Microsatellite Polymorphism in the Edaphic Spruce, *Picea asperata*, Originating from the Mountains of China

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Microsatellite variation of *Picea asperata* Mast. originating from the mountains of China was investigated by analyzing variation at seven SSR loci in 250 individuals representing ten populations. A fair degree of genetic diversity and considerable population subdivision occurred with the mean gene diversity (H) of 0.707, and genetic distances among populations varying between 0.121 and 0.224 (F_{ST}) and between 0.100 and 0.537 (R_{ST}). However, inter-population genetic distances showed no correlation with geographic distances between the population sites. This ruled out a simple isolation by distance model and suggested that migration does not have a great impact. In fact, the amount of gene flow, detected using private alleles, was very low, equaling only 0.753. Allele permutation tests revealed that stepwise-like mutations, coupled with genetic drift, could contribute to population differentiation. Moreover, significant genetic differences between populations were detected at most loci. The results indicate that natural selection, presumably through environmental stress, may be one of the main factors causing micro-geographical differentiation in the genetic structure of *P. asperata*. Based on SSR genotypes, 70% of the 250 individuals were correctly classified into their sites of origin. This suggests that microsatellites (SSRs) are effective in distinguishing genotypes of *P. asperata* originating from diverse eco-geographical sites in China.

1 Introduction

A large number of papers on the pattern and distribution of genetic variation within forest tree species has been published along with the quick development of marker techniques. Those previous studies have shown that genetic variability is critical for population persistence, especially in species that have become fragmented, bottlenecked or that have rapidly lost genetic variability (Saccheri et al. 1998, Westemeier et al. 1998). Numerous hypotheses concerning the maintenance of genetic diversity have been extensively discussed over the years (Bayer 1992, Bouza et al. 2002, Bacles et al. 2004). Random genetic drift, mutations, gene flow and mating systems are primary factors that shape population genetic structures. Spatial genetic structures may also be influenced by spatial variation in selection caused by environmental heterogeneity (Linhart and Grant 1996), such as landform, geography, climate and different biotic interactions, particularly in geographically widespread species or in species occupying fragmented habitats.

Picea asperata Mast. belongs to the flora of the alpine and canyon regions of the southwestern China, situated on the southeastern edge of the Qinghai-Tibet Plateau. It is considered to be a tertiary relic (Kuan 1981). Fossilized fragments have been found in tertiary deposits from western Sichuan and northern Yunnan (Fang 1996), confirming that *P. asperata* probably used to be much more widely distributed in the southwestern parts of China in the past. The results of pollen analyses suggest that the northern, temperate conifer forests were restricted to a refuge on the southeastern edge of the Qinghai-Tibet Plateau during the last ice age. Following the retreat of the glacier and climate warming during postglacial times, the conifer forests extended from the glacial refuge toward north and migrated into the mountainous area (Wang 1992, Liu et al. 2002). Due to the evolutionary history and topography, the present distribution of *P. asperata* is discontinuous and patchy within the mountain regions. Populations have been isolated by mountains or rivers. The typical scattered distribution may have limited or even prevented gene flow among the often isolated populations of *P. asperata* and, consequently, genetic drift may have overridden the impact of selection pressures in populations exposed to varying environmental conditions.

Compared to species with wide distribution and outcrossing breeding systems, relatively low levels of genetic diversity (Ledig and Conkle 1983, Dayanandan et al. 1999, White et al. 1999, Aguirre-Plantier et al. 2000) and increased amounts of inbreeding (Fuchs et al. 2003) have been observed in isolated populations of some tree species occupying a limited geographic range. Therefore, to secure the preservation of diversity in *P. asperata*, an in-depth investigation on the genetic diversity and population characteristics within its native range is greatly needed. Better knowledge of the level and distribution of genetic diversity among populations enables the development and utilization of appropriate conservation strategies. Isozyme studies and DNA-based analyses can contribute to genetic conservation programs by providing knowledge of genetic characteristics and understanding of population structures and differentiation. The present study was conducted to genetically characterize the populations of *P. asperata*, occurring in localities with varying climatic and geographical conditions in western China, using microsatellite markers (SSRs). The aim was to discover whether genetic divergence occurs at a micro-geographical scale and to reveal different factors that contribute to the genetic structure of *P. asperata*.

2 Materials and Methods

2.1 Tree Materials

Mature cones were collected from 250 mother trees of *P. asperata*, originating from ten populations located throughout its natural range in western China (Fig. 1) and including 25 mother trees from each population, and they were stored at 4 °C until use. All populations are pure *P. asperata* forests, and they inhabit unconnected mountainous areas within a narrow geographic range (100–105°E, 30–35°N). The vertical distribution varies from 2450 to 3300 m in altitude. The habitats occupied by the populations examined in this study represent a range of geographical and ecological conditions of altitude, hill steepness, aspect, temperature and rainfall (Table 1).
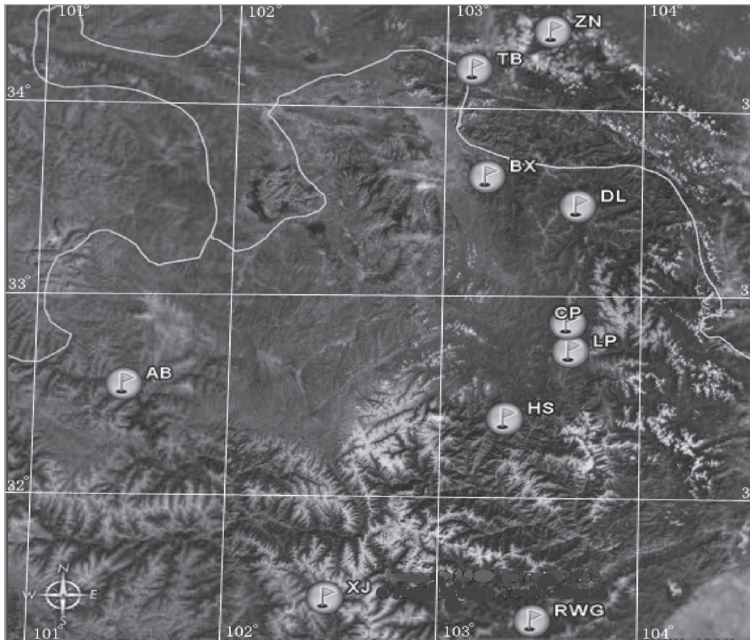
Fig.1. The locations of the populations of *P. asperata* in China (redrawn from an image 2006 Earthsat, Google Earth software). See text and Table 1 for details of each site and sample sizes.

Table 1. *P. asperata* populations surveyed, and their ecological and geographical parameters.

Population	Landform feature	Lo (E)	La (N)	Al (m)	Rn (mm)	Tm (°)	Tja (°C)	Tju (°C)	Hs (°)	As
XJ	Alpine and canyon	102°27′	31°30′	3300	614	12.0	2.2	19.9	12	N
CP	Alpine and canyon	103°37′	32°53′	3100	730	5.7	−4.3	14.5	25	SW
TB	Alpine and canyon	103°08′	34°07′	2450	570	7.0	−10.5	10.7	10	N
AB	Alpine and canyon	101°27′	32°33′	3100	712	3.3	−7.9	12.5	15	N
HS	Alpine and canyon	103°19′	32°25′	2900	833	9.0	−0.9	17.5	25	SW
ZN	Alpine and canyon	103°32′	34°20′	2800	564	4.3	−2.9	13.7	15	NW
LP	Alpine and canyon	103°38′	32°45′	3200	730	5.7	−4.3	14.5	20	NW
DL	Alpine and canyon	103°40′	33°28′	2800	553	12.7	1.7	22.2	28	N
BX	Plateau and hilly	103°13′	33°36′	2980	647	0.7	−10.5	10.7	10	SW
RWG	Plateau and mountain	103°27′	31°24′	2850	730	5.7	−4.3	14.5	25	NE

Lo, longitude; La, latitude; Al, altitude; Rn, mean annual rainfall; Tm, mean annual temperature; Tja, mean January temperature; Tju, mean July temperature; Hs, hill steepness; As, aspect.

2.2 SSR Analysis

Endosperms were carefully excised from overnight imbibed seeds. DNA was isolated from megagametophytes following the procedure of Isabel et al. (1995). Six seeds from each tree were analysed to allow the maternal genotype to be inferred. Compared with trinucleotide markers, dinucleotide markers suffer from some technical drawbacks concerning their use in genotyping, and from complex banding patterns which may make them difficult to score. Yet, based on the

available data of distances between these markers in a linkage map (Acheré et al. 2004), seven SSR dinucleotide markers were chosen from the group of markers developed by Pfeiffer et al. (1997) for the Norway spruce (*Picea abies* K.), SpAC1F7, SpAG2, SpAGC2, SpAGC1, SpAGG3, SpAGD1 and SpAC1H8, and used in the present investigation instead of trinucleotide markers (Scotti et al. 2002a) or markers available in a larger collection (Scotti et al. 2002b). The preliminary screening showed that the seven selected SSR marker loci produced simple but polymorphic patterns. The SSR reactions were based on the protocol of Pfeiffer et al. (1997) with some modifications. Following electrophoresis, the gels were silver-stained using the procedure of Panaud et al. (1996) and photographed using the Gel Doc 1000^{TM} image analysis system (Biorad) following the manufacturer's instructions. The fragment sizes were estimated using a standard molecular weight marker pUC 19 DNA/*Msp* (*Hpa*).

2.3 Data Analysis

The program *POPGENE* (Yeh et al. 1997) was used to calculate the allele frequencies, percentage of polymorphic loci (95% criterion, *P*), the mean number of alleles per locus (N_a), the effective number of alleles (N_e) and Nei's (1978) gene diversity (*H*). The *GENEPOP* version 3.4 (an updated version of *GENEPOP* version 1.2, Raymond and Rousset 1995) was used to perform tests for deviations from the Hardy-Weinberg equilibrium with the alternative hypotheses of either heterozygote deficiency or excess (Rousset and Raymond 1995). To estimate the F_{IS} value, we selected Robertson and Hill's (1984) estimate (R and H), because it has a minimum variance under the null hypothesis. The significance values were computed for each locus by unbiased estimates of an exact test using the Markov chain method through 1000 iterations. Global tests across loci and across populations were also conducted.

An exact test of allele frequency homogeneity was performed with *GENEPOP* to test the null hypothesis of no genetic differentiation among populations. The extent of genetic divergence between populations and across all sampled populations was quantified by computing pairwise

standardized R_{ST} values using differences in allele distributions based on the assumption of a strict stepwise mutation model, and F_{ST} values based on the assumption of a random mutation process with significances based on 1000 permutation processes. Also, average pairwise genetic distance values, F_{ST}/R_{ST}, for multiple loci and for each locus were computed for a set of distance classes (the space was partitioned into 6 distance classes according to Sturge's rule, 0–75 km, 76–110 km, 111–150 km, 151–200 km, 201–250 km, 251–330 km, to ensure that each class contained approximately equal numbers of locality pairs). One thousand random permutations of the allele sizes provided a distribution of pR_{ST} values, 95% confidence intervals covering the 25th to the 975th ordered values, and *P* values to test if $R_{ST} > pR_{ST}$ and to evaluate the phylogeographic pattern of *P. asperata*. The program *SPAGeDi* version 1.2 (an updated version of *SPAGeDi*, Hardy and Vekemans 2002) was used for these analyses. In order to further test isolation by distance, the relationship between the genetic and geographical distances was assessed by a Mantel test (Mantel 1967) using the program *GenAlEx* version 6 (Peakall and Smouse 2006), which tested isolation by distance based on the linear regression of pairwise genetic distance values ($F_{ST}/(1-F_{ST})$) against the logarithm of geographic distances between populations. The genetic relationships among populations were further analysed by the principal coordinate analysis (PCA) of a F_{ST} and R_{ST} matrix according to the extracted Eigen vectors in *GenAlEx* version 6. The number of migrants among populations (*N*m) were estimated both using the private allele method (Slatkin 1985) and the equation $Nm = 0.25(1-F_{ST})/F_{ST}$ and $Nm = 0.25(1-R_{ST})/R_{ST}$.

When attempting to classify individuals into their populations, a population assignment analysis was performed based on individual genotypes using Falush et al.'s (2003) "linkage model" in *Structure* version 2.0 (Pritchard and Wen 2004). Unlike the exclusion-simulation approaches (Cornuet et al. 1999) and the LOD score approaches (Paetkau et al. 1995, Banks and Eichert 2000, Campbell et al. 2003), the program, modified from Pritchard et al.'s (2000a, b) "admixture model", accounts for the possibility of correlations due to linkage and it can extract more information from the data. We

analyzed the data by a run consisting of 100 000 burn-in iterations followed by 500 000 further iterations without phased information. The data of genetic map distances between markers came from Acheré et al. (2004). The adjacent markers on the same chromosome were not especially close together, with a minimum distance of 22.3 cM and a maximum distance of 100.2 cM. The probability values in all above-mentioned tests were adjusted for multiple simultaneous table wide tests using the sequential Bonferroni adjustments (Rice 1989) to minimize Type I errors.

3   Results

3.1 Distribution of Allelic Diversity at SSR Loci

Seven microsatellite loci were assessed to characterize genetic diversity in ten populations of *P. asperata*. To ensure the validity of the analyses, PCR reactions were performed at least twice in all cases. All seven loci displayed polymorphism among the populations, with the total number of alleles ranging from thirteen at the locus SpAC1F7 to twenty-four at the loci SpAGG3 and SpAGD1. The total number of alleles per locus and the size ranges of the alleles detected are given in Table 2. A total of 139 alleles were identified, with the mean number of alleles per locus equalling 19.9. Almost half of the alleles (44.6%) were shared by at least six out of ten populations examined while the other alleles were shared by fewer populations.

3.2 Genetic Diversity within Populations

The number of alleles per locus (N_a), the effective number of alleles per locus (N_e), and the gene heterozygosity (H) were used to demonstrate the level of population genetic diversity (Table 3). In general, the populations included in the present study possessed fair levels of genetic diversity. In individual populations, the mean number of alleles per locus (N_a) ranged from 8.71 to 11.86 with the average number equalling 10.98, while N_e varied from 3.67 to 7.47 with the average of

Table 2. Characterization of the SSR loci： the repeat types and expected sizes for the SSR markers in *P. abies*, the numbers of alleles and the ranges of allele sizes for the SSR markers examined in *P. asperata*.

Locus	Repeat type	Expected size (bp)	Number of alleles	Range of allele sizes (bp)
SpAC1F7	$(AC)_{12}$	109	13	109–133
SpAG2	$(TC)_{16}$	105	18	96–135
SpAGC2	$(TA)_{11}(GA)_{20}$	126	21	80–157
SpAGC1	$(TC)_5TT(TC)_{10}$	103	17	84–129
SpAGG3	$(GA)_{24}$	136	24	110–182
SpAGD1	$(AG)_{25}$	147	24	108–210
SpAC1H8	$(GT)_{27}$	135	22	100–192

Table 3. Summary of genetic diversity indices in the populations of *P. asperata*, based on seven SSR markers.

Population	N	P (%)	N_a	N_e	H
XJ	25	100	11.71	7.47	0.7736
CP	25	100	11.86	6.89	0.7444
TB	25	100	11.14	5.79	0.6909
AB	25	100	11.57	6.14	0.6554
HS	25	100	11.00	6.75	0.7377
ZN	25	100	11.71	6.34	0.7054
LP	25	100	9.71	4.68	0.7189
DL	25	100	10.86	6.09	0.7323
BX	25	100	8.71	3.67	0.5930
RWG	25	100	11.57	7.14	0.7582
Mean	25	100	10.98	6.09	0.7072

N, sample size; P, percentage of polymorphic loci; N_a, number of alleles per locus; N_e, effective number of alleles per locus (Kimura and Crow 1964); H, Nei's (1978) gene diversity corrected for sample size.

6.09. The mean of H averaged 0.707 (range 0.593 to 0.774). Based on the values of the genetic parameters N_a, N_e and H, the highest level of diversity was detected in population XJ and the lowest diversity in population BX.

3.3 Genotype Structure

Hardy-Weinberg expectations for genotype frequencies within each population were rejected

Table 4. Summary of F-statistics (Robertson and Hill's (1984) estimate (R and H)) for seven loci in ten populations of *P. asperata*. * and ** represent significance degrees at $P<0.05$ and $P<0.01$, respectively.

Locus	XJ	CP	TB	AB	HS	ZN	LP	DL	BX	RWG
SpAC1F7	0.075	−0.035	0.193*	0.300**	0.275**	0.169*	0.458**	0.147	0.139	0.283**
SpAG2	−0.081*	0.122**	0.010	0.044	−0.049	−0.072	0.039	−0.032	−0.072	−0.107**
SpAGC2	0.049	−0.081	−0.093**	−0.031	−0.036	−0.086**	−0.082	−0.027	0.046	−0.081
SpAGC1	0.050	−0.093	−0.026	−0.309**	0.044	−0.015	0.021	−0.107**	−0.078	0.089
SpAGG3	−0.007	0.063	−0.009	−0.016	−0.031	−0.017	0.085	0.008	−0.055	−0.032
SpAGD1	0.102*	−0.058	0.035	0.002	0.060	−0.008	−0.183*	−0.058	0.183**	0.066
SpAC1H8	−0.047	−0.125**	−0.081*	−0.047	−0.183**	−0.093*	−0.047	−0.058	−0.021	−0.058

Table 5. Pairwise genetic distances (F_{ST} below, R_{ST} above) among populations of *P. asperata*.

Population	XJ	CP	TB	AB	HS	ZN	LP	DL	BX	RWG
XJ	0.000	0.214	0.439	0.431	0.351	0.423	0.537	0.266	0.494	0.480
CP	0.186	0.000	0.244	0.368	0.273	0.384	0.450	0.210	0.315	0.390
TB	0.216	0.141	0.000	0.236	0.204	0.278	0.283	0.171	0.148	0.264
AB	0.211	0.162	0.166	0.000	0.269	0.185	0.284	0.129	0.184	0.100
HS	0.221	0.160	0.180	0.201	0.000	0.220	0.230	0.209	0.220	0.347
ZN	0.157	0.132	0.135	0.161	0.178	0.000	0.353	0.227	0.303	0.260
LP	0.203	0.128	0.154	0.163	0.145	0.155	0.000	0.264	0.260	0.338
DL	0.173	0.151	0.163	0.121	0.164	0.147	0.150	0.000	0.155	0.126
BX	0.224	0.143	0.192	0.182	0.199	0.171	0.154	0.181	0.000	0.235
RWG	0.180	0.130	0.128	0.135	0.174	0.133	0.148	0.138	0.153	0.000

($P<0.05$) in 20 out of 70 cases following the algorithm of Guo and Thompson (1992). These departures occurred at least at one locus in every population and were almost equally due to heterozygote excesses (11 cases) and heterozygote deficiencies (9 cases) (Table 4). Furthermore, a half of heterozygote excess cases were attributed to a clustered surplus of heterozygotes at two loci (SpAGC2 and SpAC1H8; the P-value of a global test across populations <0.05), while heterozygote deficiency cases were attributed to a clustered deficit of heterozygotes at locus SpAC1F7. Across all loci, seven populations followed the Hardy-Weinberg equilibrium (the P-value of a global test >0.05), while three populations (XJ, LP and BX) possessed a significant heterozygote deficiency ($P<0.05$). In the whole set of *P. asperata* samples, a slight deficit of heterozygosity was detected (P-value of global test >0.05).

3.4 Genetic Differentiation

An exact test of the homogeneity of allele frequencies was used to compare differences in the allelic distribution at each locus among the ten populations. Using a Markov chain method with default values, the distribution of allele frequencies was significantly uneven among populations at all loci ($P<0.05$). These allelic differences translated into significant pairwise genetic distances, as expressed as F_{ST} (Weir and Cockerham 1984) and R_{ST} (Slatkin 1995). Permutation processes for genetic differentiation revealed significant differentiation in all pairwise comparisons. The results are given in Table 5. The mean pairwise multilocus estimates were equal to $F_{ST}=0.168$ and $R_{ST}=0.292$. The genetic distances over all loci varied between population pairs with F_{ST} and R_{ST} values showing a different pattern (Table 5).

Table 6. Allele permutation tests of genetic differentiation estimates. *, ** and *** represent the significance degrees at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, that the R_{ST} values are higher than pR_{ST}.

Locus	R_{ST}	pR_{ST} (95% C.I.)	F_{ST}
Multilocus	0.292**	0.160 (0.501–0.969)	0.168
SpAC1F7	0.194	0.197 (0.357–0.682)	0.204
SpAG2	0.401*	0.171 (0.338–0.811)	0.173
SpAGC2	0.254	0.201 (0.428–0.873)	0.205
SpAGC1	0.140	0.140 (0.563–0.801)	0.152
SpAGG3	0.319***	0.092 (0.361–0.786)	0.093
SpAGD1	0.366*	0.169 (0.463–0.938)	0.175
SpAC1H8	0.224	0.177 (0.266–0.730)	0.182

The average pairwise multilocus F_{ST} and R_{ST} values did not follow the isolation by distance pattern.

Permutation tests revealed that the multilocus global R_{ST} value as well as the estimates for three out of seven loci were significantly ($P < 0.05$) larger than the 95% range of pR_{ST} (Table 6). The multilocus pairwise R_{ST} values were significantly larger than pairwise pR_{ST} values for all six distance classes but the fifth one. The average pairwise pR_{ST} values were always somewhat lower than the pairwise F_{ST} values. The three single loci having significantly larger R_{ST} values than pR_{ST} values also had the same pattern in

different distance classes, even at shorter distances (Table 7). Mantel's test with 1000 random permutations revealed no significant correlation between pairwise genetic distance values and geographic distances among the ten populations of *P. asperata* (Fig. 2).

The overall gene flow (Nm) among populations based on private alleles was estimated to equal 0.750, which gives an estimate of the average number of migrants between all studied populations per generation. The estimate obtained from R_{ST} was quite comparable to the value obtained from the private allele method, the value equalling 0.606, while the value estimated from F_{ST} equalled 1.313. Based on pairwise estimates across all seven loci, the genetic relationships among populations were illustrated by the PCA plot of coordinates 1 and 2, respectively. The principal coordinates 1 and 2 classified populations into different main groups for both F_{ST} and R_{ST} (Fig. 3). Although the clustered populations were different in corresponding groups, two principal coordinates similarly classified population XJ alone as a group separated far from other main groups.

3.5 Population Assignment Analysis

On the basis of individual genotypes, population assignment analysis was conducted using Falush et al.'s (2003) "linkage model" with the

Table 7. Average pairwise F_{ST} and R_{ST} values among populations of *P. asperata* for a set distance, and the testing of the isolation by distance model. Each distance class contains 7–8 pairs of populations. *, ** and *** represent the significance degrees that R_{ST} values are higher than pR_{ST} after allele permutation tests at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

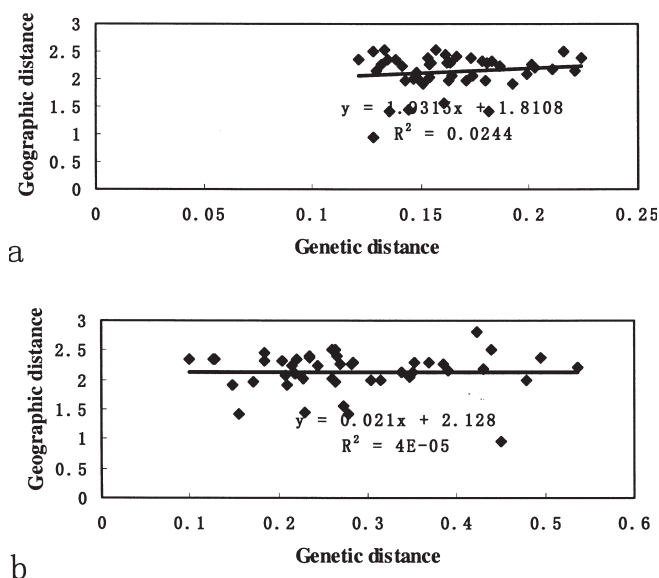| Locus	0–75 km		76–110 km		111–150 km		151–200 km		201–250 km		251–330 km	
	F_{ST}	R_{ST}	F_{ST}	R_{ST}	F_{ST}	R_{ST}	F_{ST}	R_{ST}	F_{ST}	R_{ST}	F_{ST}	R_{ST}
Multilocus	0.155	0.249*	0.169	0.288**	0.179	0.285*	0.175	0.341**	0.164	0.240	0.168	0.314*
SpAC1F7	0.168	0.217	0.137	0.185	0.229	0.206	0.276	0.162	0.176	0.183	0.205	0.196
SpAG2	0.147	0.176	0.204	0.397	0.211	0.517*	0.156	0.335*	0.155	0.319*	0.168	0.386*
SpAGC2	0.192	0.177	0.222	0.191	0.162	0.178	0.215	0.308	0.222	0.268	0.180	0.232
SpAGC1	0.139	0.172	0.134	0.162	0.124	0.109	0.162	0.143	0.163	0.130	0.177	0.190
SpAGG3	0.087	0.146	0.096	0.333***	0.092	0.262***	0.098	0.344***	0.092	0.257***	0.093	0.333***
SpAGD1	0.153	0.317**	0.218	0.321	0.163	0.282	0.162	0.378**	0.162	0.209	0.202	0.273
SpAC1H8	0.199	0.146	0.158	0.186	0.270	0.260	0.155	0.195	0.168	0.170	0.152	0.235

a

b

Fig. 2. No correlation was detected between pairwise genetic distances and geographic distances. Isolation by distance tested by a Mantel test. a) $F_{ST}/(1-F_{ST})$ *vs.* log-transformation of the geographic distance ($P=0.225$); b) $R_{ST}/(1-R_{ST})$ *vs.* log-transformation of the geographic distance ($P=0.470$).
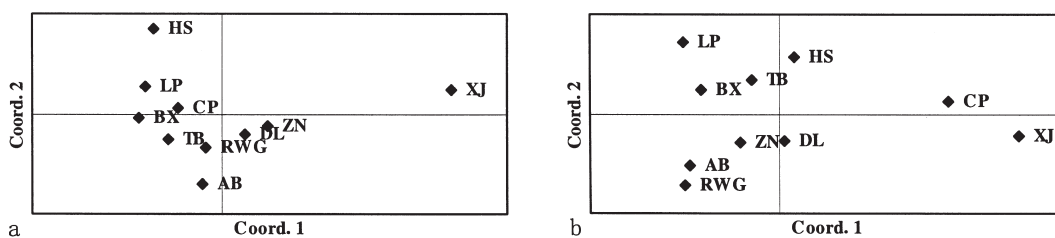

a

b

Fig. 3. The PCA plot of coordinates via covariance matrix with data standardization based on pairwise genetic distances across seven polymorphic SSR loci investigated in ten populations of *P. asperata*. a) pairwise F_{ST} PCA plot of coordinates, coordinates 1 and 2 explain 22.42% and 19.32% of the total variation, respectively; b) pairwise R_{ST} PCA plot of coordinates, coordinates 1 and 2 explain 35.44% and 21.55% of the total variation, respectively.

model of correlated allele frequencies, which assumes that frequencies in different populations are likely to be similar (probably due to migration or shared ancestry). Inferring the number of populations K = 10, the MCMC clustering algorithm correctly assigned most of the 250 individuals into their population of origin with 90% confidence intervals. The proportion of membership of individual genotypes in their respective populations according to their geographic distribution ranged between 50.4% and 85.3%, with an average of 70.4%. The assignment results are presented in Table 8. The remaining individuals of each population were mainly allocated to 2–5

Table 8. The proportion of membership of 250 individuals from ten populations of *P. asperata* into their respective populations, as revealed by a population assignment analysis, based on individual genotypes at seven SSR loci.

Pop.	XJ	CP	TB	AB	HS	ZN	LP	DL	BX	RWG	Correct classification (%)
XJ	0.741	0.034	0.015	0.044	0.054	0.011	0.017	0.013	0.016	0.052	74.1
CP	0.020	0.781	0.025	0.014	0.026	0.013	0.038	0.022	0.048	0.013	78.1
TB	0.019	0.009	0.504	0.014	0.009	0.022	0.047	0.103	0.051	0.014	50.4
AB	0.032	0.019	0.017	0.674	0.012	0.030	0.023	0.150	0.011	0.040	67.4
HS	0.040	0.018	0.013	0.011	0.837	0.016	0.023	0.012	0.015	0.015	83.7
ZN	0.037	0.020	0.092	0.027	0.013	0.690	0.015	0.038	0.045	0.024	69.0
LP	0.035	0.040	0.012	0.053	0.053	0.027	0.728	0.018	0.009	0.024	72.8
DL	0.013	0.034	0.018	0.204	0.015	0.015	0.012	0.649	0.025	0.015	64.9
BX	0.015	0.017	0.020	0.013	0.011	0.016	0.020	0.025	0.853	0.010	85.3
RWG	0.115	0.018	0.024	0.049	0.112	0.040	0.019	0.024	0.014	0.585	58.5
Mean											70.4

different populations other than that from which they originated, primarily with a tendency for the incorrectly assigned individuals to be classified into nearest populations.

4 Discussion

The main objective of the present study was to assess the level and pattern of genetic diversity, based on microsatellite markers, among local spruce populations originating from China, and to understand the reasons for the present population structure and differentiation pattern. Our results show that, despite the potential effect of genetic drift, the microsatellite variation of *P. asperata* populations is partly structured and shaped by mutations and recolonizations from different refuges. The excess of homozygotes or heterozygotes at some examined microsatellite loci and the presence of strong genetic divergence indicate that diversifying selection, caused by ecologically highly variable environment, at loci linked to the microsatellite loci or targeted at the microsatellite loci themselves, if they have a functional role, may also have contributed to the local genetic differentiation of *P. asperata* at the micro-geographical level. A comparable conclusion has been previously drawn in red spruce (*P. rubens*)

based on allozymes (Mosseler et al. 2003).

Genetic diversity is generally the result of long-term evolution and it represents the evolutionary potential of a species. To survive and adapt to an unstable environment, a species has to evolve and accumulate genetic variation. There are investigations showing that the loss of population-level genetic diversity can play a decisive role in the persistence of a species in the long term because such diversity is needed to allow a species to adapt to changing environments (Lande 1996, Oostermeijer et al. 2003). Compared to previous reports (Thomas et al. 1999, Gomez et al. 2003), the parameters presented in Tables 2 and 3 in this study clearly demonstrate the presence of fair levels of polymorphism in *P. asperata*, with the total number of alleles across the seven loci being high, equaling 139, and the mean gene diversity within populations equaling 0.707. The degree of genetic diversity maintained in the populations of *P. asperata* indicates that the species still possesses a fair level of ability to react to environmental changes. In addition, it was discovered that the distribution of genetic diversity is unequal among populations, marginal populations (TB, AB, ZN and BX) possessing slightly lower levels of genetic diversity than more central populations. The slight decline of genetic variation detected in marginal populations may be due to isolation from other populations.

The present study demonstrated that allele distributions of *P. asperata* are nonrandom within the limited geographical area where SSR loci were screened. Although no unique alleles were found in any population, the significant differences in the distribution of allele frequencies and the differentiation values $F_{ST} = 0.168$ and $R_{ST} = 0.292$ indicate that the ten populations of *P. asperata* examined differ genetically. Compared to previous studies, the amount of genetic diversity present among populations was clearly higher than the values detected in other conifer species, based primarily on isozymes (Hamrick et al. 1992, Petit et al. 1995, Zheng and Ennos 1999, Salvador et al. 2000, Prus-Glowacki et al. 2003) and microsatellites (Thomas et al. 1999), and either even or somewhat higher, depending on the differentiation measure used, than the observations based on cpSSR in *P. canariensis* ($G_{ST} = 0.190$) (Gomez et al. 2003) and in *P. pinaster* ($G_{ST} = 0.235$) (Vendramin et al. 1998).

Several evolutionary forces, such as mutations, random genetic drift, gene flow and natural selection, influence the variation patterns of genomes and populations (Gaut et al. 2000). Selection and mutations have locus-specific effects while genetic drift and gene flow act at genome-wide scale under a standard neutral model (Lewontin and Krakauer 1973, Luikart et al. 2003). Examining the spatial structuring of genetic differentiation throughout the distribution of a species can provide important insights into the relative importance of distance, migration and genetic drift (Slatkin 1993, Hutchison and Templeton 1999). When the gene flow rate is greatly reduced, population genetic differentiation will increase due to genetic drift (Hutchison and Templeton 1999). The high level of population differentiation detected at a rather small micro-geographic scale in *P. asperata* probably reflects the presence of limited gene flow. *P. asperata* inhabits a restricted and disjunctive mountain area, which includes various climate, characteristic terrain and physiognomy. Rugged mountains and fragmented habitats are significant gene exchange barriers. Especially under extensive isolation, as in the case of the marginal populations XJ and BX, increased inbreeding and more extensive genetic divergence are expected to happen due to genetic drift.

In a wind-pollinated species, the observed population genetic structure cannot be attributed to the mating system. Coupled with random genetic drift due to isolation, mutations may be another important factor contributing to genetic differentiation among populations in *P. asperata*. Microsatellite loci are typically characterized by high mutation rates. A suggested model, the stepwise mutation model (SMM), causes preferentially stepwise changes in the number of repeats and, thus, in allele sizes (Zhu et al. 2000). However, microsatellite mutations are known to deviate from an ideal SMM or GSM (Estoup and Angers 1998, Ellegren 2000, Xu et al. 2000). A comparison of the values of F_{ST} under a random mutation process and R_{ST} under a strict stepwise mutation model, computed using the same data, can provide valuable insights into phylogenetic inferences, *i.e.*, the importance of mutation *vs.* genetic drift or gene flow for population differentiation (Michalakis and Veuille 1996, Ross et al. 1997, Estoup et al. 1998, Lugon-Moulin et al. 1999). In our study, allele permutation tests, including a test of the global F_{ST}/R_{ST} value and a test of the slope (*b-lin* or *b-log* values), revealed a phylogeographic signal within populations but none among populations. The results demonstrate that stepwise-like mutations have a significant contribution to population differentiation concerning at least three loci in *P. asperata*. As reviewed by Hardy et al. (2003), a phylogeographic pattern is expected when the mutation rate is non-negligible compared to the migration rate. In agreement with this, the estimates of the linear regression of genetic distances were geographically independent. The absence of a significant relationship between geographic and genetic distances, as detected in *P. asperata*, is commonly considered as a sign of sharp local differentiation rather than as a sign of a gradual change in allele frequencies across the range of the species. Following the comments of Balloux and Goudet (2002) and Hardy et al. (2003), such results also imply that R_{ST} should be a better estimator than F_{ST} for population differentiation in *P. asperata*.

For some loci, an excess of homozygotes was found and for others an excess of heterozygotes, which is in agreement with previous studies on tropical tree species (Collevatti et al. 2001). A surplus of heterozygous individuals and negative F values may indicate that selective forces

are acting at these loci within the population (Lewontin and Cockerham 1959), while heterozygote deficiencies can be caused by inbreeding, selection against heterozygotes, Wahlund effects or selection-induced microscale differentiation (Brown 1978, Epperson 1990, Knowles 1991, Bush and Smouse 1992, Sproule and Dancik 1996). Although microsatellites are usually assumed to be neutral, there is some evidence that certain microsatellite markers show functional significance and could be subject to natural selection (Bowcock et al. 1994, Garza et al. 1995, Rico et al. 1996, Kashi et al. 1997, Kashi and Soller 1999). In this study, a significant excess of heterozygotes was detected at locus SpAGC2, which is an outlier locus in *P. abies* (Acheré et al. 2005). Moreover, the loci SpAC1F7, SpAGC1 and SpAC1H8 are located within the two profiles of an outlier locus in the linkage group 5 with approximate distances of 38.8 cM, 10.0 cM and 10.0 cM, respectively, while locus SpAG2 is closely located within a profile of an outlier locus in the linkage group 6 in *P. abies*. According to Charlesworth et al. (1997), high-level genetic differences between populations can be produced at loci present in the region of a locus subject to local selection. This may be the situation in our study, in which considerable genetic differentiation was detected at some loci.

The genetic structure of *P. asperata* populations seems to follow a mosaic-like pattern. Patchy genetic distribution may reflect multiple recolonizations, and the possible role of genetic drift and selection. Inferences from fossil evidence and pollen analyses indicate that the present distribution of *P. asperata* is a result of postglacial recolonization. The repeated formation of refuges and the successful retreat during the long duration of each glacial advance probably resulted in the formation of multiple population sites. However, there has been no reliable evidence for the presence of such refuges before this study. Perhaps the results of the population assignment analysis with the tendency to classify incorrectly assigned individuals into nearest populations may serve as evidence for this hypothesis, given the assumption of the model of correlated allele frequencies that frequencies in different populations are likely to be similar (probably due to migration or shared ancestry). According to previous studies (Paetkau

et al. 1995, Cornuet and Luikart 1996, Rannala and Mountain 1997), assignment methods can be useful in population genetic studies to identify immigrants or their descendants.

In summary, the pattern of genetic diversity detected in *P. asperata* in western China contains distinct features when compared to the variability previously found in other *Picea* species (Pfeiffer et al. 1997, Zheng and Ennos 1999, Gomez et al. 2003). The genetic structure of *P. asperata* is characterized by the following features: i) The level of genetic diversity (mean $H = 0.707$) revealed at seven SSR loci in *P. asperata* is fair and quite similar when compared with the amount of variability observed in *Pinus contorta* (average $H_e = 0.741$), based on five SSR loci (Thomas et al. 1999). ii) The presence of significant differences in allelic distributions among populations shows that the allele distribution is uneven in the populations of *P. asperata*. iii) Great genetic differentiation was found over short geographic distances. This is unlike what is generally observed in outbreeding conifers (Hamrick et al. 1992). Mutation, genetic drift and natural selection are the evolutionary forces contributing to genetic differentiation. Perhaps, postglacial recolonization can also be a factor affecting the patchy genetic distribution in *P. asperata*.

In conclusion, a fair degree of genetic diversity and considerable population subdivision was detected in *P. asperata* investigated in a spatially limited area, which, however, contains great ecological amplitude. Microsatellite polymorphisms were non-random and maybe associated with environmental stress in the populations of *P. asperata*. Their patterns were explained, at least partly, by mutation and diversifying selection. The results obtained do not only have theoretical applications but, more importantly, also practical ones, which may help in maximizing the exploitation, conservation, and utilization of local spruce species. However, *P. asperata* is a species which has been a subject to ecological, physiological and genetic studies only rarely, and the knowledge available is still limited. The on-going research on *P. asperata* strongly emphasizes the need to investigate in depth important dynamic features, such as eco-physiological parameters and demographic population structures. Only after all necessary ecological and physiological information

is available and linked to genetic information, effective management plans can be created and implemented.

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