cladistic analyses of cpDNA variation. The aims of the present study are: (1) to reconstruct a cpDNA-based phylogeny for this geographic group of species; (2) to examine whether apparent taxonomic homogeneity of species from Asia is mirrored in low differentiation at the cpDNA level.

Material and methods

Plant material. Figure 1 shows geographic distributions of the investigated taxa. Table 1 lists the species studied, their taxonomic position, and the origin of the plant material used. Bulked seed samples from one population of each species were collected in documented natural stands and grown for two vegetation periods in a greenhouse. Composite needle samples were harvested from approximately 50 seedlings of each species, and used for cpDNA extraction. In addition, needles from at least one tree from each of the 18 species were collected at the Hørsholm Arboretum, Denmark, Royal Forest Department, Bangkok, Thailand, Forestry and Forest Products Research Institute, Sapporo, Japan, Nanjing Forestry University, China, Institute of Botany, Academia Sinica, China and Institute of Forest Genetics, Placerville, U.S.A.

Our sampling strategy aimed at minimizing potential risks for deriving skewed cpDNA-based relationships. Such risks may arise due to at least one of the following causes: (i) the individual tree chosen to represent a taxon may be incorrectly classified taxonomically, (ii) the tree may be correctly classified per se, but carry a cpDNA specific to another species

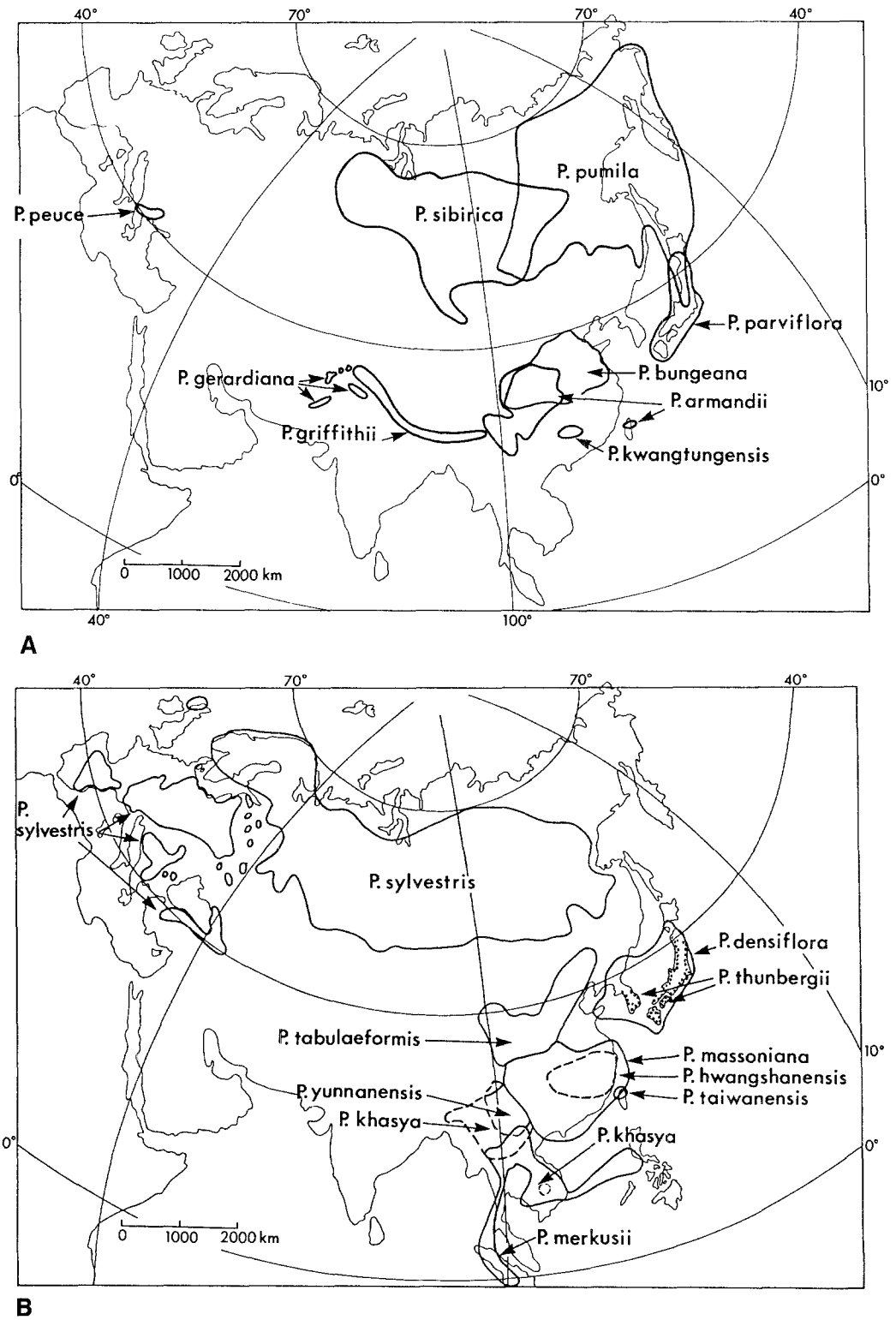

Fig. 1. Distribution of the investigated haploxylon (*A*) and diploxylon (*B*) pine species after MIROV (1967)

Table 1. List of *Pinus* species included in this study. *1* Hørsholm Arboretum, Denmark; *2* Royal Forest Department, Bangkok, Thailand; *3* Forestry and Forest Products Research Institute, Sapporo, Japan; *4* Nanjing Forestry University, China; *5* Institute of Forest Genetics at Placerville, U.S.A.; *6* Umeå, Sweden; *7* Institute of Botany, Academia Sinica, China. [a] not analysed, see Results

Subgenus	Section	Subsection	Species	Source	Distribution
Diploxylon	*Pinus*	*Sylvestres*	1. *P. densiflora* ZIEB. et ZUCC.	1, 3	Asia
			2. *P. hwangshanensis* HSIA	1, 4	Asia
			3. *P. khasya* ROYLE	2	Asia
			4. *P. massoniana* LAMB.	3, 4	Asia
			5. *P. merkusii* JUNGH et DE VRIESE	2	Asia
			6. *P. sylvestris* L.	1, 6	Eurasia
			7. *P. tabulaeformis* CARR.	7	Asia
			8. *P. taiwanensis* HAYATA	4	Asia
			9. *P. thunbergii* PARL.	1, 3	Asia
			10. *P. yunnanensis* FRANCHÉT	4	Asia
Haploxylon	*Strobus*	*Strobi*	11. *P. armandii* FRANCHÉT	7	Asia
			12. *P. griffithii* MCCLELLAND	7	Asia
			13. *P. kwantungensis* CHUN	4	Asia
			14. *P. parviflora* ZIEB. et ZUCC.	1, 3	Asia
			15. *P. peuce* GRISEB.	1	Europe
		Cembrae	16. *P. sibirica* (DU TOUR) MAYR	1	Eurasia
			–[a] *P. pumila* (PALL.) REGEL	1, 3	Asia
	Parrya	*Gerardianae*	17. *P. bungeana* ZUCC.	4, 5, 7	Asia
			18. *P. gerardiana* WALL.	5	Asia

due to past introgressive hybridization, or (iii) levels of intraspecific cpDNA variation in a single species may be too high to allow meaningful phylogenetic inference, based on assay of a single individual. By using comparative analysis of single tree and composite cpDNA samples comprising 50 individuals of each species we were able to minimize potential risks resulting from incorrect classification and intraspecific cpDNA variation.

Our choice of species was determined by the availability of material (seed and needle samples) and the prior knowledge of cpDNA variation in individual species. For instance, due to the lack of material we could not include one particularly interesting Asian species *P. krempfii* (from the monotypic subsect. *Krempfiani*). Most of the taxa included in this study occur in Asia. We also included two Eurasian species: *P. sibirica* and *P. sylvestris* and one European species: *P. peuce*. Inclusion of the latter taxon was dictated by its putative relationship with Asian haploxylon pines (MIROV 1967, FARJON 1984). On the other hand, we excluded from the present analysis two Asian taxa (*P. sylvestriformis* TAKENOUCHI and *P. densata* MASTERS) which have been found to exhibit substantial intraspecific cpDNA variation as a result of past hybridization (WANG & SZMIDT 1990, SZMIDT & WANG 1993, WANG 1992).

DNA isolation and analysis. CpDNA was purified from fresh needles following a method described by SZMIDT & al. (1986). CpDNA samples from each species were digested to completion with seven restriction enzymes: *Bam* HI, *Bcl* I, *Bgl* II, *Dra* I, *Hind* III, *Kpn* I, and *Xba* I (Boehringer®, Mannheim) according to producer's instruction. Methods for digestion, separation, DNA transfer, and hybridization were as described previously (WANG & SZMIDT

1990). The DNA size marker used was the BRL® 1 kilobase (kb) ladder. Ten probes representing non-overlapping cpDNA fragments from *P. contorta* (LIDHOLM & GUSTAFSSON 1991) were used in this study (Table 2). The probes were chosen to represent different parts of the genome. At least 70% of the cpDNA was covered by the probes used.

Interpretation of fragment patterns. The size of individual cpDNA fragments was estimated from their electrophoretic mobility using an algorithm developed by SCHAFFER & SEDEROFF (1981). Fragments smaller than approximately 0.4 kb could not be accurately scored and were omitted from further analysis. In order to avoid dependent characters, most phylogenetic analyses employing unmapped DNA fragment polymorphism exclude length mutations. However, without constructing restriction maps, it is not possible to determine with certainty, whether a fragment is a result of a length or a point mutation, and consequently whether the characters are dependent (SYTSMA & GOTTLIEB 1986, BREMER 1991, SANDBRINK & VAN BREDERODE 1991). Furthermore, both point and length mutations may lead to the creation of dependent characters (BREMER 1991). Finally, several studies have demonstrated that exclusion of putative length mutations from the data matrix subjected to cladistic analysis has usually little effect on the topology of the constructed phylogenetic trees (BREMER 1991, SANDBRINK & VAN BREDERODE 1991 and references therein). Therefore, in the present analysis each individual cpDNA fragment detected by hybridization to non-overlapping homologous probes was treated as a discrete phenotypic character of the species in question.

Statistics. Estimates of the number of cpDNA nucleotide substitutions per site (weighted $\hat{d}$ values) were calculated from restriction fragment data including all scored fragments for all pairwise species' combinations following the method of NEI (1987) (equations 5.53–5.55) and NEI & MILLER (1990), using the REAP program (MCELROY & al. 1992).

We applied two different approaches to phylogenetic reconstruction from the cpDNA restriction fragment data; one of these was phenetic and one cladistic. The phenetic approach used was the neighbour-joining method of SAITOU & NEI (1987). The unrooted tree was constructed on the basis of weighted $\hat{d}$ values using the NJTREE program (v. 2.0) of the RESTSITE package (MILLER 1991).

The choice of cladistic methods for phylogenetic reconstruction has long been matter of controversial debate and to date there is no consensus on this subject, see PENNY & al. (1992) for review. In the present study, we employed Dollo parsimony in order to cladistically analyze our data set. The method allows for a single restriction site gain on a tree, and then minimizes the number of restriction site losses and produces a rooted tree. This

Table 2. List of cpDNA probes from *Pinus contorta* used in this study (LIDHOLM & GUSTAFSSON 1991)

No.	Probe	Enzyme	Size in kb
1.	pPCH 132	*Hind* III	11.00
2.	pPCH 273	*Hind* III	11.00
3.	pPCH 220	*Hind* III	12.00
4.	pPCH 326	*Hind* III	8.50
5.	pPCH 302	*Hind* III	7.00
6.	pPCH 157	*Hind* III	4.30
7.	pPCB 28	*Bam* HI	6.30
8.	pPCK 140	*Kpn* I	9.00
9.	pPCK 32	*Kpn* I	10.50
10.	pPCK 50	*Kpn* I	5.90

algorithm has been found to be more appropriate for analysis of restriction fragment data than Wagner parsimony method which assumes that parallel gains/losses are equally probable (DEBRY & SLADE 1985). The analyses included only phylogenetically informative fragments, i.e., fragment presence or absence shared by two or more species. Both invariant and autapomorphic fragments were excluded from the total data matrix of all scored fragments using the REAP program (MCELROY & al. 1992). The reduced presence/absence matrix of informative restriction fragments was then analyzed by DOLLOP program of the PHYLIP package version 3.1 (FELSENSTEIN 1985). In order to ascertain confidence intervals using the bootstrap (FELSENSTEIN 1985), we used the DOLBOOT program of the PHYLIP package, with 100 replicate runs. To evaluate the strength of the parsimony result, we calculated the consistency index (CI) (KLUGE & FARRIS 1969). In addition, we computed the homoplasy excess ratio maximum (HERM), from which we estimated homoplasy excess ratio (HER) via equation 5 of ARCHIE (1989).

Results

Restriction cpDNA patterns. Analysis of cpDNA variation in *P. pumila* often produced incomplete digests which forced us to eliminate this species from further analysis. The number of restriction cpDNA fragments detected by individual endonuclease-probe combinations in the remaining species ranged from 32 to 118. Relatively few fragments were generated by *Kpn*I and *Hind*III (32 and 49, respectively). The fragment patterns produced by these endonucleases varied only slightly among species within each of the two subgenera. On the other hand, the remaining five endonucleases generated patterns involving more fragments (from 67 to 118). The analysis resulted in a total of 507 restriction fragments, of which 459 were variable, 48 monomorphic, and 338 deemed phylogenetically informative, as described above. The documentation of scored restriction fragments can be supplied by the authors upon request. Fragment patterns from composite samples of individual species were identical to those observed in cpDNA from single trees, indicating no discernible intraspecific variation (results not shown). A total of 18 different haplotypes were jointly detected by the endonuclease-probe combinations used in the present study which were characteristic of the 18 species investigated.

Distance analysis. The number of nucleotide substitutions per site (weighted $\hat{d}$ values) based on all 507 characters between all pairs of species ranged from 0.0003 and 0.0834 (Table 3). The greatest sequence divergence was found between haploxylon and diploxylon species (0.0601–0.0834). Except for *P. merkusii*, the smallest differences noted were those among diploxylon species of the subsection *Sylvestres* and ranged between 0.0003 and 0.0137. The $\hat{d}$ values were substantially greater among haploxylon species (0.0028–0.0195).

The neighbour-joining tree. Using $\hat{d}$ values a neighbour-joining tree illustrating differences among species was constructed (Fig. 2). As expected, the constructed tree indicated a strong bifurcation of the lineage comprising the two subgenera: haploxylon and diploxylon. In the haploxylon group, *P. bungeana* and *P. gerardiana* (sect. *Parrya* subsect. *Gerardianae*) and *P. peuce*, a rare European species, form monotypic lineages. The next lineage in the haploxylon group is also monotypic and includes *P. griffithii*. The remaining haploxylon species are separated into two weakly diverged species pairs. The first species pair was composed of *P. sibirica* and *P. parviflora*, presently assigned to two different subsections *Cembrae* and *Strobi*, respectively. The second species pair was composed of *P. kwantungensis*

Table 3. Matrix of nucleotide distances among the investigated taxa (see Table 1 for species' names)

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2	0.0086																
3	0.0100	0.0019															
4	0.0104	0.0059	0.0063														
5	0.0209	0.0220	0.0220	0.0197													
6	0.0024	0.0115	0.0128	0.0126	0.0214												
7	0.0104	0.0024	0.0031	0.0043	0.0211	0.0126											
8	0.0093	0.0012	0.0019	0.0052	0.0223	0.0114	0.0012										
9	0.0089	0.0003	0.0016	0.0056	0.0223	0.0118	0.0021	0.0009									
10	0.0114	0.0031	0.0020	0.0057	0.0226	0.0137	0.0019	0.0019	0.0028								
11	0.0799	0.0757	0.0761	0.0757	0.0813	0.0799	0.0757	0.0761	0.0761	0.0757							
12	0.0783	0.0729	0.0757	0.0741	0.0811	0.0783	0.0741	0.0745	0.0733	0.0754	0.0077						
13	0.0805	0.0763	0.0779	0.0763	0.0834	0.0792	0.0763	0.0766	0.0766	0.0775	0.0042	0.0066					
14	0.0801	0.0747	0.0763	0.0747	0.0830	0.0801	0.0747	0.0750	0.0750	0.0759	0.0060	0.0082	0.0068				
15	0.0774	0.0732	0.0749	0.0732	0.0815	0.0761	0.0732	0.0736	0.0736	0.0745	0.0095	0.0108	0.0085	0.0089			
16	0.0794	0.0728	0.0744	0.0728	0.0808	0.0794	0.0728	0.0732	0.0732	0.0740	0.0051	0.0064	0.0055	0.0028	0.0082		
17	0.0653	0.0610	0.0626	0.0601	0.0672	0.0653	0.0610	0.0614	0.0614	0.0622	0.0170	0.0186	0.0195	0.0160	0.0187	0.0157	
18	0.0735	0.0709	0.0724	0.0709	0.0785	0.0735	0.0709	0.0712	0.0712	0.0721	0.0178	0.0177	0.0176	0.0143	0.0169	0.0139	0.0100

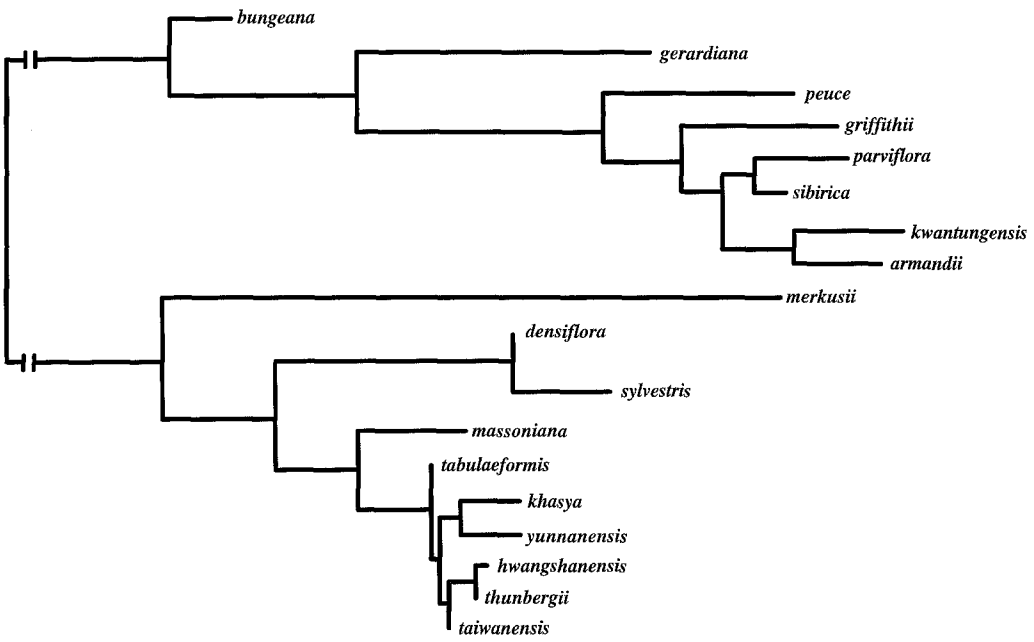

Fig. 2. Neighbour-joining tree showing genetic relationships among *Pinus* species

and *P. armandii*. In the diploxylon group, *P. merkusii* forms a monotypic and highly divergent lineage followed by a relatively distinct cluster composing *P. sylvestris* and *P. densiflora*. The next lineage is again monotypic and includes *P. massoniana*. The remaining six species are clustered together in one very weakly differentiated group.

Cladistic analysis. The DOLLOP program found three most parsimonious trees each requiring 136 reversions of which 58 occur more than once (CI = 85.35%). Homoplasy excess ratio maximum (HERM) was found to be 96.83% and the estimate of homoplasy excess ratio (HER) derived from this value was 93.08%.

Figure 3 A shows the majority-rule consensus tree resulting from the Dollo parsimony bootstrap. It identified the same major lineages as the phenetic approach, and suggested the basal ordering of haploxylon and diploxylon subgenera which is significant at the 0.01 probability level (i.e., the two clades appeared in 100% of the bootstrap samples). *P. bungeana* and *P. gerardiana* form two separate but not significantly different monotypic clades, but each of these two clades is statistically distinct from the remaining haploxylon species ($p < 0.01$). These two clades are then followed by a third statistically different monotypic clade with *P. peuce* ($p < 0.01$). The remaining haploxylon species form several clades of which only one (*P. sibirica* and *P. parviflora*) is nearly significant (95% of the bootstrap samples). Within the diploxylon subgenus, the first clade rooted near the base of the subgenus composed *P. merkusii* ($p < 0.01$). The next clade in this subgenus is composed of two statistically significant clades: one including *P. sylvestris* and *P. densiflora* ($p < 0.01$) and the other monotypic clade with *P. massoniana* ($p < 0.05$). The remaining species are grouped together with only one clade (*P. thunbergii* and *P. hwangshanensis*) significant ($p < 0.05$). Strict consensus Dollo tree shows identical grouping patterns as those found in bootstrap (Fig. 3 B).

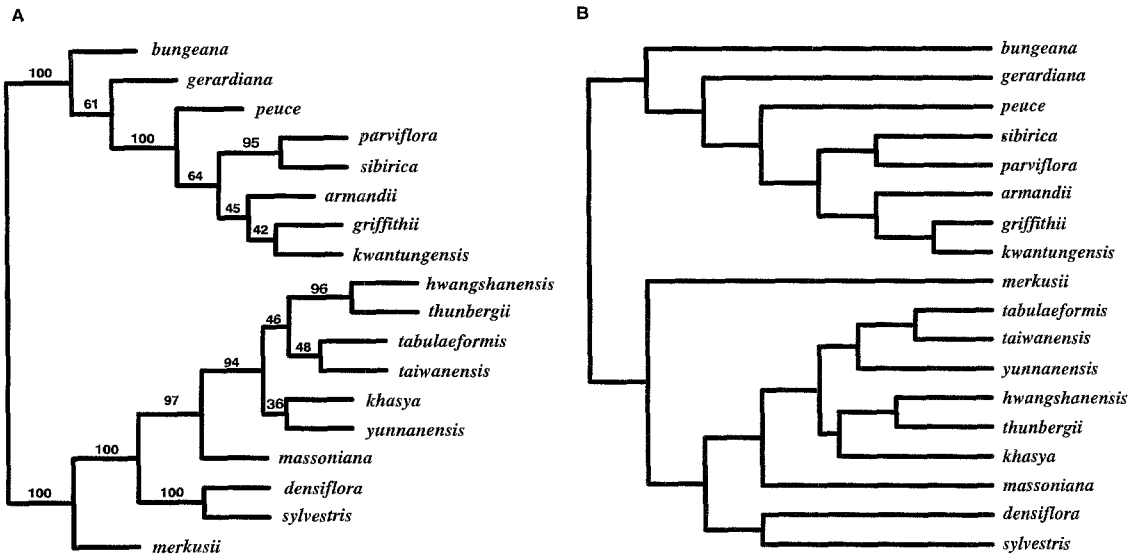

Fig. 3. Phylogenetic trees constructed using Dollo parsimony. *A* Majority-rule bootstrap tree based on 100 replicates. Numbers at nodes are percentages of the number of replicates in which the species below this node were a monophyletic group. *B* Strict consensus tree of the three equally most parsimonious trees derived from Dollo parsimony analysis

In summary, both phenetic and cladistic approaches were concordant as regards the ordering and species composition of the major cpDNA lineages. However, owing to the low differentiation within the subsection *Sylvestres* and considerable divergence between *P. bungeana* and *P. gerardiana*, the approaches yielded slightly different ordering of lineages including these species.

Discussion

Phylogenetic relationships. In the present paper, an attempt was made to assess the phylogenetic relationships among Eurasian *Pinus* species using a molecular approach, based on cpDNA variation. The obtained results were for most part congruent with earlier taxonomy based on morphological (SHAW 1914, MIROV 1967, LITTLE & CRITCHFIELD 1969, FARJON 1984) and molecular data (SZMIDT & al. 1988, STRAUSS & DOERKSEN 1990, WANG & al. 1991 b, GOVINDARAJU & al. 1992). According to paleobotanical evidence, conifers originated in the Permian period, i.e., some 250 million years BP (MIROV 1967, MILLER 1976). Already in the Cretaceous period (approximately 100 million years BP), the two subgenera, haploxylon and diploxylon, were well separated and pine species were common in the Northern Hemisphere (MIROV 1967, MILLER 1976). Distinct character of haploxylon and diploxylon species found in this study fully corroborates separate status of these two groups.

Within the haploxylon subgenus, the first two statistically significant clades ($p < 0.01$) are those of *P. gerardiana* and *P. bungeana* and are rooted near the base of the subgenus. The present finding suggests that these two species are closely related to the diploxylon subgenus. Both species are known to have some features characteristic of the other subgenus (CRITCHFIELD & LITTLE 1966). For instance,

SHAW (1914) noticed that the dentation of the ray tracheid walls in *P. bungeana* and *P. gerardiana* resembled those of the diploxylon species *P. pinea*. Both *P. bungeana* and *P. gerardiana* are intermediate between haploxylon and diploxylon species with respect to needle morphology and anatomy (KWEI & LEE 1963). Other studies revealed that *P. bungeana* differs from other haploxylon pines with respect to heartwood polyphenol composition (MIROV 1967, FARJON 1984 and references therein). Genetic distinctiveness of the section *Parrya* at the cpDNA level has been first demonstrated by SZMIDT & al. (1988). On the phylogenetic tree obtained in their study, the North American *P. aristata* (subsect. *Balfourianae*) and an Asian member of subsect. *Gerardianae: P. bungeana* formed two separate branches placed near the hypothesized root of the tree and were followed by much more homogeneous cluster composed of species from the section *Strobus*. Very similar topology of the sections *Parrya* and *Strobus* has been found in the two subsequent cpDNA-based phylogenies reconstructed by STRAUSS & DOERKSEN (1990) and WANG & al. (1991 b). The high sequence divergence between *P. bungeana* and *P. gerardiana* found in the present study ($\bar{d} = 0.0100$) provides additional evidence for the high diversity among species within sect. *Parrya* reported by other authors (MIROV 1967, FARJON 1984, SZMIDT & al. 1988, STRAUSS & DOERKSEN 1990, WANG & al. 1991 b). Our study included only Asian representatives of *Parrya*. Therefore, their relationships with North American members of sister subsections could not be re-examined. From these studies and from the present data it appears that species from the section *Parrya* are indeed genetically distinct from other haploxylon pines and diverged at the early stages of pine evolution.

In contrast to considerable diversity of sect. *Parrya* the analysis carried out by STRAUSS & DOERKSEN (1990) failed to produce statistically significant resolution of North American species from the sections *Pinus* and *Strobus*. The observed homogeneity within these two sections relative to the genus as a whole suggested that the bulk of the extant species of the genus have radiated relatively recently (STRAUSS & DOERKSEN 1990). In general, our present results are in agreement with these observations. However, the resolution obtained in the present study revealed a more detailed picture of relationships among the Asian species within these two sections.

The species of sect. *Strobus* are mostly found in Asia (subsect. *Cembrae* and half of subsect. *Strobi*) and in western North America (half of subsect. *Strobi*) (FARJON 1984). Genetic relationships among species within the section *Strobus* are not clear. According to FARJON (1984), *P. peuce, P. griffithii*, and *P. armandii* are the most distinct species in this subsection. In the present study, two significantly diverged groups of species were found within sect. *Strobus*. The first distinct, statistically supported clade composed *P. peuce*. According to fossil evidence, species closely resembling *P. peuce* in morphology were widespread in Eurasia prior to Quaternary glaciations (FARJON 1984). *P. peuce* is a montane pine growing in Macedonia and its relatives have been found in Tertiary deposits of western Europe suggesting relatively ancient, possibly Tertiary character of this taxon (MIROV 1967, FARJON 1984). The second, nearly significant group of species discerned in our study comprised *P. sibirica* and *P. parviflora*. Although the two species are currently placed in different subsections (*Cembrae* and *Strobi*, respectively) they are regarded as closely related (MIROV 1967). The high similarity of the remaining three species:

P. armandii, P. griffithii, and *P. kwantungensis* precluded any resolution within the group. In some classifications, *P. kwantungensis* is regarded as a variety of *P. parviflora* (FARJON 1984 and references therein). Distinct character of cpDNA of the latter species lends support to the separate treatment of this species proposed by MIROV (1967). Differences in needle and cone morphology between *P. kwantungensis* and *P. parviflora* have also been reported (FARJON 1984). In summary, our results indicate relatively late radiation of the section *Strobus* which, similarly as on the trees obtained in the earlier studies, appears at the lower branching levels (SZMIDT & al. 1988, STRAUSS & DOERKSEN 1990).

The subsection *Sylvestres* (sect. *Pinus*) is concentrated in Eurasia. It does not occur in western North America, and in eastern North America it is only represented by two species (FARJON 1984). Despite frequently noted phylogenetic homogeneity of this subsection, we were able to identify several statistically supported clusters of species. Unexpectedly *P. merkusii* was rooted near the base of the subgenus and formed a separate monotypic clade. On the cladogram presented by FARJON (1984), *P. merkusii* appears as the most distinct Asian species within subsect. *Sylvestres*. The species is the southernmost of all Asian pines, occurring in southern Burma, northern Thailand, Kampuchea, Vietnam, and Laos. During the Tertiary there were probably more pines adapted to tropical conditions. It is possible that *P. merkusii* represents an ancient remnant of such a group which becomes separated from the other species occurring in inland Asia as a result of frequent climatic changes at the end of the Tertiary (FARJON 1984). Another distinct, statistically supported group of species from subsect. *Sylvestres* composed *P. sylvestris* and *P. densiflora*. The two species show close ecological resemblance and form natural hybrids in northeastern Asia (FARJON 1984, WANG & al. 1991 a, SZMIDT & WANG 1993). In morphologically based phylogeny *P. sylvestris* is placed between the north and east Asian species, and its presence in western Europe may be the result of migration during the upper Tertiary and Pleistocene, favoured by a changing climate (BURGH 1984). The next significant clade within subsect. *Sylvestres* composed *P. massoniana*. The dissimilar character of this species at the cpDNA level has been demonstrated in our previous studies (WANG & SZMIDT 1990, WANG 1992). It is possible that the relatively distinct character of this taxon reflects its past isolation from other members of subsect. *Sylvestres* caused by frequent floristic changes associated with the uplift of the Tibetan Plateau (see WANG 1992 for details on this subject). On the other hand, the remaining species within subsect. *Sylvestres* formed a loose association with only one distinct statistically supported species pair: *P. thunbergii* and *P. hwangshanensis*. The resulting phylogeny of subsect. *Sylvestres* clearly indicates relatively recent differentiation of this group corroborating suggestions obtained in earlier morphological and cpDNA-based phylogenetic analyses of *Pinus* (MIROV 1967, FARJON 1984, SZMIDT & al. 1988, STRAUSS & DOERKSEN 1990, WANG & al. 1991 b). On the other hand, more pronounced divergence among some species from the section *Strobus* suggests that the radiation of this group occurred before that of subsect. *Sylvestres*.

CpDNA variation. It is evident from our present study that despite certain limitations, analysis of cpDNA variation can provide relevant information in the study of evolution. The greatest cpDNA sequence divergence was found between the two subgenera: diploxylon and haploxylon. These differences showed charac-

teristic gradual decrease when comparisons were made among species from different sections and then subsections of the genus *Pinus*. Nevertheless, even very closely related species could be resolved by their cpDNA restriction fragment patterns. This observation re-emphasizes the usefulness of cpDNA in genetic analyses requiring species-specific markers such as those aimed at seed source classification and detection of interspecific hybridization (WAGNER & al. 1987, EL-KASSABY & al. 1988, SZMIDT & al. 1988, WANG & SZMIDT 1990, WANG & al. 1991 b, SIGURGEIRSSON 1992, WANG 1992).

Another interesting observation that emerged from the present and earlier cpDNA-based reconstructions of *Pinus* phylogeny is relatively good concordance of the established relationships with phylogenies based on other data sets. Although such concordance has been noted earlier for some plant species (SYTSMA & GOTTLIEB 1986) more recent reviews on this subject emphasized frequent disagreements between cpDNA-based and other phylogenies (RIESEBERG & BRUNSFELD 1992 and references therein). These disagreements were often interpreted as evidence of cpDNA capture (i.e., replacement of cpDNA of one species by that of another) due to past hybridization (RIESEBERG & BRUNSFELD 1992 and references therein). So far, results obtained for *Pinus* species provide no supporting evidence for this phenomenon. In fact, all *Pinus* phylogenies derived from cpDNA data show very good concordance with morphological and nuclear DNA-based reconstructions (FARJON 1984, GOVINDARAJU & al. 1992). Furthermore, all previous analyses of cpDNA variation in *Pinus* clearly demonstrated distinct interspecific differences even among closely related species which suggests that cpDNA capture is not common in this group of plants. This is surprising, taking into account considerable likelihood of interspecific gene exchange in *Pinus*. However, several recent direct analyses of putative *Pinus* hybrids invariably demonstrated that interspecific gene exchange resulted in the retention of different cpDNA types in hybrids and not in the replacement of one type by another which might lead to biased phylogenies (WAGNER & al. 1987, WANG & SZMIDT 1990, SZMIDT & WANG 1993, WANG 1992). The existing evidence does not permit for unequivocal explanation of different outcomes of interspecific gene exchange in *Pinus* and other plants. It cannot be ruled out, however, that the purported frequent cpDNA capture, i.e., identity of haplotypes in two or more species results from a slower rate of cpDNA change relative to morphological and/or nuclear DNA characters. Alternatively, it may also result from insufficient genome sampling which failed to detect differences between the genomes under study and produced a false impression of cpDNA identity.

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