Genetic diversity of *Picea likiangensis* natural population at different altitudes revealed by EST-SSR markers

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Abstract

Altitude is thought to have greatly influenced current species distribution and their genetic diversity. However, it is unclear how different altitudes have affected the distribution and genetic diversity of *Picea likiangensis*, a dominant forestry species in the Qinghai-Tibetan Plateau region (QTP). In this study, we investigated the genetic diversity of *Picea likiangensis* populations which distributed in different altitudes of QTP using EST-SSR markers. The results suggested that this species has high genetic diversity at species level, with 100% of loci being polymorphic and an average Nei's gene diversity (*He*) of 0.7186 and Shannon's information index (*I*) of 1.5415. While the genetic diversity of *Picea likiangensis* at population level was lower than that at species level, with *He* and *I* being 0.6562 and 1.3742, respectively. The variation in genetic diversity of all four studied populations indicated a low-high-low pattern along the elevation gradients. The mid-elevation population (3050 m) was more genetically diverse than both low-elevation (2900 m) and high-elevation populations (3200 m and 3350 m). Nei's genetic diversity (F_{st} = 0.0809) and AMOVA analysis (*Phist* = 0.1135) indicated that a low level of genetic differentiation among populations. Gene flow among populations was 2.8384, suggesting that high gene flow is a main factor leading to high levels of the genetic diversity among populations.

Key words: Picea likiangensis, Genetic diversity, EST-SSR, Altitude.

1. Introduction

The Qinghai-Tibetan Plateau (QTP) is the highest and one of the most extensive plateau in the world (Zhang et al., 2002). The elevation of the QTP and Quaternary glaciation were key geological events that significantly affected Asian topography, atmospheric circulation, and even global climate. QTP and its adjacent regions comprise one of 'biodiversity hotspots' on the earth (Myers et al., 2000). The geological features, especially the altitude of QTP are considered to have major impacts on the species distributions and genetic structure of plants in this region (Wu et al., 1998; Hewitt, 2004; Yang et al., 2008). Elevation is a complex factor, altitudinal gradients comprise an assemblage of environmental variables, such as rapidly changing climate conditions, which markedly influence the distribution of population genetic variation of plant species (Chen et al., 2008; Hahn et al., 2012). So, an understanding of current distribution patterns of population genetic diversity and differentiation (McMahon et al., 2011) along altitudinal gradients is fundamental for the availably conservation, reasonable utilization, and establishment of management strategies for resilient species. According to thesis of elevation patterns in genetic variation of plants, the genetic and geographical structure in natural populations along elevational gradients have been strongly influenced not only by life history and ecological traits, but also by biogeographic history (Ohsawa and Ide, 2008; Gämperle and Schneller, 2002; Quiroga and Premoli, 2007; Truong et al., 2007). In recent years, many studies on genetic variation along altitudinal ranges have been done with the aid of molecular markers (Chen et al., 2008; Truong et al., 2007; McMahon et al., 2011; Hahn et al., 2012). However, up to now, relatively few investigations have been focused on the population divergence and phylogeography of plants in the QTP (Meng et al., 2007). Therefore, studies on the level of genetic diversity and its partitioning plants at varying altitudes in this region are of prime interest.

Picea likiangensis (*P. likiangensis*), an evergreen conifers tree, which is widely distributed from Western Sichuan to Tibet and from Yunnan to Qinghai, whose biogeographic history was proposed to be closely related to the uplift of the QTP and to Quaternary climate oscillations (Li et al., 2013). Previous studies mainly concerned about the speciation, forest regeneration, population dynamics and renewal of *P. likiangensis* (Guo et al., 2009; Liu et al., 2003; Zhao et al., 2012), however, the genetic diversity and genetic structure of *P. likiangensis* populations in QTP is poorly understood. Genetic diversity usually reflects the ability of a species to adapt the environment changes during evolution. The spatial distribution of the genetic structure is closely related to breeding mechanisms of the species, reflecting ecological adaptation evolution, environmental changes and natural selection effect (Dostalek et al., 2010; Ishihama et al., 2005). By using random amplified polymorphic DNA (RAPD) analysis, Peng et al. (2007) showed that there is high genetic differentiation and limited gene flow among the populations of *P. likiangensis* in the QTP, which suggested that *P. likiangensis* may have different refugia during the glacial stages in the southern region of QTP, and the northern variety may have multiple origins from these different refugia (Peng et al., 2007). Furthermore, a recent study showed that different evolutionary forces and dispersal biology together may have shaped

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the genetic architecture of *P. likiangensis* at cpDNA and mtDNA genomes across its current distributions (Zou et al., 2012). More recently, Li et al. (2013) reported that geological change and climatic oscillation during the Pliocene and Pleistocene markedly affected the evolution and demography of *P. likiangensis* in QTP by analysis the polymorphism of multiple loci, including nuclear, mitochondrial and chloroplast DNAs (Li et al., 2013).

Microsatellite, also known as simple sequence repeat (SSR), is widely used in many molecular genetic applications including genotype mapping (Acheré et al., 2004; Echt et al., 2011; Kang et al., 2010), population structure and genetic diversity analysis (Espinoza et al., 2012; Pluess et al., 2013; Lu et al., 2009), and gene flow and germplasm conservation studies (Shimono et al., 2011). SSR markers are co-dominant, abundant and evenly distributed in genomes of plants, multi-allelic, highly polymorphic and are easily detectable by simple polymerase chain reaction (PCR) technology (Hodgetts et al., 2001; Rajora et al., 2001; Peakall et al., 1998). Compared with conventional genomic SSR markers, SSRs from EST sequences have several intrinsic advantages: (1) they are embedded in functional gene sequences and directly associated with transcribed genes; (2) they have a lower cost of identification; and (3) they have a high transferability to related species

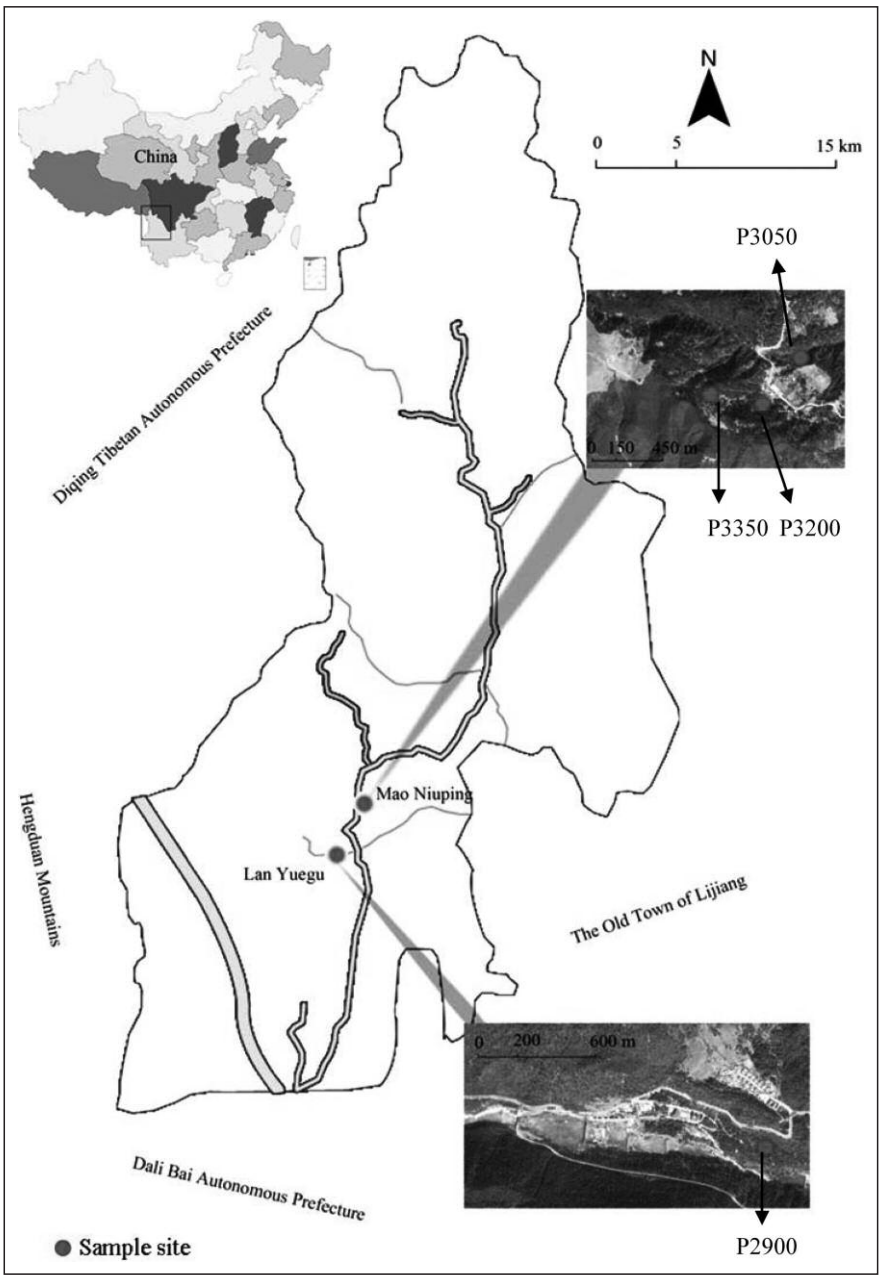

Figure 1. – Sample site surveyed in Yulong Snow Mountain Natural Reserve in Lijiang, Northwestern Yunnan.
The solid dots mean sample site. The population P2900 is labeled in the satellite map of Lan Yuegu and the populations P3050, P3200 and P3350 are labeled in the satellite map of Mao Niuping.

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(VARSHNEY et al., 2005). In this study, we investigated the polymorphism of *Picea likiangensis* populations from four different altitudes in Yulong Snow Mountain, southeast edge of QTP by using EST-SSR markers. The two specific aims of this study were to (1) describe population genetic structure spanning an elevational gradient in P. *likiangenesis* and (2) associate genetic variation with elevational and climatic gradients.

2. Materials and methods

2.1 Plant materials and DNA extraction

Needles were collected from *Picea Likiangensis* trees in 4 populations grown at different altitudes (2900 m, 3050 m, 3200 m, and 3350 m) at Yulong Snow Mountain Natural Reserve in Lijiang (*Fig. 1*), and dried on silica gel. At least 20 randomly selected individuals with height more than 10 m were sampled per population. Totally, 94 samples spaced at least 50 m apart were obtained (*Table 1*). Genomic DNA was extracted from young needles using the CTAB method (DUMOLIN et al., 1995). DNA concentration was measured by a Beckman spectrophotometer (Beckman, Fullerton, USA) and adjusted to a final concentration of 25 ng/uL in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

2.2 SSR analysis

The SSR markers were derived from the group of markers developed by PFEIFFER et al. (1997) for the Norway spruce (*Picea abies*). Preliminary screening showed that the ten selected SSR marker loci produced simple but polymorphic patterns (*Table 2*). The SSR reactions were adopted from the protocol of PFEIFFER et al. (1997) with some modifications. PCR amplifications were performed in a volume of 10 ul containing 50 ng genomic DNA, 1×Taq buffer (with 2 mM $MgCl_2$), 0.2 mM dNTPs, 0.8 uM each primer and 1 U Taq DNA polymerase (Fermentas). The reaction mixture was initially denatured at 94°C for 5 min, followed by 40 cycles of amplification at 94°C for 30 s, 56°C for 45 s and 72°C for 60 s, and a final extension at 72°C for 5 min in a GeneAmp PCR system 9700 (Applied Biosystems). The

Table 1. – Environmental conditions of *Picea Likiangensis* from 4 populations.

Population	Sample size	Altitude (m)	Location	Community type
P2900	25	2900	Lanyue Valley	Needle, broad-leaved mixed(human disturbance)
P3050	27	3050	Maoniuping	Fir,spruce mixed
P3200	20	3200	Maoniuping	Fir,spruce mixed
P3350	22	3350	Maoniuping	Fir,spruce mixed

Table 2. – Primer sequences, repeat types, PCR product sizes, and amplification conditions of spruce microsatellites (PFEIFFER et al., 1997).

SpACIH8	CCCAAGAAAAAAGTCATGGAT TCATTGGGATATGTGATACTTCC	$(GT)_{27}$	135	60
SpAC 1 F7	TTCCTCCACTGCATTCTAGC TGTTGGCCTTGCAAGTTATAG	$(AC)_{12}$	109	57
SpAGG3	CTCCAACATTCCCATGTAGC AGCATGTTGTCCCATATAGACC	$(GA)_{24}$	136	57
SpAGC2	TACCATTCAACGCAAGGG GTGTATGGTTTTCTTTTCGCA	$(TA)_{11}(GA)_{20}$	126	57
SpAGCl	TTCACCTTAGCCGAGAACC CACTGGAGATCTTCGTTCTGA	$(TC)_5TT(TC)_{10}$	103	57
SpL3AGlA4	CATACTCAATGCACCTAGATATGC AAGCAAATGAAAGCTCCTTGT	$(GA)_{21}$	103	57
SpL3AG 1 H4	GGAAAGGAGGAGGACAAGAG TAAGGATCGAGTCTCTCACTCC	$(GA)_{20}$	142	60
SpAGAl	AACGTGCACACATTCCTCTTA GTGAGGGAGGGAAGAAGGT	$(TC)_4TT(TC)_9$	134	57
SpAGDl	GTCAACCAACTTGTAAAGCCA ACTTGTTTGGCATTTTCCC	$(AG)_{25}$	147	57
SpAG2	GC1CTTCACG1GTACTTGATC TTCGAAGATCCTCCAAGATAC	$(TC)_{16}$	105	53

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PCR products were separated on 6% denaturing polyacrylamide gels. After electrophoresis, the gels were stained by as previously described (SANGUINETTI et al., 1994).

2.3 Data analysis

Only clear and polymorphic fragments that could be scored across all the samples were used in the analysis. These fragments were scored independently as present (1) or absent (0) in each population and a binary data matrix was converted to input file format for POPGENEversion 1.3.1 (YEH et al., 1999) using DataTrans 1.0 (GAI and REN, 2011). POPGENEversion 1.3.1 (YEH et al., 1999) was used to calculate a set of intra- and inter-population genetic parameters, including genetic diversity within populations (F_{is}), the total genetic diversity (F_{it}), and the relative magnitude of genetic differentiation among populations $F_{st} = (F_{is} - F_{it})/(F_{is} - 1)$. Based on the island model, gene flow was inferred indirectly using Wright's (1931) formula: $Nm = 0.25\ (1 - F_{st})/F_{st}$. Shannon's indices (LEWONTIN, 1972) were also calculated and used to characterize the gene diversity and distribution of the variation based on the formula $I = -\sum$pilog2pi, in which pi is the frequency of a given EST-SSR fragment. Following NEI (1973) using corrected allele frequencies (LYNCH and MILLIGAN, 1994), Nei's gene diversity (H_e) were calculated as the formula $H_e = 1 - \sum pi^2$. Null alleles at each locus were detected as the formula $r = (H_e - H_o)/(1 + H_e)$ (BROOKFIELD, 1996). An analysis of molecular variance (AMOVA, EXCOFFIER et al., 1992) was performed on a matrix of squared standard Euclidean distances. Input data files for the WINAMOVA v.1.55 program (EXCOFFIER et al., 1992) were generated using DCFA 1.1 (ZHANG and GE, 2002). The variance components were tested statistically by nonparametric randomization tests using 1,000 permutations. The correlation between genetic diversity index and elevation distance was detected by Mental test (MENTAL, 1967).

3. Results

3.1 Genetic diversity

Six of the 10 screened primers produced clear bands were used for the analysis the 94 samples from 4 populations. A total of 44 alleles were scored at the SSR loci with the number of alleles per locus ranged from 4 to 12 (*Table 3*). Two of the six locus, SPAC1H8 and SPL3AG1H4, maybe exist null alleles, for the frequencies of null alleles are 0.2119 and 0.2344 ($r > 0.05$), respectively (*Table 3*). *P. likiangensis* has high genetic diversity at species level, with 100% of loci being polymorphic and an average Nei's gene diversity (H_e) of 0.7186 and Shannon's information index (I) of 1.5415 (*Table 3*). Of the 44 alleles scored, 44 (100%) were polymorphic (*Table 4*). Nei's gene diversity (H_e) values varied from 0.6100 to 0.7176 and the differences between populations were not significant ($p = 0.7588$, ANOVA) (*Table 4*). Shannon's index varied from 1.2884 to 1.4997 and the differences between populations were not significant ($p = 0.8869$, ANOVA) (*Table 4*). H_e and I values indicated that genetic diversity of population was lower than the genetic diversity of species. The genetic diversity of *Picea likiangensis* estimated with Nei's gene diversity was lower than that estimated with Shannon's index of phenotypic diversity. However, the variation trend of the genetic diversity of the four populations at different altitudes, as indicated by these two indice, was generally consistent. The correlation analysis showed that the genetic diversity among populations was not significantly related to the altitude ($r_{(He,\ altitude)} = -0.7089$, $p > 0.05$; $r_{(I,\ altitude)} = -0.7475$, $p > 0.05$).

3.2 Genetic differentiation among populations

Genetic differentiation among populations (F_{st}) value (0.0809) obtained from Nei's gene diversity (*Table 5*). These results indicated that the genetic variation within populations of *Picea likiangensis* was high but differentiation among populations was low. The gene flow estimated value from F_{st} (2.8384) suggested that the degree of similarity among populations of *Picea likiangensis* was relatively high (*Table 5*) (HAMRICK et al., 1995; WANG et al., 2005).

AMOVA analysis indicated that although high variation (88.65%) ($p < 0.001$, tested using a 1,000 replication bootstrap) was found within populations, a significant proportion of the variation (11.35%) ($p < 0.001$, tested using a 1, 000 replication bootstrap) was attributable to differences among populations (*Table 5*).

Table 3. – Genetic diversity of *Picea likiangensis* based SSR markers.

Locus	N_a	N_e	I	H_o	H_e	Frequency of null alleles
SPAGG3	12	8.9660	2.2842	0.8936	0.8885	-0.0027
SPAC1H8	9	4.8858	1.8393	0.4149	0.7953	0.2119*
SPAC1F7	4	2.6715	1.1266	0.5745	0.6257	0.0315
SPAGC1	7	4.6408	1.6632	0.7021	0.7845	0.0462
SPL3AG1H4	6	2.0029	0.9264	0.1489	0.5007	0.2344*
SPAG2	6	3.5316	1.4094	0.7447	0.7168	-0.0163
Mean	7.33	4.4498	1.5415	0.5798	0.7186	0.0808*

Note: H_o mean observed heterozygosity, and H_e mean expected heterozygosity.
* Mean the estimated null allele frequency is significant (greater than 0.05).

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Table 4. – Genetic diversity of *Picea likiangensis* at different altitudes.

Population	Size	N_a	N_e	percentage of polymorphic loci(%)	I	H_o	H_e
2900	25	6.6667	3.8771	100	1.4193	0.6867	0.6767
3050	27	6.1667	4.3111	100	1.4997	0.6111	0.7176
3200	20	5.6667	3.5030	100	1.2884	0.5417	0.6100
3350	22	5.5000	3.7929	100	1.2893	0.4545	0.6204
Mean	23.5	6.0000	3.8710	100	1.3742	0.5735	0.6562
species level	94	7.3333	4.4498	100	1.5415	0.5798	0.7186

Note: N_e = Effective number of alleles (KIMURA and CROW, 1964), H_o = Observed heterozygosity, H_e = Nei's (1973) gene diversity, I = Shannon's Information index (LEWONTIN, 1972).

Table 5. – Genetic differentiation for populations of *Picea likiangensis*.

POPGEN					AMOVA					
Sample size	F_{is}	F_{it}	F_{st}	N_m	Source of variation	df	SSD	MSD	Variance components	Total variance (%)
94	0.1286	0.1992	0.0809	2.8384	Among populations	3	77.3973	25.799	0.826592	11.35 (P<0.001) [a]
					Within populations	90	581.3261	6.459	6.459179	88.65 (P<0.001) [a]

Note: Nm = estimate of gene flow from F_{st}, $Nm = 0.25(1-F_{st})/F_{st}$, df = Degrees of freedom, SSD = Sum of squares; MSD = Mean squared deviation, [a] = Significance tests after 1,000 permutations.

4. Discussion

Genetic diversity is the sum of the genetic information and is a product of long-term evolution. For a species which possess higher or much richer in the genetic diversity usually offers greater ability to adapt to the environment and easier to expand its distribution range and explore new environments. Species or potential of population evolution and the ability to adapt to the environment depend on the level of genetic diversity (GE and HONG, 1994). The level of genetic diversity can be evaluated by the percentage of polymorphic loci (P), Nei's gene diversity (H_e) and Shannon's information index (I). In this study, EST-SSR was used to reveal the genetic diversity of *Picea likiangensis* populations at different altitudes. At the species level, P was up to 100%, H_e was 0.7186, and I was 1.5415 (*Table 3*). The expected heterozygosity of *P. likiangensis* (H_e=0.7186) detected here is comparable to SSR-based estimates for species in the same family, e.g., *P. abies* (H_e=0.465) (TOLLEFSRUD et al., 2009), *P. asperata* (H_e=0.543) (WANG et al., 2005), and *P. mariana* (H_e=0.72) (RUNGIS et al., 2004). These values reflected that the total genetic diversity of *Picea likiangensis* was at a high level. In addition, the results indicated that it still has higher level of ecological adaptability, and effective population size is still sufficient to prevent genetic drift caused by the loss of genetic diversity in a long time. The natural populations of *Picea likiangensis* with high genetic diversity may be related to its biological characteristics and living habits (BENNETT et al., 2000). First of all, *Picea likiangensis* is monoecious plants with significant wind pollination characteristics. For outcrossing plants, pollination and breeding mode is conducive to help maintain a higher genetic diversity. Secondly, *Picea likiangensis* is a multi-year growth life species with overlapping generations. In the long term under the action of natural selection effect, the survival of genes have been accumulated and preserved. And the research populations located in the natural reserve can get better protection. Hence, the population itself could have a high genetic diversity.

At the population level, the percentages of polymorphic loci for four populations of *Picea likiangensis* were all 100%. The genetic diversity estimated by the Nei's gene diversity was lower than the value calculated by Shannon's information index, but both revealed the consistent trends of the genetic diversity among populations. In the present study, the distribution of genetic diversity of populations at different altitudes was not balanced, and populations at middle altitude had a higher level of genetic diversity than the populations at high altitude and low altitude. This phenomenon can be explained by the ecology zone where is the populations of *Picea likiangensis* distributed between 2, 900 m to 3, 400 m in Yulong Snow Mountain. As the altitude increases, the temperature decreased gradually and adult trees are reduced. As a result, founder effect during colonization of upper habitats was ultimately affected the gene flow and genetic diversity. And in the low

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altitude, the genetic diversity of *Picea likiangensis* was easily influenced by human activities, resulting in the loss of genetic diversity.

AMOVA analysis of molecular variance showed that high variation (88.65%) was found within populations, and only 11.35% of total variation among populations. The F_{st} value (0.0809) also indicated that the genetic variation mainly existed within populations but there was a certain degree of genetic differentiation among populations. The genetic differentiation in this study was clearly lower than the differentiation among populations observed in *P. asperata* (F_{st} = 0.223, WANG et al., 2005). Other SSR studies in the genus *Picea* showed also smaller genetic differentiation, e. g. Norway spruce at a regional (F_{st} = 0.09, ACHERÉ et al. 2005; F_{st} = 0.05, MELONI et al., 2007), sub-regional (F_{st} = 0.05, SCOTTI et al., 2006) and local level (F_{st} = 0.002, UNGER et al., 2011). Genetic differentiation of the populations is mainly affected by its own genetic characteristics (BIALOZYT et al., 2006; CHEN et al., 2008; FAYARD et al., 2009; WANG et al., 2011), such as the methods of dispersal pollen and seed and influenced by external environment changes (WANG et al., 2011), e.g. the isolation caused by human disturbance, genetic mutation, etc. The latter includes changes in the large geographical distribution and microhabitat level. Genetic differentiation in the geographical region mainly depend the capacity of diffusion gene of the species, and the genetic differentiation at microhabitat level is the spatial heterogeneity of the genotype or gene in a single population, even there is no obvious microhabitat differences, limiting gene flow can also lead to genetic differentiation. In this study, genetic variation occurred mainly within populations, and the genetic differentiation among populations was not obvious. This could be due to the widespread occurrence of wind pollination, which promotes outcrossing (BENNETT et al., 2000) and leads to conifer species display low population genetic differentiation (HAMRICK, 2004). In the relatively small geographical region, low genetic differentiation was appeared in populations of *Picea likiangensis* at the different altitudes which can be reasoned by large gene flow in homogenization. Studies have shown that if gene flow (Nm) <1, genetic drift was the main factors to affect the population genetic structure; if Nm >1, gene flow was sufficient to counteract the effect of genetic drift, and also to prevent the happening of the population genetic differentiation among populations (HAMRICK et al., 1995). *Picea likiangensis* is one of wind-pollinated plant and its pollen can be transported long distances and different populations. In addition, *Picea likiangensis* has some biological characteristics such as monoecious, high proportion of outcrossing, long life of adult trees and overlapping generations. These factors are conducive to the gene flow among populations, increasing heterogeneity among populations, and limiting the genetic differentiation among populations.

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