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Full Length Research Paper

Genetic diversity of *Stipa bungeana* populations in the Loess Plateau of China using inter-simple sequence repeat (ISSR) markers

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Inter-simple sequence repeat (ISSR) markers were used to determine the genetic diversity and genetic differentiation for eight natural populations of *Stipa bungeana* in the Loess Plateau of China. 15 primers produced stable and reproducible amplification bands which were selected from the screened 96 primers. Among a total of 390 amplified bands, 335 (85.90%) were polymorphic loci. At species level, Nei's gene diversity index and Shannon's information index were 0.1633 and 0.2703, respectively. Based on the cluster analysis, the eight populations were divided into three groups. Analysis of molecular variance (AMOVA) demonstrated that the genetic variation was found mainly among populations, which accounted for 68.71% of the total variation, but the within-population accounted for 31.29%. Mantel test revealed no significant correlation between genetic distance and geographic distance (r= -0.1345, P= 0.71). In this study, the lower genetic diversity of *S. bungeana* may be related with human activities and habit destroyed. The conservation strategies further were proposed for this plant.

Key words: *Stipa bungeana*, inter simple sequence repeats, genetic diversity, the Loess Plateau.

INTRODUCTION

Understanding the genetic diversity of a species and a population with the relation to ecological factors is a prerequisite for effective conservation and management of a species and a population. The genetic diversity and genetic differentiation of a species was considered important for species diversity and protection (Zhao et al., 2006). Genetic diversity within populations was considered highly important for possible adaptation to environmental changes for long-term survival of plant species (Bauert et al., 1998). To preserve the genetic diversity of population was considered important to stabilize population and ecosystem dynamics (Hughes et al., 2008). The genetic structure of population were affected by various environment and human activity factors, including life history, mecological traits, reproductive mode, over grazing and mowing and fire disturbance (Nybom, 2004; Mohamed et al., 2010) .

The Loess Plateau of China is located in the upper and middle of Yellow River. The total area is about 52 million hectares. At present, the Loess Plateau is a typical region of ecological fragile that is suffering from water and soil erosion and drought for a long time. The amount of annual soil erosion is estimated to be over 2200 million tons (Zhang et al., 2010). Under this weak condition, *Stipa bungeana* is the dominant and constructive species of the typical steppe in the Loess Plateau (Cheng et al., 2011). Its distribution covers some major provinces in China including Tibet, Gansu, Ningxia, Xinjiang, Qinghai, Shaanxi, Shanxi, and Inner Mongolia. In addition, *S. bungeana* is one of the major grassland types in the temperate zone of Asia. *S. bungeana* is a perennial grass which clonally grows by tiller. It mainly depends on vegetative propagation by repeatedly producing tiller

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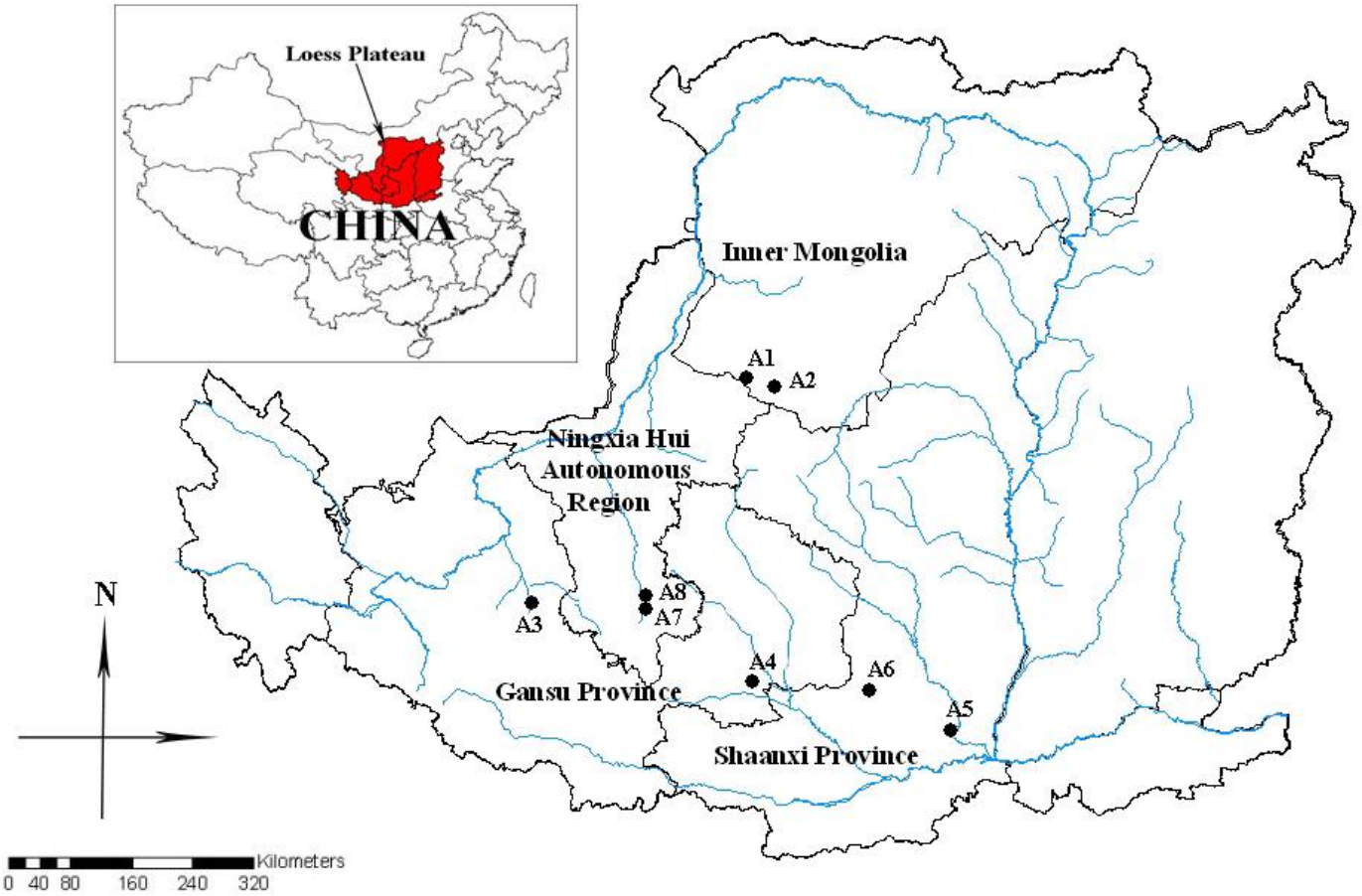

Figure 1. Geographic distribution of the eight sampled populations of S. bungeana in the Loess Plateau.

ramets from shoot base. It have large root systems and big crown width to promote few seed survival depending on the water and nutrients offered by parents. *S. bungeana* contains high level of nutrients and palatable for livestock. It tolerates drought and grazing and can be grazed by sheep in different growth stages. Therefore, it is important for ecological restoration in the Loess Plateau.

Gustafson et al. (1999) showed that the genetic variation of dominant (constructive) species could provide the information for the utilization and protection of these species and the evaluation of the ecosystem's genetic health. At present, the genetic diversity of a few *Stipa* genus plants have been reported including *Stipa grandis*, *Stipa purpurea* and *Stipa tenacissima* (Zhao et al., 2006, 2008; Wu et al., 2010; Mohamed et al., 2010; Liu et al., 2009). A number of studies have reported on *S. bungeana* ecology (Cheng et al., 2011; Huang et al., 2001a, 2001b). However, up till now, there is no report on the genetic diversity and the genetic structure of the *S. bungeana* populations from the Loess Plateau of China. Inter-simple sequence repeat (ISSR) is now established as a powerful approach for detecting species and population genetic diversity and differen-tiation (Zietkiewicz et al., 1994; Zhao et al., 2008).

In this study, ISSR molecular markers were used to analyze the genetic diversity and genetic differentiation of *S. bungeana* populations in the Loess Plateau of China. The objectives of this study were to (1) investigate the level of genetic diversity of *S. bungeana* natural populations; (2) reveal the level of genetic variations within and among populations; (3) analyze the relationship between and among genetic diversity and environmental factors; (4) provide scientific conservation strategies for *S. bungeana*.

MATERIALS AND METHODS

Sample collection

A total of 160 individuals of *S. bungeana* were collected from eight populations in Loess Plateau in September 2010 (Figure 1). Habitat conditions of the populations are shown in Table 1. 20 individuals from each population were sampled randomly, and the distance between individual plants were kept at least 10 cm within the same population. Young and fresh leaves were collected from each sampled individuals and immediately stored in zip lock bags with silica gel and taken back to the laboratory and stored at -80°C in ultra-freezer for DNA extracted.

Table 1. Locations and the habitat characters.

Location	ID	Altitude (m)	Longitude (E)	Latitude (N)	Annual mean precipitation (mm)	Annual mean temperature (°C)	Habitat
Chengchuan, Inner-Mongolia	A1	1364	107°23′	38°06′	294.1	7.4	Desert steppe
Machangjie, Inner-Mongolia	A2	1354	107°43′	38°02′	294.1	7.4	Desert steppe
Huining, Gansu	A3	1726	105°01′	35°58′	340.0	6.4	Typical steppe
Jingchuan, Gansu	A4	1305	107°31′	35°19′	555.0	10.0	Forest steppe
Pucheng, Shaanxi	A5	430	109°45′	34°53′	533.2	13.3	Forest steppe
Binxian,Shaanxi	A6	1161	108°50′	35°15′	602.3	9.2	Forest steppe
Nanshan, Yunwu Mountain,Ningxia	A7	2049	106°37′	36°25′	445.0	5.8	Typical steppe
Deteriorated grassland, Yunwu Mountain, Ningxia	A8	2047	106°37′	36°27′	423.5	6.1	Typical steppe

DNA extraction

Genomic deoxyribonucleic acid (DNA) was extracted using the modified cetyltrimethyl ammonium bromide (CTAB) method (Zhao et al., 2006). DNA concentration and purity were determined with ultraviolet-visible spectroscopy (UV–VIS) spectrophotometer and the DNA quantity was detected by using 0.8% (w/v) agarose gels. The solution was diluted to 20 ng $\mu L^{-1}$and then stored at -20°C for *polymerase chain reaction* (PCR) analysis.

Inter-simple sequence repeat (ISSR) -PCR amplification

According to the primer sequence published by University of British Columbia (UBC), a total of 96 inter-simple sequence repeat (ISSR) primer sequence were synthesized by Beijing Aoke Biological Technology Co., Ltd. Four individual plants from different populations were used for the primer screening. Finally, 15 primers (Table 2) were selected for the study based on clarity and reproducibility, and high polymorphism of amplified product bands.

PCR amplification reaction system with had total volume of 20 µl, containing 20 ng of template DNA, 10 × PCR buffer (100 Mm Tris-HC, pH 8.3; 500 mM KCl), 0.18 mM of each dNTPs, 0.75 mM of each primer, 1.87 mM of $MgCl_2$, and 1 units of *Taq* DNA polymerase (TaKaRa Biotechnology Dalian Co., Ltd., China). PCR amplification was carried out on Eppendorf PCR instrument and the PCR reaction programs as follows: initial 5 min at 94°C, followed by 35 cycles of 45 s at 94°C , 45 s annealing at 50°C (varies according to different primers), and 90 s extension at 72°C, ending with a final extension of 5 min at 72°C. PCR products were separated on 6% denatured polyacrylamide gel and detected by silver staining. Then, clear and reproducible bands were recorded and used in the analysis.

Data analysis

Clear and reproducible bands were selected for statistical analysis. Amplified bands were scored for each individual as presence (1) or absence (0). DCFA1.1 program was used to build the original document of data analysis (Zhang et al., 2002). Assuming Hardy-Weinberg equilibrium, POPGENE1.32 (Yeh et al., 1997) was used to calculate the following genetic diversity parameters: the percentage of polymorphic bands (*PPB*), Shannon's information index (*I*), Nei's gene diversity (*H*), the effective number of alleles (*ne*), observed number of alleles (*na*), gene differentiation (G_{st}) and Nei's genetic distance (Nei, 1973). The average level of gene flow (N_m) among populations was indirectly calculated using the formula: $N_m = 0.5\ (1\text{-}G_{st})/G_{st}$. (McDermott and McDonald, 1993). An unweighted pair-group method using arithmetic mean (UPGMA) dendrogram was constructed based on the matrix of Nei's genetic distance using NTSYS-pc 2.1e program (Rohlf, 2000).

In addition, analysis of molecular variance (AMOVA) procedure was used to estimate the coefficient of genetic variation among and within populations (Excofier et al., 1992). The variance components were tested statistically by nonparametric randomization tests using 1000 permutations. Meanwhile, Pearson correlation analysis was used to detect the correlation between genetic diversity parameters and environment factors, including altitude, longitude, latitude, annual mean temperature and annual mean precipitation. All these data analyses were calculated by SPSS11.0 (SPSS, 2001). Finally, the relationship between genetic distance and corresponding geographical distance among all populations was tested by Mantel's Test (Mantel, 1967).

RESULTS

Genetic diversity of *S. bungeana*

A total of 390 bands were generated using 15 primers for 160 individuals of eight populations, corresponding to an average of 26 bands per primer. Of these bands, 335 bands were polymorphic, and the percentage of polymorphic bands was 85.90% (Table 3). Amplified bands ranged in size from 100 ~ 2000 bp. Assuming Hardy-Weinberg equilibrium, Nei's gene diversity (*H*) was 0.1663, and Shannon's information index (*I*) was 0.2703 at the species level. Among the eight populations, the highest genetic diversity occurred in population A8 (*PPB* = 34.36%, *H* = 0.0981,*I* = 0.1517) and the lowest was in

Table 2. Primer sequences used in inter-simple sequence repeat (ISSR) analyses of *S. bungeana.*

Primer	Sequence (5'-3')	Tm (°C)
UBC806	$(TA)_8G$	41.5
UBC813	$(CT)_8T$	48.4
UBC814	$(CT)_8A$	50.0
UBC820	$(GT)_8C$	57.7
UBC822	$(TC)_8A$	55.7
UBC823	$(TC)_8C$	57.7
UBC827	$(AC)_8G$	56.4
UBC834	$(AG)_8YT$	56.0
UBC840	$(GA)_8YT$	54.1
UBC864	$(ATG)_5$	46.7
UBC868	$(GAA)_5$	42.6
UBC880	$(GGAGA)_3$	51.0
UBC886	$VDV(CT)_7$	57.7
UBC887	$VDV(TC)_7$	54.5
UBC891	$HVH(TG)_7$	47.1

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

population A1 (*PPB* =10.51%, *H* = 0.0306, *I* = 0.0541) (Table 3).

Genetic differentiation of *S. bungeana*

Analysis of molecular variance (AMOVA) demonstrated that the genetic variation of *S. bungeana* existed mainly among populations, which accounted for 68.71% of the total variation, but the within-population accounted for 31.29%. The coefficient of genetic differentiation (Φ_{ST}) was highly significant (P <0.001) based on the 1000 permutations (Table 4).

Genetic differentiation coefficient cannot only evaluate the degree of differentiation provenance but also indicate the relationship between provenances. In addition, genetic identity (I) and genetic distance (D) could be used to illustrate the degree of genetic differentiation further (Wang et al., 2011). POPGENE was used to calculate the genetic identity and genetic distance among populations (Table 5). Among the eight populations of *S. bungeana*, the genetic identity between A5 and A7 populations was the lowest (I = 0.7930), thus the genetic distance was the greatest (D = 0.2320). The genetic identity between A5 and A6 populations was the greatest (I=0.9806), but the genetic distance was the least (D = 0.0196).

Moreover, the level of gene flow (*Nm*) was measured to be 0.1139 individual per generation among populations.

Genetic relationship

Genetic relationship among the populations was examined by UPGMA cluster analysis. The UPGMA dendrogram (Figure 2) of eight populations was constructed based on Nei's unbiased genetic distance matrix. The dendrogram indicated that the eight populations were divided into two groups. One group consisted of the individuals from populations A7 and A8, and the other six populations were clustered in another group that can be further divided into two sub-clusters. A1 population was clustered into one separate sub-group, and the other five populations (A2, A3, A4, A5 and A6) constituted the other sub-group.

The relationship between genetic distance and corresponding geographical distance among populations was tested by Mantel's Test, and the result shows that there was no significant correlation (r= -0.1345, p = 0.71) (Figure 3). This result showed that geographical distance was not the main reason for genetic differentiation of *S. bungeana* populations.

Correlation of genetic diversity and environment factors

Pearson's correlation analysis showed that there were no significant correlation among the genetic diversity parameters (*PPB*, *H* and *I*) and environment factors (latitude, longitude, altitude, average temperature and annual rainfall) (Table 6).

DISCUSSION

Genetic diversity in *S. bungeana*

Genetic diversity refer to the level of genetic difference within species, and reflect the ability of species to adapt to the environment and the potential to be used and transformed (Wang et al., 2011). Until now, this is the first time that inter-simple sequence repeat (ISSR) markers were used on the population of *S. bungeana* from the Loess Plateau of China. It is generally acknowledged that the genetic diversity of the plant is abundant when the percentage of polymorphic bands reach about 50% at the population level (Ma et al., 2000; Liu and Jia, 2003; Sun et al., 2004). In this study, the percentage of polymorphic bands ranged from 10.51 ~ 34.36% at the population level; it was lower than at the species level. This result may be caused by some loci which only could be detected in the individual population; the other reason was that the uneven distribution of the loci in each population may lead to high polymorphism rate (Li et al., 2004). From the percentage of polymorphic bands, S. *bungeana* had a lower genetic diversity at the population level. However, the percentage of polymorphic bands could be affected by sample size and band quantity. So, the evaluation of genetic diversity only was a rough estimated value by the percentage of polymorphic bands. Therefore, Nei's gene diversity based

Table 3. Genetic diversity indexes of eight populations of *Stipa bungeana.*

Population	Polymorphic loci	*PPB*	*na*	*ne*	*H*	*I*
A1	41	10.51	1.1051	1.0604	0.0360	0.0541
A2	84	21.54	1.2154	1.1007	0.0608	0.0933
A3	93	23.85	1.2385	1.1352	0.0787	0.1184
A4	73	18.72	1.1872	1.0967	0.0583	0.0885
A5	48	12.31	1.1231	1.0757	0.0434	0.0642
A6	93	23.85	1.2385	1.1328	0.0751	0.1124
A7	109	27.95	1.2795	1.1298	0.0794	0.1231
A8	134	34.36	1.3436	1.1613	0.0981	0.1517
Mean	84.4	21.64	1.2164	1.1116	0.0662	0.1007
Species level	335	85.90	1.8590	1.2601	0.1663	0.2703

PPB = percentage of polymorphic bands; *na* = observed number of alleles; *ne* = effective number of alleles; *H* = Nei's gene diversity; *I* = Shannon's information index.

Table 4. Analysis of molecular variance (AMOVA) for eight populations of *S. bungeana.*

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation (%)	P value
Among groups	7	579.587	28.3343	68.71	<0.001
Within groups	152	12.901	12.9013	31.29	
Total	159	592.488	41.2356	100.00	

P-values were estimated by a permutation procedure based on 1000 replicate.

Table 5. Genetic similarity coefficient (above diagonal) and genetic distance (below diagonal) of *S. bungeana.*

Population	A1	A2	A3	A4	A5	A6	A7	A8
A1	0	0.8551	0.8975	0.8604	0.8280	0.8788	0.8117	0.8180
A2	0.1566	0	0.9455	0.9642	0.9540	0.9599	0.8142	0.8183
A3	0.1081	0.0561	0	0.9421	0.9256	0.9557	0.8291	0.8319
A4	0.1504	0.0364	0.0596	0	0.9736	0.9757	0.8121	0.8147
A5	0.1887	0.0470	0.0774	0.0268	0	0.9806	0.7930	0.7932
A6	0.1292	0.0409	0.0452	0.0246	0.0196	0	0.8338	0.8365
A7	0.2086	0.2056	0.1874	0.2081	0.2320	0.1817	0	0.9095
A8	0.2009	0.2006	0.1841	0.2050	0.2317	0.1785	0.0949	0

on Hardy-Weinberg supposition and Shannon's information index based on band phenotype frequency can be used to evaluate the genetic diversity of population more accurately (Qian and Ge, 2001). Nybom (2004) concluded that the average value of Nei's genetic diversity was 0.190 for monocotyledonous. In comparison to this result, our estimate for *S. bungeana* (0.0360~0.0981) was significantly lower than the average value, this result shows that the genetic diversity of *S. bungeana* was relative lower at the population level. During the process of field survey and sampling, we found that the populations with higher genetic diversity (A2, A3, A6, A7 and A8 populations) were always located in the untraversed places, the habitat protection was relatively intact and the populations were larger. In contrast to these, the populations with lower genetic diversity (A1, A4, A5) were located near the residence of farmers and herdsmen or around the traffic arteries; in these places, human and livestock destroyed the ecological environment seriously, which caused the numbers and scope of *S. bungeana* population to became smaller, and further caused the level of the genetic diversity to be relatively lower. Sun (1996) has shown that the scope of population was significant relevance with its genetic diversity. In this study, the lower genetic diversity of *S. bungeana* may be related

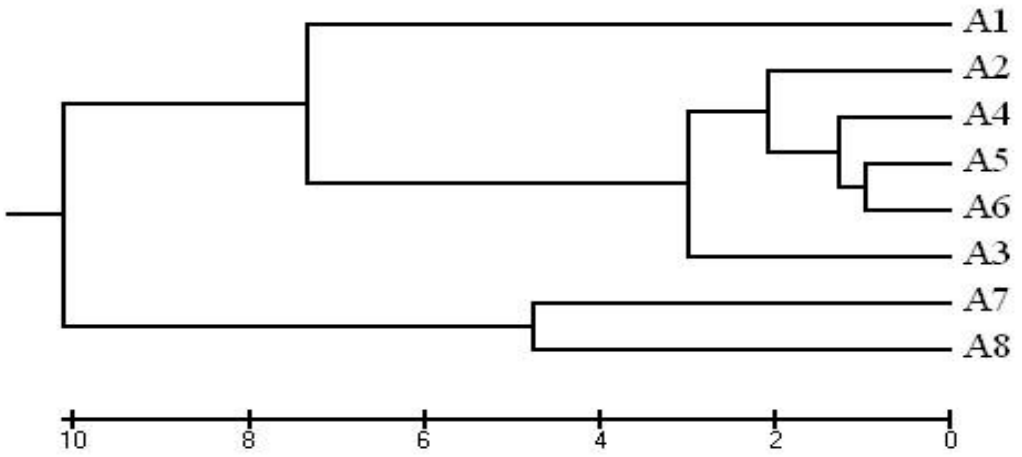

Figure 2. Dendrogram generated by UPGMA based on Nei's unbiased genetic distances for populations of S. bungeana.

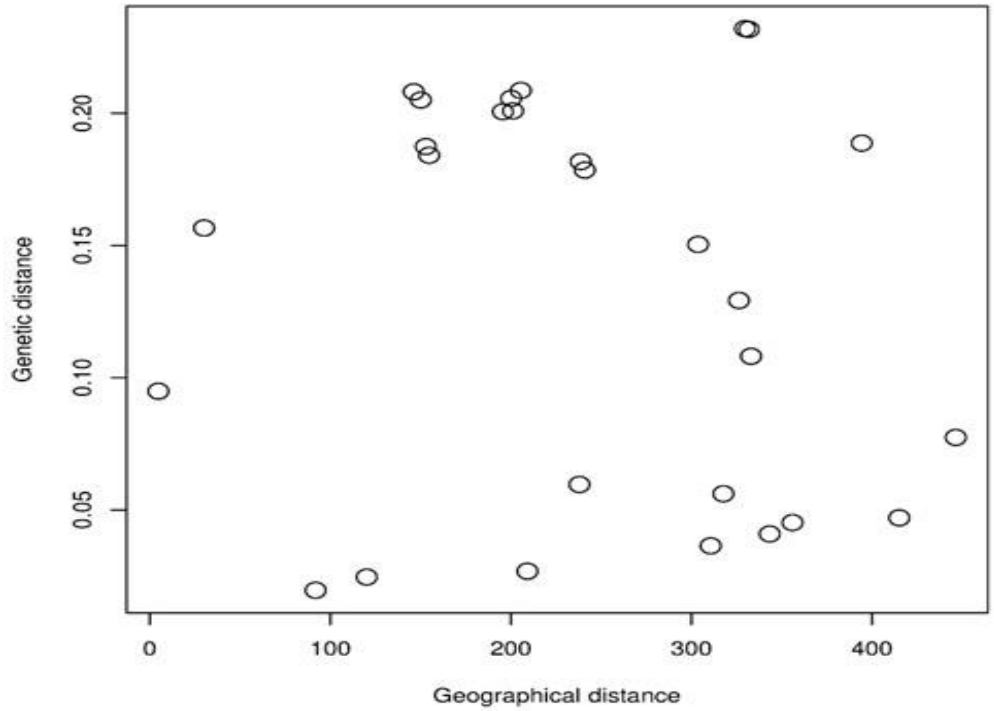

Figure 3. The correlation between genetic distance and geographic distance for populations of *S. bungeana*.

Table 6. Pearson correlation analyses for the relationships between genetic diversity index and ecological factors.

Diversity index	Altitude (m)	Longitude	Latitude	Annual mean temperature (°C)	Annual mean precipitation (mm)
PPB	0.768	-0.583	-0.066	0.059	-0.629
H	0.730	-0.596	-0.187	0.110	-0.588
I	0.749	-0.605	-0.159	0.091	-0.606

PPB =percentage of polymorphic bands; *H* = Nei's gene diversity; *I* = Shannon's information index. All data significant level P > 0.05.

with human activities and habit destroyed.

Genetic differentiation among populations

Population genetic structure was defined as the non-random distribution pattern of genetic variation of one species or population in the spatial and temporal pattern. To a large extent, it represents the evolutionary potential of a species or population (Sun, 1996). The genetic differentiation is an important parameter to evaluate the population genetic structure (Hamrick and loveless, 1989). In the present study, genetic differentiation coefficient (Φ_{ST}) among eight populations was 0.687 by the AMOVA. Population differentiation of *S. bungeana* was higher than that of long-lived perennial species (Φ_{ST} = 0.25, n = 60), out-crossing species (Φ_{ST} = 0.25, n = 73), mixed breeding species (Φ_{ST} = 0.40, n = 18) and the widespread species (Φ_{ST} = 0.34, n = 32) (Nybom, 2004). Wright (1951) pointed that the genetic differentiation was strong when coefficient was greater than 0.25. Therefore, *S. bungeana* population appeared the greater genetic differentiation. Hamrick and Godt (1990) considered that the mean value of genetic differentiation coefficient is 0.20 for cross-pollination plant, and for the selfing species is 0.51. Compared to cross-pollination plant, the selfing plant has higher genetic variation among populations and lower genetic variation within population. *S. bungeana* is a selfing species, thus the genetic variation mainly existed among the populations and the genetic variation within populations was relatively lower. First, the significant population differentiation in *S. bungeana* can be explained by the geographic environment factors. In this study, the habitat of field survey and sampling are in the Loess Plateau. There is strong wind in autumn in this area, but the terrain is complicated, and the isolation of High Mountain leads to the big geographic distances among populations, this further hinder the long-distance spread of seed and pollen and affecting the genetic information exchange among different populations in different historical periods.

Second, *S. bungeana* is an excellent grass that is favored by the cattle, sheep or goats. Under the great pressure of animals graze, it is difficult to form a strong seed bank among populations. In addition, it is hard to achieve long-distance spread depend on wind or animal carrying. Meanwhile, human over-grazing and excessive deforestation also lead to the living environment of *S. bungeana* continue to shrank and deteriorate, which result in the discontinuous distribution of populations.

Third, our field survey also found that the germination rate of *S. bungeana* was low. It grow mainly by clonal reproduction to expand population, this special reproduction increased the mating opportunity among similar individuals and lacked effective gene flow for among different populations. Therefore, we assumed that selfing breeding system, lower gene flow, seed and pollen close range spread and the limited population size may result in the greater genetic differentiation among populations of *S. bungeana*.

Hamrick and Godt (1990) concluded that plant breeding system, gene flow and seed dispersal mechanisms, reproduction mode, natural selection and other factors had a significant impact on the genetic structure of plants. Gene flow was considered as one of the main factors for homogenization of population genetic structure. The species with limited gene flow had a greater genetic differentiation than the species with wide gene flow (Hamrick and Loveless, 1989). In this study, the gene flow (N_m = 0.1139) was far less than 1, which indicated that the gene flow of *S. bungeana* populations was very limited. Wright (1951) pointed that if the estimated value of gene flow (Nm) was greater than 1, there would be certain gene flow among populations; when Nm is smaller than 1, the genetic drift was an important factor to lead to significant genetic differentiation among populations.

Implications for conservation of *S. bungeana*

The environmental degradation caused by natural and human factors exceeds the maximum limit, and then it must result in the loss of genetic diversity for a species or population (Yan et al., 2010). *S. bungeana* is widely distributed on the Loess Plateau, but with the development and utilization of grassland, the water loss and soil erosion becoming increasingly serious, which is causing the continuously destroy of natural growing environment for *S. bungeana* and further leads to the genetic diversity decreased. Therefore, it is necessary to take effective protection strategies and methods to protect the *S. bungeana* in the Loess Plateau. From this study, we known the lower genetic diversity of *S. bungeana* were related to the fragmented distribution range and the number of population continued reduction. Therefore, we should enhance *in situ* conservation of *S. bungeana* to ensure its normal growth and reproduction, and to restrain the decline trend of genetic diversity by prevent overgrazing and excessive deforestation. In addition, it is important to carry out in-depth research for seed reproduction characteristics of *S. bungeana* to improve its self-reproductive capacity by seeds in natural habitat.

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