Trees (2007) 21:457–464
DOI 10.1007/s00468-007-0138-y

ORIGINAL PAPER

Genetic variation and phylogeographic history of *Picea likiangensis* revealed by RAPD markers

Xiao-Li Peng · Chang-Ming Zhao · Gui-Li Wu · Jian-Quan Liu

Received: 17 October 2006 / Revised: 5 March 2007 / Accepted: 12 March 2007 / Published online: 4 April 2007

Abstract Repeated cycles of retreat and recolonization during the Quaternary ice ages are thought to have greatly influenced current species distributions and their genetic diversity. It remains unclear how this climatic oscillation has affected the distribution of genetic diversity between populations of wind-pollinated conifers in the Qinghai-Tibetan region. In this study, we investigated the within-species genetic diversity and phylogenetic relationships of *Picea likiangensis*, a dominant forest species in this region using polymorphic DNA (RAPD) markers. Our results suggest that this species has high overall genetic diversity, with 85.42% of loci being polymorphic and an average expected heterozygosity (H_E) of 0.239. However, there were relatively low levels of polymorphism at population levels and the differences between populations were not significant, with percentages of polymorphic bands (PPB) ranging from 46.88 to 69.76%, Nei's gene diversity (H_E) from 0.179 to 0.289 and Shannon's indices (*H*pop) from 0.267 to 0.421. In accordance with our proposed hypothesis, a high level of genetic differentiation among populations was detected based on Nei's genetic diversity (G_{ST} = 0.256) and AMOVA analysis (Phi_{st} = 0.236). Gene flow between populations was found to be limited (Nm = 1.4532) and far lower than reported for other conifer species with wide distribution ranges from other regions. No clusters corresponding to three morphological varieties found in the south, north and west, respectively, were detected in either UPGMA or PCO analyses. Our results suggest that this species may have had different refugia during the glacial stages in the southern region and that the northern variety may have multiple origins from these different refugia.

Keywords *Picea likiangensis* · Genetic diversity · RAPD · Morphological differentiation

Communicated by J. Major.

X.-L. Peng · C.-M. Zhao · G.-L. Wu · J.-Q. Liu
Key Laboratory of Arid and Grassland Ecology,
School of Life Sciences, Lanzhou University,
Lanzhou 730000, Gansu, People's Republic of China

J.-Q. Liu (✉)
Key Laboratory of Adaptation and Evolution of Plateau Biota,
Northwest Institute of Plateau Biology, The Chinese Academy
of Sciences, Xining 810001, Qinghai
People's Republic of China
e-mail: liujq@nwipb.ac.cn; zhaochm@lzu.edu.cn

Introduction

The Qinghai-Tibetan Plateau (QTP) is the largest and highest plateau in the world. Plants of this region represent a major component of the Himalayan alpine flora. The eastern area of the QTP makes up a major part of the larger South-Central 'biodiversity hotspot' recognized globally as one of 25 areas featuring exceptionally high concentrations of endemic species (Myers et al. 2000). The region comprises a series of spectacular ridges orientated north-south alternating with deep valleys, with altitudes ranging from 2,000 to 6,000 m above sea level (Li et al. 1997). These geological features are considered to have played an important role in the development of the high levels of biotic diversity currently observed in this region (Wu 1998; Wu and Wu 1996). According to this hypothesis, high levels of genetic differentiation and limited gene flow would be expected between populations. This hypothesis has been supported by several studies of insect-pollinated species from this region (e.g. Ge et al. 2005); however, less is known about the dis-

tribution of intra-specific genetic diversity in wind-pollinated species, especially of conifers that form the dominant forest vegetation in the QTP (but see Luo et al. 2005). The climatic oscillations of the Quaternary (2 MYA) resulted in the extinction of some species and the survival of others in areas (refugia) with more favorable climatic conditions, often following a severe range contraction (e.g. Hewitt 2000; Abbott and Brochmann 2003; Petit et al. 2003; Abbott and Comes 2004). During interglacial periods, significant range expansions took place from such refugia when species colonized (or recolonized) areas exposed by retreating glaciers. Although no massive ice sheet developed on the QTP during glacial periods (Shi et al. 1998), pollen fossil records suggest that forests retreated to the south and then recolonized northwards in response to the Quaternary glaciations and inter-glacial periods, respectively (Tang and Shen 1996). However, few molecular studies have focused on the effect of this geological history on the genetic diversity and morphological differentiation of tree species in this region (but see Zhang et al. 2005).

The genus *Picea* (spruce) consists of 34 species, 24 of which are native to Asia, eight in North America and two in Europe (Farjon 1990). More than half of the total number of species are distributed in the QTP, and six of these are endemic to this region. The QTP is an important secondary diversification center for *Picea*. A recently published molecular phylogeny of this genus suggests that the topographical complexity of the QTP may have contributed to its high speciation through topographical isolation of its populations (Ran et al. 2006). A detailed investigation of the intra-specific genetic distribution should help us to understand the speciation forces that have affected this genus. *Picea likiangensis* (Franch.) Pritz is one of the most important coniferous species of the QTP from both ecological and economic perspectives. This species has a wide distribution, extending from west Sichuan to Tibet and from Yunnan to Qinghai, spanning a wide range of altitudes between 2,500 and 4,000 m. Although five varieties of this species are recognized (Fu et al. 1999), only three of them, all of which have wide distributions, might represent true taxonomic units: var. *likiangensis*, var. *rubescens* Rehder and E. H. Wilson and var. *linzhiensis* Cheng et L. K. Fu. First-year branchlets in var. *likiangensis* are usually slender, sparsely pubescent, and leaves with two to four stomatal lines along each abaxial surface. Var. *rubescens* differs from this variety in that its first-year branchlets are stout, densely pubescent, with short nodes and leaves with three or four stomatal lines along each abaxial surface. In var. *linzhiensis*, first-year branchlets are usually glandular, hairy and leaves with no stomatal line along their abaxial surface. These three varieties have differing distributions in Yunnan, Sichuan and Qinghai, and Tibet, respectively. In the present study we aimed to assess the genetic diversity of *P. likiangensis* using Randomly Amplified Polymorphic DNA markers (RAPDs). The use of RAPDs has several limitations, including dominance, uncertain locus homology and the sensitivity of RAPD analysis to reaction conditions. Despite these problems, this molecular technique is still applied in the assessment of genetic variation in conifer species (Allnutt et al. 1999, 2003; Bekessy et al. 2002; Collignon and Favre 2000; Gillies et al. 1997, 1999; Isabel et al. 1995; Lee et al. 2002; Nkongolo 1999), particularly since DNA sequence information is not required prior to investigating a previously unstudied species, thereby facilitating its application to a wide range of taxa (Newton et al. 1999; Wang and Szmidt 2001). In addition, RAPDs generally provide both important information for detection of range history of a targeted species (Avise 2000) and genetic diversity estimates in spruces that are consistent with estimates obtained using co-dominant isozyme markers (Isabel et al. 1995). The two specific aims of this study were to (1) test the hypotheses that there is high genetic differentiation and limited gene flow among populations in this widely-distributed, wind-pollinated conifer in the QTP and (2) understand the phylogeographic history of this species based on RAPD variations.

Materials and methods

Plant material

Needles were collected from *P. likiangensis* trees in 11 populations in southwest China (Fig. 1; Table 1) then dried on silica gel. At least 14 randomly selected individuals were sampled per population, 228 in total, spaced at least 100 m apart. The sampled populations were located on major mountains (with altitudes all over 2,500 m a.s.l) isolated by either relatively low plateaus (altitudes lower than 2,000 m), or major valleys in southeast Tibet, southwest Sichuan and northwest Yunnan. These populations comprise pure stands or mixed forests with other conifers in alpine environments between 2,500 and 4,000 m a.s.l.

DNA extraction

Genomic DNA was extracted from 0.5 to 1.0 g samples of needles using a modified CTAB method described by Doyle and Doyle (1987). DNA concentrations were determined by comparisons with serial dilutions of standard lambda DNA.

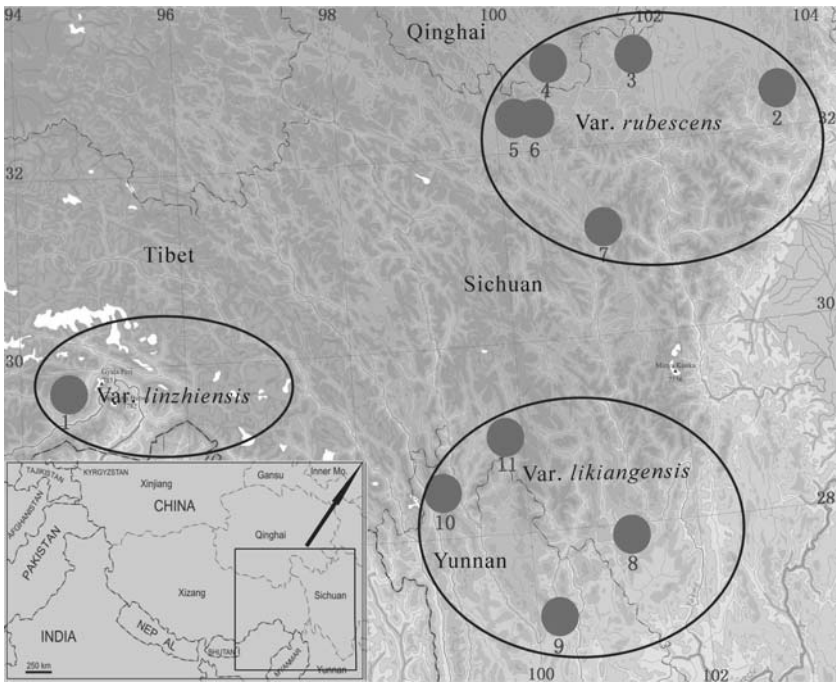

Fig. 1 The three varieties of *P. likiangensis* are, respectively, distributed in the north, south and west Qinghai–Tibetan Plateau. *Solid circles* indicate locations of the sampled populations

Table 1 The variety, latitude, longitude and altitude of populations sampled in the present study

Population	Variety	Location	Latitude	Longitude	Altitude (m)	*N*
Pop1	Var. *linzhiensis*	Linzhi, Xizang	32°47′	100°47′	3,430	23
Pop2	Var. *rubescens*	Songpan, Sichuan	32°47′	103°47′	3,200	20
Pop3	Var. *rubescens*	Aba, Sichuan	32°44′	102°05′	3,600	20
Pop4	Var. *rubescens*	Banma county, Qinghai	32°47′	100°47′	3,430	20
Pop5	Var. *rubescens*	Seda, Sichuan	31°52′	100°41′	3,560	16
Pop6	Var. *rubescens*	Seda, Sichuan	31°52′	100°41′	3,560	14
Pop7	Var. *rubescens*	Daofu, Sichuan	30°49′	101°16′	3,510	23
Pop8	Var. *likiangensis*	Muli, Sichuan	28°07′	101°19′	3,550	23
Pop9	Var. *likiangensis*	Lijiang, Yunnan	27°08′	100°14′	3,170	23
Pop10	Var. *likiangensis*	Deqin, Yunnan	28°19′	99°07′	3,900	23
Pop11	Var. *likiangensis*	Xiangcheng, Sichuan	29°13′	100°65′	4,000	23

N sample size

RAPD amplification

DNA amplification was performed according to Williams et al. (1990). Initially, 60 random decamer primers were scanned (Sangon Technologies, Shanghai, China) and nine primers (Table 2) that generated clear, reproducible banding patterns were chosen for the final analysis. To maximize the uniformity of the reaction conditions and minimize systematic errors we used the same PCR reaction kits, PCR machine and electrophoresis equipment for all of the amplifications.

The PCR reaction reagent concentrations and conditions were optimized to ensure the marker bands were reproducible. DNA amplification was performed in total reaction volumes of 20 μl consisting of 3 mM $MgCl_2$, 0.1 mM dNTPs, 0.25 μM of each primer, 1.5 units of *Taq* DNA polymerase (Casarray, Shanghai, China) and 20–40 ng of template DNA. PCR amplification was performed with the following program: three cycles of 94°C for 60 s, 36°C for 60 s and 72°C for 80 s, followed by 39 cycles of 94°C for 45 s, 37°C for 50 s and 72°C for 80 s, then a final extension step of 72°C for 7 min. Amplification products were separated by electrophoresis in a 2.0% agarose gel in 1 × TBE buffer (Sambrook et al. 1989), stained with ethidium bromide then viewed on a UV transilluminator. Their molecular weights were estimated using a 100 bp DNA ladder.

Table 2 The Randomly Amplified Polymorphic DNA (RAPD) primers used and the total number of amplified bands across all individuals sampled

Primers	Sequence 5′-3′	No. of bands studied	Primers	Sequence 5′-3′	No. of bands studied
S1382	GAGACCAGAC	8	S1450	AAGAGGCCAG	6
S1383	TTAGCGCCCC	11	S1451	CAATCGGGTC	14
S1388	TCCGCAGTAG	10	S1454	GAACGAGGGT	14
S1390	TGGTCGGGTG	9	S1510	ACTGCCCGAC	13
S1442	CTGAACCGCT	11			

Data analysis

Only clear and polymorphic fragments that could be scored across all the population 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 constructed. The RAPD-PCR fragments were analyzed as alleles, under the following assumptions. Firstly, RAPD products segregate as dominant alleles in a Mendelian fashion. Dominance has been widely observed for RAPD fragments in other conifer species (Carlson 1991; Lu et al. 1995). Secondly, the groves in this study were in Hardy–Weinberg equilibrium. Finally, the RAPD fragments represented the nuclear genome and fragments of the same apparent size in different trees were homologous. These allele frequencies were calculated from RAPD band frequencies following the methods and corrections employed by Lynch and Milligan (1994). We used POPGENE 1.31 (Yeh et al. 1999) to calculate a set of intra- and inter-population genetic parameters, including genetic diversity within populations (H_S), genetic diversity between populations (D_{ST}) and the relative magnitude of genetic differentiation among populations ($G_{ST} = H_T - H_s/H_T$). Based on the island model, gene flow was inferred indirectly using Wright's (1931) formula: $Nm = 0.5(1 - G_{ST})/G_{ST}$. Shannon's indices (Lewontin 1972) were also calculated and used to characterize the gene diversity and distribution of the variation based on the formula $H_0 = -\sum \text{pilog}_2 p_i$, in which p_i is the frequency of a given RAPD fragment. H_0 was calculated at two levels: the average diversity within populations (*H*pop) and the total diversity (*Hsp*). The proportion of diversity among populations was estimated as (*Hsp* – *H*pop)/*Hsp*. Following Nei (1973) using corrected allele frequencies (Lynch and Milligan 1994), Nei's gene diversity (*He*) were calculated.

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 AMOVA v. 1.55 program (Excoffier et al. 1992) were generated using AMOVA-PREP (Miller 1998). The variance components were tested statistically by nonparametric randomization tests using 1,000 permutations. A UPGMA (unweighted pair-group method using arithmetic average) dendrogram was constructed, based on the matrix of Nei's genetic distance using the Modified NEIGHBOR procedure of PHYLIP (Phylogeny Inference Package Version 3.75c, Felsenstein 1993). In addition, the Multivariate Statistics Package (MVSP) was used to perform a principal coordinate analysis (PCO) that provided a graphical representation of the RAPD relationships between individuals.

Results

Nine of the set of 60 screened primers produced clear RAPD banding patterns. Screening of the entire set of samples was repeated with two of the primers to assess the repeatability of the RAPD profiles and in both cases identical RAPD patterns were obtained. A total of 96 clear and repeatable RAPD bands were scored. The average number of bands was 10.6 per primer. Of the 96 RAPD bands scored, 82 (85.42%) were polymorphic, based on the 95% criterion. The percentage of polymorphic loci (*P*) ranged from 46.88% (Pop 6) to 69.76% (Pop 3) with an average value of 62.31% (Table 3). Nei's gene diversity (H_E) values varied from 0.179 (Pop 6) to 0.289 (Pop3) ($H_T = 0.3242$) and the differences between populations were not significant ($P = 0.09$, ANOVA) (Table 3). Shannon's diversity values (*H*pop) ranged from 0.267 (Pop 6) to 0.421 (Pop 3) ($D_{ST} = 0.4740$) (Table 3) with a similar non-significant difference being found between populations, although they are more sensitive measures of variation.

AMOVA analysis of pairwise RAPD distances indicated that although high variation (76.38%) was found within populations, a significant proportion of the variation ($P < 0.001$, tested using a 1,000 replication bootstrap) was attributable to differences among populations (Table 4). The G_{ST} value (0.256) obtained for Nei's gene diversity was also similar to the estimate of Phi_{st} (0.236). Pair-wise Phi_{st} values derived from AMOVA analysis suggested there were large numbers of differences between pairs of populations. All pairwise Phi_{st} comparisons were significant at the 0.05 probability level.

In order to examine relationships among populations, Nei's genetic distances were used to construct a UPGMA

Table 3 Genetic variation in 11 populations of *Picea likiangensis* (the percentage of polymorphic loci % *P*, Lynch and Milligan 1994; Nei's gene diversity H_E, Nei 1973 and Shannon's Information index H_{pop}, Lewontin 1972)

Population	*P* (%)	95% confidence interval	H_E	95% confidence interval	H_{pop}	95% confidence interval
Pop1	66.67	2.08	0.263	0.006	0.379	0.090
Pop2	67.71	2.10	0.275	0.006	0.397	0.105
Pop3	69.76	1.04	0.288	0.008	0.421	0.135
Pop4	63.54	0.00	0.244	0.007	0.360	0.070
Pop5	61.45	0.00	0.253	0.008	0.325	0.041
Pop6	46.88	4.17	0.198	0.008	0.267	0.077
Pop7	58.33	1.06	0.230	0.006	0.300	0.020
Pop8	63.54	1.04	0.264	0.006	0.374	0.074
Pop9	64.58	1.10	0.253	0.006	0.367	0.075
Pop10	66.67	1.10	0.272	0.006	0.380	0.100
Pop11	56.25	2.05	0.213	0.005	0.304	0.010
Mean	62.31	1.44	0.250	0.006	0.352	0.073

Table 4 Results of analysis of molecular variance (AMOVA) of RAPD data for 11 populations of *Picea likiangensis*

Source of variation	*df*	SSD	MSD	Variance component	Total variance (%)	*P*-value[a]
Among populations	10	901.8535	90.185	3.77	23.62	$P < 0.001$
Within populations	217	2,646.1641	12.194	12.19	76.38	$P < 0.001$

Population numbers are shown in parentheses

df Degrees of freedom, *SSD* Sum of squares, *MSD* Mean squared deviation

[a] Significance tests after 1,000 permutations

dendrogram (Fig. 2). The phylogenetic relationships indicate that the three morphological units do not cluster as single, monophylogenetic groups. Three tentative groups were identified. The first consists of two subgroups: one including the only population of var. *linzhiensis* (Pop 1) and three closely distributed populations of var. *rubescens* (Pops 2, 3 and 4); while the other sister subgroup includes two populations of var. *likiangensis* (Pops 8 and 9). The second tentative group comprises three populations of var. *rubescens* (Pops 5, 6 and 7) and one population of var. *likiangensis* (Pop 10). Most strikingly, Pop 11 differed substantially from all of the other populations sampled, forming a third isolated group. A few geographically close populations clustered together: Pops 2, 3 and 4; Pops 8 and 9; and Pops 5, 6 and 7. However, the four populations of var. *likiangensis*, which were geographically close (Fig. 1), were genetically distinct.

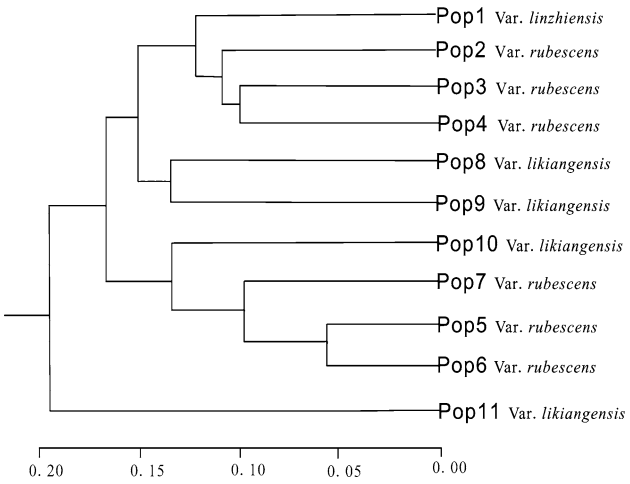

Fig. 2 UPGMA dendrogram of *Picea likiangensis* based on Nei's (1973) genetic distances, indicating the clustering relationships of sampled populations

MVSP was used for PCO analysis. The pattern obtained was broadly similar to that provided by the UPGMA dendrogram in that individual samples plotted as a continuous scatter, but population 11 formed a distinct cluster (Fig. 3). The results of the PCO are not considered here in detail because the proportion of variation accounted for in this analysis was relatively low.

Discussion

Generally, conifer species display low population differentiation (Hamrick et al. 1992; Müller-Starck et al. 1992; Hamrick 2004) due to the widespread occurrence of wind pollination, which promotes outcrossing (Bennett et al. 2000). For example, a mean G_{ST} value of only 0.07 has

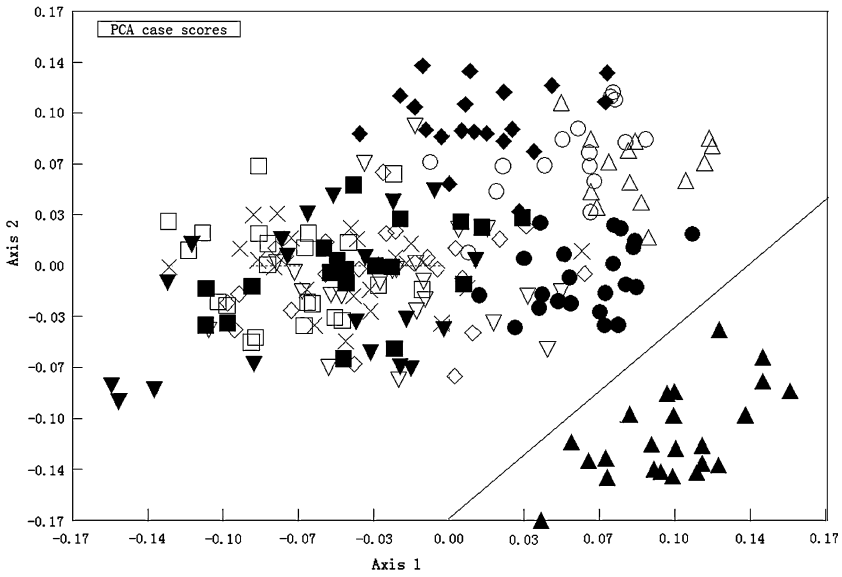

Fig. 3 PCO of *Picea likiangensis* individuals within populations (Pop 1 *filled diamond*, Pop 2 *filled inverted triangle*, Pop3 *multi-symbol*, Pop 4 *filled square*, Pop 5 *open circle*, Pop 6 *open triangle*, Pop 7 *open inverted triangle*, Pop 8 *open square*, Pop 9 *open diamond*, Pop 10 *filled circle* and Pop11 *filled triangle*)

been reported for 121 conifer species based on allozyme analyses (Hamrick and Godt 1989). This tendency for gymnosperms to display low values of population differentiation has been further confirmed by Nybom and Bartish (2000) based on an overview of recent RAPD analyses of plant species, including an examination of the relationship between values obtained from genetic analyses, and the life-history traits of the species concerned. However, geographical range also strongly affects the genetic differentiation among conifer populations (Lee et al. 2002). Wide-ranging species have less intra-specific differentiation than those with restricted ranges, although this trend is not obvious across all plant species (Nybom and Bartish 2000). The among-population differentiation of *P. likiangensis* (G_{ST} = 0.256) found here is comparable to RAPD-based estimates for narrowly distributed species in the same family, e.g., *Pinus attenuate* Lemm. (G_{ST} = 0.24), *P. muricata* D. Don (G_{ST} = 0.29), *P. radiata* D. Don (G_{ST} = 0.18) and *P. longaeva* (Wu et al. 1998). These estimates are much higher than estimates of corresponding parameters for wide-ranging species, e.g., *P. sylvestris* L. (G_{ST} = 0.019), *Pseudotsuga menziesii* (Mirb) Franco (G_{ST} = 0.05) and *Picea mariana* (Mill.) B. S. P. (F_{ST} = 0.05) (Lee et al. 1997; Szmidt et al. 1996).

The results confirmed our initial expectation that high among-population differentiation may be present in *P. likiangensis*, despite its wide distribution and wind-pollinated reproductive strategy. Luo et al. (2005) reached similar conclusions, based on allozyme analyses, for another spruce species (*P. asperata* Mast. F_{ST} = 0.31) that is widely distributed in this region. These findings suggest that complex topographies might have played a significant role in shaping the genetic structure of plants distributed in the QTP. Both high mountains and deep valleys may have prevented gene flow among populations via pollen and seed exchange and thus promoted population differentiation, even for wind-pollinated conifers. Furthermore, these complex topographies might have provided multiple, isolated refugia during glacial stages when forest trees retreated southwards (Tang and Shen 1996). These isolated populations may probably have been subject to strong bottlenecks and severe genetic drift, resulting in possible reductions in total genetic diversity and genetic differentiation among isolated populations due to the associated population and growth restrictions (Hewitt 2000). Following severe range contractions, significant range expansions have often radiated from such refuges during interglacial periods, when species colonized/re-colonized formerly occupied areas (Petit et al. 2003). Since populations growing in re-colonized areas generally originated from a few founding individuals, and thus only received a subset of the genetic diversity present in central populations, founder effects may have accelerated the reduction of their total genetic diversity and led to close phylogenetic relationships between populations in the re-colonized regions and their original refugia (Newton et al. 1999). Evidence to support this theory is provided by the clustering of the investigated populations. Four populations (Pops 8, 9, 10 and 11) from the south comprised an isolated lineage (Pop 11) and basal subgroups of the other two groups (Figs. 2, 3). This pattern seems to suggest that this species may have had at least three different refugia, and both the northern and western populations may derive from different recolonizations from populations in the southern refugia.

The three varieties of *P. likiangensis* examined in this study were mainly distinguished by morphological differences in stomatal lines and their distribution on leaves, and the presence or absence of pubescence on the first-year

branchlets (Fu et al. 1999). However, our results failed to detect such consistent morphological classification. One possible implication of this research is that the northern variety, var. *rubescens*, appears to have derived from the southern variety (var. *likiangensis*) more than once (Fig. 2) although genetic introgression may also result in such an inconsistence with morphological variations. Our ongoing studies based on the chloroplast and mitochondrial sequences supported these hypotheses: the investigated populations of var. *rubescens* from the different RAPD clades (Fig. 2) were revealed to have different haplotypes in both markers.

It is possible that the stomatal variation in this species might to be correlated with variations in photosynthetic water-use efficiency (WUE), i.e., the molar ratio of photosynthetic carbon gain to transpirational water loss (Hultine and Marshall 2000). The differences in the number of stomatal lines and their arrangement on leaves amongst the three varieties of *P. likiangensis* may be derived from their local adaptation to environmental gradients. The distinct distribution ranges of the three investigated varieties of *P. likiangensis* support this hypothesis (Fig. 1). Due to their large differences in latitude, longitude and altitude (Fig. 1; Table 1), the environments of the three varieties have distinct differences in temperature and precipitation. In order to facilitate similar WUE locally, it is possible that all of the populations of each variety with different origins probably acquired similar stomatal distribution patterns and densities in response to the same habitat selection pressures. The RAPD results presented here might only reflect the phylogeographic history of *P. likiangensis* rather than genetic bases of adaptive differentiation as suggested by the other none-functional mitochondrial and chloroplast markers (Avise 2000). Because of these limitations, further detailed comparison of mutation in genes, especially related to WUE, are required to understand genetic bases in such an ecological adaptation between different varieties of *P. likiangensis*. However, our preliminary results undoubtedly suggested that this species provides a good model system for studying the adaptive evolution of trees with the wide distribution in the QTP region.

Acknowledgments Support for this research was provided by the National Natural Science Foundation of China (30430560), FANEDD 200327 and the Chinese Academy of Sciences (Key Innovation Plan KSCX2-SW-106).

References

Abbott RJ, Brochmann C (2003) History and evolution of the arctic flora: in the footsteps of Eric Hultén. Mol Ecol 12:299–313

Abbott RJ, Comes HP (2004) Evolution in the arctic: a phylogeographic analysis of the circumarctic plant (Purple saxifrage). New Phytol 161:211–224

Allnut TR, Newton AC, Lara A, Premoli A, Armesto JJ, Vergara R, Gardner M (1999) Genetic variation in *Fitzroya cupressoides* (alerce), a threatened South American conifer. Mol Ecol 8:975–987

Allnutt TR, Newton AC, Premoli A, Lara A (2003) Genetic variation in the threatened South American conifer *Pilgerodendron uviferum* (Cupressaceae), detected using RAPD markers. Biol Conserv 114:245–253

Avise JC (2000) Phylogeography. Harvard Univsertiy Press, Cambridge, Massachusetts, London, England

Bekessy SA, Allnutt TR, Premoli AC, Lara A, Ennos RA, Burgman MA, Cortes M, Newton AC (2002) Genetic variation in the Monkey Puzzle tree (*Araucaria araucana* (Molina) K. Koch), detected using RAPD Heredity 88:243–249

Bennett KD, Haberle SG, Lumley SH (2000) The last glacial-holocene transition in South Chile. Science 290:325–328

Carlson JE (1991) Segregation of random amplified DNA Markers in F1 progeny of conifers. Theor Appl Genet 83:194–200

Collignon AM, Favre JM (2000) Contribution to the postglacial history at the western margin of *Picea abies*' natural area using RAPD markers. Ann Bot 85:713–722

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem Bull 19:11–15

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:176–191

Farjon A (1990) Pinaceae: drawing and descriptions of the genera *Abies*, *Cedrus*, *Pseudolarix*, *Keteleeria*, *Nothotsuga*, *Tsuga*, *Cathaya*, *Pseudotsuga*, *LarixandPicea*. Koeltz Scientific Books, Konigstein, Germany

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package). Department of Genetics, University of Washington, Seattle

Fu L, Li N, Mill RR (1999) *Picea*. In: Wu Z-Y, Raven PH (eds) Flora of China (4). Science Press, Beijing and Missouri Botanical Garden Press, St Louis pp 25–32

Ge XJ, Zhang LB (2005) Strong genetic differentiation of the East-Himalayan *Megacodon stylophorus* (Gentianaceae) detected by Inter-Simple Sequence Repeats (ISSR). Biol Conserv 14:849–861

Gillies ACM, Cornelius JP, Newton AC, Navarro C, Hernández M, Wilson J (1997) Genetic variation in Costa Rican populations of the tropical timber species *Cedrela odorata* L., assessed using RAPDs. Mol Ecol 6:1133–1145

Gillies ACM, Navarro C, Lowe A J, Newton AC, Hernández M, Wilson J, Corneliu JP (1999) Genetic diversity in Mesoamerican populations of *Swietenia macrophylla*, assessed using RAPDs. Heredity 83:722–732

Hamrick JL (2004) Response of forest trees to global environment changes. For Ecol Manag 197:323–335

Hamrick JL, Godt MJW (1989) Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS (eds) Population genetics and germplasm resources in crop improvement. Sinauer Associates Sunderland

Hamrick JL, Godt MJW, Sherman-Broyles SL (1992) Factors influencing levels of genetic diversity in woody plant species. New For 6:95–124

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913

Hultine KR, Marshall JD (2000) Altitude trends in conifer leaf morphology and stable carbon isotope composition. Oecologia 123:32–40

Isabel N, Beaulieu J, Bousquet J (1995) Complete congruence between gene diversity estimates derived from genotypic data at enzyme and random amplified polymorphic DNA loci in black spruce. Proc Natl Acad Sci USA 92:6369–6373

Lee SW, Kim YY, Hyun JO, Kim ZS (1997) Comparison of genetic variation in *Pinus densiflora* natural populations by allozyme and RAPD analysis. Korea J Breed 29:72–83

Lee SW, Ledig FT, Johnson DR (2002) Genetic variation at allozyme and RAPD markers in *Pinus longaeva* (Pinaceae) of the white mountains. California. Am J Bot 89:566–577

Lewontin RC (1972) The apportionment of human diversity. Evol Biol 6:381–398

Li WH (1997) Molecular evolution. Sinauer Associate Sunderland, MA

Lu MZAE, Szmidt, Wang R (1995) Inheritance of RAPD fragments in haploid and diploid tissues of *Pinus sylvestris* (L.). Heredity 74:582–598

Luo JX, Wang YH, Korpelainen H, Li CY (2005) Allozyme variation in natural populations of *Picea asperata*. Sliva Fenni 39(2):167–176

Lynch M, Milligan BG (1994) Analysis of population structure with RAPD markers. Mol Ecol 3:91–99

Miller MP (1998) AMOVA-PREP1.01. A program for the preparation of AMOVA input files from dominant-marker raw data. Northern Arizona University, Flagstaff, AZ. USA: http://www.herb. bio.nau.edu /miller/amovaprp.htm

Müller-Starck G, Baradat P, Bergmann F (1992) Genetic variation within European tree species. New For 6:23–47

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858

Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA 70:3321–3323

Newton AC, Allnutt T, Gillies ACM, Lowe A, Ennos RA (1999) Molecular phylogeography, intraspecific variation and the conservation of tree species. Trends Ecol Evol 14:140–145

Nkongolo KK (1999) RAPD variations among pure and hybrid populations of *Picea mariana*, *P. rubens*, and *P. glauca* (Pinaceae) and cytogenetic stability of *Picea* hybrids: identification of species-specific RAPD markers. Plant Sys Evol 215:229–239

Nybom H, Bartish I (2000) Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspect Plant Ecol Evol Sys 32:93–114

Petit RJ, Aguinagalde I, de Beaulieu JL (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. Science 300:1563–1565

Ran JH, Wei XX, Wang XQ (2006) Molecular phylogeny and biogeography of *Picea* (Pinaceae): implications for phylogeographical studies using cytoplasmic haplotypes. Mol Phylo Evol 41:405–419

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA

Shi YF, Li JJ, Li BY (1998) Uplift and environmental changes of Qinghai–Tibetan Plateau in the Late Cenozoic. Guangdong Science and Technology Press, Guangzhou China

Szmidt AE, Wang XR, Lu MZ (1996) Empirical assessment of allozyme and RAPD variation in *Pinus sylvestris* (L.) using haploid tissue analysis. Heredity 76:412–420

Tang LY, Shen C M (1996) Late Cenozoic vegetation history and climatic characteristics of Qinghai-Xizang Plateau. Acta Micropalaeont Sin 13:321–337

Wang XR, Szmidt AE (2001) Molecular markers in population genetics of forest trees. Scand J For Res 16:199–200

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6536

Wright S (1931) Evolution in Mendelian populations. Genetics 16:97–159

Wu CY, Wu SG (1996) A proposal for new floristic kingdom (realm). In: Zhang AL, Wu SG (eds) Floristic characteristics and diversity of East Asian plants. China Higher Education Press, Beijing Springer-Verlag Hongkong pp 3–42

Wu J, Krutovskii KV, Strauss SH (1998) Nuclear DNA diversity, population differentiation, and phylogenetic relationship in the California closed-cone pines based on RAPD and allozyme markers. Genome 42:893–908

Yeh FC, Yang R, Boyle T (1999) POPGENE. Microsoft Windows-based freeware for population genetic analysis. Release 1.31.University of Alberta Edmonton

Zhang Q, Chiang TY, Miehe G, Liu JQ, Abbott R (2005) Phylogeography of the Qinghai-Tibetan Plateau endemic *Juniperus przewalskii* inferred from chloroplast sequence variation. Mol Ecol 14:3513–3524