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Genetic diversity of wild populations of *Rheum tanguticum* endemic to China as revealed by ISSR analysis

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ABSTRACT

Rheum tanguticum is an important but endangered traditional Chinese medicine endemic to China. The wild resources have been declining. Establishing the genetic diversity of the species would assist in its conservation and breeding program. Inter-simple sequence repeats (ISSR) markers were used to assess the genetic diversity and population genetic structure in 13 wild populations of *R. tanguticum* from Qinghai Province. Thirteen selected primers produced 329 discernible bands, with 326 (92.94%) being polymorphic, indicating high genetic diversity at the species level. The Nei's gene diversity (*He*) was estimated to be 0.1724 within populations (range 0.1026–0.2104), and 0.2689 at the species level. Analysis of molecular variance (AMOVA) showed that the genetic variation was found mainly within populations (71.02%), but variance among populations was only 28.98%. In addition, Nei's differentiation coefficients (G_{st}) was found to be high (0.3585), confirming the relatively high level of genetic differentiation among populations. Mantel test revealed a significant correlation between genetic and geographic distances ($r = 0.573$, $P = 0.002$), and the unweighted pair-group method using arithmetic average (UPGMA) clustering and Principal coordinates analysis (PCoA) demonstrated similar results. Meanwhile, the genetic diversity of *R. tanguticum* positively correlated with altitude and annual mean precipitation, but negatively correlated with latitude and annual mean temperature. This result might be an explanation that the natural distribution of *R. tanguticum* is limited to alpine cold areas. We propose conservation strategy and breeding program for this plant.

1. Introduction

Rhubarb (Dahuang in Chinese), as an important traditional Chinese medicine, is derived from the dried rhizome and root of *Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., and *Rheum officinale* Baill. of the family Polygonaceae. Bitter in flavor and cold in property, it is widely used as a purgative and anti-inflammatory agent (Chinese Pharmacopoeia Commission, 2005). *R. tanguticum* is an endangered perennial herb endemic to China. It is mainly distributed in Qinghai, Gansu Provinces and west Tibetan Autonomous Region at altitudes ranging from 2300 m to 4 200 m and can be found on margins of forests, in valleys or shrubs (Yang, 1991; Liu, 1997; Li, 1998; Wu, 2004). Because of overexploitation and deterioration of its habitat, the wild resources of the plant decreased annually and rapidly (Zhang et al., 2008). In fact, it is

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considered to be threatened in China and has been listed among the important wild conservative plants list of Qinghai Province (List 1). So far, studies on *R. tanguticum* have mainly focused on chemical components (Jin and Tu, 2005; Jin et al., 2006; Komatsu et al., 2006a) and pharmacological properties (Wang et al., 1991; Zhang and Liu, 2004; Liu et al., 2008; Zhao et al., 2009). Although karyotype analysis revealed that the plant is diploid ($2n = 22$) (Hu et al., 2007) and previous works have provided preliminary data to identify *R. tanguticum* with other authentication rhubarb at molecular levels (Yang et al., 2001, 2004), little is known regarding its genetic diversity and population structure except for the SSR marker analysis (Chen et al., 2009). Analysis of genetic structure at intraspecific level of an endangered species is important to development of conservation strategies, exploration of plant genetic resources, and future breeding programs of wild plants (Hamrick and Godt, 1996).

Among various molecular marker tools, inter-simple sequence repeats (ISSR) based on PCR amplification, have been widely used for population genetic studies of various plant species, including several medicinal plants (Bornet et al., 2002; Chen et al., 2006; Fracaro and Echeverrigaray, 2006; Li and Jin, 2008). ISSR primers of repeat motifs (microsatellites) that are abundant and dispersed throughout genomes, anchor either at 5′ or 3′ end with one or few specific nucleotides and amplify the sequences between the two microsatellite loci (Wang et al., 1994, 2008; Zietkiewicz et al., 1994; Ratnaparkhe et al., 1998). Because of higher annealing temperature and longer sequence of ISSR primers, they can yield reliable and reproducible bands than RAPD (Nagaoka and Ogihara, 1997; Wolfe et al., 1998; Goulao et al., 2001; Qian et al., 2001), and the cost of the analyses is relatively lower than that of some other markers such as RFLP, SSR and AFLPs (Yang et al., 1996; Wang et al., 2008). In addition to freedom from the necessity of obtaining genomic sequence information, ISSR markers, therefore, is technically simpler than many other marker systems in genetic diversity studies of plants (Ratnaparkhe et al., 1998; Bornet and Branchard, 2001; Ye et al., 2005).

In the present study, we measured the genetic diversity within and among natural populations of *R. tanguticum* sampled from Qinghai Province using ISSR markers. The main aims of this study were to (1) assess levels of genetic diversity of natural populations; (2) reveal the partitioning of the genetic variations within and among populations; and (3) provide basic information for conservation and plant breeding of this endangered medicinal plant.

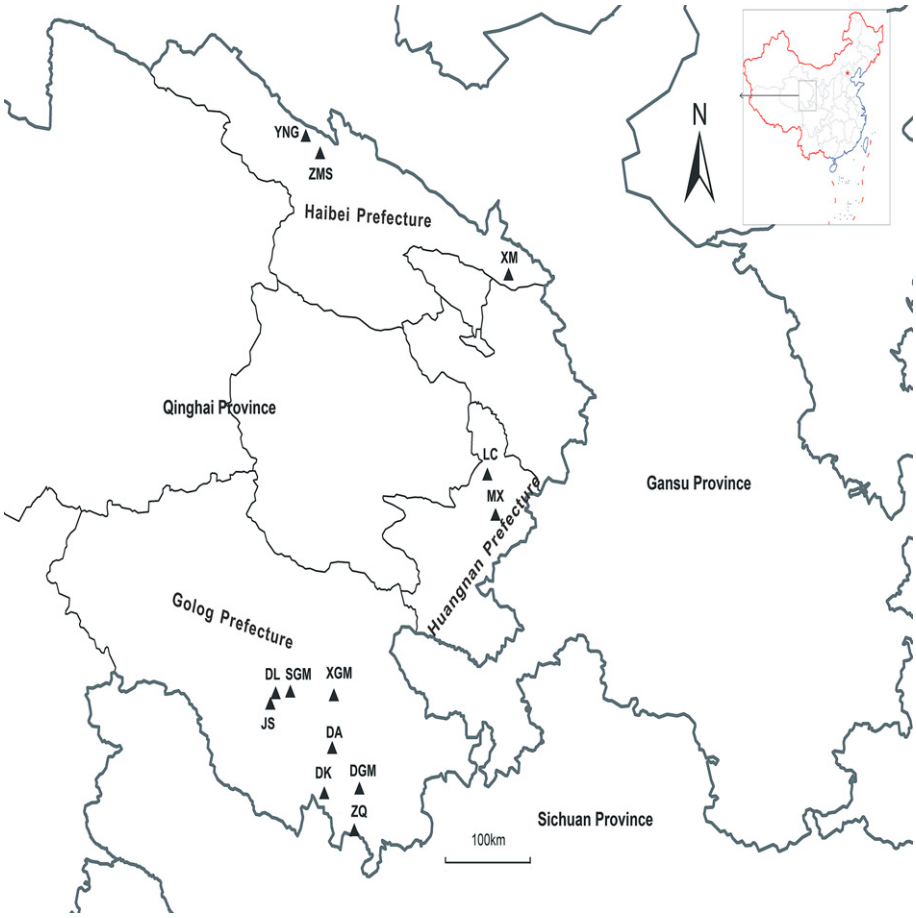

Fig. 1. Geographic distribution of the 13 sampled populations of *R. tanguticum* in Qinghai Province. For population abbreviations, see Table 1 for details.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of 160 individuals of *R. tanguticum* were collected from 13 natural populations in Qinghai Province, China, which represented the majority of wild populations in their genuine producing area. Their originations and distributions were shown in Fig. 1 and Table 1. Young leaf tissues from each sampled individuals were stored in ziplock bags with silica gel and transported back to our laboratory for DNA extraction. Genomic DNA was extracted using the modified Doyle's (Doyle and Doyle, 1987) CTAB method. The quality and quantity of DNA were determined by comparing the sample with known standards of lambda DNA in 0.8% (w/v) agarose gels. The isolated genomic DNA was diluted to 30 ng/μl and stored at −20 °C for use.

2.2. ISSR-PCR amplification

A set of 100 ISSR primers, representing di, tri, tetra and pentamer repeats, used in this study was synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., according to the primer set published by University of British Columbia (UBC) (http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/Primers_Oct2006.pdf). From the preliminary screening, over 34 primers that could amplify visible bands were selected for further examination. At last, 13 primers (Table 2), which yielded clear and reproducible banding patterns, were used in all samples of *R. tanguticum*.

PCR reactions were carried out in a volume of 20 μl consisting of 2.5 μl 10 × buffer (Tris–HCl (pH 8.3) 100 mM; KCl 500 mM), 3.0 mM $MgCl_2$, 0.1 mM dNTP, 10 pmol primer, 0.75 U Taq DNA polymerase (*TaKaRa* Biotech Co., Ltd.) and 30 ng template DNA. The amplifications were performed in a PTC-221 thermocycler (MJ Research, Bio-Rad, USA) using the following profile with an initial step of 94 °C for 5 min followed by 38 cycles of 20 s at 94 °C, 60 s at 50–58 °C (depending on primers used, Table 2) and 80 s at 72 °C with a final extension for 6 min at 72 °C. The negative control was run by replacing template DNA with ddH_2O to test for the possibility of contamination. Amplified PCR products were electrophoresed in a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide in TAE buffer and photographed on UV light. Molecular sizes of the DNA fragments were estimated using a 200 bp DNA ladder (*TaKaRa* Biotech Co., Ltd.).

2.3. Data analysis

The amplified DNA fragments were scored as presence (1) or absence (0), and only those consistently reproducible bands were scored. Smeared and weak bands were excluded. Fragments of the same molecular weight were considered as the same

Table 1
Sampling details of *R. tanguticum* populations in the present study in Qinghai Province.

Locality	Population	Longitude (E)	Latitude (N)	Altitude (m)	AMT (°C)	AMP (mm)	Habitat	Sample size
Zhiqin, Banma county, Golog Prefecture	ZQ	100°22′3.2″	32°46′2.7″	3782	0.3	648.35	Mountain root, shrub, meadow	14
Daka, Banma county, Golog Prefecture	DK	100°1′59.5″	33°3′40.9″	4010	−1.1	615.57	Mountain root, meadow	17
Duogongma, Banma county, Golog Prefecture	DGM	100°25′31.9″	33°5′54.8″	3811	0.0	624.35	Benchland	12
Shangongma, Gande county, Golog Prefecture	SGM	99°39′17.4″	33°52′12.4″	4061	−1.9	527.39	Valley, meadow	14
Xiagongma, Gande county, Golog Prefecture	XGM	100°8′23.9″	33°50′29.2″	3926	−0.9	551.06	Valley, meadow	14
Jianshe, Dari county, Golog Prefecture	JS	99°25′51.9″	33°46′24.6″	3976	−1.4	520.16	Mountain root, meadow	17
Deang, Dari county, Golog Prefecture	DA	100°7′15.1″	33°25′20.2″	4147	−1.6	591.45	Hillside, meadow	12
Dangluo, Maqin county, Golog Prefecture	DL	99°29′27.7″	33°51′27.5″	4063	−1.9	519.87	Hillside, black soil land	15
Yeniugou, Qilian county, Haibei Prefecture	YNG	99°49′35.9″	38°17′22.9″	3074	−0.8	243.49	Hillside, shrub	12
Zhamashi, Qilian county, Haibei Prefecture	ZMS	99°59′10.9″	38°9′7.8″	3269	−0.9	256.46	Hillside, forest	6
Xianmi, Menyuan county, Haibei Prefecture	XM	102°5′20.8″	37°11′9.6″	3193	0.7	333.73	Cliff, shrub	6
Maixiu, Zeku county, Huangnan Prefecture	MX	101°56′38.1″	35°16′35.6″	3128	1.3	500.12	Hillside, forest	16
Lancai, Tongren county, Huangnan Prefecture	LC	101°51′8.8″	35°35′44.4″	2848	2.1	461.19	Valley, hillside, forest	5

AMT: annual mean temperature, AMT were collected from Scientific Database, Chinese Academy of Sciences, based on 1951-1980 year after year; AMP: annual mean precipitation, AMP were collected from Scientific Database, Chinese Academy of Sciences, based on 1971-2000 year after year.

Table 2
ISSR primers used for ISSR analysis and number of bands per primer.

Primers	Sequence 5′ → 3′	T_m (°C)	No. of bands scored	No. of polymorphic bands
809	$(AT)_8T$	53.2	23	20
811	$(GA)_8C$	52.5	25	24
825	$(AC)_8T$	52.0	27	27
834	$(AG)_8YT$	53.0	29	28
836	$(AG)_8YA$	53.0	24	22
840	$(GA)_8YT$	51.0	25	21
841	$(GA)_8YC$	53.2	23	22
842	$(GA)_8YG$	53.2	25	25
868	$(GGA)_6$	51.2	25	24
888	$BDB(CA)_7$	58.5	25	21
889	$DBD(AC)_7$	55.0	25	21
890	$VHV(GT)_7$	59.0	23	22
891	$HVH(TG)_7$	56.2	27	26
Total			326	303

Y = (C, T); B = (C, G, T); D = (A, G, T); H = (A, G, T); V = (A, C, G).

locus. The resulting binary data matrix was analyzed using POPGENE Version 1.32 (Yeh et al., 1999). Genetic diversity within and among populations were measured by the percentage of polymorphic bands (PPB), the effective number of alleles (n_e), observed number of alleles (n_a), Nei's (1973) gene diversity (*He*) and Shannon's information index (*I*). At the species wide level, total genetic diversity (H_t), genetic diversity within populations (H_s) and Nei's (1973) coefficient of genetic differentiation among populations (G_{st}, $G_{st} = (H_t - H_s)/H_t$) were calculated. Corresponding estimates of gene flow (N_m), i.e. the average per generation number of migrants exchanged among populations, was calculated using the formula: $N_m = 0.5\ (1 - G_{st})/G_{st}$ (McDermott and McDonald, 1993). All these calculations assumed that populations are in Hardy-Weinberg equilibrium. To examine the genetic relationship among populations, Nei's (1978) unbiased genetic distance and genetic identity were calculated for all pairwise combinations of populations as well. An unweighted pair-group method using arithmetic average (UPGMA) dendrogram was constructed based on the matrix of Nei's unbiased genetic distance using program NTSYSpc (Rohlf, 2000).

In addition, an analysis of molecular variance (AMOVA) procedure was used to estimate the partitioning of genetic variance among regions, among and within populations. Input data files for the AMOVA 1.55 (Excoffier et al., 1992) were generated using AMOVA-PREP (Miller, 1998). The variance components were tested statistically by nonparametric randomization tests using 1000 permutations. Principal coordinates analysis (PCoA) (Kovach, 1999) was performed to ordinate relationships among population with Nei's unbiased genetic distance matrix. In order to test the correlation between genetic and geographic distances (in kilometers), which were interpreted by the latitudes and longitudes with Mapinfo 8.0 Program, among populations, Mantel test was performed using TFPGA software (Miller, 1997) (computing 999 permutations).

Meanwhile, Pearson correlation analysis was used to look for the correlation between genetic diversity and geographic factors, including altitude, latitude, longitude, annual mean temperature and annual mean precipitation. All these analyses were calculated using the SPSS 11.0 software (SPSS, 2001).

Table 3
Genetic diversity within populations of *R. tanguticum*.

Population	n_a	n_e	*He*	*I*	PPB (%)
LC	1.2577	1.1831	0.1026	0.1499	25.77
ZMS	1.3098	1.2283	0.1261	0.1830	30.98
XM	1.3252	1.2336	0.1301	0.1895	32.52
YNG	1.4663	1.2971	0.1700	0.2515	46.63
DGM	1.4785	1.2894	0.1651	0.2460	47.85
XGM	1.4816	1.3270	0.1843	0.2704	48.16
DK	1.4877	1.2988	0.1741	0.2595	48.77
MX	1.5092	1.3353	0.1898	0.2796	50.92
DA	1.5184	1.3460	0.1962	0.2886	51.84
SGM	1.5276	1.3535	0.2019	0.2969	52.76
DL	1.5337	1.3454	0.1974	0.2917	53.37
JS	1.5613	1.3312	0.1926	0.2879	56.13
ZQ	1.5890	1.3636	0.2104	0.3127	58.90
Average	1.4651	1.3025	0.1724	0.2544	46.51
Species level	1.9294	1.4374	0.2689	0.4163	92.94

n_a: observed number of alleles; n_e: effective number of alleles; *He*: Nei's (1973) gene diversity; *I*: Shannon's information index; PPB: percentage of polymorphic bands.

Table 4
Results of analysis of molecular variance (AMOVA) of ISSR data from 13 populations of *R. tanguticum*.

Source of variance	d.f.	SSD	MSD	Variance component	Total variance (%)	Fixation index	*P*-value[a]
Among populations	12	2050.96	170.91	11.67	28.98	$\Phi_{st} = 0.290$	<0.001
Within populations	147	4203.40	28.60	28.60	71.02		<0.001
Among groups	2	507.77	253.88	2.98	7.13	$\Phi_{ct} = 0.071$	<0.001
Among populations	10	1543.20	154.32	10.22	24.45	$\Phi_{sc} = 0.263$	<0.001
Within populations	147	4203.43	28.60	28.60	68.42	$\Phi_{st} = 0.316$	<0.001

d.f.: degrees of freedom; SSD: sum of squares; MSD: mean squared deviation.
[a] Significance tests after 1000 permutation.

3. Results

3.1. Genetic diversity

From 160 individuals of thirteen wild populations, 13 primers yielded 326 clearly identifiable bands ranging in size from 200 bp to 2 800 bp, corresponding to an average of 25.08 bands per primer (Table 2). Of these bands, 92.94% (303 in total) were polymorphic among all individuals, i.e. the percentage of polymorphic bands (PPB) for this species was 92.94% (Table 3). But, at the population level, the percentage of polymorphic bands (PPB) ranged from 25.77 to 58.90%, with an average of 46.51%. The average effective number of alleles per locus was 1.3025. Assuming Hardy-Weinberg equilibrium, Nei's gene diversity (*He*) varied between 0.1026 and 0.2104, with an average of 0.1724, and Shannon's information index (*I*) ranged from 0.1499 to 0.3127, with an average of 0.2544. The values of *He* and *I* showed a similar trend to PPB. When calculated at the species level, the *He* and *I* values equalled 0.2689 and 0.4163 respectively, demonstrating a relatively high level of genetic diversity. Among the 13 populations investigated, population ZQ exhibited the highest genetic variability (PPB = 58.90%; *He* = 0.2104; *I* = 0.3127), whereas the population LC exhibited the lowest variability (PPB = 25.77%; *He* = 0.1026; *I* = 0.1499) as shown in Table 3.

3.2. Genetic differentiation and relationships

According to Nei's gene diversity and AMOVA analyses, the percentages of genetic variation among populations were 35.85% (G_{st}) and 29.00% (Φ_{st}), separately, both of which indicated that the genetic differentiation was found mainly within populations. AMOVA test also proved that differentiation among groups, among populations within groups and within populations was significant ($P < 0.001$, Table 4). Furthermore, the level of gene flow (N_m) was measured to be 0.8948 individual per generation between populations, suggesting that gene exchange between populations was low.

Genetic distances between populations of *R. tanguticum* ranged from 0.0621 (between SGM and XGM) to 0.1832 (between ZMS and LC), and the average Nei's genetic identity was 0.8833 (ranging from 0.8326 to 0.9398, Table 5). The result of the Mantel test with 1000 permutations revealed that significantly correlation was found between matrices of genetic distance and of geographic distance ($r = 0.573$, $P = 0.002$) (Fig. 2).

The UPGMA dendrogram, based on Nei's (1978) unbiased genetic distance matrix (Table 5) suggested that the eight populations (ZQ, DGM, DK, JS, DL, SGM, XGM and DA) from Golog Tibetan Autonomous Prefecture grouped together firstly, then clustered with the populations from Haibei Tibetan Autonomous Prefecture (XM, ZMS and YNG). The two populations from Huangnan Tibetan Autonomous Prefecture (MX and LC) formed the other cluster (Fig. 3). Principal coordinate analysis (PcoA) produced similar results. The first three PCoA accounted for 28.20%, 20.16% and 14.77% of the total variance, respectively (Fig. 4).

Table 5
Nei's (1978) unbiased measures of genetic distance (below diagonal) and genetic identity (above diagonal) between populations.

Population	XM	ZQ	ZMS	MX	LC	DGM	DK	JS	YNG	DL	SGM	XGM	DA
XM	*****	0.8505	0.8653	0.861	0.8359	0.862	0.862	0.8697	0.8781	0.8604	0.8686	0.8686	0.8829
ZQ	0.1619	*****	0.8949	0.8965	0.8461	0.9011	0.8887	0.8984	0.8797	0.9046	0.8957	0.9022	0.9016
ZMS	0.1447	0.1110	*****	0.8466	0.8326	0.857	0.8591	0.8682	0.8659	0.8532	0.8629	0.8653	0.8747
MX	0.1497	0.1092	0.1666	*****	0.9261	0.8778	0.8825	0.8862	0.8753	0.8795	0.8849	0.8827	0.8713
LC	0.1793	0.1671	0.1832	0.0768	*****	0.8614	0.8506	0.8415	0.8393	0.8483	0.8541	0.8406	0.8487
DGM	0.1485	0.1042	0.1543	0.1303	0.1492	*****	0.8905	0.8922	0.8775	0.8948	0.8983	0.8894	0.9156
DK	0.1485	0.1180	0.1518	0.1250	0.1618	0.1160	*****	0.9008	0.8719	0.9075	0.908	0.9068	0.9091
JS	0.1396	0.1072	0.1413	0.1208	0.1726	0.1141	0.1045	*****	0.8911	0.9156	0.9192	0.9173	0.9222
YNG	0.1300	0.1282	0.1440	0.1332	0.1752	0.1307	0.1371	0.1153	*****	0.8932	0.9081	0.8919	0.8842
DL	0.1504	0.1003	0.1587	0.1284	0.1645	0.1112	0.0970	0.0881	0.1130	*****	0.9294	0.9299	0.9296
SGM	0.1408	0.1101	0.1475	0.1222	0.1577	0.1073	0.0965	0.0843	0.0964	0.0732	*****	0.9398	0.9213
XGM	0.1409	0.1029	0.1446	0.1248	0.1737	0.1172	0.0978	0.0863	0.1144	0.0727	0.0621	*****	0.9291
DA	0.1245	0.1036	0.1339	0.1377	0.1640	0.0881	0.0953	0.0810	0.1231	0.0730	0.0820	0.0735	*****

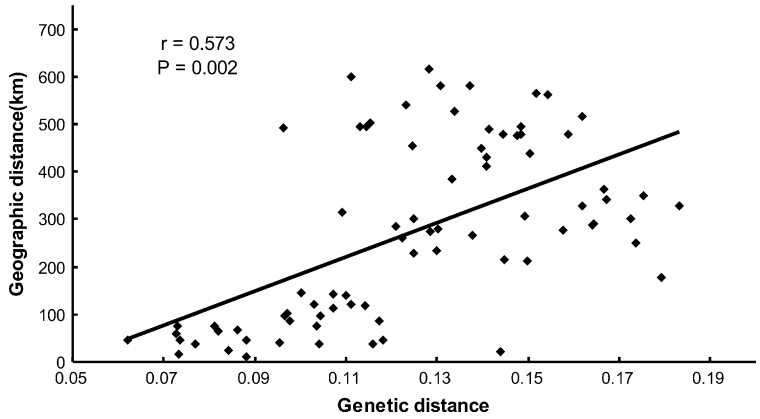

Fig. 2. Correlation between geographical distance and Nei's genetic distance revealed by the Mantel test.

3.3. Correlation between genetic diversity and geographic factors

Pearson's correlation analyses showed that a significantly positive correlation was found between altitude and genetic diversity ($r = 0.730$, $P = 0.005$ for *He*, Fig. 5 and Table 6; $r = 0.732$, $P = 0.004$ for *I*, Fig. 6 and Table 6; $r = 0.723$, $P = 0.005$ for PPB, Fig. 7 and Table 6). In contrast, genetic diversity significantly decreased with latitude ($r = -0.638$, $P = 0.019$ for *He*; $r = -0.648$, $P = 0.017$ for *I*; $r = -0.676$, $P = 0.011$ for PPB, Table 6 but figures not given). There was no significant correlation between longitude and gene diversity and Shannon's information index ($r = -0.542$, $P = 0.056$ for *He*; $r = -0.547$, $P = 0.053$ for *I*), but a reverse correlation between longitude and percentage of polymorphic bands ($r = -0.558$, $P = 0.048$). Moreover, genetic variation of *R. tanguticum* positively correlated with annual mean precipitation, but negatively correlated with annual mean temperature (Table 6).

4. Discussion

4.1. Genetic diversity

The present study revealed a relatively high level of genetic diversity in *R. tanguticum* based on ISSR markers (Table 3), compared with other species in the same family of Polygonaceae (Jin, 2007; Lu et al., 2008). Additionally, Nei's gene diversity (H_{es} and H_{ep} represent *He* at species and population levels respectively) and PPB (P_s and P_p represent PPB at species and population levels respectively) in *R. tanguticum* were considerably high when compared to long-lived perennial herbaceous

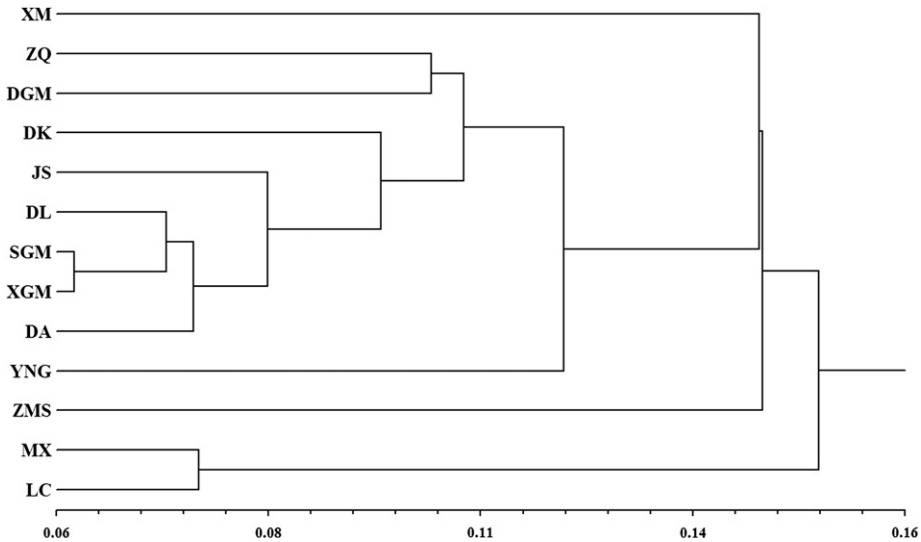

Fig. 3. UPGMA dendrogram of *R. tanguticum* based on Nei's (1978) genetic distances, indicating the clustering relationships of sampled populations.

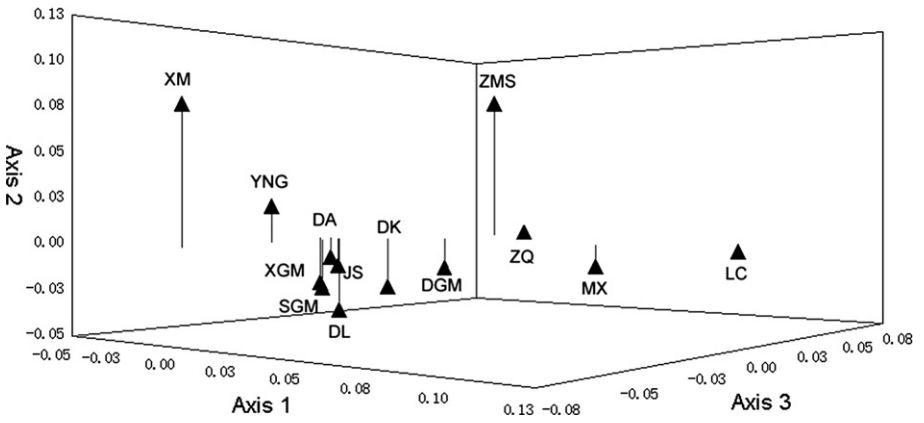

Fig. 4. Principle Coordinate analysis based on Nei's (1978) genetic distances.

($H_{es} = 0.1240$, $P_s = 39.30\%$, $H_{ep} = 0.0820$, $P_p = 21.50\%$) and dicotyledon ($H_{es} = 0.1360$, $P_s = 44.80\%$, $H_{ep} = 0.0960$, $P_p = 29.00\%$) based on isozyme analysis (Hamrick and Godt, 1989; Hamrick et al., 1992).

We accounted the possible reasons for high genetic diversity of *R. tanguticum* as followed. Firstly, mating systems have been postulated to be one of the most important factors that determine the genetic diversity in plant species (Hamrick, 1982; Hamrick and Godt, 1989). Although there was no document reporting on breeding system of *R. tanguticum*, our preliminary observations based on pollination experiments suggested that it is outcrossing. For instance, large panicles of numerous small flowers and extrorse anthers are characteristic of outcrossing plants. Moreover, *Rheum* plants are self-incompatible in nature (Komatsu et al., 2006b). Self-incompatibility is believed important in the maintenance of the high amount of genetic variability of species (Borba et al., 2001). Secondly, *R. tanguticum* could grow over twenty years and produce a great many seeds every year. The seed is of high rate of germination as well. So it is possible that the long-lived herbaceous habit providing more opportunity to accumulate mutant and abundant seeds may contribute to high amount of diversity in this species (Nybom, 2004; Zhao et al., 2007). Finally, the geographic distributing may affect the genetic diversity level of a species and in general widespread species may have higher level of genetic variability than narrowly distributed ones (Hamrick and Godt, 1996). *R. tanguticum* is narrowly distributed in Qinghai-Tibetan Plateau; however, it was once widespread in Qinghai Province (Chen, 2003).

4.2. Population genetic differentiation

Allozyme data (Hamrick and Godt, 1989, 1996) and RAPD (Nybom, 2004) markers showed that long-lived and outcrossing species typically retain most of their genetic variability within populations; on the contrary, annual and selfing species allocate more of their genetic variability among populations. The data on genetic structure of *R. tanguticum* obtained in the present study showed that the among-population differentiation coefficients ($\Phi_{st} = 0.290$ and $G_{st} = 0.3585$) were similar to, but a little higher than the average coefficients of long-lived perennial ($\Phi_{st} = 0.25$, $n = 60$; $G_{st} = 0.19$, $n = 24$) and outcrossing species ($\Phi_{st} = 0.27$, $n = 73$; $G_{st} = 0.22$, $n = 31$) (Nybom, 2004). This suggested high amount of diversity was found in intra-population and a moderate genetic differentiation among populations. The breeding system of flowering plant species greatly

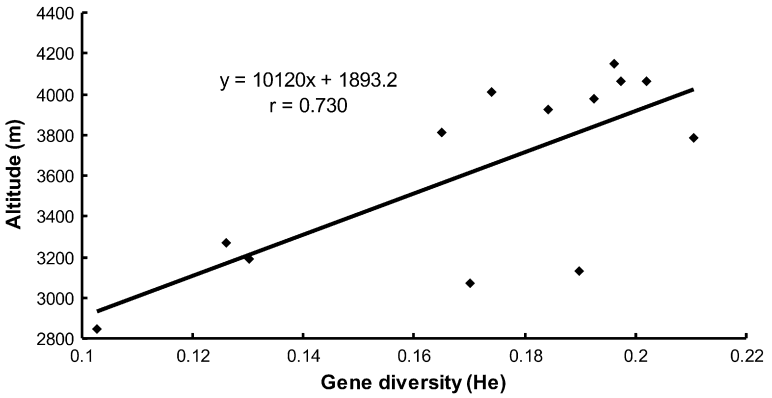

Fig. 5. The relationship between genetic diversity (*He*) and altitude.

Table 6
The relationship between genetic diversity parameters and geographic and ecological factors.

Pearson correlation	Altitude	Latitude	Longitude	AMT	AMP
He	0.730[b]	−0.638[a]	−0.542	−0.561[a]	0.580[a]
I	0.732[b]	−0.648[a]	−0.547	−0.557[a]	0.590[a]
PPB	0.723[b]	−0.676[a]	−0.558[a]	−0.523	0.619[a]

He: Nei's gene diversity; *I*: Shannon's Index; PPB: Percentage of polymorphic bands; AMT: annual mean temperature; AMP: annual mean precipitation.
[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level.

affects population genetic differentiation (Hamrick and Godt, 1989). Thus, the outcrossing breeding system probably accounted for high levels of genetic variation within populations of *R. tanguticum*. However, two other factors may have contributed to the strong population differentiation of this species. On the one hand, *R. tanguticum* is an important traditional medicinal plant and both the individuals and its habitat have decreased greatly due to human over-exploitation recently. For example, the size of some populations like ZMS and XM in this study is small, and only a few individuals were existed. The consequent reductions in its distribution and population size may have promoted genetic differentiation among isolated populations (Ellstrand and Elam, 1993). On the other hand, *R. tanguticum* is mainly distributed in forests on slopes or in valleys of Qinghai-Tibetan Plateau where high mountains and deep valleys are abundant. The complex topography of the region may have hindered gene flow via both pollen and seeds among populations, which was confirmed by the limited gene flow ($N_m = 0.8948$), thus promoted population differentiation. This interpretation was supported by the results of the cluster and principal coordinates analysis. Most populations from the same region (such as Golog and Huangnan Tibetan Autonomous Prefectures) clustered together that are in accordance to the geographic distribution, while populations from Haibei Tibetan Autonomous (YNG, ZMS and XM) were not clustered together but separated by high mountains.

In addition, previous studies of *R. tanguticum* reported that the genetic distance was not significantly correlated with geographic distance (Chen et al., 2009). However, this study showed an opposite finding. The reason for the contradiction between these two studies might be the different geographic scales of the subject investigated. The sample sites in this study nearly cover all types of habitats that *R. tanguticum* grows, while a relatively small geographic scale in the other research. Similar results were reported in previous studies that there existed no correlation between genetic and geographic distances at the microgeographic scale (Li et al., 1995, 2004; Fu et al., 2002), but a significant correlation in large scale (Yang et al., 2007; Zheng et al., 2008; Liu et al., 2009). This also suggested that the sampling strategy is an important factor in genetic diversity studies, and the samples should represent all types of habitats and distribution of the species to the greatest extent possible.

4.3. Correlation of geographic factors and genetic diversity

The genetic diversity of *R. tanguticum* increased with altitude and decreased with latitude; the possible reasons might be the environmental conditions (e.g. temperature and humidity). The relationships of genetic diversity with annual mean temperature and annual mean precipitation gave a further explanation for this phenomenon.

Increased genetic variation with higher altitude provided genetic basis for the adaption to high altitude and low temperature habitat of *R. tanguticum*. Genetic variation of other alpine plants like *Rhodiola angusta* (Yan et al., 1999) was also found negatively correlated with annual mean temperature. Our laboratory tests showed that under constant temperature conditions, the germination rate of *R. tanguticum* decreased with higher temperature (Xie, 2009). The impact of temperature on the germination rate of *R. tanguticum* further illustrated the natural distribution of this species which is endemic to alpine cold areas. At higher altitude, the lower temperature facilitates seed germination and helps to increase plants' genetic diversity. Besides, significantly positive correlation between genetic diversity of *R. tanguticum* and precipitation indicated that higher precipitation might help in seedling survival, and then have great influence on genetic diversity (Liu et al., 2009).

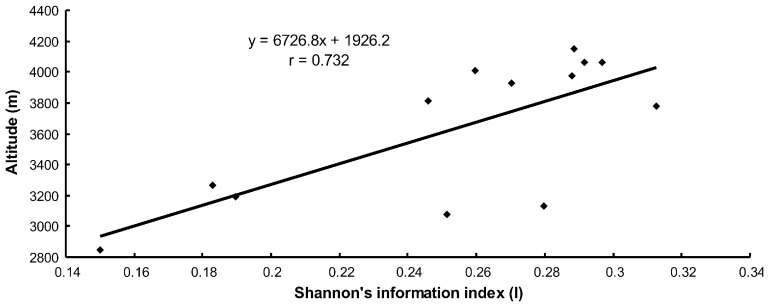

Fig. 6. The relationship between genetic diversity (*I*) and altitude.

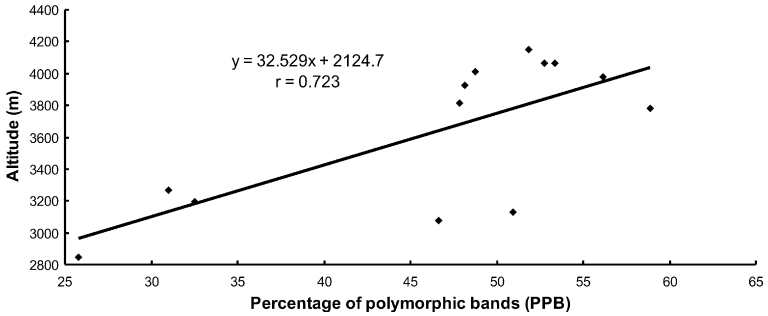

Fig. 7. The relationship between genetic diversity (PPB) and altitude.

4.4. Conservation and breeding consideration

Successful management and preservation of populations of threatened species depend on a good understanding of the distribution of genetic variation in the species (Francisco-Ortega et al., 2000; Wallace, 2002). Our findings would provide important genetic information for developing conservation strategies and cultivation of *R. tanguticum*.

One primary objective of nature conservation is to maintain genetic diversity. Considering most of the genetic variation within populations and high genetic differentiation among populations, the strategy of conservation for *R. tanguticum* should include both in situ and ex situ methods. In situ method pays more attention to restore the suitable habitats and the effective population size. For those populations with high levels of genetic variation of different regions such as ZQ, YNG and MX, we suggest that their habitats be protected and the exploitation of wild resources be forbidden. For ex situ conservation, we need to carefully design and establish a germplasm bank for this species. Collecting seeds and transferring seedlings from different populations to the suitable habitats for *R. tanguticum* and artificially increase the gene flow among populations. According to our field survey, the over-exploitation of natural populations and the extensive loss of habitats have seriously threatened the populations ZMS and XM, which should be the priority sites for ex situ conservation. Moreover, further information on cultivation, which may affect the maintenance of variation in the population, is needed before it can be accepted that this is a satisfactory conservation strategy.

Currently, the bulk demands of *R. tanguticum* for its great medicinal value cause the over-exploitation of wild resources. Numerous cultivars of *R. tanguticum* have been planted, but the good and bad are intermingled. Therefore, breeding high quality of varieties is extremely urgent. In fact, the quality of varieties is determined by the chemical constituents of medicine. So analysis of chemical constituents of these sampled populations will be studied in future. Combined with both genetic structure and chemical quality will assist in breeding excellent germplasm of *R. tanguticum*.

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