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Cytoplasmic composition in *Pinus densata* and population establishment of the diploid hybrid pine

BAO-HUA SONG,* XIAO-QUAN WANG,* XIAO-RU WANG,*† KAI-YU DING‡ and DE-YUAN HONG*
**Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, 20 Nanxincun, Xiangshan, Beijing 100093, China, †National Institute for Working Life, S-907 13 Umeå, Sweden, ‡School of Life Science, Yunnan University, Kunming 650091, China*

Abstract

Sequence and restriction site analyses of the paternally inherited chloroplast *rbc*L gene and maternally inherited mitochondrial *nad*1 fragments from the same set of populations and individuals were used to investigate cytoplasmic composition and population establishment of *Pinus densata*, a diploid pine that originated through hybridization between *P. tabuliformis* and *P. yunnanensis*. Two variable sites and three chlorotypes (TT, TC and GC) were detected on the *rbc*L gene of the three pines. *P. densata* harboured the three chlorotypes, two of which (TT, GC) were characteristic of the parental species, respectively. The third chlorotype (TC) was distributed extensively in seven of the 10 *P. densata* populations analysed, and might represent a mutation type or have been derived from an extinct parent. The distribution of chlorotypes, together with that of mitotypes, indicated that significant founder effect and backcross happened during the population establishment of the hybrid pine. *P. tabuliformis* and *P. yunnanensis* had acted as both mother and father donors, i.e. bi-directional gene flow existed between the two parental species in the past. Population differentiation of *P. densata* is high, as detected from the cytoplasmic genomes: G_{ST} = 0.533 for cpDNA and G_{ST} = 0.905 for mtDNA. The differences in cytoplasmic composition among the hybrid populations suggest that the local populations have undergone different evolutionary histories.

Keywords: chloroplast DNA, chlorotype, *rbc*L, *Pinus*, hybridization, speciation

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Introduction

Introgression is both an outcome of natural hybridization and an intermediate step for other evolutionary events (Arnold *et al*. 1991), such as race or species formation. It could also result in the breakdown of isolating barriers between two partially isolated taxa and their subsequent merger (Grant 1971). The role of introgression in plant evolution has been much discussed and debated (Anderson 1949; Stebbins 1950, 1959, 1969; Grant 1971; Rieseberg *et al*. 1988; Rieseberg & Brunsfeld 1992), as it is difficult to determine the direction and magnitude of gene flow within and among populations (Rieseberg & Brunsfeld 1992). In recent years, molecular markers have provided the best means for analysing ambiguous cases of introgression (Rieseberg *et al*. 1988; Doebley 1989). In most gymnosperms, maternally (mitochondria) and paternally (chloroplast) inherited cytoplasmic markers provide means of tracking seed and pollen dispersal, respectively. The two cytoplasmic genomes are nonrecombinant haplotypes and have a slow rate of sequence evolution (Wolfe *et al*. 1987), which makes it easy to select species-specific markers to distinguish the parental species (e.g. Rieseberg & Ellstrand 1993; Rieseberg 1997; Matos & Schaal 2000; Gugerli *et al*. 2001; Sperisen *et al*. 2001). Thus, cytoplasmic markers have been used widely to study phylogeography, hybridization, introgression and population history (e.g. Watano *et al*. 1996; Edwards-Burke *et al*. 1997; Senjo *et al*. 1999; Desplanque *et al*. 2000; Isoda *et al*. 2000; Wang *et al*. 2000; Chiang *et al*. 2001; Gugerli *et al*. 2001; Lu *et al*. 2001).

Pinus densata is a hybrid pine occurring in western Sichuan, the northwestern corner of Yunnan and southeastern Tibet (Fu *et al*. 1999), and does not form broad

Correspondence: Dr Xiao-Quan Wang. Fax: 86 10 62590843; E-mail: xiaoq_wang@ns.ibcas.ac.cn

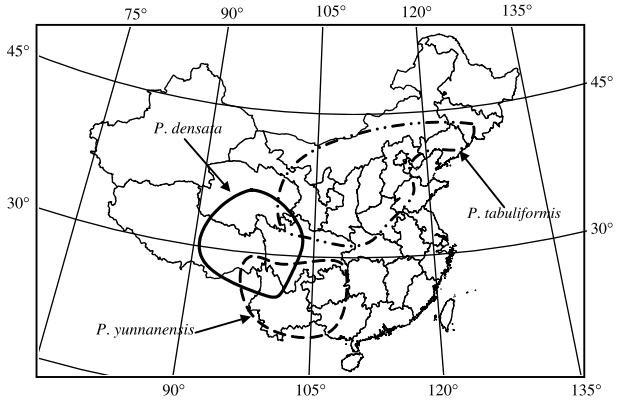

Fig. 1 The distribution of *P. densata* and its two parental species *P. tabuliformis* and *P. yunnanensis*.

overlaps in distribution with its two parental species, i.e. *P. tabuliformis* and *P. yunnanensis* (Fig. 1). The homoploid hybrid speciation of *P. densata* has been investigated through allozyme, cpDNA RFLP and mtDNA analyses (Wang & Szmidt 1990, 1994; Wang *et al*. 1990, 2001; Yu *et al*. 2000; Song *et al*. 2002). However, the previous cpDNA restriction fragment length polymorphism (RFLP) analysis did not sample *P. densata* populations from the Tibetan Plateau (Wang & Szmidt 1994). Although a comparison between mtDNA and cpDNA data was performed in Song *et al*. (2002), the cpDNA data came from RFLP analysis by Wang & Szmidt (1994) with different population sampling. Therefore, this comparison did not reveal the hybridization direction and the speciation process of the hybrid pine.

The present study was designed to investigate the paternal composition of *P. densata* populations based on chloroplast *rbc*L gene sequence. These new data were analysed in combination with the previous maternal mtDNA results from the same set of individuals and populations (Song *et al*. 2002), which allowed us to clearly define the paternal and maternal lineages for each individual and population of *P. densata*. This is the first joint cp- and mtDNA analysis performed on the same individuals of *P. densata* populations, which would give the ultimate evidence for hybrid documentation and shed light on the process and mechanism of population establishment of this high mountain pine.

Materials and methods

Population sampling

A total of 295 trees from 19 natural populations of *P. tabuliformis*, *P. densata* and *P. yunnanensis* were sampled from southwestern China. Each population was represented by 10–23 trees that were at least 50 m apart. The designation, location, elevation and sample size of each population were shown in Table 1. Among them, one

Table 1 Populations analysed in this study and their chlorotype and mitotype composition

Species	Province	Population code	Sample size	Latitude/longitude	Elevation (m)	Mitotype scored	Chlorotype scored (sites 1 + 2)
P. densata	Tibet	BS	10	30°00′ N/94°00′ E	3450	A (Pt)	TC (80.0%) GC (20.0%)
		BY	23	29°36′ N/94°12′ E	3200	A (Pt)	TC (69.6%) GC (30.4%)
		LZ	18	29°24′ N/94°40′ E	3100	A (Pt)	TC (77.8%) GC (22.2%)
	Sichuan	LXA	13	31°24′ N/103°06′ E	2000	B (Py)	TT
		LXB	11	31°24′ N/103°06′ E	2000	B (Py)	TT
		LXC	16	31°24′ N/103°06′ E	2450–2500	B (Py)	TT
		KD	15	30°00′ N/101°54′ E	3050–3150	A (Pt)	TC (46.7%) GC (46.7%) TT (6.6%)
		DB	20	30°48′ N/101°54′ E	2750–3300	B (Py)	TC (60.0%) GC (40.0%)
		SLK	19	29°52′ N/102°06′ E	3600	A (Pt)	TC (52.6%) GC (47.4%)
	Yunnan	ZD	16	27°42′ N/99°42′ E	3400	38%A (Pt), 62%B (Py)	TC (31.2%) GC (68.8%)
P. tabuliformis	Beijing	SS	16	40°48′ N/115°54′ E	1100–1500	A	TT
	Shaanxi	FP	23	33°30′ N/107°54′ E	1350–2000	A	TT
	Sichuan	GY	16	32°24′ N/105°48′ E	1300–1450	A	TT (87.5%) GC (12.5%)
P. yunnanensis	Sichuan	BX	12	30°18′ N/102°48′ E	1600	C	TT (8.3%) GC (91.7%)
		ELS	15	29°48′ N/102°12′ E	1650	B	TC (6.7%) GC (93.3%)
	Yunnan	LJ	15	26°48′ N/100°12′ E	2900	B	TC (40.0%) GC (60.0%)
		CX	10	25°00′ N/101°30′ E	2400	B	GC
		KM	13	25°00′ N/102°42′ E	2200	B	GC
		GJ	14	23°18′ N/103°06′ E	1450	B	GC
Total		19	295				

population (ZD) of *P. densata* was from a region sympatric with *P. yunnanensis*. The remaining populations of individual species do not overlap. Needles were collected from each individual tree and preserved in silica gel.

DNA analysis

Genomic DNA was extracted from silica gel dried needles using the CTAB method following the protocol of Rogers & Bendich (1988) and used as template in polymerase chain reaction (PCR). The chloroplast gene *rbc*L encoding the large subunit of ribulose bisphosphate carboxylase/oxygenase was amplified using primers P_1 and RP_5 (Wang *et al*. 1998). The PCR amplification was carried out in a volume of 25 μL, containing 5–50 ng of genomic DNA, 6.25 pmol of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$ and 0.65 Units of *Taq* DNA polymerase. Amplification was conducted in a Peltier Thermal Cycler (PTC-200, MJ Research). PCR cycles were as follows: 4 min for initial incubation at 70 °C, followed by four cycles of 2 min at 94 °C, 20 s at 50 °C, 2 min at 72 °C, followed by 36 cycles of 20 s at 94 °C, 20 s at 50 °C, and 2 min at 72 °C with a final extension step of 6 min at 72 °C. The PCR products were purified using GFX™ PCR DNA and a Gel Band Purification Kit (Pharmacia). Sequencing reactions were performed with the two PCR primers and two additional internal primers, RP_4 (Wang *et al*. 1998) and P_4 (ATC GTT ATG CAT GAC TAC CTG), to cover the whole PCR segment using ABI Prism Bigdye™ Terminator Cycle Sequencing Ready Reaction Kit. The sequencing reaction products were purified through precipitation with 95% EtOH and 3 M NaAc (pH 5.2) and then applied to the ABI 377 automatic sequencer.

We first sequenced the whole length of the PCR product of 19 individuals (one tree per population) representing the three pines, and made a sequence alignment with CLUSTAL X (Thompson *et al*. 1997). The alignment [(1377 base pairs (bp)] covers nts 31–1407 of the *P. radiata rbc*L gene (GenBank Accession no. X58134, Bousquet *et al*. 1992), in which two variable sites (site 1 and site 2), located at nts 873 (T/G) and 1253 (T/C), respectively, were detected (Fig. 2). Because the primer P_4 can cover the two variable sites, other 179 individuals of the three pines were partially sequenced with this primer. The remaining 97 individuals were analysed through amplification with primers P_4 and RP_5, followed by restriction enzyme digestions using *Mbo* II and *Fnu4H* I, respectively. The substitution from T to G at site 1 (nt 873) caused a loss of *Mbo* II recognition site ($(N)_7$ TCTTC), while the substitution from C to T at site 2 (nt1253) resulted in the loss of two *Fnu4H* I recognition sites (GCNGC). The digestions were implemented by 1-h incubation (2 h for *Fnu4H* I) at 37 °C in the presence of 1 unit enzyme and 1 × restriction buffer. The cleaved products were resolved on 2% agrose gel and visualized under UV light. Haplotypic diversity (H_E), effective number of haplotypes (N_e) and the coefficient of haplotype differentiation (G_{ST}) were calculated following Nei (1987).

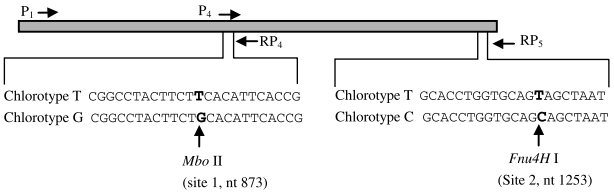

Fig. 2 Positions of primers used in the present study and chlorotypes detected from two variable sites of the *rbc*L gene of *P. tabuliformis*, *P. densata* and *P. yunnanensis*. The recognition sites of *Mbo* II and *Fnu4H* I are shown.

Results

Based on the results of sequence or restriction site analyses of all individuals sampled, no other variable sites were detected except nts 873 (site 1) and 1253 (site 2) found in the first round of sequence analysis of 19 trees. Site 1 (T/G) is the third position of the 291st codon of the *rbc*L gene, while site 2 (T/C) is the second position of the 418th codon. When the two sites were combined, a total of three chlorotypes (TT, TC, GC) were detected in the three pines. The distribution of these chlorotypes in the 19 populations was shown in Table 1 and Fig. 3. All individuals of *P. tabuliformis* had chlorotype TT except that two from GY, a population near *P. densata*, possessed chlorotype GC. In contrast, *P. yunnanensis* was dominated by chlorotype GC (89.87%), although seven individuals (8.86%) from populations at the margins of the range (six in LJ, one in ELS) had chlorotype TC and one from population BX had TT. In *P. densata*, 41 (25.5%) individuals possessed chlorotype TT, 72 (44.7%) possessed TC and 48 (29.8%) had GC. Most *P. densata* populations possessed chlorotypes TC and GC, while LXA-C had pure chlorotype TT.

The frequency of each chlorotype and diversity measures for the sampled populations of the three pines are presented in Tables 1 and 2. The average chlorotype diversity within populations was 0.306, 0.073 and 0.126 for *P. densata*, *P. tabuliformis* and *P. yunnanensis*, respectively. In *P. densata* populations, the haplotype diversity (H_E) ranged from 0 to 0.599, and the effective number of haplotypes (N_e) ranged from 1.000 (LXA-C) to 2.268 (KD). The analysis of the apportionment of the haplotype diversity within and among *P. densata* populations revealed that the total diversity was 0.655, of which 53.3% was attributed to differences among populations. Compared to the chlorotype diversity, the mitotypes within populations of the three pines were mainly monomorphic, except the sympatric population ZD of *P. densata*. This produced a high $G_{ST} = 0.905$.

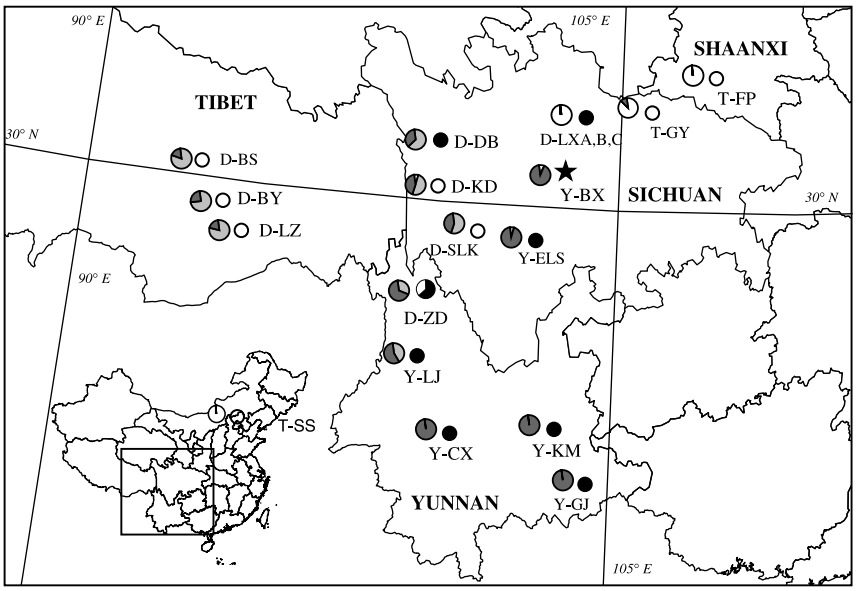

Fig. 3 The distribution and frequency of chlorotypes and mitotypes detected from 19 populations of *P. densata*, *P. tabuliformis* and *P. yunnanensis*. The prefixes T-, D-, Y- represent *P. tabuliformis*, *P. densata* and *P. yunnanensis*, respectively. Data from Song *et al*. (2002). ○ Mitotype A* ● Mitotype B* ★ Mitotype C*
*(Data from Song *et al*. 2002)

Discussion

As shown in Fig. 3 and Table 1, *P. densata*, the hybrid species, possesses three frequent chlorotypes TT, GC and TC. The former two are identical to those found in the central populations of its two parental species, *P. tabuliformis* (TT) and *P. yunnanensis* (GC). The third one, i.e. TC, is absent from *P. tabuliformis* and the central populations of *P. yunnanensis*. The extensive distribution of chlorotype TC in *P. densata* could be explained by four hypotheses.

First, the chlorotype TC may be descended from the polymorphism of ancestral cpDNA. If this is the case, the common ancestor of *P. tabuliformis* and *P. yunnanensis* must possess the chlorotype TC in addition to chlorotypes TT and GC. However, the absence of chlorotype TC from *P. tabuliformis* and the central populations of *P. yunnanensis* renders this hypothesis less likely. The TC chlorotype in a few individuals from the marginal populations of *P. yunnanensis* has come most probably from the backcross between *P. densata* and *P. yunnanensis*. Second, this chlorotype might have stemmed from the intermolecular recombination between chlorotypes TT and GC, but this hypothesis seems unreasonable because the chloroplast is uni-parentally inherited and cpDNA recombination was very rarely documented. Nevertheless, the possibility for a recombinant type cannot be ruled out completely. Third, the chlorotype TC could have been derived from another extinct species, which might have been involved in the speciation of *P. densata*. In the cpDNA-RFLP analysis, Wang & Szmidt (1994) also found that a novel chlorotype existed in all the hybrid populations analysed. Fourth, this chlorotype could be produced by point mutation.

Comparison of chloroplast gene diversity in the three pines has revealed the highest value in *P. densata*, the lowest in *P. tabuliformis* and intermediate in *P. yunnanensis*. The population differentiation (G_{ST}) of *P. densata* is 0.533, a value much higher than those detected previously by cpDNA-RFLP (18.1%, Wang & Szmidt 1994) and by allozyme analysis (8.6%, Wang *et al*. 2001), which may be attributed to sampling difference among these studies. By excluding populations LXA-C, which were fixed for chlorotype TT, the G_{ST} value in *P. densata* would decrease to 10.56%. Hence, the high population differentiation in *P. densata* is caused mainly by the fixation of one chlorotype in populations LXA-C and its absence from other populations. The high G_{ST} indicates limited gene exchange among *P. densata* populations from different regions. Most *P. densata* populations occupy very high habitats (over 3000 m), while LXA-C are from lower altitudes (2000–2500 m) with different climatic and ecological conditions, which may have led to the isolation of these populations. Based on the mitotype distribution, a much higher G_{ST} = 0.905 has been observed among these *P. densata* populations due to the fixation of alternative mitotypes. In pines, cpDNA and mtDNA are transmitted via pollen and seeds, respectively. The pollen migration rate is much higher than seeds (Ennos 1994). Thus, higher G_{ST} for mtDNA is often observed among conifer populations (Wang & Szmidt 2001). The very different maternal and paternal composition and the high G_{ST} among *P. densata* populations imply different origins and strong founder effect.

The joint cp- and mtDNA analysis shows that bi-directional gene flow existed between *P. yunnanensis* and *P. tabuliformis* during the initial hybridization and speciation of *P. densata*, although populations of the high mountain

Table 2 Measures of haplotypic diversity (H_E) and effective number of haplotypes (N_e) in *Pinus densata*, *P. tabuliformis* and *P. yunnanensis*

	Population																
	Pinus densata									*P. tabuliformis*			*P. yunnanensis*				
Parameter	BS	BY	LZ	LXA	LXB	LXC	KD	DB	SLK	ZD	SS	FP	GY	BX	ELS	LJ	CX	KM	GJ
n	10	23	18	13	11	16	15	20	19	19	16	23	16	12	15	15	10	13	14
cp-*rbc*L																			
H_E	0.320	0.423	0.346	0.000	0.000	0.000	0.559	0.480	0.499	0.432	0.000	0.000	0.219	0.153	0.124	0.480	0.000	0.000	0.000
N_e	1.471	1.733	1.529	1.000	1.000	1.000	2.268	1.923	1.996	1.761	1.000	1.000	1.280	1.181	1.142	1.923	1.000	1.000	1.000
mt-*nad*1*																			
H_E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.471	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

n indicates the sample size *Data from Song *et al*. (2002).

pine now have a stabilized hybrid nature (Wang *et al*. 2001). For example, populations LXA-C of *P. densata*, having pure chlorotype TT together with mitotype B, should have *P. tabuliformis* as their paternal ancestor and *P. yunnanensis* as their maternal one. On the other hand, some individuals of populations BY, BS, LZ and SLK have *P. yunnanensis* as their paternal lineage and *P. tabuliformis* as their maternal one considering the existence of chlorotype GC and pure mitotype A in these populations (Fig. 3, Table 1). DB, a typical *P. densata* population located far from the present distribution centre of *P. yunnanensis*, had not only the pure mitotype (B) of *P. yunnanensis* (Song *et al*. 2002) but also chlorotype GC characteristic of *P. yunnanensis* (Fig. 3, Table 1). The unique cytoplasmic composition of this population may be attributed to extensive pollen gene flow between DB and *P. yunnanensis* after the hybridization *P. yunnanensis* × *P. tabuliformis* during geographical changes caused by several uplifts of the Tibetan Plateau.

In the homoploid speciation, the mechanisms such as rapid chromosomal evolution and the availability of a suitable hybrid habitat become critical (Anderson 1948; Templeton 1981; McCarthy *et al*. 1995; Rieseberg 1997; Buerkle *et al*. 2000; Welch & Rieseberg 2002). Some regions, such as the Tibetan Plateau and the Himalayas (Ruddiman & Kutzbach 1991; Song *et al*. 2002), the Hawaiian Islands (Baldwin & Robichaux 1995; Lowrey 1995) and the northern Andes (Monsterio & Sarmiento 1991), where drastic changes (elevation or repeat glaciation) have been experienced in geological history, have become hotspots for studying mechanisms and history of speciation. In fact, all well-documented examples of homoploid hybrid plant species occur in habitats that are different from those of their parental species (Rieseberg 1997). The speciation of the homoploid hybrid *P. densata* is very probably related to the uplift of the Tibetan Plateau. Drastic geographical and climatic changes provided the hybrid with completely different habitats to colonize and expand. As a result, *P. densata* occupies mainly very high habitats (2700–4100 m), where neither of its parents can grow, and forms extensive pure forests. The unique evolutionary history of population DB, the different paternal and maternal lineages and the high G_{ST} among populations all support the complex of population development histories related to the regional geography. The three *P. densata* populations (BS, BY and LZ) from the Tibet Plateau have similar cytoplasmic composition with chlorotype TC in predominance, indicating that geographical radiation has possibly happened in the speciation process of *P. densata* during the uplift of the Tibetan Plateau and the chlorotype TC might have certain adaptive advantage. To clarify the adaptive nature of the hybrid and molecular mechanisms of adaptation requires extensive investigation, including cytogenetic and coding nuclear genes analyses, QTL mapping, transplantation and controlled crossing tests.

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The authors collaborate on projects on molecular systematics and evolution of gymnosperms and on population dynamics of conifer species. Our laboratory specializes in plant taxonomy, molecular phylogeny, cytogenetics and population biology of plants. This work is a part of B-H. Song's PhD programme.
