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GENE FLOW AND SPECIES DELIMITATION: A CASE STUDY OF TWO PINE SPECIES WITH OVERLAPPING DISTRIBUTIONS IN SOUTHEAST CHINA

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Species delimitation detected by molecular markers is complicated by introgression and incomplete lineage sorting between species. Recent modeling suggests that fixed genetic differences between species are highly related to rates of intraspecific gene flow. However, it remains unclear whether such differences are due to high levels of intraspecific gene flow overriding the spread of introgressed alleles or favoring rapid lineage sorting between species. In pines, chloroplast (cp) and mitochondrial (mt) DNAs are normally paternally and maternally inherited, respectively, and thus their relative rates of intraspecific gene flow are expected to be high and low, respectively. In this study, we used two pine species with overlapping geographical distributions in southeast China, *P. massoniana* and *P. hwangshanensis,* as a model system to examine the association between organelle gene flow and variation within and between species. We found that cpDNA variation across these two pine species is more species specific than mtDNA variation and almost delimits taxonomic boundaries. The shared mt/cp DNA genetic variation between species shows no bias in regard to parapatric versus allopatric species' distributions. Our results therefore support the hypothesis that high intraspecific gene flow has accelerated cpDNA lineage sorting between these two pine species.

KEY WORDS: cpDNA, gene flow, introgression, lineage sorting, mtDNA, *Pinus*.

The level of gene flow among conspecific plant populations is important in regard to maintaining them as units of the same species (Levin 1979; Rieseberg et al. 2006). Genetic variation that unites intraspecific but differentiates interspecific populations is especially important for diagnosing and delimiting species (e.g., Davis and Nixon 1992; Bobola et al. 1996; DeSalle et al. 2005; Brower 2006). However, introgression between hybridizing species can disturb interspecific boundaries and result in a mixed gene pool (e.g., Belahbib et al. 2001; Mallet 2005, 2007). Such introgression is promoted between species with incomplete intrinsic reproduction isolation in situations of parapatry or sympatry that can arise, for example, when geographical and/or ecological isolating barriers are lost following environmental change and species' range expansions (Melo-Ferreira et al. 2005; Baack and Rieseberg 2007; Rieseberg et al. 2007).

It is often thought that a capacity for high levels of intraspecific gene flow will translate into frequent interspecific introgression between hybridizing species and possibly the erosion

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of species boundaries (Levin 1979). However, recent modeling shows that introgression frequency between species is negatively related to rates of intraspecific gene flow (Currat et al. 2008). This is because introgressed genes are not diluted by gene flow from populations of the recipient species and may thus rapidly increase in frequency in a recipient species' gene pool. In contrast, when alleles of a recipient species show high rates of gene flow, introgressed alleles will remain at low frequency in an introgressed population, due to high levels of immigration of alleles from non-introgressed populations. Thus, high intraspecific gene flow may override introgression and produce diagnostic genetic differences between species (Petit and Excoffier 2009). Alternatively or in addition, high rates of intraspecific gene flow may promote rapid lineage sorting such that alleles exhibiting high dispersal rapidly become species specific (Wright 1943; Hoelzer 1997).

In this study, we attempted to distinguish between these two hypotheses, i.e., that high intraspecific gene flow promotes molecular divergence between species because it limits the spread of introgressed genes—the "introgression" hypothesis, or because it hastens lineage sorting—"lineage sorting" hypothesis, through a comparison of mtDNA and cpDNA variation in two interfertile pine species, *P. massoniana* Lamb. and *P. hwangshanensis* Hisa. These two species are morphologically distinguished and have nearly overlapping distribution ranges in southeast China (Fig. 1). The former species has a much wider distribution than the other and possesses lank needles with curling tips and dorsal peripheral resin canals, while *P. hwangshanensis* has stout needles with straight tips and 3–7 resin canals positioned centrally. In addition, trunks of *P. massoniana* are dark brown while those of *P. hwangshanensis* are reddish-brown. In all populations examined in October, the cones of *P. massoniana* had not yet opened, while those of *P. hwangshanensis* had opened and contained mature seeds. The two species also differ in altitudinal preference with *P. hwangshanensis* occurring above 700 m, while *P. massoniana* is usually distributed at lower elevations (Fu et al. 1999). At altitudes between 700–1000 m where the two species may occur parapatrically, they can form hybrid zones as evidenced by studies recording morphological and RAPD variation at such sites (Xin et al. 1992; Luo et al. 2001). As in other pine species, the pollen of these two pines is wind-dispersed while seeds are dispersed by animals (Fu et al. 1999). It is unknown whether the two taxa are sister species, but previous phylogenetic studies of *Pinus* suggest they are closely related, being placed in the same subgenus, section and subsection among 32 species occurring in China (Wang et al. 1999; Gernandt et al. 2005). Within their distribution ranges, no other pine species from the same subgenus are present, suggesting that present-day hybridization with other pine species does not occur. A phylogeographic analysis using cpDNA sequence data has been conducted previously on *P. hwangshanensis* (Chiang et al. 2006), but not on *P. massoniana.*

In all pine species examined, chloroplast (cp) genomes exhibit paternal inheritance while mitochondrial (mt) genomes exhibit complete or high levels of maternal inheritance (Wagner et al. 1987; Neale and Sederoff 1988; Mogensen 1996; Guo et al. 2005). Thus mt gene flow occurs mainly via seed and cp gene flow via pollen and seed. MtDNA and cpDNA sequences have been widely used as genetic markers to estimate intraspecific diversity and gene flow in the Pinaceae (e.g., Dong and Wagner 1993, 1994; Tsumura and Suyama 1998; Tsumura et al. 2000; Burban and Petit 2003; Chiang et al. 2006; Jaramillo-Correa et al. 2006; Aizawa et al. 2007; Meng et al. 2007; Naydenov et al. 2007; Chen et al. 2008; Jaramillo-Correa et al. 2008) and all available studies show that the combined pollen- and seed-mediated gene flow of cpDNA markers is greater than mainly seed-mediated gene flow of mtDNA markers (Petit et al. 2005). Therefore, a comparison of cpDNA and mtDNA variation within and between pine species is appropriate for further empirical testing of the proposed correlation between rate of gene flow and species delimitation (Du et al. 2009).

In this study, we examined the genetic structure and evolutionary relationships among a total of 285 individuals from nine localities where *P. massoniana* and *P. hwangshanensis* occurred together (parapatrically distributed) and 12 localities where only one of them was present (allopatrically distributed). In doing so, we aimed to address the following questions: (1) How is cpDNA and mtDNA variation distributed within and between the two species? (2) Is cpDNA variation more species specific than mtDNA variation? If so, is this likely to be due to dilution of introgressed cpDNA alleles in parapatric populations caused by high levels of intraspecific gene flow (introgression hypothesis) or might it be caused by rapid lineage-sorting as evidenced by the total intraspecific gene pool becoming homogenized in both allopatric and parapatric populations (lineage sorting hypothesis)?

Materials and Methods

SPECIES AND POPULATION SAMPLING

We sampled natural populations of *P. massoniana* and *P. hwangshanensis* throughout their current distribution range in southeast China (Table 1). In total, 30 populations from 21 localities were sampled. At nine localities, both species occurred as parapatric populations, while at the remaining localities only one species was present such that there were 10 allopatric populations of *P. massoniana* and two allopatric populations of *P. hwangshanensis* sampled (Fig. 1, Table 1). When two species were present in the same locality (e.g., on the same mountain), we avoided sampling from possible hybrid zones by collecting individuals of different species separated by a minimum of 100 m difference in altitude. From each population, needles of 6–13 individual adult trees spaced at least 200 m apart were collected and dried in silica

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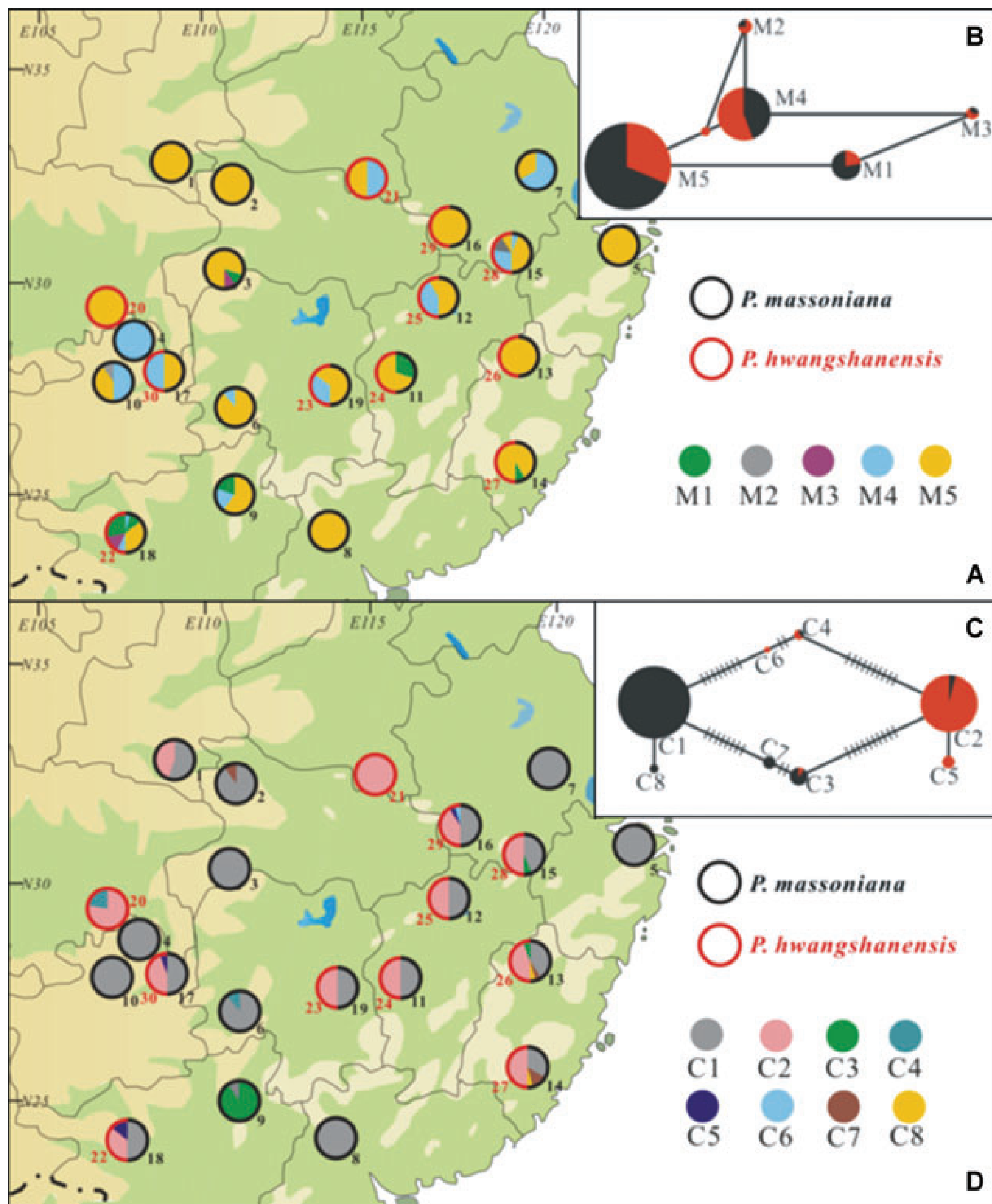

Figure 1. Distributions (A and D) and networks (B and C) of mitotypes and chlorotypes recorded in *Pinus massoniana* (marked by the black circle lines) and *P. hwangshanensis* (marked by the red circle lines). Locations where both species were collected, but from different altitudes, are represented by circles that are half red and half black. The black and red segments within circles in (B) and (C) represent the relative proportions of each mitotype (B) or chlorotype (C) in *Pinus massoniana* and *P. hwangshanensis,* respectively.

gel. The latitude, longitude, and altitude of each site sampled were measured by Extrex GIS (Garmin).

DNA ISOLATION, AMPLIFICATION AND SEQUENCING

Total DNA was isolated from approximately 40 mg of silica-gel dried, leaf-needle material per sample according to a hexadecetyltrimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle 1987) modified for use with an electric tissue homogenizer (QIAGEN, manufactured by Retsch). Intron 1 of subunit 5 of the mitochondrial NADH dehydrogenase gene (*nad*5 intron 1) and intron 3 of subunit 4 of the same gene (*nad*4/3–4), were amplified and sequenced, using primers described by Gamache et al. (2003) and Dumolin-Lapegue et al. (1997) respectively. In addition, three cpDNA fragments, *rpl*16F71-*rpl*16R15, *trn*S-trnG and *rbc*L, were amplified and sequenced following Small et al. (1998), Demesure et al. (1995) and Wang et al. (1998). Polymerase chain reaction (PCR) was performed in a 25-µL volume, containing 10–40 ng plant DNA, 50 mm Tris-HCl, 1.5 mm $MgCl_2$, 250 µg/mL BSA, 0.5 mm dNTPs, 2 µm of each primer, and 0.75 U of *Taq* polymerase. PCR products were purified using

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Table 1. Sample sites and haplotype distribution of two species collected from their allopatric and parapatric regions.

Code	Population/ location	Latitude (E)	Longitude (N)	Alt (m)	*N*	Mitotype (*nad*5, *nad* 4/3–4)					Chlorotype (*rpl*16, *rbcl* and *trn*S-*trn*G)							
						M1	M2	M3	M4	M5	C1	C2	C3	C4	C5	C6	C7	C8
P. massoniana																		
Allopatric populations																		
1	Ankang SX	32°40.274′	109°02.074′	477	9	0	0	0	0	9	5	4	0	0	0	0	0	0
2	Shengnongjia HB	31°45.203′	110°48.338′	612	10	0	0	0	0	10	9	0	0	0	0	0	1	0
3	Badong HB	30°37.993′	110°19.693′	1124	10	1	0	1	0	8	10	0	0	0	0	0	0	0
4	Daozhen GZ	28°52.583′	107°35.437′	751	10	0	0	0	10	0	10	0	0	0	0	0	0	0
5	Simingshan ZJ	29°43.638′	121°.5.465′	625	10	0	0	0	0	10	10	0	0	0	0	0	0	0
6	Dongkou HUB	27°04.333′	110°32.926′	347	9	0	0	0	1	8	8	0	0	1	0	0	0	0
7	Nanjing JS	32°09.166′	118°57.632′	174	9	0	0	0	6	3	9	0	0	0	0	0	0	0
8	Zhaoqing GD	23°10.026′	112°33.327′	179	10	0	0	0	0	10	10	0	0	0	0	0	0	0
9	Guilin GX	25°02.995′	110°18.198′	365	10	2	0	0	2	6	1	0	9	0	0	0	0	0
10	Meitan GZ	27°46.122′	107°26.518′	807	10	0	1	0	5	4	10	0	0	0	0	0	0	0
Subtotal					97	3	1	1	24	68	82	4	9	1	0	0	1	0
Parapatric populations																		
11	Yichun JX	27°33.605′	114°21.193′	540	10	6	0	0	0	4	10	0	0	0	0	0	0	0
12	Jiujiang JX	29°37.109′	116°01.474′	753	6	0	0	0	0	6	6	0	0	0	0	0	0	0
13	Wuyi FJ	27°40.432′	117°55.678′	275	11	0	0	0	0	11	9	0	0	0	0	0	1	1
14	Longyan FJ	25°14.152′	117°06.010′	655	12	2	0	0	0	10	8	0	0	0	0	0	3	1
15	Huangshan AH	30°06.694′	118°11.457′	563	8	0	0	0	1	7	7	0	1	0	0	0	0	0
16	Qiqnshan AH	30°43.823′	116°26.607′	539	9	0	0	0	0	9	9	0	0	0	0	0	0	0
17	Yinjiang GZ	27°54.741′	108°39.658′	1537	9	0	0	0	0	9	9	0	0	0	0	0	0	0
18	Wuming GX	23°30.469′	108°26.034′	1287	10	3	0	0	1	6	10	0	0	0	0	0	0	0
19	Henyang HU	27°17.282′	112°41.488′	737	8	0	0	0	0	8	8	0	0	0	0	0	0	0
Subtotal					83	11	0	0	2	70	76	0	1	0	0	0	4	2
Subtotal					180	14	1	1	26	138	158	4	10	1	0	0	5	2
P. hwangshanensis																		
Allopatric populations																		
20	Nanchuan CQ	29°10.092′	107°05.437′	529	9	0	0	0	0	9	0	7	0	2	0	0	0	0
21	Xinyang HU	31°48.060′	114°04.416′	743	10	0	0	0	5	5	0	10	0	0	0	0	0	0
Subtotal					19	0	0	0	5	14	0	17	0	2	0	0	0	0
Parapatric populations																		
22	Wuming GX	23°30.469′	108°26.034′	1287	7	4	0	2	1	0	0	5	0	0	2	0	0	0
23	Henyang HU	27°17.282′	112°41.488′	1120	10	0	0	0	7	3	0	10	0	0	0	0	0	0
24	Yichun JX	27°35.594′	114°18.330′	1269	11	0	0	0	0	11	0	11	0	0	0	0	0	0
25	Jiujiang JX	29°37.760′	116°01.797′	442	6	0	0	0	5	1	0	6	0	0	0	0	0	0
26	Wuyi FJ	27°56.099′	117°50.326′	752	9	0	0	0	0	9	0	8	1	0	0	0	0	0
27	Longyan FJ	25°15.346′	117°02.870′	1433	10	0	0	0	0	10	0	10	0	0	0	0	0	0
28	Huangshan AH	30°06.694′	118°11.457′	1426	11	0	3	0	6	2	0	11	0	0	0	0	0	0
29	Qiqnshan AH	30°43.823′	116°26.607′	892	13	0	0	0	0	13	0	11	0	0	1	1	0	0
30	Yinjiang GZ	27°54.741′	108°39.658′	2020	9	0	0	0	9	0	0	8	0	0	1	0	0	0
Subtotal					86	4	3	2	28	49	0	80	1	0	4	1	0	0
Subtotal					105	4	3	2	33	63	0	97	1	2	4	1	0	0
Total					285	18	4	3	59	201	158	101	11	3	4	1	5	2

Abbreviation: HB, Hubei; SX, Shaanxi; GZ, Guizhou; HN, Henan; GX, Guangxi; GD, Guangdong; JX, Jiangxi; FJ, Fujian; AH, Anhui; ZJ, Zhejiang; JS, Jiangsu; CQ, Chongqin; *N*, samples size; Alt, altitude.

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a TIANquick Midi Purification Kit following the recommended protocol (Tiangen Biotech, Beijing, China). Sequencing reactions were performed with the PCR primers described above and two additional internal primers, *nad*4/IF705 (ATA GCGAAACACC-CGTAATG) and *nad*5/IR558 (ACTACGGTCGGGCTATCA), to cover the whole PCR segment using ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). DNA sequences were aligned with Clustal X (Thompson et al. 1997) and double checked by eye. A matrix of combined sequences was constructed for the 285 individuals examined, in which five different mtDNA sequences (mitotypes) and eight different cpDNA sequences (chlorotypes) were identified. These mitochondrial and chloroplast fragment sequences have been deposited in the EMBL GenBank under accession numbers: FJ906698-FJ906722.

DATA ANALYSIS

Phylogenetic relationships among mtDNA and cpDNA haplotypes were reconstructed using NETWORK version 4.2.0.1 (Bandelt et al. 1999; available at http://www.fluxus-engineering.com). Average gene diversity within populations (H_S), total gene diversity (H_T), and the coefficients of differentiation G_{ST} and N_{ST} were estimated for each species, separately and combined, for both mtDNA and cpDNA markers, using permut (available at http://www.pierroton.inra.fr/genetics/labo/ Software/Permut/). The two estimates of population divergence, G_{ST} (coefficient of genetic variation over all population; Nei 1973) and N_{ST} (coefficient of genetic variation influenced by both haplotype frequencies and genetic distances between haplotypes), were compared using a permutation test with 1000 permutations. One sample *t*-tests were used to evaluate whether G_{ST} and N_{ST} values differed significantly between the two species. Hierarchical partitioning of diversity among species, populations and individuals was examined by AMOVA (Excoffier et al. 1992) using the program ARLEQUIN version 3.0 (Excoffier et al. 2005), with significance tests based on 1000 permutations. We used the Mantel test to assess the significance of isolation by distance between populations with 1000 random permutations on matrices of pairwise population F_{ST} values and the natural logarithm of geographical distances (Rousset 1997).

The presence of loops and alternative links within a minimum-spanning tree can result from recombination and/or recurrent mutation (Templeton and Sing 1993). To examine this, we applied the four-gametic criterion defined by Hudson and Kaplan (1985), to infer possible recombination genotypes between two polymorphic loci. For every pair of polymorphic nucleotide sites, when all four possible combinations of alleles (i.e., Ab, AB, aB, and ab between two genotypes AB and ab) are observed, these haplotypes are assumed to have originated by recombination of alleles at the two different sites ("loci").

Results

mtDNA VARIATION

We recorded five mitotypes (M1–M5) across *P. massoniana* and *P. hwangshanensis* from examining mtDNA *nad*5 intron 1 and *nad*4/3–4 sequence variation (Table 2). Sixteen populations were fixed for a particular mitotype, while the remaining 14 populations were polymorphic (Table 1 and Fig. 1A). The most frequent mitotype, M5, was present in 27 of the 30 populations sampled across 20 of the 21 localities, and was absent from only one population of *P. massoniana* and two populations of *P. hwangshanensis*. Mitotype M4 was also widespread and shared by both species. The other three mitotypes (M1, M2 and M3) occurred at low frequency in several populations of both species. We failed to find a single species-specific mitotype and all mitotypes were shared among allopatric and parapatric populations of both species. The mitotype network was circular (Fig. 1B) and relationships between mitotypes remained unclear.

Total mtDNA diversity was higher in *P. hwangshanensis* ($H_T = 0.605$) than *P. massoniana* ($H_T = 0.384$), while average within-population diversity was similar in both species ($H_S = 0.213$ in *P. massoniana*, $H_S = 0.243$ in *P. hwangshanensis*). Both G_{ST} and N_{ST} were higher in *P. hwangshanensis* (0.597, 0.684) than in *P. massoniana* (0.444, 0.453) ($P < 0.01$) and there was no significant difference between G_{ST} and N_{ST} (Table 3) in either species. The results of Mantel tests with 1000 permutations revealed that genetic divergence of populations was not significantly correlated with geographic distance for either species ($r = 0.017$ and 0.051, $P > 0.\ 01$) (Table 3). Analysis of molecular variance (AMOVA) showed that most variation was partitioned within species (95.80%), with negligible variation present between species (4.20%, Table 4). However, within both species, between-population variation was significant and accounted for 45% and 66% of total variation in *P. massoniana* and *P. hwangshanensis*, respectively (Table 4).

cpDNA VARIATION

Three indels and 18 base substitutions in the three cpDNA fragments examined (Table 2) resolved eight chlorotypes with two of them recorded as frequent (C1 and C2) and six as relatively rare (C3–C8) (Table 1 and Fig. 1C). In *P. massoniana,* chlorotype C1 was fixed in 12 populations, was present at high frequency in five other populations, and occurred at low frequency in the remaining two populations surveyed (i.e., in populations 1 (Ankang) and 9 (Guilin). In contrast, in *P. hwangshanensis* chlorotype C1 was absent, and instead, chlorotype C2 was fixed in seven of 11 populations surveyed and occurred at high frequency in the remaining four populations. Chlorotype C2 was recorded in only one population of *P. massoniana* (at Ankang). Of the remaining six chlorotypes (C3–C8), C7 and C8 were recorded only in

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Table 2. The variable sites in aligned mtDNA and cpDNA sequences which gave rise to five mitotypes and eight chlorotypes recorded across *Pinus massoniana* and *P. hwangshanensis*. Dashes indicate missing nucleotides; ○ indicates sequence "ATAT"; ◇ indicates sequence "GAA"; ☆ indicates sequence "GGGTTCTCTATCTATTTA".

Mitotype	Nucleotide variable positions			
	*nad*5		*nad*4/3–4	
	266	288	188	210
M1	GCCCCCCCAAATTAAGTCAAAAAAAG		GGTGGGGGGGCTTAT--------------G	
M2	GGTGGGGGGGCTTAT--------------G		GCCCCCCCAAATTAAGTCAAAAAAAG	
M3	GCCCCCCCAAATTAAGTCAAAAAAAG		GCCCCCCCAAATTAAGTAAAAAAAAG	
M4	GGTGGGGGGGCTTAT--------------G		GCCCCCCCAAATTAAGTAAAAAAAAG	
M5	GGTGGGGGGGCTTAT--------------G		GGTGGGGGGGCTTAT--------------G	

Chlorotype	Nucleotide variable positions																				
	rpl16		*rbc*L								*trn*S-*trn*G										
	18	214	342	626	627	715	717	965	1076	1301	20	27	31	38	64	67	77	88	113	190	376
C1	○	G	C	A	A	T	G	G	C	C	T	T	C	◇	C	C	T	T	C	–	C
C2	–	A	T	C	C	C	A	T	T	G	C	G	G	–	A	G	A	A	G	–	A
C3	○	G	C	A	A	T	G	G	C	C	C	G	G	–	A	G	A	A	G	–	A
C4	–	A	T	C	C	C	A	T	T	G	T	T	C	◇	C	C	T	T	C	–	C
C5	–	A	T	C	C	C	A	T	T	G	C	G	G	–	A	G	A	A	G	☆	A
C6	○	G	T	C	C	C	A	T	T	G	T	T	C	◇	C	C	T	T	C	–	C
C7	○	G	C	A	A	T	G	G	C	C	C	G	G	–	A	G	A	A	G	–	C
C8	–	G	C	A	A	T	G	G	C	C	T	T	C	◇	C	C	T	T	C	–	C

P. massoniana, while C5 and C6 were present only in *P. hwangshanensis*. Chlorotypes C3 and C4 occurred in both species in some allopatric and parapatric populations (Fig. 1C). The minimum-spanning network for chlorotypes (Fig. 1D) was circular in form and two distinct clades (C1, C8 vs C2, C5) were identified. Average within-population cpDNA diversity was similar for both species (Table 3), while G_{ST} and N_{ST} differed significantly between species, being higher in *P. massoniana* (0.448 and 0.458,

Table 3. Genetic diversity estimates and Mantel tests for mtDNA and cpDNA variations in *Pinus massoniana* and *P. hwangshanensis*. H_S, average gene diversity within populations; H_T, total gene diversity; G_{ST}, interpopulation differentiation; N_{ST}, the number of substitution types. *indicates that N_{ST} is significantly different from G_{ST} (**$P<0.001$), ns=not significantly different; NC=not computed due to small sample size.

Species	H_S	H_T	G_{ST}	N_{ST}	Mantel test $r(p)$
mtDNA variation					
P. massoniana	0.213 (0.0587)	0.384 (0.0895)	0.444 (0.1270)	0.453 (0.1268) ns	0.017 (0.440)
P. hwangshanensis	0.243 (0.0884)	0.605 (0.0921)	0.597 (0.1086)	0.648 (0.0811) ns	0.051 (0.318)
Total	0.224 (0.0485)	0.474 (0.0709)	0.527 (0.0754)	0.559 (0.0694)ns	0.011 (0.520)
cpDNA variation					
P. massoniana	0.121 (0.0426)	0.219 (0.0877)	0.448 (0.1723)	0.458 (0.1447) ns	0.009 (0.451)
P. hwangshanensis	0.146 (0.0547)	0.154(0.0556)	0.054 (NC)	0.059 (NC) ns	0.237 (0.099)
Total	0.148 (0.0353)	0.584 (0.0496)	0.747 (0.0530)	0.867 (0.0440)**	0.073 (0.927)

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Table 4. Analysis of molecular variance (AMOVA) of mtDNA and cpDNA variation in *Pinus massoniana* and *P. hwangshanensis*. d.f., degrees of freedom; SS, sum of squares; VC, variance component; **P<0.001; 1000 permutations.

Species	Source of variation	d.f.	SS	VC	V%	F-statistics
Mitotype						
All	Among species	1	54.235	0.205	4.20	F_{CT}=0.042
	Among populations within species	28	734.837	2.543	52.05	F_{SC}=0.543**
	Within population	255	544.998	2.137	43.75	F_{ST}=0.562**
	Total	284	1334.070	0.049		
P. massoniana	Among populations	18	350.464	1.821	44.87	F_{ST}=0.449**
	Within populations	161	360.181	2.237	55.13	
P. hwangshanensis	Among populations	10	384.373	3.835	66.11	F_{ST}=0.661**
	Within populations	94	184.818	1.967	33.89	
Chlorotype						
All	Among species	1	1392.200	10.455	88.19	F_{CT}=0.882**
	Among populations within species	28	147.840	0.457	3.86	F_{SC}=0.327**
	Within population	255	275.374	0.943	7.96	F_{ST}=0.920**
	Total	284	2109.780	0.119		
P. massoniana	Among populations	18	130.123	0.674	44.34	F_{ST}=0.443**
	Within populations	161	136.305	0.846	55.66	
P. hwangshanensis	Among populations	10	17.718	0.069	5.91	F_{ST}=0.059**
	Within populations	94	104.424	0.109	94.09	

respectively) than in *P. hwangshanensis* (0.054 and 0.059 respectively). In neither species was N_{ST} significantly greater than G_{ST} ($P < 0.001$). Mantel tests failed to reveal a significant association ($r = 0.009$ and 0.237, $P > 0.01$) between genetic differences and geographical distances between populations across either species' distribution (Table 3). AMOVA showed that (88.19%) of the total cpDNA variation was distributed between species. Between-population variation was significant and accounted for ~44% of the total variation in *P. massoniana,* but was not significant in *P. hwangshanensis* where it accounted for only ~6% of total variation (Table 4).

Discussion

Our comparison of maternally inherited mtDNA and paternally inherited cpDNA sequence variation in two closely related pine species, *P. hwangshanensis* and *P. massoniana,* with overlapping geographical distributions in China, showed that cpDNA variation was much more effective at distinguishing the two species. Whereas ~88% of the total cpDNA variation recorded was partitioned between species, only ~4% of mtDNA variation was partitioned in this way. Thus, our results are in agreement with the recent proposal that paternally inherited genome markers, which are expected to show high levels of intraspecific gene flow, are less likely to be shared between closely related and interfertile species than maternally inherited genome markers that show much lower levels of intraspecific gene flow (Du et al. 2009).

ORGANELLE DNA POLYMORPHISM AND DISTRIBUTION WITHIN AND BETWEEN *P. HWANGSHANENSIS* AND *P. MASSONIANA*

A high level of mtDNA polymorphism was detected across both *P. hwangshanensis* and *P. massoniana*. Within-population diversity was relatively low in both species ($H_S = 0.213$ for *P. massoniana,* and $H_S = 0.243$ for *P. hwangshanensis*), and most within species diversity was due to variation between populations. Within both species, the five mitotypes recorded were distributed randomly among populations although M5 was widespread. Thus mitotype diversity across all 21 populations was associated neither with species nor with geography ($r = 0.017$ and 0.051, $P > 0.01$).

In contrast, the two pine species could be largely distinguished according to chlorotype. In *P. massoniana* 158 of 180 individuals sampled (~88%) possessed the C1 chlorotype, while in *P. hwangshannensis* 97 of 104 trees examined (~94%) had the C2 chlorotype. C1 was not found in *P. hwangshannensis,* while C2 was recorded in only four individuals of *P. massoniana,* all from the allopatric population at Ankang. Because chlorotypes C1 and C2 occurred at high frequency they were considered to represent the most ancient haplotypes (see Watterson and Guess 1977; Donnelly and Tavaré 1986; Crandall and Templeton 1993) while the four chlorotypes (C3, C4, C6 and C7), positioned between C1 and C2 in the network, were assumed to be recombinants of C1 and C2, according to the "four gametic criterion" (Hudson and Kaplan 1985; Jaramillo-Correa and Bousquet 2005). Of these remaining six chlorotypes recorded, all of which occurred at low

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frequency, four were species specific (C5 and C6 to *P. hwangshanensis*, and C7 and C8 to *P. massoniana*). A greater proportion of between population cpDNA diversity was recorded in *P. massoniana* (44%) relative to that in *P. hwangshannensis* (6%), but this was largely due to the presence of the C2 haplotype at intermediate frequency in the Ankang population, the presence of C3 at high frequency in the Guilin population, and the presence of C7 in a quarter of *P. massoniana* trees surveyed at Longyan. It is feasible that these three populations are derived from populations existing in isolated refugia during the Last Glacial Maximum (LGM, ~18,000 years ago) at the same or neighboring sites where they are now located, and that intraspecific gene flow has not yet homogenized their divergent cpDNA signatures.

Because of the low mutation rates of cpDNA and mtDNA in pines (Wolfe et al. 1987; Willyard et al. 2007), it is likely that most of the mitotype and chlorotype variation recorded across both species originated before the LGM. The lack of geographic structure in the distribution of chlorotype and mitotype variation according to the IBD analyses, might suggest that neither species experienced a history of range-wide retreat and recolonization during and since the LGM as appears to have been the case in some other Chinese conifers (e.g., Zhang et al. 2005; Meng et al. 2007). However, a high proportion of between-population differentiation for mtDNA in both species and cpDNA variation in *P. massoniana*, coupled with some cases of fixation or near fixation of different mitotypes and chlorotypes in disjunct populations (Table 1, Fig. 1), indicate that multiple glacial refugia may have existed for both species during the LGM (Hewitt 2000; Petit et al. 2003; Godbout et al. 2005, 2008; Chen et al. 2008). Multiple glacial refugia have been proposed for other plant and animal species in southeast China based on their genetic structures (e.g., Gao et al. 2007; Li et al. 2009). Also, palaeoclimatic and palaeovegetation evidence indicates that glacial climates might have fragmented the distributions of conifers and other forest trees into multiple refugia in southeast China (Liu 1988; Yu et al. 2007).

CAUSES OF DIFFERENCES IN SPECIES DIVERGENCE FOR DIFFERENT ORGANELLE MARKERS

Most importantly, our results support the view that in conifers variation in paternally inherited cpDNA, is more species specific than variation for maternally inherited mtDNA (Du et al. 2009). This effect is believed to stem from intraspecific gene flow being more extensive for paternally inherited cpDNA than for maternally inherited mtDNA. Thus, one possible hypothesis to explain the effect (the introgression hypothesis) is that in situations where hybridization occurs between species the spread of an introgressed chlorotype in a recipient population will be countered by a high level of immigration of intraspecific chlorotypes into the same population from nonintrogressed populations. This will keep the introgressed chlorotype in check at low frequency in the recipient population and therefore maintain a high probability that it will eventually be lost through drift. In contrast, an introgressed mitotype in a recipient population will not face the diluting effect of high levels of mitotype immigration from nonintrogressed populations, because of low levels of intraspecific mtDNA flow, and consequently is more likely to be maintained and to spread in the recipient population, and in turn the species (Currat et al. 2008). However, from the results of the present study it is evident that mitotypes shared between *P. hwangshannensis* and *P. massoniana* show no biased frequency in regard to their parapatric or allopatric distributions. If the shared mitotype variation were due to introgression between species, it would be expected to be concentrated geographically in parapatric populations (Palme et al. 2004; McGuire et al. 2007), which was not the case for mtDNA in the present study. Thus, the sharing of mitotypes between the two pines seems not to result from relatively recent interspecfic introgression occurring following postglacial range expansions of both species. In fact, from these results we can rule out that recent introgression of mtDNA, and by extension cpDNA, has occurred between the two species except possibly within the narrow hybrid zones that exist between parapatric populations. Mitotype sharing between species seems more likely, therefore, to derive from the retention of ancestral polymorphisms because of slow lineage sorting (lineage sorting hypothesis). A low level of intraspecific mtDNA gene flow will increase/maintain genetic divergence between populations due to genetic drift and therefore be expected to result in a greater amount of time before lineage sorting is completed between species (Wright 1943; Hoelzer 1997; Petit and Vendramin 2006).

The possibility that cpDNA variation is more species specific than mtDNA variation in these two pine species because of a faster rate of cpDNA intraspecific gene flow, gains further support from the analysis of the origin and distribution of chlorotypes across species. Four recombinant chlorotypes (C3, C4, C6 and C7) derived from the ancestral chlorotypes (C1 and C2) were detected and shown to be scattered widely across the geographical ranges of the two species. The widely scattered distributions of these recombinant chlorotypes suggest that recombination between C1 and C2 probably occurred early in the history of these two species rather than following recent contacts between them. In addition, the occurrence of C2 in *P. massoniana* at a very low frequency may suggest that chlorotypes C1 and C2 might have been present in both species early in their history. It is unlikely that C2 was introgressed into *P. massoniana* recently because the one population of this species that contains C2 (at Ankang) is allopatric to *P. hwangshanensis,* which is entirely fixed for C2. One possibility is that the common ancestor of the two pine species was polymorphic for chlorotypes C1 and C2 and that rapid chlorotype lineage sorting, promoted by high intraspecific gene flow, accompanied the origin and evolution of *P. hwangshanensis* and *P. massoniana.*

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In contrast, both species have maintained the mtDNA polymorphism present in their common ancestor.

Other possible causes of the different patterns of cpDNA and mtDNA variation within and between these two pine species cannot at this stage be ruled out. For example, an alternative cause of the greater cpDNA divergence variation between the two pine species could stem from mutation rate heterogeneity between cpDNA and mtDNA (Wolfe et al. 1987) resulting in nonuniform retention of chlorotype and mitotype polymorphism across the species (Sloan et al. 2008). A faster mutation rate can accelerate lineage sorting and result in rapid fixation of species-specific alleles through genetic drift. Thus, if the mutation rate of cpDNA is faster than that of mtDNA in these two pine species, this could be a cause of rapid cpDNA divergence between them, rather than a higher rate of intraspecific cpDNA gene flow. Currently, we have no information on rates of mutation for cpDNA and mtDNA in these pine species and, consequently, are not able to evaluate whether mutation rate heterogeneity might have been a cause of the cpDNA divergence between them.

Irrespective of the exact cause of the great difference in partitioning of mtDNA and cpDNA variation within and between species recorded in this study, it is clear from our results and those of others (e.g., Du et al. 2009) that DNA markers which are subject to high intraspecific gene flow are often more species specific. Thus, paternally inherited cpDNA markers in conifers and other gymnosperms are likely to be suitable for species delimitation, more so than maternally inherited mtDNA. This, of course, will not be the case in most angiosperms where cpDNA is known to be maternally inherited and therefore likely to exhibit a low level of gene flow (Mogensen 1996). In fact, a limited number of such case studies based on maternal cpDNA in angiosperms suggest extensive sharing of chlorotypes across morphologically distinct species due to low rates of lineage sorting of ancestral polymorphisms and/or introgression (e.g., Palme et al. 2004). Further population studies of genetic variation and gene flow are needed in conifers to confirm that the patterns we have reported here are frequently present within and between closely related and interfertile species. If they are, then cpDNA variation will be a useful tool for distinguishing such coniferous species at the molecular level.

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