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Allopatric divergence and regional range expansion of *Juniperus sabina* in China

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Abstract In this study, we aimed to study the phylogeographic pattern of *Juniperus sabina*, a shrub species commonly occurring in the northern, northwestern and western China. We sequenced three chloroplast DNA fragments (*trn*L-*trn*F, *trn*S-*trn*G, and *trn*D-*trn*T) for 137 individuals from 16 populations of this species. Five chloroplast DNA chlorotypes (A, B, C, D, and E) were identified and they showed no overlapping distribution. The population subdivision is very high ($G_{ST} = 0.926$, $N_{ST} = 0.980$), suggesting a distinct phylogeographic structure ($N_{ST} > G_{ST}$, $P < 0.05$). Phylogenetic analyses of the five chlorotypes were clustered into three clades, consistent with their respective distributions in three separate regions: northern Xinjiang, western Xinjiang, and northern-northwestern China. However, within each region, the interpopulation differentiation is extremely low. These results as well as statistical tests suggested distinct allopatric differentiations between regional populations and independent glacial refugia for postglacial recolonization. The deserts that developed during the late Quaternary might have acted as effective barriers to promote genetic differentiation among these regions. However, the low diversity dominated by the single chlorotype within each fragmented region suggested that all current populations were derived from a common regional range expansion.

Key words cpDNA, genetic diversity, *Juniperus sabina*, phylogeography, range expansion.

The climatic oscillations during the Quaternary led to repeated range contraction and expansion of most organisms and such changes undoubtedly left genetic signatures in their current populations (Hewitt, 1996; Abbott et al., 2000; Avise, 2000; Schonswetter et al., 2006). The glacial refugia and postglacial recolonization routes of most woody species inferred from the geographic distributions of genetic variations are largely congruent with those from fossil evidence (Petit & Grivet, 2002; Burban & Petit, 2003; Hamper et al., 2003; Kropf et al., 2003; Jaramillo-Correa et al., 2004; Marquardt & Epperson, 2004; Godbaut et al., 2005). However, because of the lack of fossil records, genetic evidence has become the only information for deciphering the historical changes in distribution range and glacial refugia of a few woody species (Petit et al., 2003, 2005; Anderson et al., 2006; Afzal-Rafii & Dodd, 2007). Although no unified glacial sheet had occurred in Asia, paleodata suggested that the distribution ranges of woody species in this region were similarly changed by the Quaternary climatic oscillations. For example, during the Last Glacial Maximum (LGM), approximately 18,000 years ago, steppe and even desert vegetation were thought to have replaced the current coniferous and deciduous forests in northern and northwestern China (Yu et al., 2000; Harrison et al., 2001; Ni et al., 2006). The available studies support the hypothesis that the Quaternary climatic oscillations had triggered regional range contraction and expansion of the woody species in this region, but retained multiple refugia during the LGM (Chen et al., 2008). In addition, a few geographical barriers (for example, formation of the deserts) were revealed to have further blocked the genetic exchanges between isolated populations distributed in a few regions of northwestern China (Meng et al., 2007). However, between northern-northwestern China and Xinjiang of the western China, more and larger deserts had formed before or at the beginning of the Quaternary (Sun et al., 1998; Yang et al., 2006). Plants within the same genus (for example, *Picea*) isolated by them were recognized as different species (Meng et al., 2007) while a few widespread species are disjunctly distributed through these regions. It is desirable to know whether populations in these disjunct regions were derived from a common refugium or from independent refugia that occurred within each region during the Quaternary glaciations.

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Juniperus sabina L. of the Cupressaceae is such a species, with disjunct distributions in northern Xinjiang, western Xinjiang and northern-northwestern China. It is a small shrub with widespread occurrence in arid rocky and sandy habitats (Adams et al., 2007). Previous studies of other species from Cupressaceae (Neale et al., 1989, 1991; Wagner, 1992; Mogensen, 1996; Kondo et al., 1998) have shown that chloroplast DNA (cpDNA) is paternally inherited in members of this family. Available evidence suggests that cpDNA sequence variation is highly effective in revealing glacial refugia and postglacial recolonization patterns of a few species in this family (Hwang et al., 2003; Zhang et al., 2005; Opgenoorth et al., 2010). In this study, we sequenced three cpDNA fragments (*trn*L-*trn*F, *trn*S-*trn*G, and *trn*D-*trn*T) for 137 individuals from 16 populations of *J. sabina*. Based on the genetic variation of these sequence fragments, we aimed to: (i) to determine whether there is any inter-regional genetic differentiation; and (ii) to find out whether there were independent glacial refugia for this species in northern Xinjiang, western Xinjiang and northern-northwestern China isolated by deserts.

1 Material and methods

1.1 Population sampling

Altogether, 137 trees of *J. sabina* were sampled from 16 populations, with 4–15 individuals (spaced more than 100 m apart) from each population. These populations covered the entire geographic distribution of this species. The latitude, longitude, and altitude at each collection center were measured using an Etrex GIS monitor (Garmin, Taiwan, China). The leaf needles of samples, above 10 g, were immediately dried in silica gel until DNA extraction and voucher specimens for all populations were deposited in Lanzhou University Herbarium (LZU).

1.2 DNA extraction, amplification and sequencing

Total genomic DNA was extracted from approximately 50 mg of silica gel-dried leaf-needles per sample according to the modified CTAB method (Doyle & Doyle, 1987). In initial screening, three pairs of cpDNA primers (*trn*L-*trn*F, *trn*S-*trn*G, and *trn*D-*trn*T), which were designed by Taberlet et al. (1991), Hamilton (1999), and Demesure et al. (1995), respectively, were used for examining sequence variants from different populations. Polymerase chain reaction (PCR) was carried out in a 25-μL volume, containing 10–40 ng plant DNA, 50 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L dNTPs, 2 μmol/L of each primer, and 0.75 U Taq DNA polymerase. For primers *trn*L-*trn*F, the PCR program was designed as follows: initial template denaturation at 94 °C for 5 min, then 36 cycles of denaturation at 94 °C for 1 min, annealing at 56.5 °C for 45 s, extension at 72 °C for 1.5 min, then a final extension at 72 °C for 7 min. The other two PCR programs were similar to that for *trn*L-*trn*F primers, except that the annealing temperatures were changed to 56 °C. The PCR products were purified using a CASpure PCR Purification Kit following the recommended protocol (Casarray, Shanghai, China). Sequencing reactions were carried out using the PCR primers and ABI Prism Bigdye terminator cycle ready reaction kit (Applied Biosystems, Foster City, CA, USA). The sequencing products were analyzed on an automated sequencer (3130xl; Perkin Elmer, Applied Biosystems). All DNA sequences determined for this study were submitted to GenBank under the accession numbers GU395998–GU396006.

1.3 Data analysis

The CLUSTALX program version 1.8.1 (Thompson et al., 1997) was used for aligning the DNA sequences with subsequent manual adjustments. A matrix of combined sequences was constructed for 137 trees from 16 populations and from the matrix, different sequences were identified as the chlorotypes.

Phylogenetic analysis of cpDNA chlorotypes were carried out by maximum parsimony, using PAUP version 4.0 b10 (Swofford, 2002). In all analyses, the mononucleotide repeats were excluded due to their highly variable evolutionary rate in the microsatellite region and all gaps (indels) were coded as binary states (0 or 1) using the Gapcoder program (Young & Healy, 2003). The maximum parsimony heuristic search parameters were random addition of sequence (1000 replicates) with tree-bisection-reconnection branch swapping, MULTREES, and COLLAPSE options on. The phylogenic tree was tested by bootstrap analysis and the bootstrap values were estimated from 1000 replicates to assess the relative support for relationships between haplotypes (Felsenstein, 1985; Maddison, 1991). For further examining the relationships among chlorotypes, the chlorotype median-joining network was also constructed using the program network version 4.2.0.1 (available at http://www.fluxus-engineering.com) (Bandelt et al., 1999).

Estimates of unbiased genetic diversity (H_E) were calculated for each population based on haplotype composition (Nei, 1987). The parameters of population diversity (H_S, H_T) and differentiation (G_{ST}, N_{ST}) were estimated using PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software/PermutCpSSR), as described in Pons & Petit (1996). Whereas G_{ST} only

considers haplotype frequencies, N_{ST} takes into account both haplotype frequencies and their genetic distances. For assessing whether the overall population differentiation showed geographic structure, two different parameters (G_{ST} and N_{ST}) were compared using a permutation test with 1000 permutations. Only when the value of N_{ST} is significantly larger than the value of G_{ST} can the presence of phylogeographic structure be assumed (Pons & Petit, 1996). The significance of difference between G_{ST} and N_{ST} was made by a comparison using the U-statistic, which is approximated by a Gaussian variable by taking into account the covariance between N_{ST} and G_{ST}, and a one-sided test (Pons & Petit, 1996). The genetic differentiation within, among populations and among subregions were evaluated by analysis of molecular variance (AMOVA) using ARLEQUIN software version 2.0 (Schneider et al, 2000) and the levels of significance were tested by a nonparametric permutation procedure with1000 permutations.

We estimated the coalescence times (the most recent common ancestor, TMRCA) of all cpDNA haplotypes using the program BEAST 1.5.3 (Drummond & Rambaut, 2007). The mutation rate in *Juniperus* remains unknown. Graur & Li (2000) reported an average mutation rate of 1.2–1.7 $\times$ 10^{-9} substitution per site per year for cpDNA in plants and we therefore chose the middle mutation rate of 1.45 $\times$ 10^{-9} to calibrate our dataset. We treated each indel as a mutation, as is usually the case in such phylogeographic analyses (e.g. Wang et al., 2009). We used the HKY model of nucleotide substitution with estimated base frequencies and a molecular clock with uncorrelated log normal distribution of branch lengths. Following a burn-in of 500,000 cycles, all parameters were sampled once every 500 generations from 5,000,000 Markov chain Monte Carlo steps. Convergence of the chains to the stationary distribution was checked by visual inspection of plotted posterior estimates using the program Tracer (Rambaut & Drummond, 2007), and the effective sample size for each parameter sampled from the Markov chain Monte Carlo analysis was almost always found to exceed 100, usually by an order of magnitude. We used the likelihood ratio test (LRT) to compare pairs of phylogeographic hypotheses. When the likelihood of the more complex model is significantly greater than that of the simpler model (as judged by an X^2 statistic), the complex model is chosen. All of these analyses were carried out in PAML 4.1 (Yang, 2007), in which we compared the likelihood values of the hypothesized phylogenetic trees with the recovered haplotypes situated on the different positions.

2 Results

2.1 Sequence variation and chlorotype distribution

Altogether, five different sequences were identified from sequencing the *trn*L-*trn*F, *trn*S-*trn*G, and *trn*D-*trn*T regions of 137 trees sampled from 16 populations across the entire geographic range of *J. sabina* (Table 1). More variable sites were found in the *trn*S-*trn*G region, where two insertions and two single nucleotide substitution mutations occurred, and the sequences ranged between 763 and 768 bp in length. In the *trn*L-*trn*F region, two insertions were found and the sequences ranged between 640 and 660bp. In the *trn*D-*trn*T region only one insertion was found. In combination, these polymorphisms defined five chlorotypes: A, B, C, D, and E (Table 1).

Chlorotype A (populations 7–16, Table 2, Fig. 1) was fixed in all populations in northern-northwestern China, with the only exception being population 13 which contained another chlorotype, D, at low frequency. Only chlorotype B was fixed in all populations (populations 2–6, Table 2, Fig. 1) in northern Xinjiang, and two chlorotypes, C and E (at low frequency), were fixed in population 1 in western Xinjiang. No chlorotype was shared between these regional distributions.

2.2 Phylogenetic analyses of the cpDNA chlorotypes

We used PAUP version 4.0 b10 to establish the maximum parsimonious tree of chlorotypes with one

Table 1 Variable sites of the aligned sequences of chloroplast DNA fragments in five chlorotypes of *Juniperus sabina*

	Variable positions										
	*trn*L-*trn*F					*trn*S-*trn*G					*trn*D-*trn*T
	4	5	5	6	7	8	1	1	1	1	1
Chlorotype	7	3	4	9	9	0	0	0	2	5	5
	2	3	1	0	8	4	8	9	8	0	1
							4	0	8	0	2
A	—	TGTTATTG		C	T———T		G———T		G	G———G	
B	⋆	T———G		T	T———T		G———T		A	G———G	
C	—	T———G		C	T———T		GAAAAGT		G	G———G	
D	—	TGTTATTG		C	TATCTTT		G———T		G	G———G	
E	—	T———G		C	T———T		GAAAAGT		G	GCGTGTGCCGGGG	

Sequences are numbered from 5′ to 3′ ends in each region. —, deletion; ⋆, CTGTATATAACATACACAAA.

Table 2 Origin of materials and sample numbers in 16 populations of *Juniperus sabina*

Population no.	Location	Latitude (°N)	Longitude (°W)	Altitude (m)	*n*	Chlorotype
1	Ili, Xinjiang, China	44° 06.46′	80° 46.23′	852	7	C, E
2	Habahe, Xinjiang, China	47°20.79′	86°11.59′	1250	7	B
3	Tieliekti, Xinjiang, China	48°38.27′	86°48.44′	1350–2500	7	B
4	Burqin,Xinjiang,China	48°48.02′	86°55.09′	1782	7	B
5	Altay, Xinjiang, China	48°00.04′	88°20.47′	2029	7	B
6	Altay Xinjiang, China	47°13.67′	89°55.14′	1367	11	B
7	Zhangye, Gansu, China	38°40.12′	100°28.09′	2760	15	A
8	Shandan, Gansu, China	38°27.15′	102°20.54′	3160	7	A
9	Jingtai, Gansu, China	37°26.73′	103°44.64′	2620–3160	13	A
10	Gangcha, Qinghai, China	36°45.18′	100°46.27′	3270	6	A
11	Helanshan, Ningxia, China	38°30.76′	105°10.46′	2018	4	A
12	Helanshan, Ningxia, China	38°43.88′	105°55.18′	1868	10	A
13	Wushen banner, Nei Mongol, China	38°57.16′	109°16.65′	1370	10	A, D
14	Baiyinaobao, Nei Mongol, China	43°31.96′	117°12.47′	1868	7	A
15	Chifeng, Nei Mongol, China	42°56.01′	119°01.36′	636	15	A
16	Jagdaqi, Heilongjiang, China	50°25.33′	124°00.21′	472	4	A

sample of *Juniperus pseudosabina* as an outgroup. A single tree was obtained (Fig. 2: A) and three clades (I, II, and III) were identified. Clade I included two chlorotypes (HapA, HapD) distributed in northern-northwestern China, clade II comprised two chlorotypes (HapC, HapE) from western Xinjiang, and clade III consisted of a single chlorotype (HapB) in northern Xinjiang. The minimum spanning network analyses also clustered all chlorotypes into three groups with the same topological relationships (Fig. 2: B).

2.3 Genetic diversity and genetic structure

We estimated chlorotype diversity based on haplotype frequencies for each population (Table 1). The level of total genetic diversity H_T (0.577, Table 3) across all populations was much higher than the average within-population H_S (0.043) and, consequently, population differentiation across the entire range of this species was very high. The level of N_{ST} (0.980) was significantly higher than G_{ST} (0.926; $P < 0.05$), indicating significant phylogeographic structure across the species' range (Table 3) (Pons & Petit, 1996). AMOVA analyses supported the three-region divergence of the species with approximate 85.97% variation attributed to this pattern of differentiation (Table 4). However, when two assumed regions of Xinjiang (northern Xinjiang, western Xinjiang) were considered together, H_T, H_S, and G_{ST} were lower in the former than the latter region (Table 3).

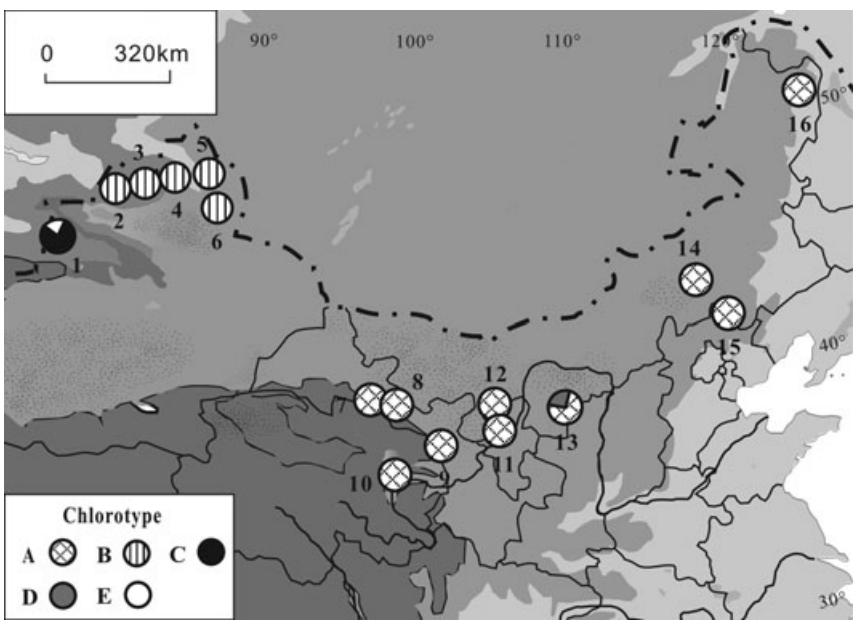

Fig. 1. Distribution of the recovered chlorotypes in *Juniperus sabina*.

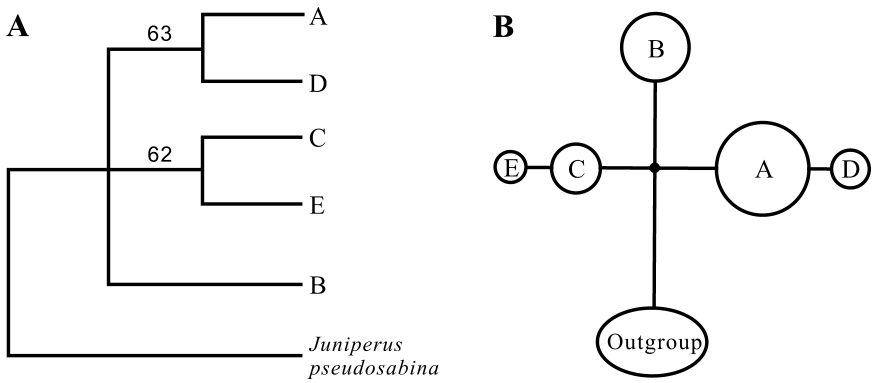

Fig. 2. **A,** Single most parsimonious tree (length = 27, confidence interval = 1.000, retention index = 1.000) based on sequence data of five chlorotypes in *Juniperus sabina*; numbers above branches indicate bootstrap values. **B,** Median-joining network for five chlorotypes.

2.4 Coalescence analyses of chlorotypes and statistical test of glacial refugia

Bayesian estimates of TMRCA of all haplotypes suggested that they coalesced 0.78 mya with a 95% highest posterior density of 0.26–1.43 mya. The divergence times between haplotypes A and D and between haplotypes C and E were similarly dated at approximately 0.16 mya with a 95% highest posterior density of 0.02–0.41 mya or 0.02–0.42 mya. We assumed that all current populations of the species derived from a single refugium as the null hypothesis (Fig. 3: A). In this regard, all populations and haplotypes were derived from a single refugium. We then compared this hypothesis with the phylogeographic inference that the three clades represented three separate refugia (the complex model) (Fig. 3: B). The chlorotype relationships were consistent with the phylogenetic tree (Fig. 2). Our LRT test clearly supported the complex model based on the phylogenetic locations of chlorotypes on the assumed tree (Fig. 3: A) ($-2 \ln \mathrm{LR} = 26.87$, $\mathrm{df} = 3$, $P < 0.0001$).

3 Discussion

3.1 Allopatric divergence and independent glacial refugia

In this study, we recovered five chlorotypes from 16 populations across the entire geographic distribution of *J. sabina* in China. Phylogenetic analyses of these chlorotypes resolved three distinct clades (Fig. 2), which are consistent with their respective distributions in three separate regions: northern Xinjiang, western Xinjiang, and northern-northwestern China (Table 1). It is interesting that these chlorotypes show no geographic overlap (Table 2, Fig. 1). This significant phylogeographic structure across the species range was also supported by the comparison tests between N_{ST} and G_{ST} ($P < 0.05$) (Table 3) (Pons & Petit, 1996). In some conifers, genetic differentiation based on the cpDNA variation is relatively low between regional populations (Petit et al., 2005), e.g., *Pinus flexilis* (Latta & Mitton, 1997), *Pinus banksiana*, and *Pinus contorta* (Dong & Wagner, 1994), and *Cunninghamia* spp. and Cupressaceae (Hwang et al., 2003). However, in other species, genetic variation was revealed with strong geographic correlations, for example, *Pinus muricata* (Hong et al., 1993), *Juniperus przewalskii* (Zhang et al., 2005), and *Juniperus tibetica* (Opgenoorth et al., 2010).

The high level of differentiation as well as the three distinct geographic clades recovered here suggested that independent glacial refugia might have been maintained in *J. sabina*. This inference was further supported by the LRT test. In addition, our coalescence analyses suggested that the three distinct clades (Fig. 2) probably diverged approximately 0.78 mya. These results suggested that regional genetic differentiation of this species resulted mainly from geographic isolation posed by the large deserts that developed in western China. For

Table 3 Estimation of average gene diversity within populations (H_S), total gene diversity (H_T), interpopulation differentiation (G_{ST}), and the number of substitution types (N_{ST}) (mean ± SE in parentheses) of *Juniperus sabina* across the entire distribution within Xinjiang, China and northern-northwestern China

Regions	H_S	H_T	G_{ST}	N_{ST}
Northern-northwestern China (populations 7–16)	0.040 (0.0395)	0.044 (0.0425)	0.111 (nc)	0.111 (nc)
Xinjiang (populations 1–6)	0.048 (0.0476)	0.333 (0.2358)	0.857 (nc)	0.966 (nc)
Total distribution	0.043 (0.0293)	0.577 (0.0847)	0.926 (0.0470)	0.980 (0.0138)*

nc, not computed due to small sample size. *, N_{ST} is significantly different from G_{ST}, $0.01 < P < 0.05$.

Table 4 Analysis of molecular variance of genetic variation in *Juniperus sabina* populations found in northern-northwestern China and Xinjiang, China

Source of variation	Degrees of freedom	Variance components	Percentage of variation	Fixation index
Among regions	1	0.41535	85.97	$F_{RT} = 0.975^{**}$
Among populations	13	0.04675	9.68	$F_{SR} = 0.195^{*}$
Within populations	117	0.02100	4.35	$F_{ST} = 0.168$

F_{RT}, correlation of haplotypes within groups relative to the total; F_{SR}, correlation within populations relative to the region; F_{ST}, correlation within populations relative to the total. *, $P < 0.05$; **, $P < 0.01$; 1000 permutations.

example, the Gurbantunggut Desert had formed approximately before the Quaternary, but enlarged during the middle Pleistocene (Sun et al., 1998; Yang et al., 2006). This enlargement perhaps finally isolated populations in northern Xinjiang from those in western Xinjiang. Similarly, the Badain Jaran-Tengger Desert, which initiated in the Pliocene and enlarged greatly during the middle Pleistocene (Sun et al., 1998; Yang et al., 2006), isolated the populations occurring in Xinjiang from those in northern-northwestern China. This species probably occurred widely across China before the Pliocene (Adams et al., 2007), but was gradually divided into three separate regions by the developing deserts. Within two of the three regional clades, one more chlorotype was found and their origins were dated to approximately 0.16 mya, earlier than the LGM initiated approximately 0.02 mya (Hewitt, 2000). However, each of them was fixed in only one population. This distribution suggested that each of the three separate regions comprised a single unit to respond to the subsequent climatic changes. The low diversity within each of the three regions might result from regional range expansion and/or recolonization at the end of the LGM.

3.2 Postglacial range expansion across northern-northwestern China

Juniperus sabina is widely distributed across northern-northwestern China. The populations seen today might be relics of a widespread distribution of this species before the Quaternary. If we assumed that there were polymorphic cpDNA chlorotypes during that time, the current populations would more likely be fixed or nearly fixed for different chlorotypes due to random effects of genetic drift in fragmented populations under the multiple-refugia scenario. However, our results suggested that the single HapA was almost fixed for all populations, except for population 13 containing HapD at a very low frequency. This chlorotype originated approximately 0.16 mya and it should have had enough time to expand into the other populations under the neutral hypothesis without selective and demographic pressures. It is highly likely that all populations in northern China had retreated into this population (glacial refugium) during the subsequent glacial stage and recolonized the other regions at the end of the LGM. This hypothesis is highly consistent with previous reports that the new recolonized areas usually have low levels of genetic diversity both within and among populations and the number of chlorotypes should be decreased gradually from the refugium (Hewitt, 2000; Heuertz et al., 2004; Petit et al., 2005). It is interesting that these findings are largely consistent with paleorecords (mostly pollen fossil) that most species in northern China might have migrated to a southern refugium during the LGM (Yu et al., 2000; Harrison et al., 2001) and recolonized the current areas. In addition, our phylogeographic analyses of *J. sabina* suggest that this postglacial expansion might have extended to northwestern China across the Tengger Desert that isolates populations 7–10 in Gansu and Qinghai from those (populations 11–16) in northern China. This desert, formed approximately at the start of the Quaternary (Yang et al., 2006), was shown to have blocked the seed-mediated gene flow of *Picea crassifolia* (Meng et al., 2007). In contrast, the higher arid tolerance of *J. sabina* might have facilitated its recent dispersals around or across the Tengger Desert during the postglacial recolonization. This expansion resulted in the populations in Gansu and Qinghai, as well as in northern China, which were fixed for a common haplotype (A) with the exception of population 13 (Fig. 1).

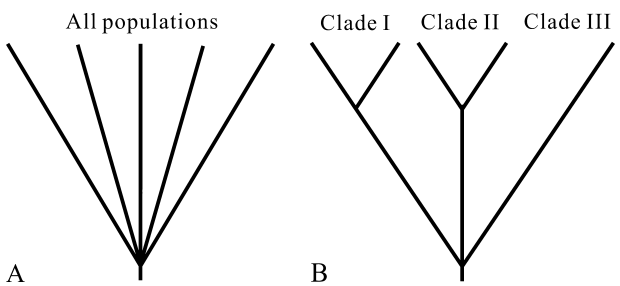

Fig. 3. Models used to test glacial refugia hypotheses. **A,** Single refuge hypothesis. **B**, Three refugia hypothesis.

The population structure of cpDNA variation in *J. sabina* in northern China is very different from that revealed previously for two species (*Ostryopsis davidiana* and *Pinus tabulaeformis*) in northern China. Both

of these species are characterized by geographically differentiated predominant genotypes in different regions of northern China (Chen et al., 2008; Tian et al., 2009), indicating multiple isolated refugia. A preliminary comparison of *J. sabina*, *O. davidiana*, and *P. tabulaeformis* in northern China investigated to date shows that they do not share a common phylogeographic history. Therefore, more similar studies are needed before a general conclusion can be drawn on how plants in northern China responded to the Quaternary climatic oscillations.

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