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HYBRIDIZATION AND CHLOROPLAST DNA VARIATION IN A *PINUS* SPECIES COMPLEX FROM ASIA

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Abstract.—Heterologous hybridization of chloroplast DNA (cpDNA) involving 30 endonuclease-probe combinations was used to analyze cpDNA variation in multiple individuals and populations of *Pinus tabulaeformis* (Carr.), *Pinus yunnanensis* (Franchèt) and *Pinus massoniana* (Lamb.). Restriction fragment patterns detected by several combinations distinguished among the three species. The obtained cpDNA markers were subsequently used to examine cpDNA variation of *Pinus densata* (Masters), a putative tertiary hybrid between *P. tabulaeformis* and *P. yunnanensis*. The analysis demonstrated that *P. densata* populations harbor three different haplotypes. Two of these haplotypes are characteristic of *P. tabulaeformis* and *P. yunnanensis*. However, the third haplotype found in *P. densata* appears to be absent in other extant Asian *Pinus* species. It is suggested that the observed cpDNA composition of *P. densata* populations is a result of past hybridization involving *P. tabulaeformis, P. yunnanensis,* and a third unknown or extinct taxon. Chloroplast DNA polymorphism in *P. densata* was much greater than that for nuclear allozyme markers in this and the other *Pinus* species. Population differentiation was also substantial in *P. densata* and exceeded that for allozyme markers. In contrast, no cpDNA polymorphism was detected in populations of *P. tabulaeformis, P. yunnanensis,* and *P. massoniana*. The study suggests that interspecific gene exchange may lead to the creation of stable cpDNA polymorphism in conifer hybrids.

Key words.—Chloroplast DNA variation, evolution, *Pinus,* species hybridization.

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Because of the uniparental and asexual inheritance of chloroplast DNA (cpDNA), groups of associated restriction sites are not separated by recombination, and the ancestry of individual haplotypes may remain recognizable even after many generations of sexual reproduction (Whittemore and Schaal 1991). These features of cpDNA have prompted frequent use of cpDNA markers in studies of gene exchange among plant populations (Rieseberg and Brunsfeld 1992 and references therein). Although results from these studies have provided many valuable insights into plant evolution, the ultimate consequences of gene exchange upon the levels and patterns of cpDNA variation are still controversial. In some studies, interspecific gene exchange was found to result in the replacement of cpDNA of one species by cpDNA of another (Rieseberg et al. 1991 and references therein). In other instances, however, a substantial increase of cpDNA polymorphism has been observed as a result of such exchange (Wagner et al. 1987; Szmidt et al. 1988; Wang and Szmidt 1990; Wagner et al. 1991; Sigurgeirsson 1992).

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Pinus species are particularly interesting for studies of interspecific gene exchange and its effects on cpDNA polymorphism and plant evolution. They are long-lived, wind-pollinated, predominantly outcrossing, woody perennials with a broad ecological amplitude ranging from subarctic to subtropical regions. The genus includes many closely related, crossable and sympatric species. In contrast to most angiosperms, cpDNA transmission and dispersal in *Pinus* occurs through pollen (Neale and Sederoff 1989; Wagner et al. 1989; Dong et al. 1992; Wagner et al. 1992). Natural hybridization occurs frequently among related species (Mirov 1967 and references therein; Wagner et al. 1987; Wang and Szmidt 1990; Wagner et al. 1991; Szmidt and Wang 1993). Artificial hybridization experiments revealed that, unlike many other plants, interspecific *Pinus* hybrids are often fertile, have normal meiosis, and sometimes surpass parental species in growth performance (Little and Righter 1965; Kormut'ák and Lanáková 1988 and references therein).

In this study, we conducted a survey of paternally inherited cpDNA variation in a complex of four *Pinus* species from Asia: *P. tabulaeformis* (Carr.), *P. yunnanensis* (Franchèt), *P. densata* (Masters), and *P. massoniana* (Lamb.). Accord-

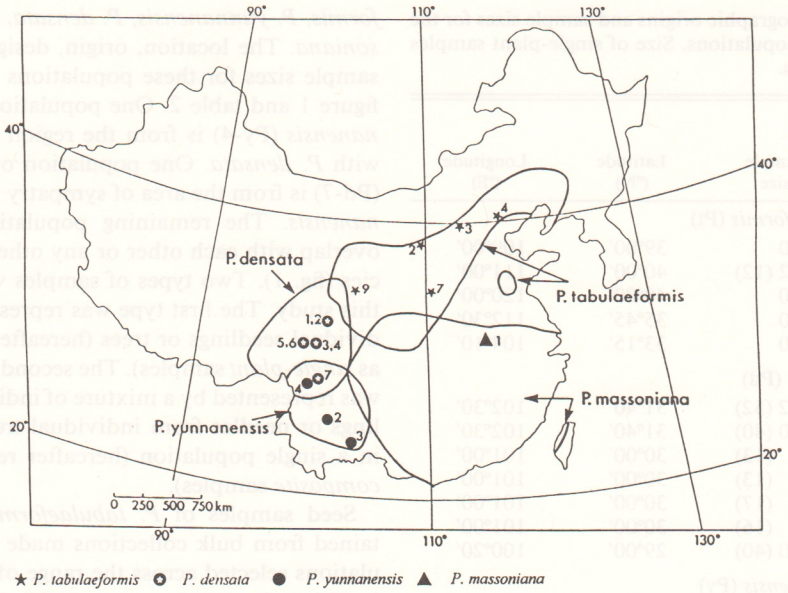

FIG. 1. Distribution of the investigated species and the locations of the sampled populations (see table 2 for further details).

ing to some authors, one of these species, *P. densata,* arose through hybridization between *P. tabulaeformis* and *P. yunnanensis* (Wu 1956; Mirov 1967). Our recent studies employing allozyme and cpDNA markers have provided new evidence supporting the hybrid origin of this taxon (Wang and Szmidt 1990; Wang et al. 1990). *Pinus densata* is morphologically and anatomically intermediate between its two putative parents (table 1). However, the three species have very different ecological requirements and do not form broad overlaps (Mirov 1967; Cheng 1983; Li and Liu 1984; fig. 1). *Pinus densata* occurs in western Sichuan and the eastern part of the Tibetan Plateau. It is endemic to high mountain elevations from 2700 m to 4200 m, where neither of the potential parents can normally grow (Guan 1981; Li and Liu 1984). *Pinus tabulaeformis* grows over

TABLE 1. Comparison of *Pinus tabulaeformis, Pinus densata,* and *Pinus yunnanensis* (Wu 1956; Kwei and Lee 1963; Saylor and Smith 1966; Saylor and Koenig 1967; Cheng and Fu 1978; Yang 1987).

Character	Species		
	P. tabulaeformis	*P. densata*	*P. yunnanensis*
Attitudinal range (m)	0–2600	2700–4200	600–3100
Distribution	31°N–43°N 103°E–125°E	27°N–34°N 95°E–104°E	23°N–29°N 98°E–105°E
Chromosome number (2*n*)	24	24	24
Number of needles	2	2 and/or 3	3
Length of needles (cm)	6–15	6–15	10–30
Needle diameter (mm)	1.5	1.0–1.5	1.0–1.2
Needle cross-cut	half circle	triangle	triangle
Number of resin ducts	5–8	3–7	4–5
Cone length (cm)	4–9	5–6	5–11
Cone diameter (cm)	4–9	4–5	4–7
Seed length (mm)	6–8	4–6	4–5
Number of hypocotyls	8–12	—*	6–8
Apophysis	swollen	swollen	flattened
Branchlet	glaucous	glabrous	stout

* No data available.

TABLE 2. Geographic origins and sample sizes for the investigated populations. Size of single-plant samples in parentheses.

Species/ population number	Sample size	Latitude (°N)	Longitude (°E)
Pinus tabulaeformis (Pt)			
2.	50	39°00′	109°00′
3.	62 (12)	40°00′	111°00′
4.	50	40°00′	120°00′
7.	50	35°45′	112°30′
9.	50	33°15′	104°10′
Pinus densata (Pd)			
1.	112 (52)	31°40′	102°30′
2.	90 (40)	31°40′	102°30′
3.	(13)	30°00′	101°00′
4.	(13)	30°00′	101°00′
5.	(17)	30°00′	101°00′
6.	(16)	30°00′	101°00′
7.	90 (40)	29°00′	100°20′
Pinus yunnanensis (Py)			
2.	50	24°30′	102°30′
3.	65 (15)	23°00′	105°00′
4.	30	29°00′	100°00′
Pinus massoniana (Pm)			
1.	50	32°00′	117°00′
2.	(5)	unknown	

a vast area in northern and central China and is separated from *P. yunnanensis* by *P. densata* and *P. massoniana* with which it forms narrow overlapping zones (Wu 1956; Mirov 1967; Guan 1981; fig. 1). *Pinus yunnanensis* occurs in southern Sichuan and throughout Yunnan, except the alpine region of the extreme northwestern corner, which is occupied by *P. densata* (Mirov 1967). In this region, *P. densata* grows above 2900 m, whereas *P. yunnanensis* occupies lower elevations and attains best development at 1600 m to 2600 m (Li and Liu 1984).

In this study, we have surveyed several different portions of the chloroplast genome in many individuals from different populations of *P. tabulaeformis, P. yunnanensis, P. densata,* and *P. massoniana.* Using this information, we address the following questions. (1) What are the levels and patterns of cpDNA variation in the investigated species? (2) Are our new cpDNA data consistent with the suggested hybrid origin of *P. densata*?

MATERIALS AND METHODS

Plant Material

We sampled 17 populations of *Pinus tabulaeformis, P. yunnanensis, P. densata,* and *P. massoniana.* The location, origin, designations and sample sizes for these populations are given in figure 1 and table 2. One population of *P. yunnanensis* (Py-4) is from the region of sympatry with *P. densata.* One population of *P. densata* (Pd-7) is from the area of sympatry with *P. yunnanensis.* The remaining populations do not overlap with each other or any other *Pinus* species (fig. 1). Two types of samples were used in this study. The first type was represented by individual seedlings or trees (hereafter referred to as *single-plant* samples). The second sample type was represented by a mixture of individual seedlings or needles from individual trees collected in a single population (hereafter referred to as *composite* samples).

Seed samples of *P. tabulaeformis* were obtained from bulk collections made in five populations selected across the range of this species in China (fig. 1). More than 100 individual trees per population were included in these collections. Similar seed samples were obtained from three populations of *P. densata* (Pd-1, Pd-2, and Pd-7), two populations of *P. yunnanensis* (Py-2 and Py-3), and one population of *P. massoniana* (Pm-1) (fig. 1). In addition, seed samples representing open-pollinated, half-sib families were collected from 12 individual trees in one *P. densata* population (Pd-2). All seed collections were made in documented stands. Random seed samples were taken from each of these collections, sown separately in a greenhouse, and grown for 6 mo. Composite samples composed of 50 individual seedlings were randomly taken from each collection. Furthermore, individual seedlings were randomly taken from *P. densata* populations Pd-1, Pd-2, and Pd-7 and populations of *P. tabulaeformis* (Pt-3) and *P. yunnanensis* (Py-3) and used for cpDNA extraction.

We have collected, in addition, needle samples from individual trees in four populations of *P. densata* (Pd-3 through Pd-6) at localities in western Sichuan (fig. 1). The region represents the southern part of the *P. densata* distribution and its average altitude is 3000 m. *Pinus densata* is the only *Pinus* species growing here and occurs as pure, sparsely distributed, naturally regenerated stands. Samples Pd-3 and Pd-4 were collected in two dense, even-aged stands separated by approximately 10 km. Sample Pd-5 was collected in a sparse stand growing on a dry sandy site. Sample Pd-6 was taken from a multiage stand growing on a steep north facing slope. In

each of these four stands, needles were collected from 13 to 17 individual trees at approximately 40-m intervals along a transect of some 700 m. A further composite needle sample was collected from 30 trees in one experimental stand (Py-4) of *P. yunnanensis* growing on the Erlangshan mountain in Sichuan. The stand was established artificially with seeds collected in the natural population of this species from southern Sichuan. In addition, needle samples were collected from five individual trees of *P. massoniana* from Japan. All needle samples were collected from several different parts of the crown of each individual tree. Following collection, needles were stored at −20°C until cpDNA extraction.

DNA Analysis

Purified cpDNA from single-plant and composite samples of all species was prepared using protocol described by Szmidt et al. (1986). In a preliminary survey, we analyzed cpDNA variation in *P. tabulaeformis* and *P. yunnanensis* using 14 different restriction endonucleases (Wang and Szmidt 1990; Wang unpubl. data). Restriction fragment differences were observed for only 3 of these 14 restriction endonucleases (*Bcl*I, *Bgl*II, and *Dra*I) indicating considerable cpDNA similarity of the two species. The same three restriction endonucleases detected many polymorphic sites characteristic of *P. massoniana* (Wang and Szmidt 1990; Wang unpubl. data). Three of the polymorphic *Dra*I fragments were found to hybridize to the *psb*D probe representing an internal fragment of the *psb*D gene from *Spinacia oleracea* (Alt et al. 1984; Lidholm et al. 1988). The fragments were characteristic of the three species in question (Wang and Szmidt 1990).

To identify probes detecting additional polymorphic restriction fragments that distinguish among *P. tabulaeformis, P. yunnanensis,* and *P. massoniana,* and to further survey cpDNA variation in these species, single-plant and composite cpDNA samples were digested separately with *Bcl*I, *Bgl*II, and *Dra*I, and hybridized to *psb*D probe and nine nonoverlapping cpDNA clones from *P. contorta*: pPCH132 (11 kb), pPCH273 (11 kb), pPCH220 (12 kb), pPCH157 (4.3 kb), pPCH302 (7.0 kb), pPCH326 (8.5 kb), pPCK140 (9.0 kb), pPCK32 (10.2 kb), and pPCK50 (5.9 kb) (Lidholm and Gustafsson 1991). The probes used in this study covered about 65% of the chloroplast genome. Methods for digestion, separation, DNA transfer, and hybridization were as described previously (Wang and Szmidt 1990). The DNA size marker used was the 1-kb ladder (BRL®).

Statistical Analysis

Measures of haplotypic diversity ($\hat{h}$) and their standard errors (SE_h), effective number of haplotypes (n_e), and the coefficient of haplotype differentiation (G_{ST}) were calculated using methods described by Nei (1987). The statistical significance of differences in haplotypic diversities among populations were evaluated by *t*-tests (Nei 1987).

RESULTS

CpDNA Variation in Pinus tabulaeformis, P. yunnanensis, and P. massoniana

Of the 30 endonuclease-probe combinations employed in this study, 20 combinations detected identical cpDNA restriction fragment patterns (hereafter referred to as cpDNA variants) in all samples and species. Four combinations detected cpDNA variants distinguishing *Pinus tabulaeformis, P. yunnanensis,* and *P. massoniana.* Because of the similar size of cpDNA fragments in *P. tabulaeformis* and *P. yunnanensis,* cpDNA variants detected by one of these four combinations (*Bcl*I/pPCH326) were difficult to distinguish. Therefore, polymorphism detected by this combination was not surveyed further. Chloroplast DNA variants detected by the other three combinations *Dra*I/*psb*D, *Dra*I/pPCK32, and *Bgl*II/pPCH132 in single-plant samples from *P. tabulaeformis, P. yunnanensis,* and *P. massoniana* are presented in fig. 2A, B, and C, respectively. *Pinus tabulaeformis* had A_1, B_1, C_1 variants detected by *Dra*I/*psb*D, *Dra*I/pPCK32, and *Bgl*II/pPCH132, respectively, whereas *P. yunnanensis* and *P. massoniana* had A_2, B_2, C_2 and A_3, B_3, C_3 variants, respectively (fig. 2A, B, and C). The remaining six combinations distinguished *P. massoniana* from *P. tabulaeformis* and *P. yunnanensis* but not between the latter two species. One of these combinations, *Dra*I/pPCH132, was used as an additional marker in population analyses made in this study. Chloroplast DNA variants detected by this combination in *P. tabulaeformis* and *P. massoniana* are shown in fig. 2D. Two different variants were detected by this combination, the first variant (D_1) was shared by *P. tabulaeformis* and *P. yunnanensis,* whereas the second variant (D_2) was restricted to *P. massoniana.* The three different haplotypes detected jointly by the *Dra*I/*psb*D,

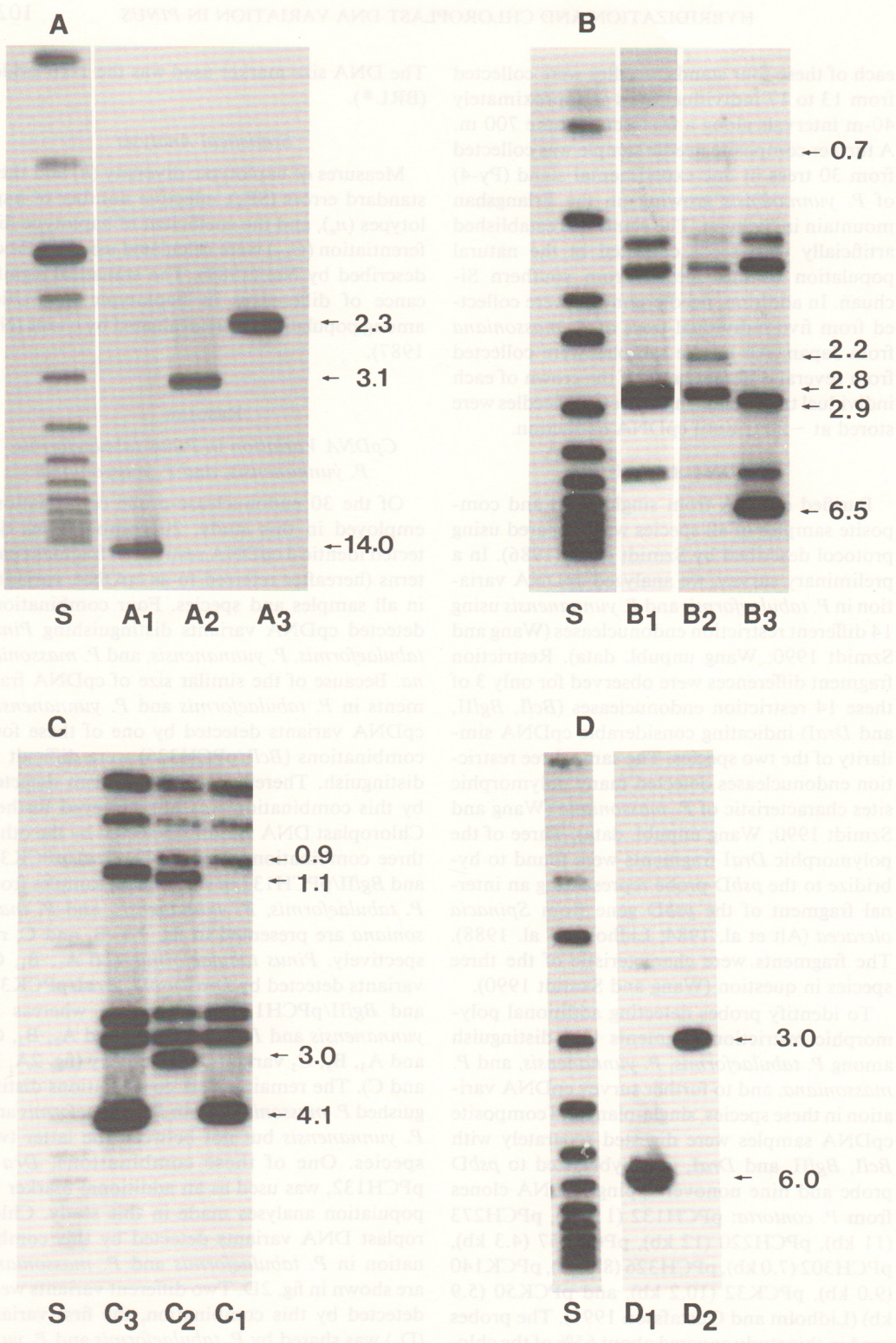

FIG. 2. Hybridization patterns detected by *Dra*I/*psb*D (A); *Dra*I/pPCK32 (B); *Dra*I/pPCH132 (C); and *Bgl*II/pPCH132 (D). A_1, B_1, C_1, and D_1: *Pinus tabulaeformis*; A_2, B_2, C_2: *Pinus yunnanensis*; A_3, B_3, C_3, and D_2: *Pinus massoniana*; S: DNA standard. Numbers indicate the sizes (in kb) of polymorphic fragments.

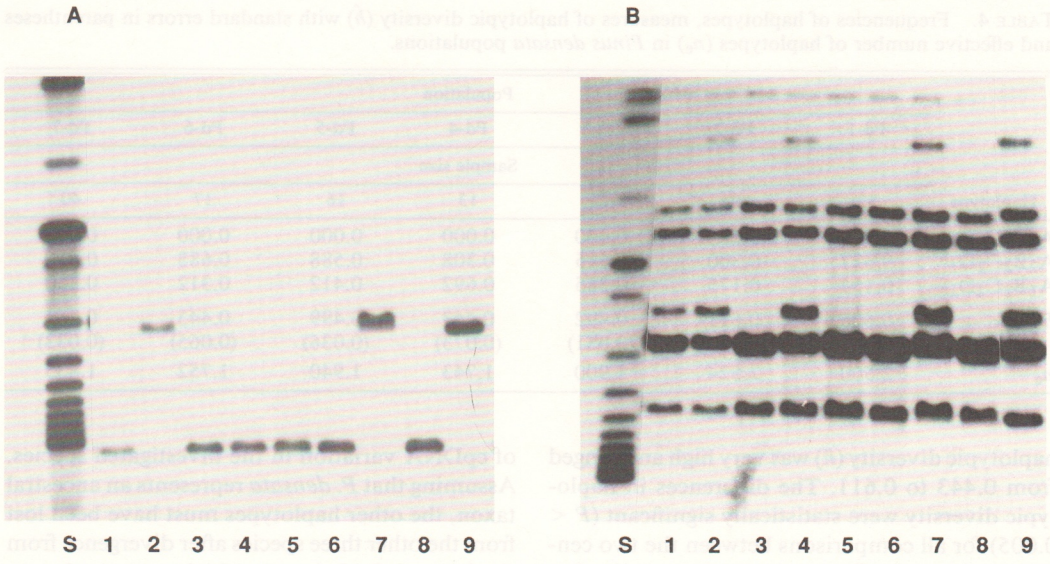

FIG. 3. Hybridization patterns detected by *Dra*I/*psb*D (A) and *Dra*I/pPCK32 (B) in *Pinus densata* individuals. Note individuals 3, 5, 6, and 8 with *Pinus tabulaeformis* patterns (A_1B_1); individuals 2, 7, and 9 with *Pinus yunnanensis* patterns (A_2B_2), and individuals 1 and 4 with novel haplotype (A_1B_2). S, DNA standard.

*Dra*I/pPCK32, *Bgl*II/pPCH132, and *Dra*I/pPCH132 combinations in single-plant samples of *P. tabulaeformis, P. yunnanensis,* and *P. massoniana* are given in table 3.

Chloroplast DNA variants detected in composite samples from *P. tabulaeformis, P. yunnanensis,* and *P. massoniana* were identical with those observed in corresponding single-plant samples (results not shown). Only one cpDNA variant per endonuclease-probe combination was observed, which implies the presence of only one haplotype in each of those three species.

CpDNA Variation in Pinus densata

Two haplotypes were observed in the five southern populations of *P. densata* (Pd-3 through Pd-7). One of these haplotypes ($A_2B_2C_2D_1$) was identical to that found in *P. yunnanensis* (table 3). The second haplotype ($A_1B_2C_2D_1$) was different from haplotypes found in *P. tabulaeformis, P. yunnanensis,* and *P. massoniana* and combined the A_1 cpDNA variant detected by *Dra*I/*psb*D in *P. tabulaeformis,* the B_2, and C_2 variants detected by *Dra*I/pPCK32 and *Bgl*II/pPCH132 in *P. yunnanensis* and the D_1 variant detected by *Dra*I/pPCH132, which was shared by *P. tabulaeformis* and *P. yunnanensis* (table 3, fig. 3). Three haplotypes were found in the two central *P. densata* populations (Pd-1 and Pd-2). Two of these haplotypes were identical with those found in the southern *P. densata* populations. The third haplotype ($A_1B_1C_1D_1$) was identical to that found in *P. tabulaeformis.* The $A_3B_3C_3D_2$ haplotype characteristic of *P. massoniana* was absent from all *P. densata* populations. No additional polymorphism was detected in *P. densata* by any other endonuclease-probe combination used in this study.

Frequencies of individual haplotypes and diversity measures for the *P. densata* populations are presented in table 4. The effective number of haplotypes (n_e) ranged from 1.743 to 2.524. The

TABLE 3. Haplotypes observed in the investigated species.

Species	Haplotype		
Pinus tabulaeformis	$A_1B_1C_1D_1$		
P. yunnanensis	$A_2B_2C_2D_1$		
P. massoniana	$A_3B_3C_3D_2$		
P. densata	$A_1B_1C_1D_1$	$A_1B_2C_2D_1$	$A_2B_2C_2D_1$

TABLE 4. Frequencies of haplotypes, measures of haplotypic diversity ($\hat{h}$) with standard errors in parentheses and effective number of haplotypes (n_e) in *Pinus densata* populations.

	Population						
	Pd-1	Pd-2	Pd-3	Pd-4	Pd-5	Pd-6	Pd-7
	Sample size						
Haplotype	52	40	13	13	16	17	40
$A_1B_1C_1D_1$	0.519	0.525	0.000	0.000	0.000	0.000	0.000
$A_2B_2C_2D_1$	0.327	0.300	0.615	0.308	0.588	0.688	0.650
$A_1B_2C_2D_1$	0.154	0.175	0.385	0.692	0.412	0.312	0.350
$\hat{h}$	0.606 (0.027)	0.611 (0.033)	0.492 (0.051)	0.443 (0.073)	0.499 (0.036)	0.443 (0.065)	0.461 (0.033)
n_e	2.500	2.524	1.900	1.743	1.940	1.752	1.835

haplotypic diversity ($\hat{h}$) was very high and ranged from 0.443 to 0.611. The differences in haplotypic diversity were statistically significant ($P < 0.005$) for all comparisons between the two central populations (Pd-1 and Pd-2) and the five southern *P. densata* populations (Pd-3 through Pd-7). Analysis of the apportionment of the observed haplotypic diversity within and among *P. densata* populations revealed that the total diversity was 0.606, of which 18.1% was attributable to differences among populations.

All 12 composite samples representing open-pollinated, half-sib families of *P. densata* harbored cpDNA variants characteristic of *P. tabulaeformis* and *P. yunnanensis* (results not shown). Chloroplast DNA variants diagnostic for *P. massoniana* were absent from these families. Only composite samples were available for analysis. Therefore, the frequencies of individual haplotypes could not be scored in this material.

DISCUSSION

Our present results demonstrate that the levels and patterns of cpDNA variation within and among Asian *Pinus* species are highly variable. A characteristic feature of cpDNA variation observed in this study was the occurrence of three species with no detectable intraspecific cpDNA variation separated by another taxon (*Pinus densata*) showing an unusually high cpDNA diversity. The occurrence of dissimilar and uniform taxa separated by more variable intermediates can represent the ancestral species from which the extremes were differentiated (Barber and Jackson 1957; Heiser 1973). However, our earlier (Wang and Szmidt 1990; Wang et al. 1990) and present results provide strong evidence suggesting that other factors than primary intergradation were responsible for the observed patterns of cpDNA variation in the investigated species. Assuming that *P. densata* represents an ancestral taxon, the other haplotypes must have been lost from the other three species after divergence from *P. densata*. Such loss would be extremely unlikely. We have no convincing evidence that populations of *Pinus tabulaeformis, Pinus yunnanensis,* and *Pinus massoniana* have undergone any severe bottlenecks that could cause loss of cpDNA variation through random genetic drift. To the contrary, each of these three species is characterized by a wide, continuous distribution suggesting good opportunities for gene flow among populations likely to reduce effects of drift. Similarly, independent acquisition of haplotypes characteristic of *P. tabulaeformis* and *P. yunnanensis* by *P. densata* through convergent evolution does not appear as likely explanation. Unlike morphological characters that may converge when exposed to similar selection pressures, molecular markers are likely to be neutral and thus not prone to convergence (Kimura 1982). It is even less likely that convergence would affect all markers simultaneously. Therefore, it appears that other phenomena such as interspecific gene exchange, mutation, or recombination are more likely causes of the observed high levels of haplotypic diversity in *P. densata* populations.

Previous morphological and molecular studies have suggested that *P. densata* arose through hybridization between *P. tabulaeformis* and *P. yunnanensis* (Wu 1956; Mirov 1967; Wang and Szmidt 1990; Wang et al. 1990). The occurrence of haplotypes characteristic of these two species in central *P. densata* populations corroborates this suggestion. Results from analysis of *P. densata* half-sib families indicate that *P. densata* does not merely represent a mixture of different reproductively isolated species carrying distinct

haplotypes. However, the absence of *P. tabulaeformis* haplotype in southern populations and the occurrence of the third novel haplotype in all *P. densata* populations indicate that the evolution of this taxon was more complicated than previously thought.

At least three hypotheses can be advanced to explain the occurrence of the third novel haplotype in *P. densata.* First, it may be caused by leakage of maternal cpDNA followed by intermolecular recombination between maternal and paternal cpDNAs typical for *P. tabulaeformis* and *P. yunnanensis.* A second explanation involves a loss of the *Dra*I site detectable by the *psb*D probe, but retention of *Dra*I and *Bgl*II sites detectable by the pPCK32 and pPCH132 probes. Such an event would result in the formation of only one additional haplotype identical with that observed in this study. Several authors have predicted the origin and maintenance of novel genetic variants in hybrid zones and expanding populations (Morgan and Strobeck 1979; Golding and Strobeck 1983; Maruyama and Fuerst 1984). For instance, more haplotypes incorporating cpDNA variants typical of the genomes of both parental species within single DNA have been found in a hybrid zone involving *Pinus banksiana* and *Pinus contorta* (Govindaraju et al. 1989). Explanations involving recombination or mutation are compatible with the fragment composition of the novel haplotype found in *P. densata.* However, our other results argue against such origins of this haplotype. First, recombination between two different cpDNA types would lead to the creation of heteroplasmic individual(s) similar to those found in some other *Pinus* species (Govindaraju et al. 1988; White 1990). However, despite intensive sampling including entire plants or different parts of the crown of many individuals and populations of four different taxa, we did not find any evidence for heteroplasmy or other haplotypes that could arise through recombination or mutation. Furthermore, assuming an increased mutation frequency in *P. densata* caused by hybridization, we would expect to find more atypical haplotypes. In contrast to such an expectation, only three different haplotypes were found in *P. densata* of which two were also present in *P. tabulaeformis* and *P. yunnanensis,* respectively. The third explanation may be that the novel haplotype found in *P. densata* was contributed by another species. The novel haplotype is entirely different from the haplotype characteristic of *P. massoniana,* which eliminates this taxon as a potential source. As revealed by other studies, this haplotype does not occur in any other extant *Pinus* species from Asia (Wang 1992). Therefore, if the presence of the third novel haplotype in *P. densata* populations is caused by past hybridization it must have been derived from an unknown or extinct taxon. The *P. tabulaeformis* haplotype is found only in the two *P. densata* populations that are closest to areas of sympatry between *P. densata* and *P. tabulaeformis.* However, the other two haplotypes are found in all *P. densata* populations. This may suggest that *P. densata* has initially arisen through hybridization between *P. yunnanensis* and an unknown taxon. It is also possible that an expanding hybrid has contributed to the extinction of the unknown taxon by replacing it at higher elevations. Subsequently, expanding *P. densata* came into contact with *P. tabulaeformis,* and secondary hybrids were formed, which introduced the *P. tabulaeformis* cpDNA into northern populations. Based on the present results, it is difficult to definitely rule out any of these explanations. Nevertheless, the fact that the novel haplotype was detected only in *P. densata* indicates that its presence is somehow connected with the history of this species.

As revealed by artificial crossing experiments, intrinsic reproductive barriers between *P. tabulaeformis* and *P. yunnanensis* appear to be absent, and the two species are still crossable (Institute of Forest Genetics at Placerville, California, unpubl. data). Temporary relaxation of ecological barriers in the past could enable gene exchange among *P. tabulaeformis, P. yunnanensis,* and a third unknown taxon. The hybrid populations might then spread into habitats inaccessible to the parental types. Such origin of *P. densata* is consistent with a large body of fossil and geological evidence that testifies to substantial physiographic, climatic, and floristic instability caused by the land uplifts and repeated glaciations of the Tibetan Plateau in the Tertiary (Florin 1963; Frenzel 1968; CLIMAP 1976; Ruddiman and Kutzbach 1991; Harrison et al. 1992). Consequently, frequent displacements and extinctions developed among *Pinus* species occurring in this region (Mirov 1967; van der Burgh 1984). Such conditions were particularly favorable for the creation of zones of sympatry among migrating taxa and new habitats that could be filled by the arising hybrids. *Pinus densata* is fully fertile and shows vigorous natural reproduction forming extensive pure forests. The natural re-

generation of the species is particularly efficient at fire sites and can reach 6000 individuals per hectare within 5 yr after fire (Jitai Peng pers. comm. 1991). Analysis of seed anatomy and viability does not reveal any anomalies as compared with the putative parents (Wang unpubl. data). All these characteristics indicate that *P. densata* represents a distinct stable taxon well adapted to its present environment. A characteristic feature of *P. tabulaeformis, P. densata,* and *P. yunnanensis* is their distinct ecological separation that is likely to reduce opportunities for gene exchange among extant populations of these taxa (Wu 1956; Cheng 1983; Li and Liu 1984). Allopatric distribution of the majority of *P. densata* populations and the lack of cpDNA polymorphism in sympatric populations of *P. tabulaeformis* and *P. yunnanensis* also argues against such exchange. In the present zones of overlap, only backcrossing can occur as the other parent is absent. Offspring from such backcrosses may be at a disadvantage as compared with the hybrid or pure parent, which would prevent spread of cpDNA polymorphism outside the range of sympatry. A similar situation was observed in a complex of *Carduus* species in which cpDNA polymorphism was restricted to hybrid populations (Warwick et al. 1989). However, the patterns of cpDNA polymorphism in *P. densata* differ from those observed in some other plant hybrids. For instance, Arnold et al. (1991) have studied cpDNA variation in two hybrid *Iris* species and found that only one population harbored haplotypes characteristic of both parents. In addition, no novel haplotypes were found in this population. A similar situation was reported by Rieseberg (1991) for three putative hybrids of *Helianthus.* In this study, only one hybrid (*Helianthus anomalus*) harbored both parental haplotypes. Wendel et al. (1991) has studied cpDNA based phylogeny in a complex of *Gossypium* species and concluded that one of the investigated taxa has originated through hybridization but retained cpDNA of only the maternal parent. It thus appears that interspecific gene exchange in plants may result either in sustained cpDNA polymorphism or in fixation of one of cpDNA type.

Measures of haplotypic diversity obtained for individual populations of *P. densata* (0.443–0.611) are much higher than diversity for nuclear markers in this species (0.203–0.257; Wang unpubl. data) and exceed cpDNA diversity found in other *Pinus* species (Wagner et al. 1987; Wang et al. 1990; Wagner et al. 1991; Szmidt and Wang 1993). Intraspecific variation of pollen-transmitted asexual genomes such as cpDNA was also detected in other conifer species (Wagner et al. 1987; White 1990; Ali et al. 1991; Wagner et al. 1991). In addition to the pronounced intraspecific cpDNA variation in *P. densata,* considerable differentiation was found among individual populations of this species. The proportion of haplotypic diversity caused by differences among *P. densata* populations was 18.1% whereas it was only 3.8% for allozymes (Wang unpubl. data). It must be noted, however, that the high differentiation observed among populations of *P. densata* was caused largely by the absence of the *P. tabulaeformis* haplotype from southern populations. Nevertheless, the proportion of haplotypic diversity among the southern group of populations (7.5%) was still higher than for allozymes.

In *Pinus* and many other conifers cpDNA transmission and long-distance dispersal occur through pollen (Neale et al. 1986; Szmidt et al. 1987; Szmidt et al. 1988; Wagner et al. 1989; Muona 1990 and references therein; Dong et al. 1992). The size of the pollen (cpDNA) gene pool is large in this group of plants. Therefore, loss of alternative haplotypes in a population because of drift will be counteracted by pollen flow from other populations. Backcrossing is also expected to quickly eliminate cpDNA of one of the two parental species (Birky et al. 1983; Avise and Saunders 1984). However, in a hybrid population, with little or no backcrossing such as *P. densata,* cpDNA polymorphism derived from parental species could persist. It has been suggested that the uniparental inheritance and nonrecombinant character of cytoplasmic genomes lead to effective population sizes lower than those of corresponding nuclear polymorphisms (Birky et al. 1989), which enhances differentiation among populations (Karl et al. 1992). Because of the larger effective size of the cpDNA gene pool than for the maternally transmitted mitochondrial DNA (mtDNA) pool, population differentiation may be less pronounced for cpDNA than for mtDNA. In fact, interpopulational cpDNA diversity in *P. densata* is lower than that observed in other *Pinus* species for maternally transmitted mtDNA markers (Dong and Wagner unpubl. data).

Concluding Remarks

Contrasting levels and patterns of cpDNA variation within and among populations have

been frequently observed (Soltis et al. 1992 and references therein). Our present study provides an additional striking example of such patterns. *Pinus densata* occupies a vast area and harbors substantial cpDNA polymorphism that does not extend beyond the species' boundaries. The present study provides suggestive evidence that interspecific hybridization may lead to sustained cpDNA polymorphism. More rigorous and exhaustive genome and species sampling is clearly needed to permit better understanding of the nature of the cpDNA polymorphism in plant populations. Limited genome and population sampling may lead to underestimation of the existing cpDNA variation (Soltis et al. 1992). It may also lead to overestimation of this variation in cases in which few but very variable cpDNA regions are analyzed.

Our present findings warn against the use of single cpDNA markers in genetic analysis of purported hybrid taxa. Furthermore, they also imply that analysis of cpDNA variation requires information about the linkage among individual mutations used as markers. The lack of complete linkage is indicative of the presence of additional haplotypes that will go undetected if all diagnostic mutations are not scored simultaneously in each individual. As demonstrated by the present study and others, different haplotypes consisting of cpDNA variants characteristic of different species may occur. Unfortunately, except for a few studies, for example, Rieseberg et al. (1990), from the existing reports on cpDNA variation in plants employing more than one diagnostic mutation it is often difficult to infer whether individual mutations were linked.

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