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Genetic diversity of the endangered and medically important *Lycium ruthenicum* Murr. revealed by sequence-related amplified polymorphism (SRAP) markers

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ABSTRACT

Sequence-related amplified polymorphism (SRAP) markers were used to assess the genetic diversity and population genetic structure in fourteen wild populations of *Lycium ruthenicum* from Northwestern China. Thirty-one selected primer combinations produced 468 discernible bands, with 398 (85.04%) being polymorphic, indicating relatively high genetic diversity at the species level. Analysis of molecular variance showed that the genetic variation was found mainly within populations (84.45%), but variance among populations was only 15.55%. And there was a moderate genetic differentiation ($G_{st} = 0.2155$) among populations. Mantel test revealed a significant correlation between genetic and geographic distances ($r = 0.303$, $P = 0.004$), and the unweighted pair-group method using arithmetic average clustering and principal coordinates analysis demonstrated similar results. A total of nine significant ($P < 0.05$) correlations were detected between three indices of genetic diversity and seven ecogeographic factors. Also recommendations for conservation of the endangered species resources and breeding program are proposed.

1. Introduction

Lycium L. (Solanaceae) is a genus of approximately 80 species distributed worldwide, but particularly concentrated in South America, southwestern North America and southern Africa (Hitchcock, 1932; Chiang-Cabrera, 1981; Bernardello, 1986; Venter, 2000; Miller, 2002; Levin and Miller, 2005). *Lycium* species are long-lived perennial shrubs and many inhabit arid to semiarid environments, though some are found in coastal saline habitats. The majority of species are hermaphroditic, have perfect flowers, and produce red or purple, fleshy berries. *Lycium ruthenicum* Murr. is a unique nutritional and medicinal food, which mainly distributes in salinized desert of Northwestern China. Its special physiological characteristics of drought-resistance and salt-resistance make it an ideal plant for preventing soil desertification and alleviating the degree of soil salinity–alkalinity, which are very important for the ecosystem and agriculture in the remote area (Zhang et al., 2007; Zheng et al., 2011). In addition to that, *L. ruthenicum* has been recorded in Tibetan medical classic "Jing Zhu Ben Cao" as a traditional herb. Its ripe fruits had been used for treatment of heart disease, abnormal menstruation and menopause. Because of overexploitation and deterioration of its habitat, the number of populations and individuals of this species has dropped

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considerably in recent decades to the point where it is now considered to be threatened in China and has been listed among the important wild conservative plants list of Qinghai Province (Yang et al., 2007a; Chen et al., 2008b). So far, studies on *L. ruthenicum* have mainly focused on chemical components (Sheidai et al., 1999; Kosar et al., 2003; Altintas et al., 2006; Li et al., 2009; Zheng et al., 2011; Liu et al., 2012) and pharmacological properties (Li et al., 2007; Feng et al., 2009; Wang et al., 2009a; Zhao et al., 2011). Analysis of the genetic diversity and structure of an endangered species is an important prerequisite for conservation as it reflects the status and survival potential of populations (Lande, 1988). Although karyotype analysis revealed that the plant is diploid ($2n = 24$) (Sheidai et al., 1999; Chen et al., 2008a), the genetic diversity analysis among and within populations of *L. ruthenicum* remains unknown, and it is difficult to implement effective conservation strategies.

Sequence-related amplified polymorphism (SRAP), is a new molecular marker first introduced by Li and Quiros (2001), which aims at the amplification of open reading frames (ORFs). SRAP has several advantages: simplicity, reproducibility, reasonable throughput rate, easy isolation of bands for sequencing and it targets open reading frames (ORFs). Budak et al. (2004) compared four marker systems in buffalograss and found the values of revealing genetic diversity power as: SRAP > SSR > ISSR > RAPD. SRAP has been applied extensively in molecular identification, genetic linkage map construction, gene tagging, genomic and cDNA fingerprinting, genetic diversity analysis and comparative genetics of different species (Li and Quiros, 2001; Ferriol et al., 2003; Budak et al., 2004; Espósito et al., 2007; Ding et al., 2008; Wang et al., 2009b; Li et al., 2010; Cai et al., 2011; Masoud et al., 2012).

In the present study, we measured the genetic diversity within and among natural populations of *L. ruthenicum* sampled from Northwestern China using SRAP markers. The main objectives 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 elementary information for conservation and plant breeding of this endangered medicinal plant.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of 174 individuals of *L. ruthenicum* were collected from 14 natural populations in northwest 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

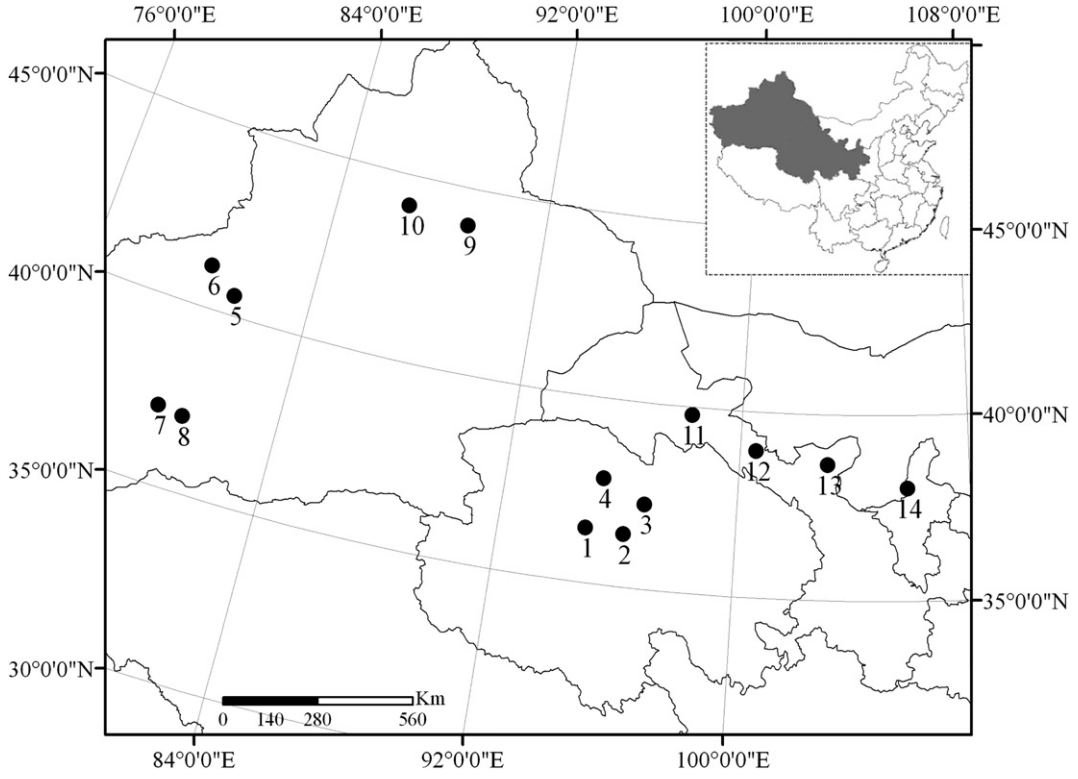

Fig. 1. Geographic distribution of the 14 sampled populations of *L. ruthenicum* in northwest China. For names of the numbered populations see list in Table 1.

Table 1
Sampling details of *L. ruthenicum* populations in the present study in northwest China.

No.	Locality	Pop.	Sample size	Lt[a] (N)	Ln[b] (E)	Al[c] (m)	AMT[d] (°C)	AMP[e] (mm)	AMH[f] (%)	AMSH[g] (h)
1	Geermu, Qinghai Province	GEM	17	36.47	94.95	2782	5.3	42.2	31.9	3096
2	Nuomuhong, Qinghai Province	NMH	20	36.41	96.24	2770	4.4	150.9	33.0	3094
3	Delingha, Qinghai Province	DLH	12	37.26	96.85	2814	3.7	176.1	34.7	3169
4	Dachaidan, Qinghai Province	DCD	12	37.84	95.36	3147	1.8	83.0	34.9	3257
5	Alaer, Xinjiang Province	ALE	11	40.53	81.29	1080	10.7	52.0	52.0	2915
6	Wensu, Xinjiang Province	WS	8	41.13	80.21	1135	10.3	74.4	52.0	2913
7	Moyu, Xinjiang Province	MY	5	37.11	79.91	1432	12.5	35.5	42.7	2587
8	Cele, Xinjiang Province	CL	11	37.01	80.80	1456	11.9	33.2	41.8	2580
9	Qitai, Xinjiang Province	QT	6	43.99	89.05	761	5.2	192	61.3	2987
10	Hutubi, Xinjiang Province	HTB	16	44.18	86.70	584	6.9	286.2	58.3	2523
11	Jiayuguan, Gansu Province	JYG	16	39.80	98.23	1715	8.0	80.5	43.3	3031
12	Zhangye, Gansu Province	ZY	13	38.94	100.56	1470	7.4	124.1	51.8	3085
13	Minqin, Gansu Province	MQ	13	38.65	103.08	1363	8.3	113.0	44.5	3073
14	Qingtongxia, Ningxia Province	QTX	14	38.03	105.86	1211	8.6	186.6	53.0	2906

The climatic data were collected from China Meteorological Data Sharing Service System, based on 1971–2000 year after year.
[a] Latitude.
[b] Longitude.
[c] Altitude.
[d] Annual mean temperature.
[e] Annual mean precipitation.
[f] Annual mean humidity.
[g] Annual mean sunshine hours.

back to our laboratory for DNA extraction. Genomic DNA was extracted using the modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The quality and quantity of DNA were determined by comparing the sample with known standards of lambda DNA in 1.0% (w/v) agarose gels. The isolated genomic DNA was diluted to 30 μg/ml with TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and stored at −20 °C for use.

2.2. SRAP analysis

SRAP analysis was carried out according to previously established protocols described by Li and Quiros (2001) with minor modifications. One hundred and eighty-two SRAP primer combinations were initially screened in eight representative samples (Table 2). Primer combinations were excluded from the study if their banding patterns were difficult to score or if they failed to amplify consistently in all lines. Of these 182 SRAP primer pairs, thirty-one primer combinations that produced consistent amplifications and clear polymorphic bands were selected to amplify the rest of the samples (Table 3). All samples were amplified at least three times if the initial amplification failed.

The PCR reaction mixtures (20 μl total volume) containing 2.0 μl of 10× PCR buffer (2.0 mM of $MgCl_2$), dNTPs 300 μmol, forward primer 0.4 μmol, reverse primer 0.4 μmol, Taq DNA polymerase 1 U (*TaKaRa* Biotech Co., Ltd., Dalian, China) and template DNA 30 ng. The amplifications were performed in a PTC-221 thermocycler (MJ Research, Bio-Rad, USA) using with following PCR program: 94 °C for 5 min, followed by 5 cycles of three steps: 1 min denaturing at 94 °C, 1 min annealing at 35 °C, and 1 min elongation at 72 °C. In the following 35 cycles the annealing temperature was increased to 50 °C, with a final elongation step at 72 °C for 10 min. The negative control was run by replacing template DNA with ddH_2O to test for the possibility of contamination. Amplification PCR products were electrophoresed in a 1.7% agarose gel containing 0.5 mg/ml

Table 2
The forward and reverse SRAP primers used in this study.

Forward primer (5′→3′)		Reverse primer (5′→3′)	
me1:	TGAGTCCAAACCGGATA	em1:	GACTGCGTACGAATTAAT
me2:	TGAGTCCAAACCGGAGC	em2:	GACTGCGTACGAATTTGC
me3:	TGAGTCCAAACCGGAAT	em3:	GACTGCGTACGAATTGAC
me4:	TGAGTCCAAACCGGACC	em4:	GACTGCGTACGAATTTGA
me5:	TGAGTCCAAACCGGAAG	em5:	GACTGCGTACGAATTAAC
me6:	TGAGTCCAAACCGGACT	em6:	GACTGCGTACGAATTGCA
me7:	TGAGTCCAAACCGGACA	em7:	GACTGCGTACGAATTCAA
me8:	TGAGTCCAAACCGGAAC	em8:	GACTGCGTACGAATTCTG
me9:	TGAGTCCAAACCGGAGA	em9:	GACTGCGTACGAATTCGA
me10:	TGAGTCCAAACCGGACG	em10:	GACTGCGTACGAATTCAG
me11:	TGAGTCCAAACCGGTCC	em11:	GACTGCGTACGAATTCCA
me12:	TGAGTCCAAACCGGTAG	em12:	GACTGCGTACGAATTATG
me13:	TGAGTCCAAACCGGTGC	em13:	TGTGGTCCGCAAATTTAG
me14:	TGAGTCCAAACCGGTCA		

Table 3
Polymorphism revealed by 31 SRAP primer combinations.

Primer combination	Total band	Polymorphic band	Percentage of polymorphic band
me1/em3	11	9	81.82
me2/em3	21	19	90.48
me2/em5	19	17	89.47
me3/em9	12	9	75.00
me4/em5	10	7	70.00
me4/em8	16	13	81.25
me4/em11	9	7	77.78
me5/em5	11	10	90.91
me5/em13	20	19	95.00
me6/em2	14	10	71.43
me6/em9	14	12	85.71
me7/em3	18	17	94.44
me7/em7	10	9	90.00
me7/em10	21	17	80.95
me8/em5	18	15	83.33
me8/em9	5	4	80.00
me8/em10	14	12	85.71
me8/em11	16	14	87.50
me8/em13	12	11	91.67
me9/em13	12	10	83.33
me10/em3	19	17	89.47
me10/em9	20	19	95.00
me11/em6	16	13	81.25
me11/em9	20	19	95.00
me11/em10	17	15	88.24
me12/em2	15	12	80.00
me12/em4	10	7	70.00
me12/em10	18	15	83.33
me13/em7	16	12	75.00
me14/em1	16	14	87.50
me14/em10	18	14	77.78
Total	468	398	85.04

ethidium bromide in TAE buffer and photographed on UV light (Gel Dox XR+, Bio-Rad USA). Molecular sizes of the DNA fragments were estimated using a DL5000 marker (*TaKaRa* Biotech Co., Ltd., Dalian, China).

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 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 (H_e) and Shannon's information index (I) (Lewontin, 1972). 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 were 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 with arithmetic mean (UPGMA) dendrogram was constructed based on the matrix of Nei's unbiased genetic distance using program NTSYSpc version 2.1 (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 (PCA) (Kovach, 1999) was performed to ordinate relationships among population with Nei's unbiased genetic distance matrix. The correlation between genetic distances and geographic (measured in kilometers) distances among populations was investigated by the Mantel test (Mantel, 1967).

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

3. Results

3.1. Genetic diversity

From 174 individuals of fourteen wild populations, 31 primers yielded 468 clearly identifiable bands with an average of 15.10 bands per primer combination (Table 3). Of these bands, 85.04% (398 in total) were polymorphic among all individuals, i.e. the percentage of polymorphic bands (PPB) for this species was 85.04% (Table 4). But, at the population level, the percentage of polymorphic bands (PPB) ranged from 36.54 to 64.53%, with an average of 49.02%. The average effective number of alleles (N_e) per locus was 1.2862. Assuming Hardy–Weinberg equilibrium, Nei's gene diversity (H_e) varied between 0.1360 and 0.2162, with an average of 0.1666, and Shannon's information index (I) ranged from 0.2007 to 0.3230, with an average of 0.2497. The values of H_e and I showed a similar trend to PPB. When calculated at the species level, the H_e and I values equaled 0.2112 and 0.3329 respectively, demonstrating a relatively high level of genetic diversity. Among the 14 populations investigated, population ZY exhibited the highest genetic variability ($H_e = 0.2162$; $I = 0.3230$; PPB = 62.39%), whereas the population QT exhibited the lowest variability ($H_e = 0.1360$; $I = 0.2007$; PPB = 36.54%) as shown in Table 4.

3.2. Genetic differentiation and relationships

According to Nei's gene diversity and AMOVA analysis, the percentages of genetic variation among populations were 21.55% (G_{st}) and 15.50% (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 5). Furthermore, the level of gene flow (N_m) was measured to be 1.8199 individual per generation between populations, suggesting that gene exchange between populations was relatively high. Genetic distances between populations of *L. ruthenicum* ranged from 0.0144 (between GEM and NMH) to 0.0934 (between DLH and QT), and the average Nei's genetic identity was 0.9502 (ranging from 0.9108 to 0.9857, Table 6). The result of the Mantel test with 1000 permutations revealed that significantly correlation was found between matrices of genetic distance and of geographic distance ($r = 0.303$, $P = 0.004$) (Fig. 2). The UPGMA dendrogram, based on Nei's (1978) unbiased genetic distance matrix (Table 6) suggested that the seven populations (GEM, NMH, DLH, DCD, ALE, WS and CL) from Qinghai and Xinjiang Province grouped together firstly, four populations (JYG, MQ, ZY and QTX) from Gansu and Ningxia Province grouped together secondly, and then clustered with the population (MY) from Xinjiang Province. The two populations from Xinjiang Province (QT and HTB) formed the other cluster (Fig. 3). The principal coordinate analysis (PCA) results indicated the first three characteristic vectors accounted for 77.69% of the variation (Fig. 4), which basically accorded with the clustering results by UPGMA.

3.3. Correlation between genetic diversity and ecogeographic variables

The Spearman rank correlations between the genetic indices (H_e, I and PPB) and ecogeographic variables were analyzed by the SPSS 11.0 software (SPSS, 2001) (Table 7). The results showed that a significantly positive correlation was found between altitude and genetic diversity ($r_s = 0.684$, $P = 0.007$ for H_e; $r_s = 0.678$, $P = 0.008$ for I; $r_s = 0.688$, $P = 0.007$ for PPB), and

Table 4
Genetic diversity within populations of *L. ruthenicum*.

Population	N_a[a]	N_e[b]	H_e[c]	I[d]	PPB[e] (%)
GEM	1.5043	1.2650	0.1588	0.2415	50.43
NMH	1.6453	1.3185	0.1911	0.2931	64.53
DLH	1.5470	1.3320	0.1918	0.2863	54.70
DCD	1.5321	1.3029	0.1771	0.2669	53.21
ALE	1.5064	1.3142	0.1799	0.2672	50.64
WS	1.4252	1.2530	0.1464	0.2191	42.52
MY	1.4103	1.2671	0.1531	0.2268	41.03
CL	1.4637	1.2729	0.1584	0.2372	46.37
QT	1.3654	1.2409	0.1360	0.2007	36.54
HTB	1.4444	1.2545	0.1481	0.2219	44.44
JYG	1.4786	1.2693	0.1589	0.2396	47.86
ZY	1.6239	1.3741	0.2162	0.3230	62.39
MQ	1.4509	1.2722	0.1579	0.2356	45.09
QTX	1.4658	1.2703	0.1581	0.2372	46.58
Population average	1.4902	1.2862	0.1666	0.2497	49.02
Species level	1.8504	1.3415	0.2112	0.3329	85.04

[a] Observed number of alleles.
[b] Effective number of alleles.
[c] Nei's (1973) gene diversity.
[d] Shannon's Information index.
[e] Percentage of polymorphic bands.

Table 5
AMOVA analysis of genetic structure.

Source of variance	d.f.[a]	SSD[b]	MSD[c]	Variance component	Total variance (%)	Fixation index	*P*-value[d]
Among populations	13	1721.05	132.39	7.45	15.55	$\Phi_{st} = 0.155$	<0.001
Within populations	160	6478.75	40.49	40.49	84.45		<0.001
Among groups	3	688.74	229.58	2.85	5.85	$\Phi_{ct} = 0.059$	<0.001
Among populations	10	1032.30	103.23	5.28	10.85	$\Phi_{sc} = 0.115$	<0.001
Within populations	160	6478.75	40.49	40.49	83.29	$\Phi_{st} = 0.167$	<0.001

[a] Degrees of freedom.
[b] Sum of squares.
[c] Mean squared deviation.
[d] Significance tests after 1000 permutation.

between annual mean sunshine hours and genetic diversity ($r_s = 0.626$, $P = 0.017$ for H_e; $r_s = 0.640$, $P = 0.014$ for I; $r_s = 0.684$, $P = 0.007$ for PPB). In contrast, genetic diversity significantly decreased with annual mean humidity ($r_s = -0.559$, $P = 0.038$ for H_e; $r_s = -0.575$, $P = 0.032$ for I; $r_s = -0.568$, $P = 0.034$ for PPB). There was no significant correlation between four eco-geographic factors (latitude, longitude, annual mean temperature and annual mean precipitation) and genetic diversity. Moreover, genetic variation of *L. ruthenicum* positively correlated with longitude, but negatively correlated with latitude, annual mean temperature and annual mean precipitation (Table 7).

4. Discussion

4.1. Genetic diversity

SRAP markers were applied to assess the level and pattern of genetic diversity in fourteen populations of *L. ruthenicum* in this research. Unique traits of the *Lycium* plants such as deceptive pollination strategies and dust-like, wind and small animals (birds and rodents) dispersed seed maintain high population numbers in some species while promoting outbreeding and gene flow between populations (Levin and Miller, 2005). Such traits are associated with high levels of within population diversity and lower genetic differentiation between populations (Hamrick and Godt, 1996). The genetic diversity between populations at species level ($H_e = 0.2112$) was very similar to the average genetic diversity level between populations ($H_e = 0.22$) estimated by Nybom (2004) based on RAPD, ISSR and AFLP analysis. Additionally, Nei's gene diversity (H_{es} and H_{ep} represent H_e at species and population levels respectively) and PPB (P_s and P_p represent PPB at species and population levels respectively) in *L. ruthenicum* were considerably high when compared to the average of Solanaceae plant ($H_{es} = 0.0940$, $P_s = 32.00\%$) (Hamrick and Godt, 1996), long-lived perennial woody plants ($H_{es} = 0.1770$, $P_s = 65.00\%$, $H_{ep} = 0.1480$, $P_p = 49.00\%$) 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). Also comparing with these Solanaceae plants of similar life history characteristics (Zhao et al., 2010; Cheng et al., 2000; Adam et al., 1995), *L. ruthenicum* possesses as much genetic diversity as the same family of other plants.

There were some possible reasons we accounted for high diversity of *L. ruthenicum* as followed. Firstly, the life history characteristics of species, especially breeding system, have an important effect on both genetic diversity and population structure (Hamrick, 1982; Hamrick and Godt, 1989; Nybom and Bartish, 2000; Nybom, 2004). *L. ruthenicum* is pollinated by insects and wind and the seeds are mainly spread by birds and rodents. Self-incompatibility is believed important in the maintenance of the high levels of genetic variability of species (Borba et al., 2001). Although there was no document reporting

Table 6
Nei's (1978) unbiased measures of genetic distance (below diagonal) and genetic identity (above diagonal) between populations.

Population	GEM	NMH	DLH	DCD	ALE	WS	MY	CL	QT	HTB	JYG	ZY	MQ	QTX
GEM	****	0.9857	0.9648	0.9667	0.9648	0.9611	0.9454	0.9596	0.9260	0.9358	0.9543	0.9432	0.9498	0.9438
NMH	0.0144	****	0.9785	0.9746	0.9733	0.9683	0.9544	0.9630	0.9294	0.9451	0.9636	0.9589	0.9574	0.9529
DLH	0.0358	0.0217	****	0.9643	0.9643	0.9565	0.9398	0.9503	0.9108	0.9264	0.9415	0.9477	0.9388	0.9324
DCD	0.0339	0.0257	0.0363	****	0.9624	0.9591	0.9315	0.9484	0.9245	0.9411	0.9535	0.9441	0.9510	0.9476
ALE	0.0359	0.0270	0.0364	0.0383	****	0.9751	0.9545	0.9695	0.9261	0.9444	0.9556	0.9571	0.9523	0.9467
WS	0.0397	0.0322	0.0445	0.0418	0.0252	****	0.9492	0.9690	0.9226	0.9406	0.9586	0.9460	0.9506	0.9470
MY	0.0562	0.0467	0.0620	0.0710	0.0466	0.0521	****	0.9497	0.9286	0.9376	0.9419	0.9570	0.9370	0.9283
CL	0.0412	0.0377	0.0510	0.0530	0.0310	0.0315	0.0516	****	0.9209	0.9400	0.9533	0.9485	0.9476	0.9412
QT	0.0769	0.0732	0.0934	0.0785	0.0768	0.0805	0.0741	0.0824	****	0.9753	0.9369	0.9294	0.9323	0.9434
HTB	0.0664	0.0564	0.0765	0.0607	0.0572	0.0612	0.0645	0.0619	0.0250	****	0.9593	0.9454	0.9518	0.9570
JYG	0.0468	0.0371	0.0603	0.0476	0.0454	0.0423	0.0599	0.0478	0.0652	0.0415	****	0.9660	0.9732	0.9663
ZY	0.0585	0.0420	0.0537	0.0575	0.0438	0.0555	0.0440	0.0529	0.0732	0.0562	0.0346	****	0.9615	0.9561
MQ	0.0515	0.0435	0.0631	0.0502	0.0489	0.0506	0.0651	0.0538	0.0701	0.0494	0.0272	0.0393	****	0.9609
QTX	0.0579	0.0482	0.0700	0.0539	0.0548	0.0545	0.0745	0.0606	0.0583	0.0439	0.0343	0.0449	0.0399	****

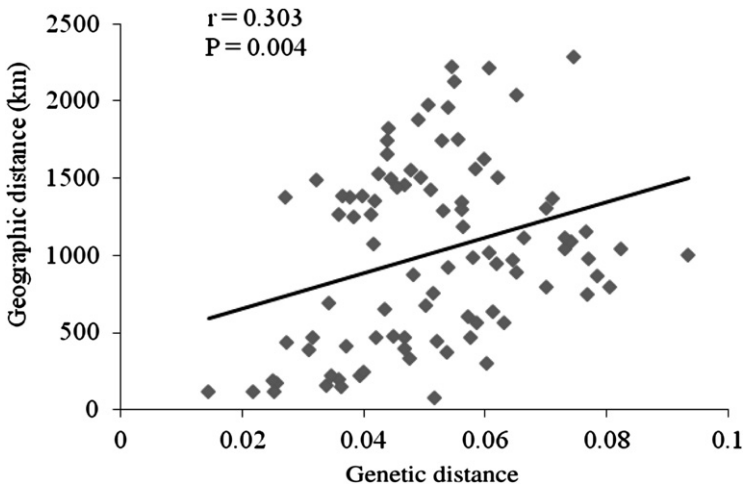

Fig. 2. Correlation between geographical distance and Nei's genetic distance revealed by the Mantel test.

on breeding system of *L. ruthenicum*, our preliminary observation through pollination experiments suggested that it is self-incompatible, which was consistent with the results through analysis of partial S-RNase sequences from *Lycium parishii* and investigation of self-incompatibility in this species (Savage and Miller, 2006). In addition, numerous long-lived perennials with mixed mating systems have higher gene diversity compare to plants that are annuals or short-lived perennials (Hamrick and Godt, 1996; Nybom and Bartish, 2000; Nybom, 2004). *L. ruthenicum* is a perennial shrub. Its long life span may contribute to the high level of genetic diversity at species level (Cai et al., 2011; Nybom, 2004). Finally, the geographical distribution 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). Previous investigation showed that *L. ruthenicum* was widely

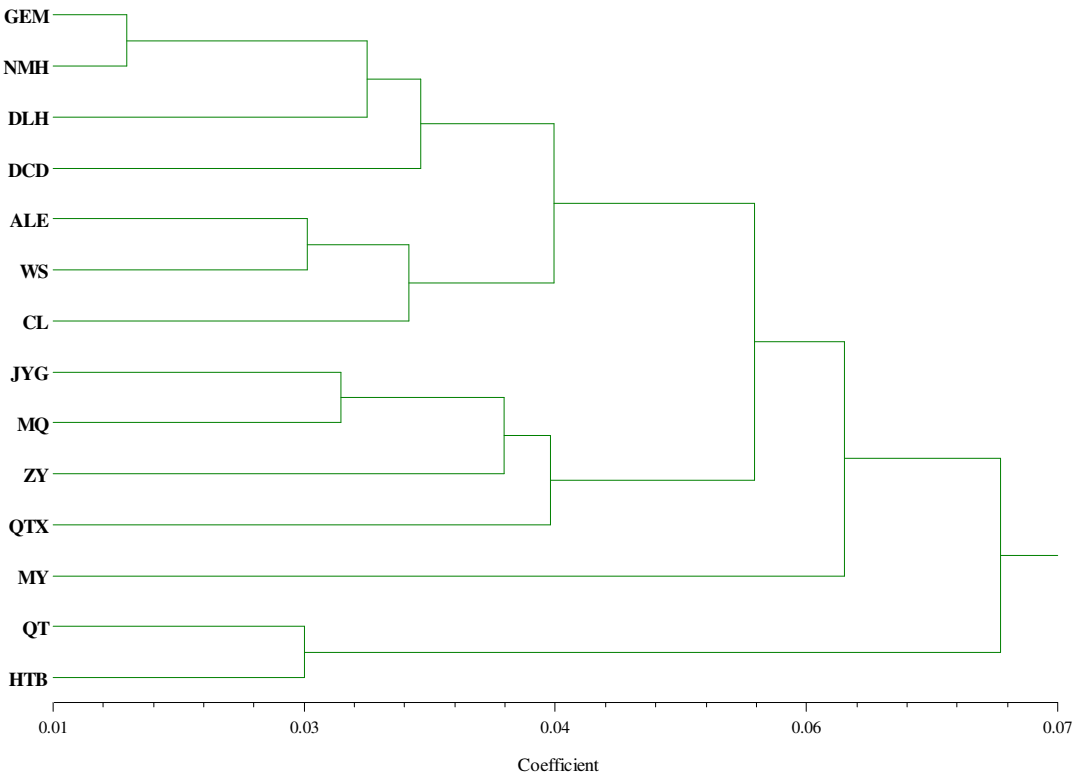

Fig. 3. UPGMA dendrogram of *L. ruthenicum* based on Nei's (1978) genetic distances, indicating the clustering relationships of sampled populations.

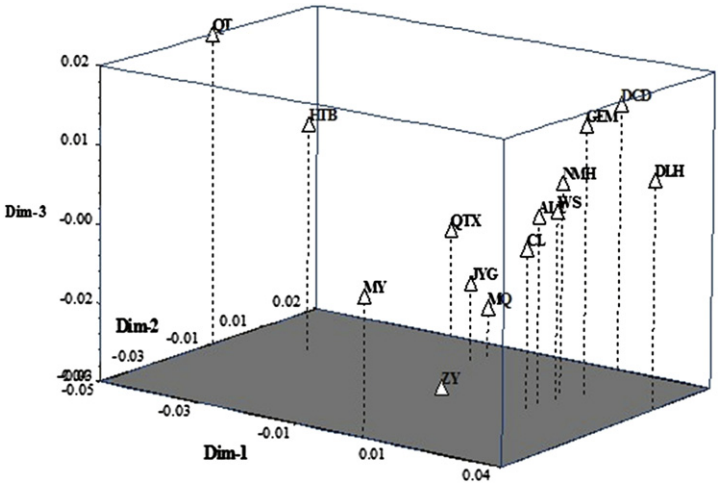

Fig. 4. Principle of coordinate analysis based on Nei's (1978) genetic distance.

distributed in northwest China (Dong et al., 2008; Zheng et al., 2011), but it reduced severely since 1960s because of habitat deterioration and human over exploitation (Gan et al., 1997; Chen et al., 2008a,b).

4.2. Population genetic structure

Gene differentiation and gene flow are important indices to estimate the population genetic structure of species. Significant heterogeneity in the genetic structure of *Lycium* populations was recently documented in a comprehensive review by Yin et al. (2005) and Miller et al. (2008). The value of Φ_{st} and G_{st} in this species were 0.1550 and 0.2155, respectively, indicating that our results about estimates of genetic structuring were consistent with the studies of Zhao et al. (2010). Also Zhao et al. (2010) found moderate genetic differentiation among populations of *Lycium* accessions. In addition, the genetic among-population differentiation coefficient for this species was similar to, but a little lower than the coefficients of long-lived perennial and outcrossing species (Hamrick and Godt, 1989, 1996). This differentiation was also confirmed by the AMOVA analysis, which indicated that around 15.55% of the total molecular variance was attributable to among-population diversity and 84.45% was attributable to within-population diversity (Table 5).

Gene flow, the movement of gene within and between populations, is negatively correlated with gene differentiation (Grant, 1991). Solanaceae seeds are very small and light and dispersed by wind and birds for long distance and this may be important in promoting gene flow (Flora Reipublicae Popularis Sinicae, 1978; Levin and Miller, 2005). The gene flow estimate was 1.8199 among populations and this level of migration will prevent continued divergence among populations (Wright,

Table 7
Spearman rank correlations (r_s) between genetic diversity parameters and ecogeographic factors.

		Lt[a]	Ln[b]	Al[c]	AMT[d]	AMP[e]	AMH[f]	AMSH[g]
H_e[h]	r_s	−0.437	0.424	0.684**	−0.341	−0.064	−0.559*	0.626*
	p	0.118	0.131	0.007	0.233	0.829	0.038	0.017
I[i]	r_s	−0.482	0.427	0.678**	−0.356	−0.057	−0.575*	0.640*
	p	0.081	0.128	0.008	0.211	0.846	0.032	0.014
PPB[j]	r_s	−0.451	0.477	0.688**	−0.477	0.051	−0.568*	0.684**
	p	0.106	0.085	0.007	0.085	0.864	0.034	0.007

** Correlation is significant at the 0.01 level.
* Correlation is significant at the 0.05 level.
[a] Latitude.
[b] Longitude.
[c] Altitude.
[d] Annual mean temperature.
[e] Annual mean precipitation.
[f] Annual mean humidity.
[g] Annual mean sunshine hours.
[h] Nei's (1973) gene diversity.
[i] Shannon's Information index.
[j] Percentage of polymorphic bands.

1951). This can be explained by the fact the *L. ruthenicum* is still common within the study area with numerous populations scattered around. The outcrossing system usually promotes high gene flow between populations, reduces between-population differentiation and maintains a higher level of overall genetic diversity (Hamrick and Godt, 1989). So a second explanation can be found in the breeding system of the species. *L. ruthenicum* is a cross-pollination plant and seeds can be dispersed very long-distance by wind and birds (Li et al., 2011; Levin and Miller, 2005). In natural conditions, millions of seeds mature and escape from the fruits in the late October. At the same time, the sand-blowing wind and sandstorms usually reach the distribution areas of *L. ruthenicum* in Northwestern China, which will be help to disperse the light seeds. However, *L. ruthenicum* mainly distributes in salinized desert, and high mountains and deserts are common in the region (Dong et al., 2008). The complex topography may have hindered gene flow via both pollen and seeds among populations, which was confirmed by the limited gene flow, and 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 Qinghai and Gansu provinces) clustered together that are in accordance to the geographic distribution, while populations from Xinjiang province (ALE, WS MY, CL, QT and HTB) were not clustered together but separated by Tianshan Mountains and the Taklimakan Desert.

In summary, our genetic analysis indicated that the *L. ruthenicum* populations were characterized by a high level of genetic diversity and moderate population differentiation (Tables 3–5). Three possible factors may account for such a genetic pattern. Firstly, *L. ruthenicum* is monoecious and is a cross-pollination species. Its pollen is dispersed by the wind and insects over long distances, and this is likely to promote gene flow between isolated populations. This is undoubtedly likely to enhance intraspecific diversity and reduce inter-population differentiation (Hedrick, 2004). Secondly, majority of individuals of this species are long-lived and have partial clonal growth (Wang, 2011; Dong et al., 2008). The current stands in most populations are, therefore, likely to be well established for a long time through cloning reproductions while these daughter ramets maintained the same genetic diversity as their parents (Vonlanthen et al., 2010). Thirdly, the long isolation between the sampled populations may have occurred recently because of climatic changes and human disturbance. For example, most shrubberies composed of this species were cut for agricultural purposes during the period from 1950 to 1960 (Chen et al., 2006). In addition, during the past 100 years, precipitation in the northwest has declined dramatically and most rivers have dried up (Zhang et al., 2003). The shortage of water and expansion of the desert resulted in the death of *L. ruthenicum* at a number of sites. However, at the other sites, a few populations persist due to the availability of waters in the microhabitats. The long life cycle and clonal reproduction of this species may have helped these current fragmented populations to retain their historical genetic diversity when these populations were connected. Therefore, it is imaginable that the fragmentations are too short to cause the genetic differentiations between populations. Overall, our findings suggest that the endangered status of the species is mainly due to anthropologic and environmental effects rather than a lack of genetic diversity.

In addition, there were some previous researches reported that the genetic distance was not significantly correlated with geographic distance (Cuevas-Arias et al., 2008; Shang et al., 2010). However, this study showed an opposite finding. The reason for the contradiction between these studies might be the different geographic scales of the subject investigated. The sample sites in this study nearly cover all types of habitats that *L. ruthenicum* grows, while a relatively small geographic scale in the other research. Also similar results were reported in related previous studies that there existed no correlation between genetic and geographic distances at the microgeographic scale (Fu et al., 2002; Li et al., 2004; Chen et al., 2009; Dong et al., 2010), but a significant correlation in large scale (Yang et al., 2007b; Zheng et al., 2008; Liu et al., 2009; Hu et al., 2010). 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 genetic diversity and ecogeographic variables

The genetic diversity of *L. ruthenicum* 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 adaptation to high altitude and low temperature habitat of *L. ruthenicum*. Genetic variation of other alpine and desert plants (e.g., *Rheum tanguticum* (Hu et al., 2010), *Rhodiola angusta* (Yan et al., 1999) and *Populus euphratica* (Wang et al., 2011)) was also found negatively correlated with annual mean temperature. The impact of temperature and humidity on the germination rate of *L. ruthenicum* further illustrated the natural distribution of this species which is adaptation to alpine cold and dry desert environment. At higher altitude, the lower temperature facilitates seed germination and helps to increase plants' genetic diversity. Besides, significantly positive correlation between annual mean sunshine hours and genetic diversity of *L. ruthenicum* indicated that more sunshine hours might help in seedling survival and growth of plants, and then have great influence on genetic diversity (Arbabi, 2011; Xie et al., 2009; Chloupek et al., 2004).

4.4. Implications for conservation

Genetic diversity is critically important for a species to maintain its evolutionary potential to cope with an ever changing environment; loss of genetic diversity is often associated with reduced fitness. The maintenance of genetic variation is a major objective within conservation plans for endangered species (Avise and Hamrick, 1996). Information obtained in this study provides valuable baseline data on population genetics, which can contribute to conservation plans or be used to optimize the

artificial propagation of this endangered species. Firstly, in situ conservation strategies should be adopted to protect and restore all existing populations of *L. ruthenicum*. Anthropogenic habitat destruction should be prevented, in order to help seedling establishment and recruitment of new individuals, increasing the population size through natural regeneration. Also cutting adult trees and digging seedlings must be prohibited to maintain effective population sizes. Population size is an important restoration consideration in endangered and rare species (Allendorf, 1986). A further management measure should aim at increasing the number of plants in small populations by reintroduction from nursery. Secondly, the construction of an in situ conservation area is an ideal way to protect wild *L. ruthenicum* genetic resources. It will result in effective conservation of their genetic resources and the evolution of the resources under natural environments. Thirdly, 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 *L. ruthenicum* 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 MY and QT, 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. Fourthly, Hybridizing between cultivars and wild *L. ruthenicum* resources must be prevented and reducing interference of cultivars should take place to ensure that the genes of wild resources are excellent and homozygous.

However, the bulk demands of *L. ruthenicum* for its great medicinal value cause the over-exploitation of wild resources in recent years. Numerous cultivars of *L. ruthenicum* 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 *L. ruthenicum*.

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