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Molecular systematics and genetic differentiation of *Pinus sylvestris* (L.) and *P. densiflora* (Sieb. et Zucc.)

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Summary. Allozyme variation was examined in 22 populations of *Pinus densiflora* (Sieb. et Zucc.) and four geographic varieties of *P. sylvestris* (L.): var 'lapponica' (Fries, Hartman), var 'armena' (Komarov), var 'mongolica' (Litvinov) and var 'sylvestriformis' (Takenouchi). In addition, we developed paternal chloroplast (cp) DNA markers that distinguish *P. densiflora* from var 'lapponica', var 'armena' and var 'mongolica'. UPGMA cluster analysis based on Nei's distances between all pairwise combinations of the 22 populations revealed patterns corresponding strictly to geographic origin and taxonomic status. Analysis of allozyme variation in var 'lapponica', var 'armena' and var 'mongolica' demonstrated a high level of intrapopulational variability but a low level of interpopulational differentiation. It appears that the late Pleistocene blending of genetically diverse populations was responsible for the observed variation patterns. The constructed phylogenetic trees also showed late divergence of these three varieties. The var 'sylvestriformis' was genetically distinct from the other three *P. sylvestris* varieties. The genetic distances separating var 'sylvestriformis' from *P. densiflora* and the other taxa lend support to a separate taxonomic status for var 'sylvestriformis' and a close relation with *P. densiflora*. We found that var 'sylvestriformis' harbors admixtures of allozymes and cpDNA from both *P. sylvestris* and *P. densiflora*, which suggests an introgressive nature of this variety. Levels of intrapopulational variability were similar in *P. sylvestris* and *P. densiflora*, but interpopulational differentiation was much higher in *P. densiflora*. In the constructed phylogenetic trees, populations of this species were characterized by relatively long internode distances and branch lengths. The present results suggest that *P. densiflora* has a more advanced evolutionary age than *P. sylvestris*.

Key words: Allozymes – Chloroplast DNA – Introgression – Evolution

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Introduction

Pinus sylvestris (L.) is characterized by the most extensive continuous range of all species in the family *Pinaceae* and spreads over a distance of about 14,000 km (Boratyński 1991). The species is noted for its immense phenotypic variability and its systematic division is still unclear (Molotkov and Patlaj 1991). In the study reported here we adopted the classification proposed by Ruby and Wright (1976) and Cheng et al. (1975). At the southern limit of its distribution in the Caucasus, *P. sylvestris* is represented by var 'armena' (Komarov) (var 'armena', hereafter), a scrubby pine, which is regarded as a glacial refugium of *P. sylvestris* in Europe (Ruby and Wright 1976). It extends from the Black Sea coast across the main Caucasian range and continues as far as 38°30′N (Mirov 1967). The *P. sylvestris* occurring at the northern limit of its distribution is considered to be of a very recent, post-glacial origin and belongs to var 'lapponica' (Fries, Hartman) (var 'lapponica', hereafter) (Ruby and Wright 1976). Two geographic varieties, 'mongolica' (Litvinov) (var 'mongolica', hereafter) and 'sylvestriformis' (Takenouchi) (var 'sylvestriformis', hereafter), are recognized at the eastern limit of the *P. sylvestris* distribution (Cheng et al. 1975). Variety, 'mongolica' occurs in the mountainous areas of extreme northeastern China, while var 'sylvestriformis' occurs in a very limited region in the Changbai mountains where it grows at elevations between 800 and 1,600 m, partly overlapping

with *Pinus densiflora* (Sieb. et Zucc.), which occurs below 900 m (Cheng and Fu 1978). It does not overlap, however, with the distribution of var 'mongolica', which is confined to the northeastern extremes of China (Cheng and Fu 1978). The systematic position of var 'sylvestriformis' is not settled, but there is general agreement among authors that it is morphologically intermediate between var 'mongolica' and *P. densiflora* (Cheng and Fu 1978; Takenouchi 1942). *P. densiflora* is widely distributed in Japan, Korea and southeastern Manchuria and on the Shantung Peninsula in China (Cheng and Fu 1978; Mirov 1967). The distribution and genetic differentiation of *P. densiflora* in inland Asia has not been well studied.

Allozyme variation has been useful in discerning patterns of genetic differentiation in closely related conifer taxa (Muona 1990 and references therein). On the other hand, allozyme markers alone have often been insufficient in resolving ambiguous cases of introgression, because diagnostic alleles of putative parents are rarely available (Szmidt 1991 and references therein). Recent studies on chloroplast (cp) DNA variation in conifers have furnished paternal markers that permit easy determination of the genetic composition of purported hybrid taxa (Szmidt 1991 and references therein; Wang and Szmidt 1990).

In the study presented here we have analyzed genetic variation in populations of var 'lapponica', 'mongolica', 'sylvestriformis' and 'armena' and *P. densiflora*. Our specific goals were: (1) to examine the relatedness of var 'sylvestriformis' and *P. densiflora* and to test whether the introgression of genes from var 'mongolica' and/or *P. densiflora* has affected the genetic makeup of this variety; (2) to test an earlier hypothesis suggesting the recent divergence of *P. sylvestris* varieties occurring in northern Eurasia; and (3) to examine genetic variation in populations of *P. densiflora*. To achieve these goals we examined nuclear, biparentally-inherited allozyme markers in populations of these five taxa. In addition, we developed paternally inherited cpDNA markers diagnostic for *P. sylvestris* and *P. densiflora* and used them for genetic documentation of var 'sylvestriformis'.

Material and methods

Plant material

The geographic distributions of the investigated taxa and the locations of the sampled regions are shown in Fig. 1. The seed material used for allozyme analysis came from 22 populations, including 3 populations of var 'lapponica' (Pl-1 through Pl-3) from Sweden, 4 populations of var 'armena' (Pa-1 through Pa-4) from Turkey, 4 populations of var 'mongolica' (Pm-1 through Pm-4) and 5 populations of var 'sylvestriformis' (Ps-1 through Ps-5) from China and 6 populations of *P. densiflora* from Japan (Pd-1 through Pd-5) and China (Pd-6). Each population was represented by a bulked seed sample. All seed collections were made in documented natural stands. From 100 to 135 seeds were randomly taken from each collection and regarded as random samples of the zygote population.

Populations of var 'armena' and var 'mongolica' analyzed for cpDNA variation were represented by composite seedling samples from populations Pa-2 and Pm-1, respectively. For var 'lapponica' we collected a composite needle sample from 50 trees in a natural stand near Umeå, Sweden. Variety 'sylvestriformis' was represented by a seedling sample from population Ps-2 and by a composite needle sample collected from 12 trees in a natural stand (Ps-6) of this variety in China. One composite seedling sample of *P. densiflora* was taken from population Pd-6. All composite seedling samples used for cpDNA extraction comprised 100 individuals per population and were raised from the same seed collections used for allozyme analysis. Furthermore, we obtained needles from six individual trees of *P. densiflora* in the Hørsholm Arboretum, Denmark and in the Forestry and Forest Products Research Institute, Sapporo, Japan.

Allozyme and cpDNA analysis

Isoenzyme extraction, separation and staining procedures have been described previously (Szmidt 1984). Nine enzyme systems

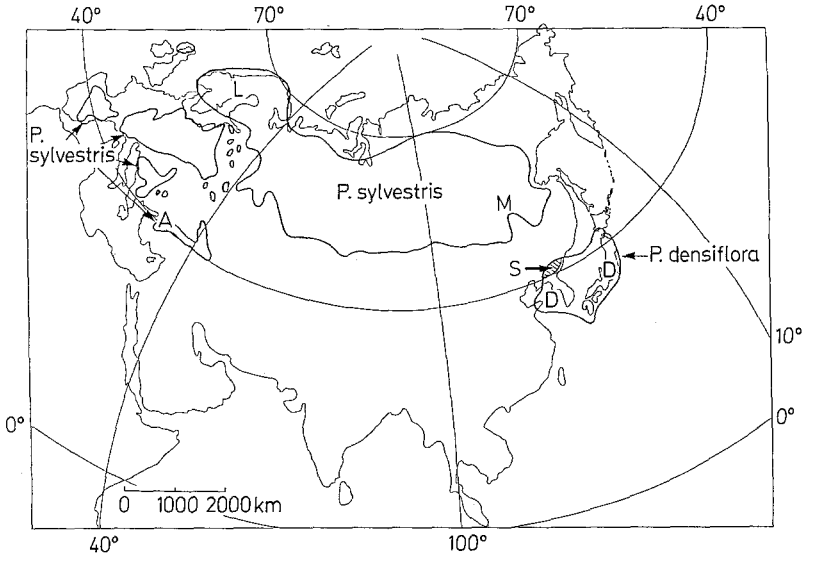

Fig. 1. Distribution of *P. sylvestris* and *P. densiflora* in Eurasia (after Mirov 1967), and the locations of the sampled regions for var 'lapponica' (*L*), var 'armena' (*A*), var 'mongolica' (*M*), var 'sylvestriformis' (*S*) and *P. densiflora* (*D*)

encoded by 14 loci were analyzed as described previously (Szmidt 1984; Wang et al. 1991). All 14 loci were assayed simultaneously in each macrogametophyte and the corresponding embryo. In this report only embryo data are utilized. Chloroplast DNA was extracted from fresh needles (Szmidt et al. 1986). The cpDNA samples were digested separately with *Aval*, *Bam*HI, *Bcl*I, *Dra*I, *Hin*dIII and *Xba*I (Boehringer, Mannheim). Methods for digestion, separation, DNA transfer and hybridization were as described previously (Wang and Szmidt 1990). The probes used in this study were five cpDNA fragments representing different parts of the *P. contorta* chloroplast genome – pPCB28 (6.4 kb), pPCH132 (11.0 kb), pPCK32 (10.5 kb), pPCK140 (9.2 kb) and pPCH157 (4.3 kb) (Lidholm and Gustafsson 1991) – and a 21.0-kb *Pst*I fragment from *Petunia hybrida* cpDNA (Palmer et al. 1983). Restriction fragment patterns observed in the composite cpDNA samples were compared to the patterns from reference individual trees of vars 'lapponica', 'armena' and 'mongolica' and of *P. densiflora* available in our laboratory.

Statistical methods

Allozyme frequencies, observed and unbiased expected heterozygosities, unbiased genetic distance measures (Nei 1987), chord distance (Cavalli-Sforza and Edwards 1967) and cluster analysis using the unweighted pair-group method algorithm (UPGMA) and the Wagner procedure (Sneath and Sokal 1973) were computed for all 14 loci using release 1.7 of the BIOSYS-1 program (Swofford and Selander 1981). Gene diversity analyses followed Nei (1987) using the 11 loci that were polymorphic in all of the taxa examined. A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95.

The UPGMA analysis based on Nei's distances was used to permit comparison of our data with those from other studies. The disadvantage of this method in phylogenetic reconstruction is that it assumes equal evolutionary rates in all taxa (Nei 1987). To infer phylogenetic relationships among the investigated taxa from the allozyme data we employed two phenetic methods that are free of this assumption. The first method was the Wagner distance procedure based on chord distance. The maximum number of partial networks saved during tree construction was 30, and the tree was rooted using the midpoint method of Farris (1972). Populations were added according to the multiple addition criterion (Swofford 1981). The second method was the neighbor-joining procedure developed by Saitou and Nei (1987). The neighbor-joining tree was constructed from the matrix of chord distances for the investigated populations using a REST-SITE computer package (Miller 1991).

The relative contribution of allozymes (m) from var 'mongolica' and *P. densiflora* to the investigated populations of var 'sylvestriformis' was estimated using a least-squares procedure (Roberts and Hiorns 1965; Elston 1971). The standard errors of m (SE_m) were calculated as described by Wheeler and Guries (1987). All calculations were made using mean allozyme frequency data for populations of var 'mongolica' and *P. densiflora*, including frequencies of all of the alleles scored.

Results

All statistics and detailed origins for the individual populations can be obtained upon request from the authors. Of the 14 loci examined, 12 loci were polymorphic in at least one variety of *P. sylvestris* and 10 loci were polymorphic in at least one population of *P. densiflora*. While no locus was fixed for alternative alleles in the taxa examined, allele frequencies at several polymorphic loci formed distinct clines and were intermediate in var 'sylvestriformis' (results not shown). Mean variability measures based on all 14 loci are summarized in Table 1. The percentage of polymorphic loci in vars 'lapponica', 'armena' and 'mongolica' ranged between 54.3 and 71.4 and was lowest for var 'mongolica'. Mean expected (H_e) and observed (H_o) heterozygosities were high and ranged between 0.187–0.244 and 0.184–0.230, respectively, in these three varieties. Slightly higher amounts of genetic variation were found for var 'sylvestriformis' and *P. densiflora* (Table 1). Distinct differences were found among the investigated taxa with respect to the apportionment of gene diversity within and among their popu-

Table 1. Mean values for the percentage of polymorphic loci (P), average number of alleles per locus (A), expected (H_e) and observed (H_o) heterozygosity based on all loci, and gene diversity estimates based on 11 loci polymorphic in all investigated taxa (standard errors in parentheses)

Number	Taxon	Number of populations	Mean sample size/locus	A	P	H_o	H_e	H_T	H_S	D_{ST}	G_{ST}
	P. sylvestris										
1	var 'lapponica'	3	399.5 (0.8)	3.2 (0.4	71.4	0.193 (0.044)	0.220 (0.051)	0.269	0.268	0.001	0.004
2	var 'armena'	4	398.0 (1.2)	3.0 (0.3)	64.3	0.230 (0.048)	0.244 (0.050)	0.304	0.302	0.002	0.007
3	var 'mongolica'	4	477.2 (0.7)	2.9 (0.4)	54.3	0.184 (0.042)	0.187 (0.043)	0.237	0.235	0.002	0.008
	1+2+3	11	1247.7 (2.3)	4.1 (0.5)	64.3	0.201 (0.043)	0.220 (0.047)	0.277	0.268	0.008	0.026
4	var 'sylvestriformis'	5	551.1 (4.6)	3.8 (0.5)	71.4	0.254 (0.056)	0.287 (0.065)	0.349	0.338	0.011	0.026
5	*P. densiflora*	6	603.9 (2.6)	3.5 (0.4)	64.3	0.255 (0.066)	0.275 (0.070)	0.321	0.298	0.024	0.058

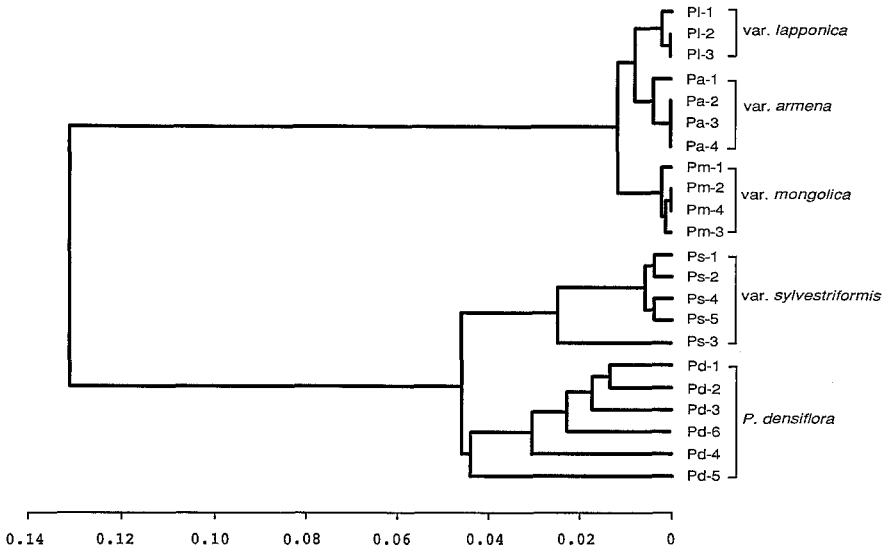

Fig. 2. UPGMA phenogram based on Nei's unbiased genetic distance for the investigated populations

Table 2. Allozyme admixtures from var 'mongolica' and *P. densiflora* in populations of var 'sylvestriformis'

Taxon population	var 'mongolica'		*P. densiflora*	
	m	SE_m	*m*	SE_m
Ps-1	0.253 ± 0.031		0.747 ± 0.029	
Ps-2	0.256 ± 0.025		0.744 ± 0.024	
Ps-3	0.367 ± 0.026		0.633 ± 0.024	
Ps-4	0.277 ± 0.023		0.723 ± 0.022	
Ps-5	0.373 ± 0.021		0.627 ± 0.020	

lations. The gene diversity due to differences among populations (G_{ST}) ranged between 0.004 and 0.008 in vars 'lapponica', 'armena' and 'mongolica'. More population differentiation was found for var 'sylvestriformis' (0.026) and for *P. densiflora* (0.058) (Table 1).

Estimates of allozyme admixtures in populations of var 'sylvestriformis' are given in Table 2. All examined populations of this variety harbored allozymes for both var 'mongolica' and *P. densiflora*. On the other hand, the *m* values for individual populations of var 'mongolica' and *P. densiflora* ranged between 0.000–0.025 and 0.941–1.000, respectively, indicating that they are reasonably pure representatives of the taxa in question (analysis not shown).

UPGMA cluster analysis of Nei's distances between all pairwise combinations of the 22 populations revealed patterns that corresponded strictly to geographic origin and taxonomic status (Fig. 2). Populations were split into two major clusters of which one comprised all of the sampled populations of var 'sylvestriformis' and *P. densiflora*. The other cluster comprised all of the sampled populations of vars 'lapponica', 'armena' and 'mongolica'. The first of these two clusters was further divided into one cluster of var 'sylvestriformis' populations and one cluster of *P. densiflora* populations. The second major cluster was split into three distinct but much less diverged clusters, each comprising all of the populations of the remaining varieties of *P. sylvestris*.

As noted by Ritland and Eckenwalder (1992) the occurrence of introgressive hybridization can bias phylogenetic inference by causing departures from the assumption of uniform rates of evolutionary divergence. The analysis of allozyme and cpDNA variation in var 'sylvestriformis' indicated the introgressive nature of this variety. Therefore, var 'sylvestriformis' was excluded from the phylogenetic reconstructions. The shortest Wagner tree (1.096) obtained after removal of var 'sylvestriformis' is shown in Fig. 3. Similar to what appeared on the UPGMA phenogram populations of *P. densiflora* clustered separately from populations of vars 'lapponica', 'armena' and 'mongolica'. The *P. sylvestris* varieties were further clustered by variety. The topology of the neighbor-joining tree was identical with that of the Wagner tree and therefore is not presented.

All cpDNA fragments detected by hybridizations were clearly visible as distinct bands in the restriction patterns of purified extracts, which indicates their chloroplast origin. Both restriction and hybridization patterns found in composite samples were concordant with patterns of the reference individuals used in this study. Two endonuclease-probe combinations (*Bcl*I/pPCH132 and *Dra*I/pPCK32) detected fragment patterns that distinguished *P. densiflora* from vars 'lapponica', 'armena' and 'mongolica'. The *Bcl*I/pPCH132 combination detected two (4.7 and 7.3 kb) fragments in the *P. densiflora* pattern and one 12-kb fragment in these three *P. sylvestris* varieties (Fig. 4a). The *Dra*I/pPCK32 combination detected one 2.7-kb fragment in *P. densiflora* that was replaced by two (1.0 and 1.7 kb) fragments in vars 'lapponica', 'armena' and 'mongolica' (Fig. 4b).

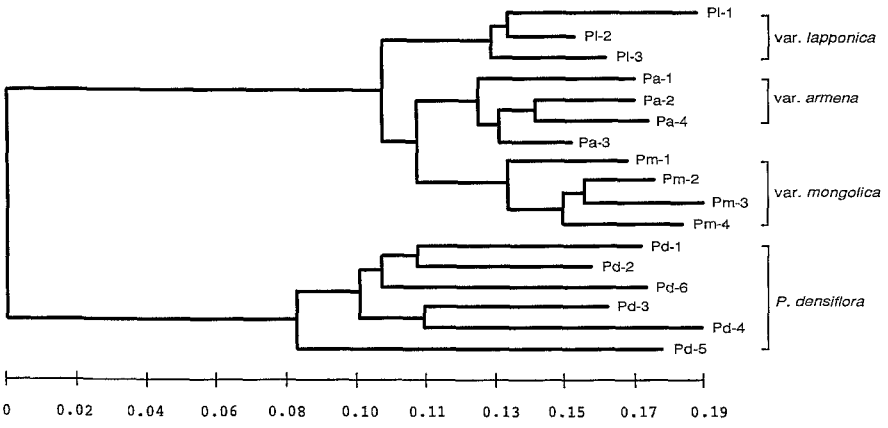

Fig. 3. Phylogenetic tree produced using the Wagner procedure with chord distance for the investigated populations of var 'lapponica', var 'armena', var 'mongolica' and *P. densiflora*

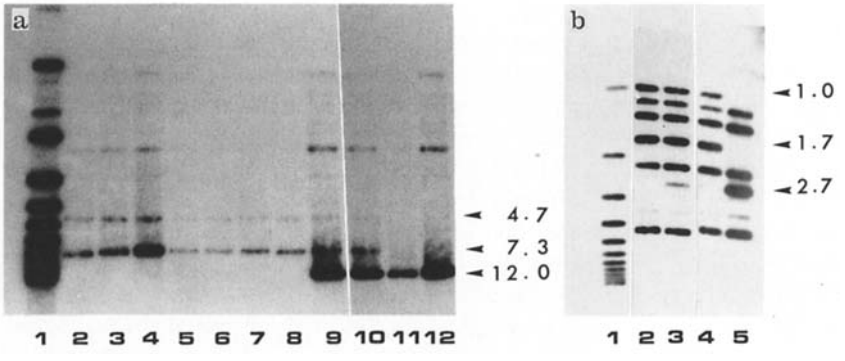

Fig. 4 a, b. Hybridization patterns detected by *Bcl*I/pPCH132 (**a**) and *Dra*I/pPCK32 (**b**) endonuclease-probe combinations in var 'lapponica' (Pl), var 'mongolica' (Pm), var 'sylvestriformis' (Ps) and *P. densiflora* (Pd). *Numbers to the right* are sizes (in kb) of diagnostic fragments. **a** *Lane 1* DNA standard, *2* Pd-6, *3–8 P. densiflora* individuals, *9* Ps-2, *10* Ps-6, *11* Pm-1, *12* Pl-4. **b** *Lane 1* DNA standard, *2* Pl-4, *3* Ps-2, *4* Pm-1, *5* Pd-6

The relative sizes of these fragments are consistent with the occurrence of two independent, restriction-site gains or losses. Both samples of var 'sylvestriformis' harbored *Bcl*I and *Dra*I fragments of two types: those diagnostic of *P. densiflora* and also those of the three varieties of *P. sylvestris* (Fig. 4a, b). Intraspecific variation in *P. densiflora* was detected by the *Bam*HI/pPCB28 combination. One individual of this species had an unique 7.2-kb *Bam*HI fragment, whereas all remaining samples of *P. densiflora* and varieties of *P. sylvestris* shared different 6.4-kb fragment. The difference detected by this combination was apparently due to length mutation. Hybridization patterns for the remaining endonuclease-probe combinations were identical in all taxa.

Discussion

Earlier studies revealed that var 'sylvestriformis' is morphologically intermediate between var 'mongolica' and *P. densiflora* (Cheng and Fu 1978; Cheng et al. 1975; Takenouchi 1942). This intermediacy has made var 'sylvestriformis' recalcitrant for taxonomic classification. The taxon was first described by Takenouchi (1942) as a form of *P. densiflora*. However, the Chinese Flora regards it as a variety of *P. sylvestris* (Cheng and Fu 1978). The genetic distances separating var 'sylvestriformis' from *P. densiflora* and the other varieties of *P. sylvestris* lend support to a separate taxonomic status for var 'sylvestriformis'. UPGMA analysis places this variety in a separate group, but with stronger affinity to *P. densiflora* than to any of the remaining varieties of *P. sylvestris*. These results are best reconciled with the classification proposed by Takenouchi (1942), which treats this taxon as a form of *P. densiflora*.

In our previous study we proposed that the genetic distinctiveness of var 'sylvestriformis' may be due to past hybridization between var 'mongolica' and *P. densiflora* (Wang et al. 1991). Our present findings provide the first genetic evidence supporting this suggestion. First, var 'sylvestriformis' harbors substantial admixures of allozymes from var 'mongolica' and *P. densiflora*. Second, despite a very limited geographic range, populations of var 'sylvestriformis' have considerable genetic variability that may represent additive effect of gene introgression from other, genetically distinct taxa. The introgressive character of var 'sylvestriformis' is further substantiated by our analysis of cpDNA variation, which showed that populations of this variety contain cpDNA genotypes from *P. densiflora* as well as *P. sylvestris*. An ubiquitous question inherent to all studies of introgression is whether observed patterns of genetic variation in the purported hybrid actually depict effects of interspecific gene exchange or reflect rather the ancestral character of the taxon in question. The observed concordance be-

tween the allozyme and cpDNA composition of var 'sylvestriformis' renders the latter possibility highly unlikely. Since the Pleistocene, the distributions of *P. sylvestris* and *P. densiflora* in Asia have undergone numerous expansions and contractions as a result of changing climate (Mirov 1967). These migrations could have led to the creation of temporary zones of contact followed by gene exchange between the two species. *P. sylvestris* and *P. densiflora* are crossable (Kosiński 1991 and references therein). Extant populations of var 'sylvestriformis' are generally sympatric with *P. densiflora* and separated only by altitude. On the other hand, var 'sylvestriformis' and var 'mongolica' have non-overlapping ranges (Cheng and Fu 1978). Therefore, while var 'sylvestriformis' may still receive genes from *P. densiflora*, it is presently spatially isolated from the other putative parent. The large proportions of allozymes in var 'sylvestriformis' that are derived from *P. densiflora* are concordant with this expectation. Only composite samples of var 'sylvestriformis' were analyzed for cpDNA variation, which precludes estimation of cpDNA admixtures in this variety. It is not clear whether (a) var 'sylvestriformis' is currently reverting to its sympatric parent or (b) it represents a stabilized introgressant like that found in another Asian *Pinus* complex (Wang and Szmidt 1990). The altitudinal isolation of var 'sylvestriformis' from *P. densiflora* lends some support to the latter suggestion. However, more detailed genetic documentation involving additional populations of var 'sylvestriformis' is required to resolve this issue.

Pines like *P. sylvestris* are known to have occurred in the Tertiary period, and thus it is likely that *P. sylvestris* was already well differentiated in pre-Pleistocene times (Mirov 1967). However, the repeated invasions of glaciers during the late Pleistocene period eliminated *P. sylvestris* from northern Europe and northern Asia, and the remnants survived only in southern Europe and west-central Asia (Mirov 1967). Therefore, most contemporary populations of *P. sylvestris* must be of post-glacial origin. The range of *P. sylvestris* repeatedly changed during the Pleistocene (Molotkov and Patlaj 1991). Consequently, displaced origins and new genetic combinations developed in Eurasian populations. The Pleistocene blending of genetically diverse populations likely increased the genetic variability in post-glacial populations, which may explain the observed high levels of intrapopulational allozyme variability in *P. sylvestris*. This blending would also tend to reduce previous differentiation, which may explain the low levels of genic diversity among the varieties. Gene flow among expanding post-glacial populations could further reduce their genetic divergence. A rough approximation of the time since divergence between taxa can be estimated with the formula: $t = 5 \times 10^6 \times D$; where t is the divergence time in years, and D is the average distance between taxa (Nei 1987). The estimated times of divergence between pairs of individual populations of vars 'lapponica', 'armena' and 'mongolica' (5,000–10,000 years) agree well with other lines of evidence suggesting a late post-glacial distribution of *P. sylvestris* (Mirov 1967). The constructed phylogenetic trees also showed that the divergence of vars 'lapponica', 'armena' and 'mongolica' has occurred later than that among *P. densiflora* populations.

Comparison between patterns of phenotypic and allozyme variation among these varieties gives a somewhat puzzling picture. On one hand, the weak differentiation between vars 'lapponica' and 'mongolica' observed in this study is concordant with results from provenance trials that show that despite geographic separation both these varieties are very similar with respect to various phenotypic traits (Giertych 1991 and references therein; Ruby and Wright 1976). On the other hand, however, var 'armena' differs from the other two varieties with respect to a number of phenotypic traits (Giertych 1991 and references therein; Ruby and Wright 1976). The observed discordance of allozyme and phenotypic data between var 'armena' and the other two varieties may result from the fact that phenotypic traits respond more rapidly to selective pressures than neutral allozymes (Kimura 1982). Peripheral populations of *P. sylvestris*, such as those investigated in this study, occur at the ecological limits of species distribution and may be exposed to strong and diverse selective pressures. Populations of var 'armena' occur in a warm and arid climate, while those of vars 'lapponica' and 'mongolica' occur in a cool boreal climate. Thus, var 'armena' and the other two varieties are likely affected by strong and contrasting selection. Our study provides the first detailed allozyme information about populations from the southern periphery of *P. sylvestris* in Turkey and from the eastern periphery in China. Nevertheless, a continuing lack of data for other parts of the *P. sylvestris* distribution precludes precise reconstructions of migration history and evolutionary divergence in this species. Comparable studies with central European and Siberian populations are clearly required to alleviate this problem.

Levels of intrapopulational variability were similar in *P. sylvestris* and *P. densiflora*, but interpopulational differentiation was much higher in *P. densiflora*. In addition, *P. densiflora* was the only taxon investigated in this study that showed intraspecific cpDNA variation. The considerable genetic richness of this taxon (Son et al. 1989; this study) indicates that it has not experienced severe bottlenecks. Plausible factors which could contribute to high interpopulational differentiation include limited gene flow, small effective population size, introgression of genes from other taxa, and long periods of time since the divergence of individual populations. Some of these factors may be particularly important in the evolution of *P. densiflora*. Most of the analyzed pop-

ulations are from the islands of Japan and are spatially isolated, which likely diminishes gene flow among populations. The islands of Japan became separated from the continent during the Miocene period (Mirov 1967). *P. densiflora* survived Pleistocene glaciations, which implies that the time lapsed since divergence of the Japanese populations of *P. densiflora* may be considerable (Mirov 1967). A more advanced evolutionary age of *P. densiflora* in comparison with *P. sylvestris* gains further support from the phylogenetic analysis, which showed that *P. densiflora* was characterized by longer internode distances and branch length than populations of *P. sylvestris*. Nevertheless, a more extensive survey that would include Chinese and Korean populations of *P. densiflora* is required to better explain evolutionary processes that have shaped the genetic makeup of this species.

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