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Assessment of AFLP-Based Genetic Variation in the Populations of *Picea asperata*

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Summary

Picea asperata Mast., which occurs in a restricted habitat in western China, has a wide ecological amplitude. In the present study, ten natural populations of *P. asperata* were studied using AFLP markers to investigate the population genetic structure and the level of genetic diversity. Of the 210 loci identified with two EcoRI/MseI primer combinations, 142 loci were found to be polymorphic. Yet, the level of genetic diversity observed within populations was quite low. The averages of NEI's gene diversities (h) and Shannon's indices of diversity (I) calculated across populations equaled 0.156 and 0.227, respectively. The coefficient of gene differentiation among populations, based on the estimate G_{ST} and the unbiased estimate Φ_{st}, equaled 0.340 and 0.231, respectively. The mean genetic distance (D) between population pairs was 0.119 (range 0.050-0.156). Such high values indicate that there is significant differentiation among populations of *P. asperata*. Several factors could have contributed to the strong population differentiation, including relatively limited gene flow between populations (N_m = 0.968). Variation in environmental conditions and consequent selection pressures may be other factors attributing to the high level of genetic differentiation among populations. In addition, it was discovered that the geographic distances are not correlated with the genetic distances between the populations of *P. asperata.*

Key words: AFLP markers, genetic structure, genetic diversity, *Picea asperata* Mast.

Introduction

Picea asperata Mast. is an endemic spruce species, which mainly occurs in the northwestern Sichuan province and the southern Gansu province, approximately within the geographical range of 100.1–106.8 °E and 30.2–34.6 °N (LIU et al., 2002). *P. asperata* is one of the most important trees for the production of wood pulp and lumber, as well as one of the keystone species of

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subalpine coniferous forests in southwestern China (WU and RAVEN, 1994). Therefore, the conservation of the genetic resources of *P. asperata* is of both ecological and economical importance.

The occurrence of *P. asperata* in disjunctive mountainous areas with a narrow geographic range may have influenced its genetic structure. During its evolutionary history, *P. asperata* has been exposed to long-term geographic isolation and later also to artificial selection (WU, 1959), which may have resulted in a decrease in genetic diversity. A low level of genetic variation is problematic for further adaptation to changing environments. The present study of genetic diversity in the native range of *P. asperata* is relevant for the planning of conservation actions to secure the maintenance of the species' long-term variability and viability. Better knowledge of the level and distribution of the genetic diversity among populations enables more realistic conservation and restoration strategies in *P. asperata.*

Molecular markers, which can be used to analyze large numbers of loci distributed throughout the genome (BROWN et al., 1990), have proven to be powerful tools in the assessment of genetic variation both within and among plant populations. The amplified fragment length polymorphism (AFLP) technique described originally by VOS et al. (1995) combines two strategies widely used for DNA fingerprinting: RFLP and the power of polymerase chain reaction. Due to the high multiplex ratio (number of polymorphic markers generated in a single PCR experiment), high reproducibility, and the small quantity and quality requirements for DNA (JONES et al., 1998; PEJIC et al., 1998), AFLP has great potential to detect genetic variation in any part of the genome. Therefore, it is a suitable tool for population genetic studies (TRAVIS et al., 1996; LERCETEAU and SZMIDT, 1999), including the evaluation of genetic variability (ROA et al., 1997; ESTELLE and ALFRED, 1999; WANG et al., 2003).

In this study, the main goal is to estimate the levels and patterns of genetic variation and genetic differences within and among natural populations of *P. asperata* throughout its natural range of occurrence using AFLP analyses. This work will provide valuable information on plant genetic resources which can be used to aid practical conservation actions.

2. Materials and Methods

2.1. Plant materials

Plant material of 250 mother trees of *P. asperata* from ten populations distributed throughout their natural range was sampled in western China (*Fig. 1*). The geographical and ecological parameters of the sampled trees are shown in *Table 1.*

2.2. DNA extraction

DNA was isolated from 0.5 g of megagametophytes using the protocol of Doyle and Doyle (1987) with 1% β-mercaptoethanol (v/v) and 1% PVP 40000 (w/v) added to the CTAB extraction buffer. The extractions were performed using chloroform-isoamyl alcohol (24:1), and the resulting DNA pellet was washed with 70% ethanol before the final suspension step. DNA concentrations

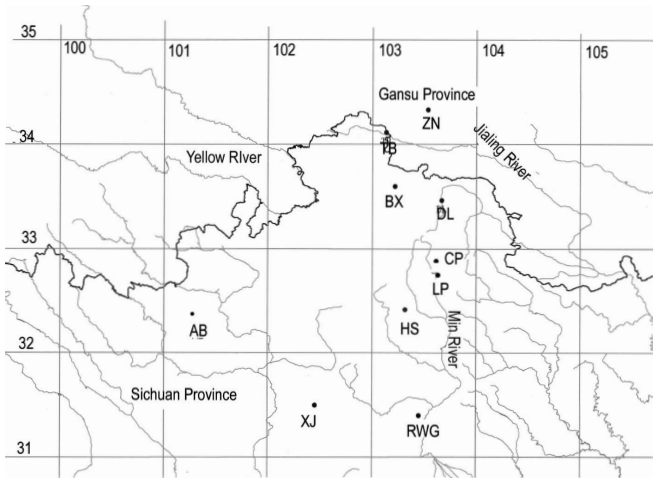

Figure 1. – The geographical distribution of *P. asperata* in China. The locations of the natural population sampled in this study are shown. Population characteristics are given in *Table 1.*

Table 1. – Locations and ecological variables of the sampled populations of *P. asperata.*

Population	Latitude	Longitude	Altitude (m)	Annual average temperature (°C)	January average temperature (°C)	July average temperature (°C)	Annual precipitation (mm)	Hill steepness (°)
XJ	31°30′	102°27′	3300	12.0	2.2	19.9	614	12
HS	32°25′	103°19′	2900	9.0	-0.9	17.5	833	25
ZN	34°20′	103°32′	2800	4.3	-2.9	13.7	564	15
AB	32°33′	101°27′	3100	3.3	-7.9	12.5	712	15
DL	33°28′	103°40′	2800	12.7	1.7	22.2	553	28
RWG	31°24′	103°27′	2850	5.7	-4.3	14.5	730	25
CP	32°53′	103°37′	3100	5.7	-4.3	14.5	730	25
LP	32°45′	103°38′	3200	5.7	-4.3	14.5	730	20
BX	33°36′	103°13′	2980	0.7	-10.5	10.7	648	10
TB	34°07′	103°08′	2450	7.0	-10.5	10.7	570	10

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were determined by comparison with a serial dilution of standard lambda DNA.

2.3. AFLP analysis

AFLP reactions were performed according to the procedure described by VOS et al. (1995) with some modification. All amplification reactions were performed in a GeneAmp® PCR System 9700 (perkin Elmer Corp., Norwalk, CT, USA). Six plants were used in the initial primer test including 72 combinations of selective bases of EcoRI and MseI primers with three selective bases. Two primer combinations (E-ACC/M-CCA and E-AGG/M-CAG) that resulted in clear amplification profiles were selected for the complete analysis. After the selective amplification, the products of the polymerase chain reaction (PCR) were mixed with an equal volume of loading buffer [98% (v/v) formamide, 10 mM EDTA, 0.05% (v/v) xylene cyanol, and 0.05% (v/v) bromophenol blue] and denatured at 95°C for 6 min and directly placed on ice. 5 µl of the mixture was loaded on a 6% (v/v) polyacrylamide gel (30 x 34 cm) containing 7 M urea, which was pre-warmed for 2 h. Gels were run with 0.5 x TBE electrophoresis buffer [50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.3] at 70 W constant power for 2 h. After electrophoresis, gels were stained with 0.1% silver nitrate (BASSAM et al., 1991).

A reproducibility test was performed for six trees with all primer pair combinations. For each of those samples, three independent DNA extractions were carried out, and AFLP fingerprints were generated using all different DNA templates. The test proved the high reproducibility of AFLP fingerprints.

2.4. Population genetic analysis

The fragments amplified by AFLP primers were scored as either present (1) or absent (0) on the basis of size in comparison with external standards using the Gel Doc 1000™ image analysis system (Biorad). Bands smaller than 80 bp in size were excluded from the analyses because they appeared unstable in the AFLP experiment. Amplification of high molecular weight fragments may be influenced by differences in the quality of DNA or in the conditions of the PCR reactions, causing them to be less reproducible than the smaller fragments. Therefore, bands longer than 600 bp were excluded from the analyses as well. The two primer combinations used produced a total of 210 bands, of which EcoRI + ACC and MseI + CCA, and EcoRI + AGG and MseI + CAG produced 106 and 104 bands, respectively, ranging from 80 to 600 bp in size.

The following parameters of genetic variation were assessed for each population: gene diversity (h) (NEI, 1973), Shannon's information index (I) (LEWONTIN, 1972), observed number of alleles (N_a) and effective number of alleles (N_e). Genetic divergence between populations was further investigated using NEI's (1978) unbiased genetic distance (GD) and genetic identities (GID). NEI's (1978) unbiased genetic distances were calculated for all population pairs and used to construct a phylogenetic tree (UPGMA). All above mentioned calculations were performed using POPGENE version 1.32 (YEH et al., 1997).

The relationship between genetic and geographical distances was assessed by a Mantel test (MANTEL, 1967) using the program GENALEX version 5 (PEAKALL and SMOUSE, 2001), which tested isolation by distance based on the linear regression of pairwise genetic distance values between populations against straight-line geographical distances between population pairs. The partitioning of total genetic variation was assessed by a hierarchical analysis of molecular variance using the software package AMOVA-PREP and AMOVA1.55 (EXCOFFIER, 1993). The significance of variance components was tested using permutation tests (1000 permutations). An estimate of gene flow among populations (Nm) was computed using MCDERMOTT and MCDONALD's, 1993; formula $N_m = (1\text{-}G_{ST})/2G_{ST}$. By using the program SPSS, PEARSON's correlations between the genetic diversities within populations and the eco-geographical factors were determined.

3. Results

3.1. Genetic variation within populations

The parameters of genetic variability among populations are presented in *Table 2*. In individual populations, NEI's gene diversities (h) and SHANNON's indices of diversity (I) varied from 0.140 to 0.175, and from 0.213 to 0.255, respectively. For the whole set of populations, the h and I values were 0.237 and 0.354, respectively, and the number of alleles per locus (N_a) and the effective number of alleles per locus (N_e) equaled 1.68 and 1.41, respectively. Across populations, the average NEI's gene diversity (h) and SHANNON's index of diversity (I) were 0.156 and 0.227, respectively, and the mean number of alleles per locus (N_a) and the effective number of alleles per locus (N_e) equaled 1.37 and 1.15, respectively. The highest levels of diversity were detected in populations DL and CP, and the lowest diversity in population AB, with both h and I showing the same tendency. In addition, most populations followed the pattern that genetic diversities within populations increased with

Table 2. – Genetic variation parameters of *P. asperata.* The mean values are calculated across populations while the total values are calculated across the species.

Population	N	h	I	N_a	N_e
XJ	25	0.1500	0.217	1.357	1.266±0.384
HS	25	0.1594	0.233	1.391	1.279±0. 370
ZN	25	0.1682	0.242	1.386	1.306±0. 403
AB	25	0.1400	0.200	1.314	1.258±0. 395
DL	25	0.1747	0.255	1.424	1.306±0. 377
RWG	25	0.1513	0.222	1.381	1.260±0. 356
CP	25	0.1745	0.251	1.405	1.315±0. 401
LP	25	0.1504	0.218	1.357	1.268±0. 378
BX	25	0.1513	0.217	1.343	1.277±0. 398
TB	25	0.1416	0.213	1.352	1.258±0. 368
Mean	25	0.156	0.227	1.370	1.150
Total	250	0.237	0.354	1.676	1.412±0. 387

N, sample size; N_a, observed number of alleles per locus (KIMURA and CROW, 1964); N_e, effective number of alleles per locus (KIMURA and CROW, 1964); h, NEI's (1978) gene diversity; I, Shannon's Information index.

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Table 3. – Genetic distances among populations of *P. asperata*, based on NEI's (1978) unbiased measurements.

POP	XJ	HS	ZN	AB	DL	RWG	CP	LP	BX	TB
XJ	****									
HS	0.1443	****								
ZN	0.1355	0.0682	****							
AB	0.1546	0.0954	0.0520	****						
DL	0.1190	0.1050	0.1361	0.1311	****					
RWG	0.1555	0.1184	0.1401	0.1472	0.1032	****				
CP	0.1326	0.0848	0.0905	0.0912	0.0840	0.0982	****			
LP	0.1454	0.0990	0.0964	0.0945	0.1018	0.1074	0.0568	****		
BX	0.1046	0.1211	0.1147	0.1236	0.1129	0.1199	0.1055	0.0733	****	
TB	0.1312	0.1014	0.1268	0.1215	0.0498	0.1069	0.0975	0.0931	0.1144	****

hill steepness: The correlation coefficients between the hill steepness and Nei's gene diversity, and hill steepness and Shannon's indices of diversity were both significantly positive, equaling 0.688 ($p < 0.05$, d.f. = 8), 0.655 ($p < 0.05$, d.f. = 8), respectively.

3.2. Population genetic structure

When all populations were compared, a high level of genetic differentiation was detected (G_{ST} = 0.340). The total gene diversity (H_t) and gene diversity within populations (H_s) equaled 0.237 and 0.157, respectively. The hierarchical analysis conducted using AMOVA revealed that 23.1% of the total variance was attributable to differences between populations and 76.9% to variation between individuals within populations. This hierarchical analysis further indicates the presence of a high degree of population differentiation among populations over short geographic distances. The overall rate of gene flow (N_m) among populations was quite low, equaling 0.968.

3.3. Genetic distances and relationships

Genetic distances were calculated for each pair of populations to estimate the extent of divergence (*Table 3*). The average genetic distance among populations equaled 0.119. The lowest genetic distance (0.0498) was found between populations DL and TB, and the greatest genetic distance (0.1555) was detected between populations XJ and RWG. The estimates of GD further confirmed the sharp genetic differentiation among populations over short geographic distances. The UPGMA cluster analyses based on NEI's (1978) unbiased genetic distances was performed to further show the genetic relationships among populations (*Fig. 2*). The dendrogram grouped the populations in the following way: the first group consisted of populations CP, LP, DL, TB, BX and RWG while the second cluster consisted of populations AB, HS and ZN. Population XJ differed considerably from the other populations and was clustered alone as a third group. A Mantel test with 1000 random permutations revealed no correlation between pair-wise genetic distance values and geographical distances (*Fig. 3*).

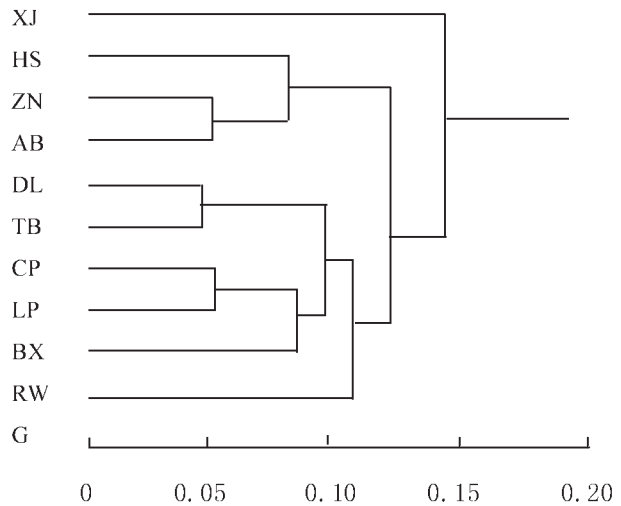

Figure 2. – Dendrogram of genetic distances among ten populations of *P. asperata.*

4. Discussion

Our results show that the populations of *P. asperata* investigated in Western China possess low degrees of genetic diversity. This result is in good agreement with comparable studies based on allozymes (LUO et al., 2005) and RAPDs (XUE et al. unpublished), which also revealed low levels of genetic variation in *P. asperata.* However, the present estimates, based on AFLPs, were lower than those based on SSR variation in the same ten populations of *P. asperata* (WANG et al., 2005). Also

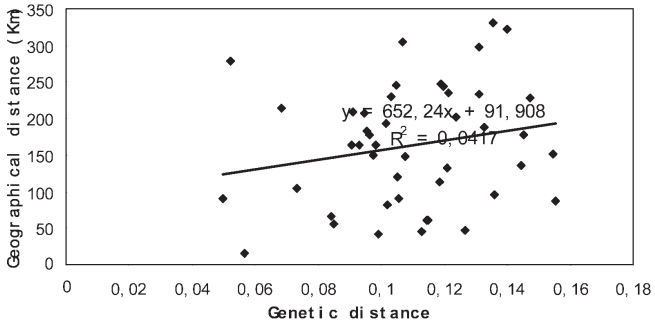

Figure 3. – No correlation was detected between pair-wise genetic distance values and geographical distances. Isolation by distance tested by a Mantel test.

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in previous studies, a similar tendency in variation depending on the method used has been observed. RAPD and AFLP generally appeared to yield similar estimates (PALACIOS et al., 1999; DIAZ et al., 2001; WACHIRA et al., 2001; ZAWKO et al., 2001; NYBOM, 2004), whereas SSR-derived estimates of within-population variation were usually considerably higher and also had a wider range in values than those obtained by methods based on dominant markers (THOMAS et al., 1999; DIAZ et al., 2000; MARIETTE et al., 2001; MAGUIRE et al., 2002; TURPEINEN et al., 2003). The difference is likely to result from the ability of SSRs to detect greater levels of polymorphism in general when compared with other marker analyses (POWELL et al., 1996; MAGUIRE et al., 2002). The genomic distribution of AFLP markers generated by EcoRI/MseI restriction digestion is reported to be biased towards centromeric regions in plants (YOUNG et al., 1999).

The ten investigated populations of *P. asperata* possessed a low level of genetic diversity, similar to the levels obtained in other species possessing restricted occurrences (WRIGHT, 1977; SCHOEN and BROWN, 1991; GITZENDANNER and SOLTIS, 2000; BROADHURST and COATES, 2002; MARGARET, 2002). The present study confirms the assumption that restricted species maintain less genetic diversity than do more widespread species (KARRON, 1987; HAMRICK and GODT, 1989). The relationship between low levels of genetic diversity and restricted geographic distribution is likely to be a consequence of habitat specificity and stochastic processes associated with population size fluctuations, and inbreeding may also be important (BARRETT and KOHN, 1991; ELLSTRAND and ELAM, 1993). In our study, the low level of genetic diversity present in *P. asperata* may be due to a number of factors. Fluctuations in population size, caused by habitat destruction and landscape fragmentation, may have a strong effect. Habitat specificity is also a likely factor: *P. asperata* occurs in rugged alpine and canyon regions, which is likely to decrease migration between populations but also between patches within populations. In addition, the reduced levels of genetic variation in *P. asperata* can be caused by other events, such as strong directional selection, founder effects associated with relatively recent divergence and isolation, and environmental stochasticity resulting from, e.g., the invasion of exotic species or altered climatic conditions.

P. asperata inhabits a limited area in western China, which has a characteristic terrain, physiognomy and climate. The species has been exposed to long-term geographical isolation, and also to artificial selection (WU, 1959). As a result of human interference, large areas of the natural range of *P. asperata* have been destroyed, and only a few natural populations remain. In addition to possessing limited genetic variation within populations, *P. asperata* also exhibits considerable levels of differentiation among populations. This latter pattern is also in agreement with previous studies on many rare species of flowerings plants (HAMRICK and GODT, 1989; KARRON, 1991).

The amount of genetic differentiation present among populations was considerable (G_{ST} = 0.340) despite the relatively short geographic distances between the examined populations. The value was clearly higher than those detected in other conifer species in studies based primarily on isozymes. In a meta analysis based on information of 121 species of gymnosperms (HAMRICK et al., 1992), the mean level of differentiation (G_{ST}) obtained equaled only 0.073. The G_{ST} value detected here for *P. asperata* is higher than the differentiation among populations observed in *P. pinaster* (G_{ST} = 0.102, MARIETTE et al., 2001), *P. oocarpa* (G_{ST} = 0.07, DIAZ et al., 2001), and *P. abies* (G_{ST} = 0.190, WANG et al., 2003), also based on AFLP. For estimates of among-population variation, a good agreement is generally found between AFLP and RAPD, while the SSR-derived values are usually lower (NYBOM, 2004). In the case of *P. asperata*, the G_{ST} value based on AFLP fingerprinting was similar to the value obtained by both allozymes (0.311, LUO et al., 2005) and RAPDs (0.341, XUE et al., unpublished) but higher than that obtained by SSR (0.223, WANG et al., 2005).

When populations are small and isolated, genetic drift influences the genetic structure and increases differentiation among populations (BARRETT and KOHN, 1991; ELLSTRAND and ELAM, 1993). Genetic differentiation in general can be expected to increase when gene flow is restricted and natural selection is operative (SLATKIN, 1985, 1987). The divergence among *P. asperata* populations may be explained partly by limited gene flow (N_m = 0.968). Rugged mountains and habitat fragmentation in the study area are significant gene flow barriers for *P. asperata*.

As observed in this study, the pattern of genetic differentiation does not correlate with geographic distance in *P. asperata* and, consequently, adjacent populations do not group together in the analysis of genetic distances. This result indicates that geographical distance as such is not an important factor influencing the genetic relatedness among populations in *P. asperata*. On the other hand, there have been suggestions that, on smaller scales, genetic variation may be more strongly related to elevational variation (YEH et al., 1985; REHFELDT, 1988). Yet, the range of elevation in this study is only 850 m (from 2450 m to 3300 m), which may limit the chance to detect genetic differences possibly related to elevation. In all, three causes, sporadic selection pressures, genetic drift and limited gene flow are the likely elements to explain the pattern of genetic differentiation in the populations of *P. asperata*. In addition, founder effects may contribute to the high level of genetic differentiation present in *P. asperata*.

The extensive genetic differentiation detected among *P. asperata* populations has a number of implications for the management and conservation of the species. From a conservation point of view, each population should be managed and maintained separately because they possess partially unique genetic characteristics. In such a situation, the loss of any single population would lead to a loss of genetic variation for the species as a whole (SHRESTHA et al., 2002). However, this is not a practical approach to conservation. The data suggest that conservation measurements should focus on the conservation of different populations in or *ex situ*. In order to secure genetic sources, an obvious approach is to establish seed

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orchards of *P. asperata*. Besides seed populations in possession of high genetic diversity (e.g., DL and CP), the use of genetically differing populations, such as XJ, as a seed source would promote a high level of genetic diversity among plantations. Also, regular monitoring of the genetic diversity present in plantations is important when aiming to ensure that the wide genetic base is maintained.

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