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Intraspecific divergences of *Rhodiola alsia* (Crassulaceae) based on plastid DNA and internal transcribed spacer fragments

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In the present study, we used two maternally inherited plastid DNA intergenic spacers, *rpl20-rps12* and *trnS-trnG*, and the biparentally inherited nuclear ribosomal internal transcribed spacer (ITS) region to explore genetic variation and phylogeographical history of *Rhodiola alsia*, a herb endemic to the Qinghai-Tibetan Plateau (QTP). Based on range-wide sampling (18 populations and 227 individuals), we detected 45 plastid DNA haplotypes and 19 ITS sequence types. Only three plastid DNA haplotypes were widespread; most haplotypes were restricted to single sites or to neighbouring populations. Analysis of molecular variance revealed that most of the genetic variance was found within populations (51.24%) but that populations were also distinct (F_{ST} = 0.48759). We found three areas with relatively high plastid DNA diversity and these could further be recognized as potentially isolated divergence centres based on the ITS sequence type distribution. These represent three potentially isolated glacial refugia for *R. alsia*: one of them has long been recognized as an important refugium on the south-eastern edge of the QTP, whereas the others are new and located in the north and south of the Tanggula Mountains on the plateau platform. Divergence time estimates based on ITS suggest that the main lineages of *R. alsia* diverged from each other 0.35–0.87 Mya, indicating that climatic oscillations during the Pleistocene may have been an important driver of intraspecific divergence in *R. alsia*. *Rhodiola alsia* probably experienced a phylogeographical history of retreat to isolated glacial refugia during Quaternary glaciations that led to different degrees of allopatric intraspecific divergence. *Botanical Journal of the Linnean Society*, 2012, **168**, 204–215.

ADDITIONAL KEYWORDS: cpDNA – glacial refugia – intraspecific divergence – phylogeography – Qinghai-Tibetan Plateau.

INTRODUCTION

Species on the Qinghai-Tibetan Plateau (QTP) have most likely been more affected by Quaternary glaciations than those in other regions of similar latitude (Yang *et al*., 2008). However, the effects of the uplift of the QTP and of Quaternary glaciations on population genetic structure and distribution range of species in this area are still understudied. Most phylogeographical studies conducted so far on the QTP tend to indicate that the south-eastern edge of the QTP was a refugium for alpine species during Quaternary glaciations, and that these species expanded their ranges on the platform during interglacial and postglacial periods (Qu *et al*., 2005; Zhang *et al*., 2005; Meng *et al*., 2007; Chen *et al*., 2008a, b; Yang *et al*., 2008). Alternatively, suitable microenvironments might have existed in some restricted ice-free areas on the central plateau platform and might have allowed cold-tolerant herbs to survive *in situ* at least during the last glaciation (70–10 000 years ago; Zheng, Xu & Shen, 2002). Indeed, growing evidence indicates that Quaternary glaciations were

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asynchronous with variable impacts in different regions of the QTP (Owen *et al*., 2005; Thompson *et al*., 2006), and that no massive ice sheet developed on the QTP during glaciations (Shi *et al*. 1995; Shi, Zheng & Yao, 1997; Shi, Li & Li, 1998; Zheng & Rutter, 1998). Only two phylogeographical studies conducted on QTP alpine plants (within a distribution range covering both the south-eastern edge and central platform of the QTP) indicated the importance of the Pleistocene period for the evolution of intraspecific divergence. These studies were performed with *Pedicularis longiflora* Rudolph (Yang *et al*., 2008) and *Aconitum gymnandrum* Maxim. (Wang *et al*., 2009a), of which only the latter revealed a multi-refugia pattern on the central plateau platform during glaciations (Wang *et al*., 2009a). Further phylogeographical studies of QTP alpine plants are required to obtain a better understanding of the evolutionary history of species in this area and to uncover to what extent Pleistocene was an important period for the intraspecific divergence of QTP alpine plants.

Rhodiola alsia (Fröd.) S.H.Fu (Crassulaceae) is a perennial herbaceous species endemic to the QTP (Fu & Ohba, 2001). It is an important element of siliceous alpine and subnival massifs. This alpine plant is widespread in the high mountains of the QTP between its south-eastern edge and the west of the plateau platform at 3400–4800 m a.s.l. *Rhodiola alsia* forms multiramet cushions and mats, which consist of hemispherical rosettes, 6–10 cm in diameter. This species grows on alpine slopes and in shrubby habitats with a discontinuous distribution pattern. By contrast to previously studied species, *R. alsia* reproduces both clonally and sexually (Xia *et al*., 2005). Field investigation indicates that natural populations of *R. alsia* are small, usually consisting of 30–50 individuals. Genetic distribution pattern of *R. alsia* has preliminarily been studied using a plastid DNA fragment, *rpl20-rps12*, and this revealed that most genetic variation was found within populations (52.58%) as opposed to between populations (47.42%; Gao *et al*., 2009). Because this fragment revealed only limited divergence, inferences on phylogeographical history of *R. alsia* were not sufficiently supported. Furthermore, because plastid DNA is usually inherited from only one of the parents (Birky, 2001; Xu, 2005), plastid DNA markers may only partly recover the phylogeographical history of a species (Mäder *et al*., 2010). The additional use of biparentally inherited nuclear DNA markers could therefore contribute greatly to uncovering the evolutionary history of this species. In the present study, we used the nuclear ribosomal internal transcribed spacer (ITS) sequence data and two plastid DNA intergenic spacers, *rpl20-rps12* and *trnS-trnG*, to explore the genetic variation and to retrieve the phylogeographica; history of *R. alsia*. Our study addresses the following questions: (i) was the Pleistocene an important period for intraspecific divergence of *R. alsia* and (ii) was there a single refugium at the south-eastern plateau edge, or did multiple refugia exist for this species, possibly located at the central QTP platform?

MATERIAL AND METHODS

Population sampling

We obtained samples of *R. alsia* from populations from almost the entire distribution range of the species, which encompasses the central area and the south-eastern edge of the QTP. Because natural populations of *R. alsia* are rather small, we collected as many individuals as possible in all populations. Fresh leaves of six to 24 individuals (according to the population size) separated by at least 10 m were collected from each population. In total, 227 individuals from 18 populations were sampled (Fig. 1 and Table 1). Collected leaf materials were dried in silica gel and stored at room temperature. Voucher specimens of all populations are deposited in the Herbarium of the Northwest Institute of Plateau Biology, Xining, Qinghai Province.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Total genomic DNA was extracted from silica-dried leaves using a modified cetyl trimethylammonium bromide method (Doyle & Doyle, 1987). The plastid DNA *rpl*20-*rps*12 and *trn*S-*trn*G intergenic spacers were amplified for all collected *R. alsia* individuals and an additional outgroup, *Sedum oreades* Raym.-Hamet, using primers previously described by Hamilton (1999). The PCR mixture and amplification program followed Gao *et al*. (2009). For ITS, primers ITS1 and ITS4 as reported by White *et al*. (1990) were used for amplification. PCR was performed in a 25-μL volume, containing 0.6 μL (approximately 20 ng) plant DNA, 2.5 μL of 10 × PCR buffer (without Mg^{2+}), 1.5 μL of 25 mM $MgCl_2$, 0.3 μL of 10 mM dNTPs, 1.0 μL of 5 pM of each primer and 0.3 μL (1.5 units) of *Taq* polymerase. The amplification profile was 3 min at 94 °C, followed by 31 cycles of 1 min at 94 °C, 1 min at 57 °C, 70 s at 72 °C, with a final 7-min extension at 72 °C. Samples were then stored at 4 °C until further processing. PCR products were purified using a CASpure PCR Purification Kit in accordance with the manufacturer's instructions (CASarray). Sequencing reactions were carried out in a Biometra thermocycler using DYEnamic Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc.) also in accordance with the manufacturer's instructions. Sequencing products were separated

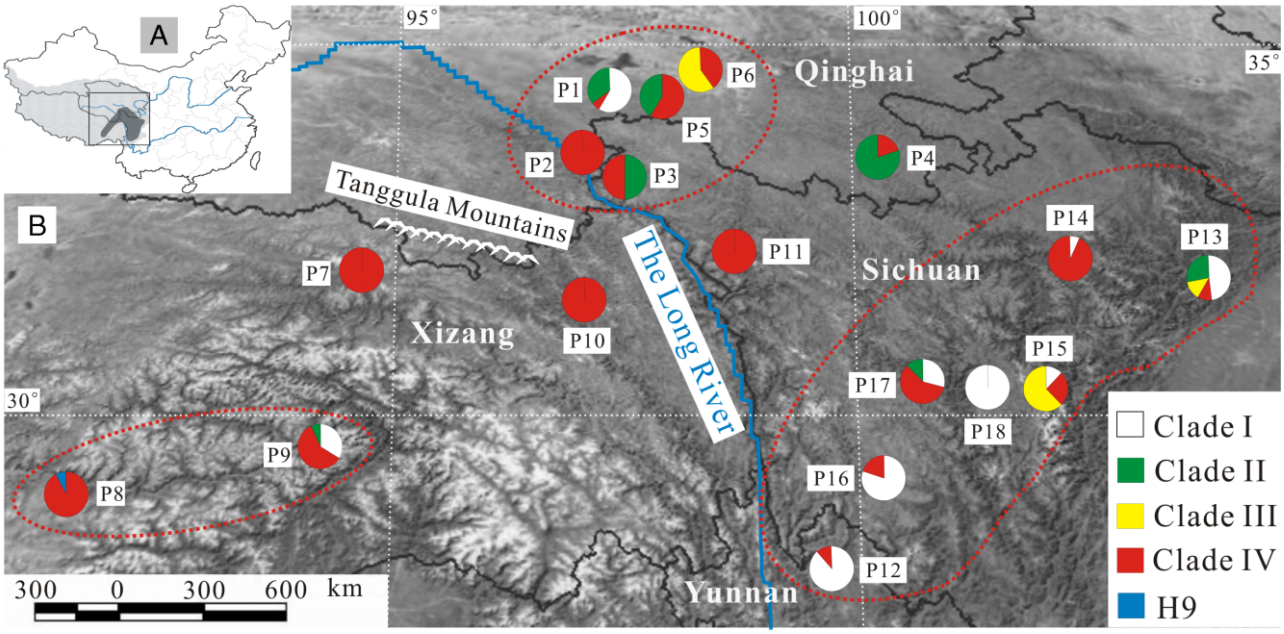

Figure 1. A, map of China, indicating the Qinghai-Titetan Plateau (lightly shaded) and the distribution range of *Rhodiola alsia* (darkly shaded). B, map showing the sampled populations of *R. alsia* and the distribution of four plastid DNA haplotype clades (I–IV) and H9 as identified by the maximum parsinomy analysis in Fig. 2. Areas indicated by red broken lines represent three potentially glacial refugia for *R. alsia*.

and analyzed on a MegaBACE 500 Automated Sequencer (Amersham Pharmacia Biotech Inc.). The ITS sequence of *Rhodiola yunnanensis* (Franch.) Fu, which was used as outgroup, was retrieved from GenBank (accession number AB088602).

Data analysis

The DNA sequences were aligned using the software CLUSTAL X (Thompson *et al.*, 1997), with minor subsequent corrections. The most parsimonious relationships among *R. alsia* plastid DNA haplotypes with *S. oreades* as outgroup were reconstructed using PAUP* 4.0b10 (Swofford, 2002). In the maximum parsinomy analysis, all characters were equally weighted and treated as unordered. An heuristic search strategy with 1000 random addition sequence replicates was implemented, in combination with ACCTRAN character optimization, MULPARS, tree bisection–reconnection (TBR) branch swapping and STEEPEST DESCENT options on. Bootstrap analysis (BS) was used to assess branch support of clades found in the most parsimonious trees. BS was calculated from 1000 replicates using an heuristic search with simple addition with TBR and MULPARS options on. The relationships among detected plastid DNA haplotypes were examined via a haplotype network constructed with NETWORK, version 4.5.0.0 (http://www.fluxustechnology.com/; Weir, 1996). Total haplotype diversity (h_T), average within-population diversity (h_S), G_{ST} (Nei, 1987) and N_{ST} (Grivet & Petit, 2002) were calculated using the software PERMUT (http://www.pierroton.inra.fr/genetics/labl/software/PermutCpSSR; Pons & Petit, 1996) to estimate differentiations between populations. To statistically compare G_{ST} and N_{ST} (Burban *et al.*, 1999), a permutation test with 1000 random permutations of haplotype identities was implemented using the same software. The software SAMOVA, version 1.0 (http://web.unife.it/progetti/genetica/isabelle/samova.html; Dupanloup, Schneider & Excoffier, 2002) was used to define groups of populations that are geographically homogeneous and maximally differentiated from each other. Gene diversity (h) and nucleotide diversity (π) of each population were calculated in ARLEQUIN, version 3.01 (Excoffier, Laval & Schneider, 2006). Differentiation within populations, between populations within groups, and between groups were estimated by analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992), also implemented in ARLEQUIN. For ITS sequences, a site was classified as heterozygous when double peak occurred in the same position in both strands, with the weakest signal reaching at least 25% of the strength of the strongest (Fuertes-Aguilar, Rosselló & Nieto-Feliner, 1999; Fuertes-Aguilar & Nieto-Feliner, 2003). For heterozygous individuals, ITS sequence types were estimated with PHASE version 1.0 (Stephens, Smith

Table 1. Sampling data, estimates of gene diversity (h) and nucleotide diversity (π), as well as haplotype composition, for the plastid DNA dataset from the 18 populations of *Rhodiola alsia*

Population code	Location	Latitude (N)	Longitude (E)	Altitude (m)	N	h	π (10×)	Plastid DNA haplotypes (N)
P1	Qingshuihe, QH	34°05′	97°37′	4670	14	0.5824	0.2899	H1 (8) H2 (5) H3 (1)
P2	Xiewu, QH	33°12′	97°26′	4350	12	0.0000	0.0000	H4 (12)
P3	Shiqu, SC	33°08′	97°33′	4390	6	0.7333	0.0279	H4 (1) H8 (2) H14 (3)
P4	Dari, QH	33°17′	100°23′	4370	10	0.3556	0.0151	H8 (2) H14 (8)
P5	Maduo, QH	34°17′	97°58′	4550	12	0.6818	0.0247	H3 (5) H38 (2) H39 (5)
P6	Chengduo, QH	34°52′	98°28′	4200	10	0.9333	0.1693	H3 (2) H40 (2) H41 (1) H42 (2) H43 (1) H44 (1) H45 (1)
P7	Dingqing, XZ	31°41′	94°55′	4900	11	0.0000	0.0000	H5 (11)
P8	Jiacha, XZ	29°01′	92°21′	4820	13	0.7949	0.0677	H6 (3) H7 (5) H8 (3) H9 (1) H10 (1)
P9	Linzhi, XZ	29°41′	94°43′	3470	15	0.7048	0.3391	H3 (1) H8 (8) H11 (1) H12 (3) H13 (1) H14 (1)
P10	Changdu, XZ	31°17′	97°20′	4590	12	0.0000	0.0000	H8 (12)
P11	Dege, SC	31°56′	98°54′	4560	6	0.6000	0.0043	H8 (3) H15 (3)
P12	Zhongdian, YN	28°34′	99°49′	4330	17	0.8750	0.2849	H8 (2) H16 (2) H17 (5) H18 (3) H19 (2) H20 (2) H21 (1)
P13	Maoxian, SC	31°39′	103°56′	3500	21	0.8476	0.3690	H8 (2) H14 (6) H22 (3) H23 (4) H24 (4) H25 (2)
P14	Hongyuan, SC	31°52′	102°40′	3970	14	0.5934	0.0709	H8 (2) H19 (1) H26 (9) H27 (1) H28 (1)
P15	Kangding, SC	30°04′	101°48′	4290	8	0.6429	0.1370	H3 (1) H29 (1) H30 (5) H31 (1)
P16	Xiangcheng, SC	29°08′	100°01′	4520	10	0.6444	0.1823	H8 (2) H19 (1) H32 (6) H33 (1)
P17	Yajiang, SC	30°09′	100°40′	4290	24	0.7971	0.3136	H3 (1) H8 (10) H33 (1) H34 (3) H35 (4) H36 (2) H37 (2) H38 (1)
P18	Yajiang, SC	30°03′	101°20′	4280	12	0.4848	0.1796	H24 (4) H25 (8)

QH, Qinghai; SC, Sichuan; XZ, Xizang; YN, Yunnan.

& Donnelly, 2001). Nucleotide diversity at the species level was estimated using the computer software MEGA, version 3.1 (Kumar, Tamura & Nei, 2004). The ITS dataset was then used to estimate divergence times across detected *R. alsia* sequence types. *Rhodiola yunnanensis* was used as the outgroup. A strict molecular clock assumption was not rejected showing almost no rate variation among branches. We used BEAST, version 1.5.3 (Drummond & Rambaut, 2007) employing a Bayesian Markov chain Monte Carlo chain to estimate the divergence timescales of the main lineages across the detected ITS sequence types of *R. alsia*. Under the general time-reversible substitution model with a gamma distribution and four rate categories, a coalescent tree model assuming constant size was implemented with a strict molecular clock model. Because reliable calibration points are not available for *Rhodiola* L., we used the substitution rate of 5.69×10^{-9} substitution per site per year ($s\ s^{-1}\ y^{-1}$) for *Aichryson* Webb & Berthel. (Mes, van Brederode & t'Hart, 1996), which is close to *Rhodiola*. The same approach was successfully used to estimate divergence times in many species, such as *Pedicularis longiflora* (Yang *et al*., 2008) and *Aconitum gymnandrum* (Wang *et al*., 2009a). Posterior distributions of parameters were approximated using two independent ten million generation runs, sampled every 1000 generations, after discarding the first quarter of generations as burn-in. The effective sample size of all parameters were obtained with TRACER, 1.2.1 (Drummond & Rambaut, 2007). The software TREEANNOTATOR, version 1.5.3, was employed to summarize output results. A tree with ages for each node and their 95% highest posterior density are displayed in FIGTREE, version 1.3.1 (Drummond & Rambaut, 2007).

RESULTS

Plastid DNA dataset

The sequence length of the *rpl20-rps12* intergenic spacer of *R. alsia* was in the range 797–835 bp. The alignment length was 840 bp, including 36 variable sites: 28 were caused by point mutations and eight were the results of indels. Twenty-nine different sequences were identified based on these polymorphic sites. The total alignment length of the *trnS-trnG* region of *R. alsia* was 660 bp. There were a 9–13-bp tyrosine homopolymer at site 230 and an 8–15-bp adenine homopolymer at site 310 in the *trnS-trnG* intergenic spacer. The length of the nucleotide fragment between these homopolymers varied from 48 to 67 bp and showed two different types; therefore, we treated this nucleotide fragment as one variable site. We thus obtained 33 polymorphic sites, which defined 23 different sequences. The 29 *rpl20-rps12* sequences and 23 *trnS-trnG* sequences recorded in *R. alsia*, together with the *rpl20-rps12* and *trnS-trnG* sequences obtained from one individual of *S. oreades*, have been submitted to GenBank under accession numbers FJ797968–FJ797970, FJ797972–FJ797976, FJ797978–FJ797998, HQ840970–HQ840992, HQ841012 and HQ841013. The combined data of both *rpl20-rps12* and *trnS-trnG* sequences identified 45 different plastid DNA haplotypes (H1–H45). Only a few haplotypes (e.g. H3, H8 and H14) were widespread across the species distribution range, whereas 36 out of the 45 haplotypes were restricted to single or neighbouring population(s) (Table 1). The seven populations (P12–P18) from the south-eastern QTP, harboured 26 haplotypes (58% of the total), 22 of which (H16–H37) were endemic. The remaining 11 populations, occupying the QTP platform, harboured 23 haplotypes, 19 (H1, H2, H4–H7, H9–H13, H15, H39–H45) of which were endemic. Gene diversity (h) and nucleotide diversity (π) among the 18 sampled populations ranged from zero to 0.9333 and zero to 0.03690, respectively (Table 1). With seven haplotypes, six of which were endemic, the northernmost population P6 was the most variable population ($h = 0.9333$). Populations P12, P13 and P17 from the south-eastern QTP and populations P3, P8 and P9 from the QTP platform also contained high levels of gene diversity. Population P13 from the south-eastern QTP had the highest level of nucleotide diversity ($\pi = 0.03690$), although other populations, such as P12, P15, P16, P17 and P18 from the south-eastern QTP and P1, P6 and P9 from the QTP platform, also had high nucleotide diversity. Phylogenetic analysis using the most parsimony method and network reconstruction for the 45 plastid DNA haplotypes allowed us to recognize five clades (I–IV and H9) (Fig. 2). The distribution of these five clades is shown in Figure 1. Clade I, including 18 haplotypes, occurred in all populations from the south-eastern QTP (P12–P18) and two populations (P1 and P9) from the QTP platform. Clade II, containing only four haplotypes, was mainly distributed in the QTP platform populations (P1, P3–P5 and P9) and also in two populations P13 and P17 from the south-eastern QTP. Six haplotypes comprised clade III that was sparsely distributed in three populations, two (P13 and P15) from the south-eastern QTP and P6 from the QTP platform. Clade IV was widespread across all sampled populations except P18. H9 formed an independent clade and was only fixed in the westernmost population P8 from the QTP platform. Total haplotype diversity (h_T) was estimated to be 0.950, and average within-population diversity (h_S) was 0.571. The values for N_{ST} and G_{ST} were 0.461 and 0.399, respectively. A permutation test showed that the N_{ST} value was not significantly higher than the associated G_{ST} value ($P > 0.05$, 1000 permutations), indicating an absence of phylogeographical structure across the distribution of *R. alsia*. Spatial analysis of molecular variance (SAMOVA) indicated that the genetic differentiation among groups was the highest ($F_{CT} = 0.49665$, $P < 0.001$) when the sampled populations pooled into two groups, one containing three populations (P12, P16 and P18) from the south-eastern-most edge of the QTP (Fig. 1), and the other comprising the remaining 15 populations. AMOVA suggested that 51.24% of the total genetic variation was found within populations and 48.76% between populations. The pairwise F_{ST} value was 0.48759 (Table 2).

ITS dataset

The alignment length of ITS sequences was 614 characters. Thirty-nine polymorphic sites were found, of which 15 were detected in the ITS1 region, three in the 5.8S gene, and 21 in ITS2. Thirty-eight percent of the 39 polymorphic sites were a result of heterozygosity in some individuals. Using these polymorphic sites, 19 genotypes were identified among the sampled 18 populations with GenBank accession numbers (HQ840993–HQ841011). Sequence types of heterozygous individuals were then estimated with PHASE and 19 sequence types (S1–S19) were identified in total. The estimated nucleotide diversity of *R. alsia* based on the ITS dataset was 0.0054 at the species level. The distribution of these sequence types showed a clear geographical pattern (Table 3). Sequence types S1 and S2 were exclusively restricted in northern populations (P1–P7) on the plateau platform. Sequence types S11 and S13–S19 were exclusively fixed in the south-eastern populations (P12–P18) with the exception of S11, which also occurred in P10 from the QTP platform. Six sequence types (S4–S9) were endemic to the two westernmost populations

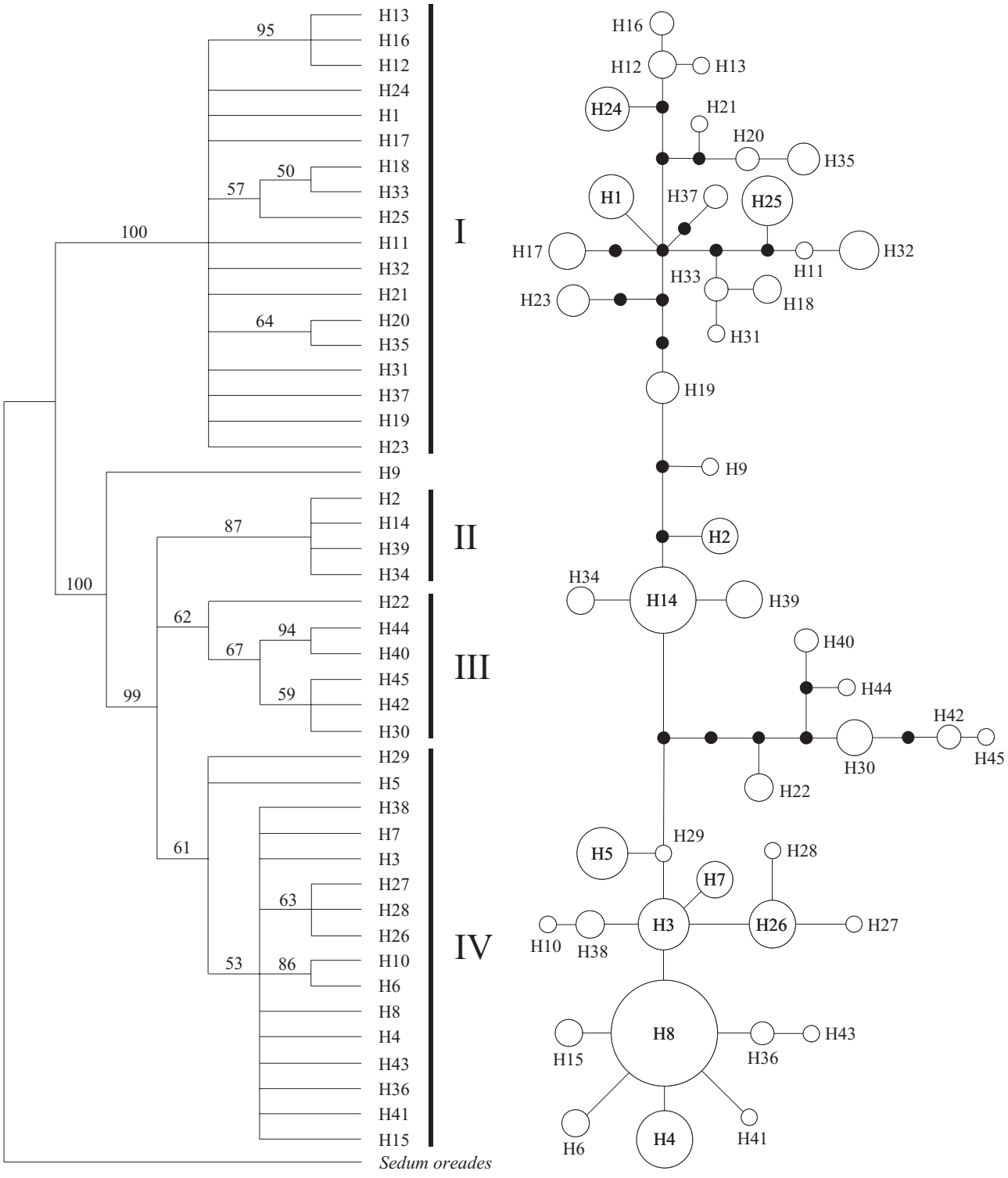

Figure 2. The maximum parsinomy tree topology (length = 338, consistency index = 0.9438, retention index = 0.9681; left) and network (right) of the 45 plastid DNA haplotypes detected from *Rhodiola alsia*. Numbers on the branches indicate the bootstrap values (1000 replicates). The relative sizes of the circles in the network are proportional to haplotype frequencies and black dots represent missing haplotypes.

Table 2. Results of analysis of molecular variance of plastid DNA sequence data for populations and population groups of *Rhodiola alsia*

Source of variation	d.f.	SS	VC	PV (%)	Fixation indices
(i) Total populations					
Among populations	17	1039.933	4.50855 Va	48.76	F_{ST} = 0.48759*
Within populations	209	990.244	4.73801 Vb	51.24	
Total	226	2030.177	9.24655		
(ii) (12, 16, 18) versus (1–11, 13–15, 17)					
Among groups	1	1065.868	15.57058 Va	49.67	F_{ST} = 0.60708*
Among populations within groups	16	886.108	3.46214 Vb	11.04	F_{SC} = 0.21939*
Within populations	209	2574.527	12.31831 Vc	39.29	F_{CT} = 0.49665*
Total	226	4526.502	31.35103		

Population numbers in parentheses represent the grouping pattern identified by spatial analysis of molecular variance. d.f., degrees of freedom; SS, sum of squares; VC, variance component; F_{ST}, correlation within populations relative to total; F_{SC}, correlation within populations relative to groups; F_{CT}, correlation of haplotypes within groups relative to total; *$P < 0.001$, 10000 permutations.

Table 3. Internal transcribed spacer sequence type composition for the 18 populations of *Rhodiola alsia*

Population code	*N*	Sequence types (*N*)
P1	14	S1/S2 (14)
P2	12	S1/S1 (12)
P3	6	S1/S1 (5); S1/S2 (1)
P4	10	S1/S1 (10)
P5	12	S1/S1 (7); S1/S2 (5)
P6	10	S1/S1 (1); S1/S2 (9)
P7	11	S1/S1 (11)
P8	13	S3/S4 (4); S5/S5 (2); S5/S7 (1); S6/S6 (5) S8/S8 (1)
P9	15	S3/S9 (15)
P10	12	S3/S9 (4); S10/S11 (8)
P11	6	S12/S12 (6)
P12	17	S11/S11 (6); S11/S13 (9); S13/S13 (2)
P13	21	S3/S14 (6); S3/S15 (7); S3/S18 (2); S14/S16 (4); S15/S17 (2)
P14	14	S11/S11 (5); S11/S13 (8); S13/S13 (1)
P15	8	S19/S19 (8)
P16	10	S11/S11 (10)
P17	24	S11/S11 (24)
P18	12	S11/S11 (1); S11/S13 (8); S13/S13 (3)

Population codes are the same as those given in Table 1.

P8 and P9, except one sequence type S9, which was shared by the intermediate population P10. S10 and S12 were restricted to the two intermediate populations P10 and P11, respectively. The remaining sequence type, S3, was shared by four populations, three from the plateau platform (P8–P10) and one from the south-eastern edge (P13). However, the clustering of the 19 detected ITS sequence types was not completely consistent with their geographical distribution pattern. Moreover, the pattern of relationships between lineages received low bootstrap support (Fig. 3). Divergence time estimation suggested that *R. alsia* diverged from *R. yunnanensis* approximately 3.01 Mya (Fig. 3). The divergence times of sequence types S6 and S19 were estimated to be 1.31 Mya and 0.96 Mya, respectively. However, the main lineages of *R. alsia* ITS sequence types were estimated to have a divergence time of < 0.87 Mya.

DISCUSSION

Genetic structure and ITS sequence diversity of *R. alsia*

AMOVA analyses based on the plastid DNA dataset suggested that > 48% of the total genetic variation was found between populations, indicating the high genetic differentiation across the sampled region. Most previous studies have suggested that high genetic differentiation between populations within a species was usually coupled with the distinct phylogeographical structure (Avise, 2004; Zhang *et al*., 2005). However, haplotype identification permutation tests indicated that N_{ST} was not significantly larger than G_{ST}, suggesting an absence of phylogeographical structure across the distribution range of *R. alsia*. Within *R. alsia*, it is therefore peculiar that despite the high interpopulation genetic differentiation, the phylogeographical structure remained unclear. In addition, genetic analyses indicated that both total and within-population diversities were high in *R. alsia* (h_T = 0.950, h_S = 0.571). In other studies on QTP alpine species that revealed a high interpopulation differentiation, the genetic diversity index was relatively low, as in *Pedicularis longiflora* (h_T = 0.770, h_S = 0.332; Yang *et al*.,

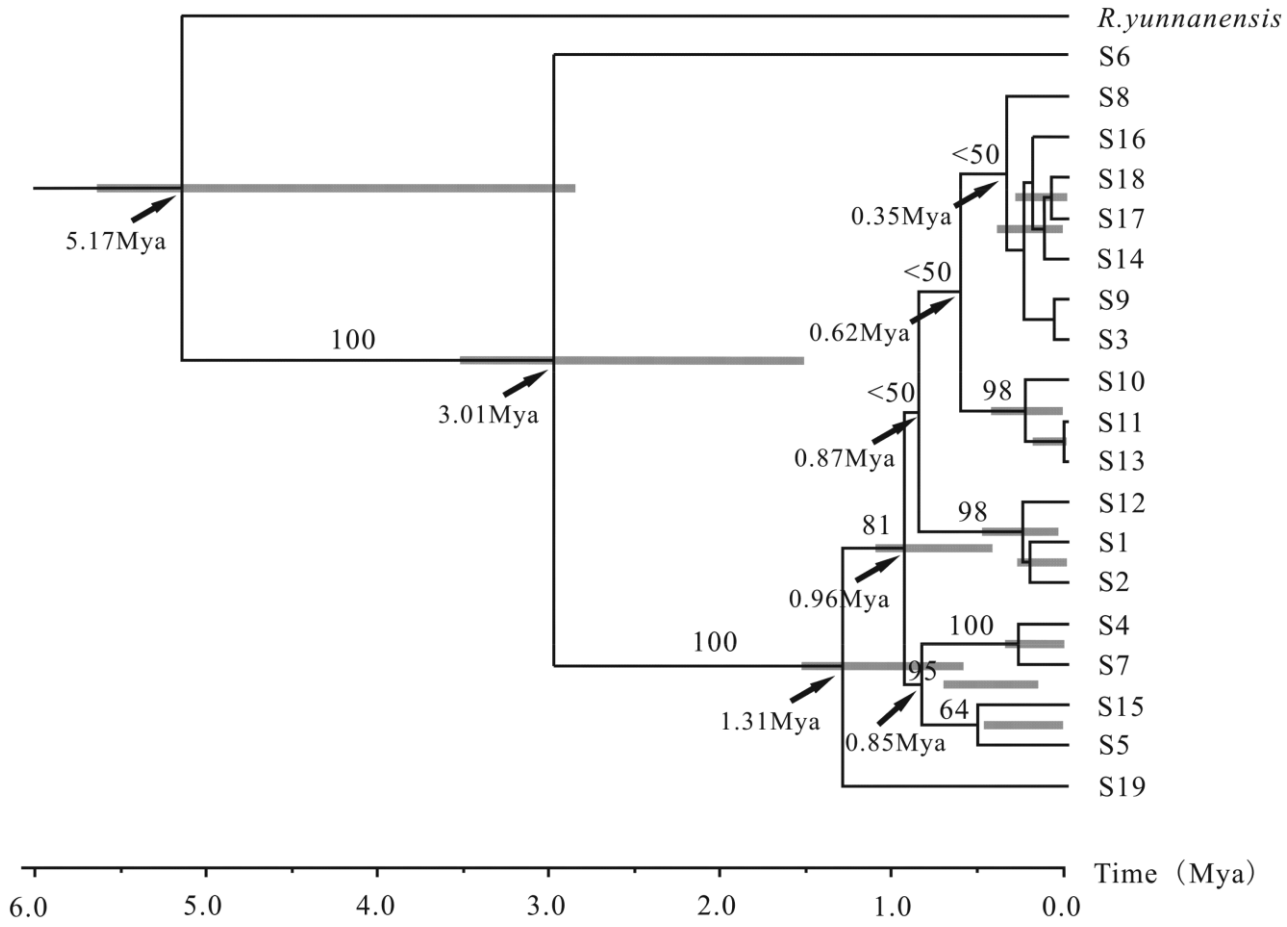

Figure 3. Majority rule consensus phylogenetic tree of the 19 detected internal transcribed spacer sequence types of *Rhodiola alsia* based on the Bayesian inference and their divergence times estimated with the substitution rate of 5.69×10^{-9} s s^{-1} y^{-1}. Numbers on the branches indicate the percentage values of the Bayesian posterior probability. The node age estimates are marked under branches. The length of the grey bars represents 95% highest posterior density.

2008) and *Aconitum gymnandrum* ($h_T = 0.739$, $h_S = 0.207$; Wang *et al*., 2009a). In *R. alsia*, the genetic distribution pattern of only a few widespread haplotypes (e.g. H3, H8 and H14) compared to a large proportion of endemic haplotypes may result in the apparent absence of phylogeographical structure, high levels of total haplotype diversity, and average within-population diversity of this species. The same genetic structure was also observed in two other QTP alpine species that harbours a high number of local haplotypes: *Potentilla fruticosa* L. (Li *et al*., 2010) and *Potentilla glabra* Lodd. (Wang *et al*., 2009b).

The ITS dataset within *R. alsia* revealed high levels of nucleotide diversity (0.0054), indicating that the process of concerted evolution is not fast enough to homogenize different ITS copies (Lorenz-Lemke *et al*., 2005). This process acts through unequal crossing over during meiosis and biased gene conversion (Dover, 1994; Liao, 1999). Inter-chromosomal exchange seems to be more common towards telomeres, as occur in *Gossypium* L., *Paeonia* L and *Thinopyrum* Á. Löve (Wendel, Schnabel & Seelanan, 1995; Zhang & Sang, 1999; Li & Zhang, 2002). High ITS polymorphism in *R. alsia* may indicate that nrDNA is not located terminally on chromosomes, resulting in a slow down in the homogenization of different ITS copies. Additionally, high levels of ITS polymorphism in *R. alsia* may be a result of vegetative reproduction. *Rhodiola alsia* reproduces both sexually and clonally (Xia *et al*., 2005). A severe growing environment (a short growing season of 120 days and a mean annual temperature between –5 °C and 0 °C) might favour clonal propagation in *R. alsia*. Vegetative reproduction may extend generation time, and this may slow the process of recombination through which concerted evolution or homogenization can occur (Sang, Crawford & Stuessy, 1995; Buckler & Holtsford, 1996). The same phenomenon was also revealed in species of *Passiflora* L., as described by Lorenz-Lemke *et al*. (2005) and Mäder *et al*. (2010).

Glacial refugia and inference of demographic history

Given our sampling strategy, genetic diversity of natural populations of *R. alsia* is likely to have been well estimated in the present study. According to Petit *et al*. (2003), plant populations in refugia have high genetic divergence and uniqueness. The results of the present study revealed three areas with relatively high plastid DNA genetic diversity. These areas could further be recognized as three potentially independent divergence centers for *R. alsia* based on ITS sequence type distribution. This distribution of genetic diversity of both plastid DNA and ITS dataset might suggest three potentially isolated glacial refugia for *R. alsia* (Fig. 1).

One region with high plastid DNA genetic diversity is located on the south-eastern edge of the QTP, an area already known as an important refugium for most studied plant species (Zhang *et al*., 2005; Meng *et al*., 2007; Chen *et al*., 2008a, b; Yang *et al*., 2008; Wang *et al*., 2009a). The seven populations (P12–P18) from the south-eastern QTP generally have high gene diversity (h) and/or nucleotide diversity (π), and most (85%) plastid DNA haplotypes occurring in this region are endemic. In addition, nine (S3, S11 and S13–S19) ITS sequence types occurred in this region and seven (S13–S19) were endemic, which may indicate a divergence center for *R. alsia*. Therefore, we infer this region as a potential glacial refugium for *R. alsia*. The south-eastern edge of the QTP, especially the Hengduan Mountains area, harbouring a high concentration of palaeo- and neo-endemics (Tao, 1992; Ying, Boufford & Zhang, 1993; Wang & Zhang, 1994) and high intraspecific gene diversity (Zhang *et al*., 2005; Meng *et al*., 2007; Chen *et al*., 2008a, b; Yang *et al*., 2008; Wang *et al*., 2009a), has long been treated not only as an important Tertiary center of species diversity, but also as an important glacial refugium for many plants, including some taxa of Laurasian angiosperms, such as species of *Rhododendron* L., *Rhodiola*, *Gentiana* L. (Ho, Chen & Liu, 2002) and *Circaeaster* Maxim. (Wang, 1992).

A second area with high plastid DNA genetic diversity is located north of the Tanggula Mountains on the plateau platform. The northernmost population P6 has the highest gene diversity ($h = 0.9333$) among the 18 populations investigated and has relatively high nucleotide diversity ($\pi = 0.01693$). Other populations from this region, such as P1 ($\pi = 0.02899$) and P3 ($h = 0.7333$), also have relatively high nucleotide diversity or gene diversity. High levels of genetic diversity in this region might be the result of two alternative biogeographical scenarios. Either a glacial refugium existed in this region and allowed *R. alsia* to survive climate oscillations and to accumulate genetic diversity (Tzedakis *et al*., 2002) or, in contrast, this region acted as an area that mixes organisms from different origins, resulting in higher genetic diversity than the original sources (Petit *et al*., 2003). If the latter hypothesis is true, then haplotypes detected in this region should form a subset of haplotypes of the multiple original sources, although ten out of the 14 plastid DNA haplotypes in these populations are endemic and do not appear in the remaining distribution range of *R. alsia*. Furthermore, ITS sequence types S1 and S2 were restricted to this area, indicating another divergence centre of *R. alsia*. The most parsimonious explanation is consequently the presence of a glacial refugium for *R. alsia* north of the Tanggula Mountains at least during the last glaciation. In addition, postglacial and/or interglacial migration from the south-eastern edge of the QTP to the platform as proposed by two other QTP alpine herbs, *Metagentiana striata* (Maxim.) T.N. Ho, S.W. Lo & Shi L. Wen (Chen *et al*., 2008a, b) and *Pedicularis longiflora* (Yang *et al*., 2008) appears unlikely in *R. alsia* because no evidence of a reduction in plastid DNA and ITS genetic diversity was detected from the south-eastern QTP to platform.

Third, a small restricted refugium might have existed in the south of the Tanggula Mountains. Populations in this region are geographically isolated from the remaining populations by the Long River and the Tanggula Mountains, indicating little possibility of colonization from the other two potential refugia during interglacial periods. In addition, high plastid DNA genetic diversity was detected in the two westernmost populations, P8 and P9, with ten fixed haplotypes, of which seven were endemic. Similar results were found in a related study on *Spiraea alpina* Pall. and *Sibiraea angustata* (Rehder) Hand.-Mazz., two shrub species endemic to the QTP (Y. Duan and F. Zhang, unpubl. data).

Postglacial or interglacial expansion from refugial populations might have occurred within both refugia in the south-eastern edge of the QTP and north of the Tanggula Mountains. However, populations south of the Tanggula Mountains might have been strongly reduced during glacial periods. Only small and isolated populations, such as P8 and P9, survived in restricted ice-free regions and range expansion within this refugium appears to be restricted during postglacial or interglacial periods. The four between-refugium populations, P4, P7, P10 and P11, which only fixed widespread or single endemic plastid DNA haplotypes, might be the result of a recent colonization event from refugia. In these four populations, founder effects and genetic drift might have lead to low levels of haplotype and nucleotide diversity. Because the single sequence type (S1) occurring in P4 and P7 was shared with populations from the north of

the Tanggula Mountains, populations P4 and P7 might be the result of long-distance recolonization from the refugium located in the north of the Tanggula Mountains. However, it is difficult to determine the original refugium of P10 and P11 because no populations are available in areas between these two populations and the three inferred refugia.

Allopatric intraspecific divergence in *R. alsia*

Because most variation (54%) occurred in the ITS2 region for *R. alsia*, we used the substitution rate (5.69×10^{-9} s s^{-1} y^{-1}) of ITS2 for *Aichryson* (Mes *et al*., 1996), a closely-related group to *Rhodiola*, to estimate the divergence times between lineages of detected ITS sequence types of *R. alsia*. Based on this substitution rate, the main lineages of *R. alsia* ITS sequence types had divergence times in the range 0.35–0.87 Mya. Although these estimates of divergence dates should be treated with caution, they correspond well with the glacial and interglacial history of the QTP (Zheng *et al*., 2002). The divergence times of the main lineages of ITS sequence types detected in *R. alsia* mostly fall within the interglacial period between the most extensive Naynayxungla Glaciation at 0.50–0.72 Mya and the Guxiang (the Penultimate) Glaciation at 0.13–0.30 Mya (Zheng *et al*., 2002). This result indicates that climatic oscillations caused by glacial and interglacial repeats during the Pleistocene might be an important driving factor for intraspecific divergence in *R. alsia*. Other phylogeographical studies on alpine plants of the QTP have also highlighted the importance of the Pleistocene period for the generation of intraspecific differentiation: intraspecific divergence time for *P. longiflora* was in the range 0.138–0.667 Mya (Yang *et al*., 2008) and was < 1.7 Ma for *A. gymnandrum* (Wang *et al*., 2009a). It is likely that repeated glacial and interglacial cycles during the Pleistocene created allopatric fragmentation and isolation of populations of *R. alsia*, which promoted allopatric intraspecific divergence. Only three plastid DNA haplotypes (H3, H8 and H14) were found to be widespread across the distribution range of *R. alsia*. Moreover, these haplotypes occupy interior nodes of the haplotype network and can be considered as ancestral according to the coalescent theory (Posada & Crandall, 2001). However, some of these inferred ancestral haplotypes (e.g. H3 and H8) were not clustered in a basal or ancestral phylogenetic position. This discrepancy might be a result of the different statistical criteria implemented in the software PAUP* 4.0b10 (Swofford, 2002) and NETWORK, version 4.5.0.0 (Weir, 1996). The same discrepancy also occurred in many phylogeographical studies (Yang *et al*., 2008; Wang *et al*., 2009a; Xu *et al*., 2010). It is possible that *R. alsia* was widespread across the QTP before the Quaternary glaciations, widely fixing ancestral haplotypes (e.g. H3, H8 and H14). The populations of *R. alsia* were then geographically isolated by the subsequent glacial and interglacial repeats and experienced an independent evolution. The existence of three isolated areas harbouring relatively high plastid DNA genetic diversity and showing an almost exclusive distribution pattern of ITS sequence types further suggests an allopatric intraspecific divergence of *R. alsia*. A large proportion of endemic plastid DNA haplotypes indicates that gene flow among populations of *R. alsia* must be low or absent. More generally, it is premature to determine whether Pleistocene intraspecific differentiation is a general rule for plant species on the QTP. Further studies of species with differing distributions and life-history traits are needed.

CONCLUSION

The present study has contributed to our understanding of the phylogeography of alpine species on the QTP. *Rhodiola alsia* does not share a common phylogeographical history with the other three QTP alpine species investigated to date. Our results revealed that *R. alsia* retreated into three isolated refugia during the Quaternary glaciations: one has long been recognized as an important glacial refugium in the southeastern edge of the QTP, and the others, located in the north and south of the Tanggula Mountains on the plateau platform, are newly considered. During glaciations, allopatric fragmentation isolated the distribution range of *R. alsia* into disjuncted areas in where populations evolved independently, which resulted in a large proportion of endemic plastid DNA haplotypes. Pleistocene climatic oscillations may be of great importance for the generation of intraspecific divergence in *R. alsia*. However, large-scale phylogeographical studies of alpine species on the QTP are still scarce; therefore, it is still premature to infer general trends about the evolutionary history of the QTP flora during the Quaternary. In conclusion, the present study underlines that more phylogeographical studies are required across a wider range of species, possibly with contrasting distribution, habitat and life-history characteristics, to interpret whether the phylogeographical history of plants on the QTP depends on their habitat or life strategy, or whether it is random.

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