Genetic diversity pattern of *Stipa purpurea* populations in the hinterland of Qinghai–Tibet Plateau

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**Abstract**

*Stipa purpurea* is among constructive endemic species in alpine meadow and steppe on the Qinghai–Tibet Plateau. To reveal the genetic diversity of this species and its relationship with geographic distribution pattern, we sampled eight populations across a northward transect with an increasing aridity gradient in the hinterland of Qinghai–Tibet Plateau. Their genetic diversity was quantified using eight intersimple sequence repeat (ISSR) primers. We found that *S. purpurea* had relatively low genetic diversity (*Hₑ = 0.135*) but significant genetic differentiation among populations (*Gₛₜ = 0.391*), indicating relatively more genetic diversity retained within populations. A Mantel test revealed a significant relationship between genetic and geographic distance in the *S. purpurea* populations. The genetic diversity tended to decrease with increasing latitude and longitude, while no significant relationship was found between genetic diversity and altitude, suggesting the possible influences of humidity and temperature on genetic diversity of alpine plant. We propose conservation measures for this plant on the plateau.

**Introduction**

The Qinghai–Tibet Plateau, with a mean altitude of more than 4000 m, is known as ‘the third pole of the earth’ (Wu, 1980; Zhang *et al.*, 2002). As the highest place in the world, the habitats in the hinterland of the plateau are characterised by harsh environmental conditions, such as strong wind, high ultraviolet radiation, poor nutrient soil, low temperature and precipitation (Wu, 1980; He *et al.*, 2007). The precipitation decreases from southeast to northwest because of uplift of the Himalaya that blocks the humidity from the Indian Ocean and the temperature also decreases with latitude and elevation (Bai *et al.*, 2004). Under these harsh conditions, alpine steppe and alpine meadow are two of the dominant vegetation types in the hinterland of this plateau (QPIST, 1988; Li & Zhou, 1998), which cover more than 60% of the area on the plateau (Sun, 1996).

Genetic diversity within populations was considered highly important for possible adaptation to environmental changes, and consequently, for long-term survival of plant species (Bauert *et al.*, 1998). Genetic variation could be influenced by reproductive mode and breeding system (Hamrick & Godt, 1989; Nybom, 2004). The environmental conditions (e.g. temperature and precipitation) as well as human activities could affect genetic diversity as well. Among those ecological factors, the pattern of snow melting could determine the growing season of the vegetation (Gugerli *et al.*, 1999), and the microclimates, which are affected by high level of environmental heterogeneity in alpine areas, might induce adaptation within single populations. The populations, fragmented by human disturbance in alpine landscape, might particularly affect the partitioning of genetic diversity among populations (Young *et al.*, 1996). All the fragmented environmental conditions would exert strong selection...
forces on and might strengthen population differentiation (Gugerli et al., 1999; Pluss & Stöcklin, 2004). Therefore, the current genetic structure could reflect the interaction between plant and environment.

Stipa purpurea Griseb., as a constructive species in the alpine steppe and alpine meadow on the Qinghai–Tibet Plateau (Wu, 1980), is a species endemic to the Qinghai–Tibet Plateau and the Pamirs (Guo & Sun, 1982; Lu & Wu, 1996). Its distribution covers alpine areas of five provinces in China including Tibet, Gansu, Xinjiang, Qinghai and Sichuan and mainly is distributed from 1900 to 5150 m in altitude (QPIST, 1988). The habitats in which it often grows include alpine meadows and steppes, the valleys of alpine rivers and fluvial terraces. This plant contains high levels of crude proteins and fats and is favoured by livestock. S. purpurea is a perennial grass with a typical phalanx growth form by tillering. It clonally grows by repeatedly producing tillering ramets from shoot base in which circular clones spread in a tightly advancing front. It seems that the aboveground part of a clone was usually constrained in a space less than 30 cm in diameter (unpublished data). This plant flowers and sets seeds during the period from July to October (Wu, 1987). Apart from this, little was known about its breeding system and population genetic diversity. However, this plant has recently suffered natural and anthropogenic impacts. Human activities including yak trampling and livestock overgrazing as well as sod removal for construction result in vast areas of ‘black soil’ (Zhao et al., 2006a), and, once degraded, these ecosystems are not easy to restore. This would also disturb the growth of S. purpurea and other plants.

As a powerful tool in measuring genetic diversity and differentiation of plant population, the PCR base DNA marker system intersimple sequence repeats (ISSRs) potentially provides a much higher number of markers than allozyme analysis (Esselman et al., 1999; Li & Ge, 2001; Liston et al., 2003) and is much more reliable than random amplified polymorphic DNA PCR (Wolfe et al., 1998; Esselman et al., 1999). ISSR is now well established as a sensitive approach for detecting species and population genetic diversity and differentiation (Meimberg et al., 2006; Angelone et al., 2007; Culley et al., 2007; Liu et al., 2007) in spite of being a dominant marker system (Nybomb, 2004).

In the present study, we measured the genetic diversity of S. purpurea populations sampled from different local sites along a latitudinal gradient in the hinterland of the Qinghai–Tibet Plateau, using ISSR markers. The objective of this study was to reveal the pattern and level of genetic diversity of S. purpurea populations and their possible relationship with geographic/environmental factors. Such information could help understanding the molecular mechanisms of S. purpurea to adapt to the plateau environment and would also support the conservation strategies/policies of the ecosystem on the plateau.

Materials and methods

Sampling design

We selected a northward transect for S. purpurea sampling with an increasing aridity and latitudinal gradient in the hinterland of the Qinghai–Tibet Plateau which was separated by Tanggula Mountains (north region and south region). Along this transect, eight sites (populations) were selected, among them five (P1–P5) were located in the north region and three (P6–P8) in the south region of Tanggula Mountains (Fig. 1; Table 1). Leaves of S. purpurea with flowers were collected using line transects. At each site, three line transects of 10 m, separated by at least 20 m, were set up with one sample picked every 0.5 m along the line. The resulting populations P1, P2, P4, P6, P7, P8 and population P3, P5 were sampled in August 2004 and August 2005, respectively. A total of 475 individuals were sampled. All samples were dried with silica gel immediately and brought back to the laboratory and stored at −70°C until DNA extraction.

Intersimple sequence repeat polymerase chain reaction assay

Individual genomic DNA was extracted from about 500 mg of leaf tissues using the modified method of Doyle & Doyle (1987). In total, 111 ISSR primers [55 from the Biotechnology Laboratory, University of British Columbia (UBC) and the others from Suo (2006)] were screened against a test panel comprised of one random individual from each population. From the preliminary screening, over 60 primers that could amplify visible bands were selected for further examination. Eventually, eight ISSR primers that produced clear and reproducible bands were chosen for the amplification of all DNA samples (Table 2). Reproducibility of ISSR banding pattern was tested by repeated amplifications of five individuals. Each PCR reaction was carried out in a total volume of 25 μL, containing 1.5 mM of MgCl₂, 0.2 mM of dNTPs, 0.2 μM of primer, 1.5 U of Taq DNA polymerase (Takara, Dalian, China), 1 × Taq DNA polymerase buffer and approximately 25 ng of template DNA. The amplifications were performed by a PTC-200 thermocycler (MJ Research, Watertown, MA, USA). Amplification was carried out as follows: preheating 2.0 min at 72°C, 3.0 min at 94°C; 35 cycles for 40 s at 94°C, 45 s at 50°C and 1.5 min at 72°C; ending with 8 min at 72°C for extension, and then soaked at 4°C. The amplification products were separated by
electrophoresis on 1.5% agarose gels in 0.5× TAE (Tris-acetate–ethylenediaminetetraacetic acid) buffer, stained with ethidium bromide. After running for approximately 3 h at 80 V (4.0 V cm⁻¹), the gel was photographed by an Alpha Ease FC Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA). Molecular sizes of the fragments were estimated using a 200-bp DNA ladder (marker provided by Tiangene Ltd., Beijing, China). The negative control was run by replacing template DNA with ddH₂O.

Data analysis

The amplified DNA polymorphic fragments (bands) were scored as presence (1) or absence (0), and the data matrix of the ISSR phenotypes were assembled for further analysis. The percentage of polymorphic bands (PPB), effective allele number (Aₑ), Nei’s expected heterozygosity (i.e. genetic diversity, Hₑ) and the Shannon index (I) were calculated to estimate the level of genetic diversity. Population differentiation was analysed for polymorphism between populations by Gₛ. Gene flow (Nₑ) was estimated from Nₑ = (1/4)(1 – Gₛ)/Gₛ (Nei, 1987). All these calculations were performed by using POPGENE program ver. 1.32 (Yeh et al., 2000). The analysis of molecular variance (AMOVA) program version 1.5 was also used to describe genetic structure and variability among populations with the aid of the ARLEQUIN version 3.0.1 software (Excoffier et al., 2005). The total sum of squared deviations (SS) were partitioned into...
components for variation within populations, variation among populations within regions and variation among regions. The corresponding mean squared deviations (MS) were then obtained by dividing each SS by the appropriate degrees of freedom (d.f.). The variance components of each hierarchical level were extracted by equating the MS to their expectations (Excoffier et al., 1992). The number of permutations for significance testing was set at 5000. The effectiveness of the genetic distance in describing the separation of the populations was tested by a Mantel test (Mantel & Valand, 1970) conducted on the matrices of genetic distance and geographic distance. The Mantel test was performed with 1000 random permutations with NTYSYS 2.02 (Rohlf, 1997). A cluster tree of the unweighted pair group method with arithmetic mean (UPGMA) (Nei, 1978) was constructed using NTYSYS 2.02 to examine the relationship of the populations.

Multilocus linkage disequilibrium gives an estimation of the degree of association among loci or the amount of recombination because of meiosis, and therefore, an evaluation of the occurrence of sexual reproduction. Indeed, if outbreeding is the main method of reproduction, no linkage among loci is expected; but high linkage value is expected if inbreeding is dominating. Multilocus linkage disequilibrium \( r_d \) was calculated using the index of association modified to remove the dependency of sample size (Agapow & Burt, 2001). Statistic calculations and significance tests by randomisation were performed with the programme MULTILOCUS 1.3 (http://www.agapow.net/software/multilocus).

Correlation analyses were performed to reveal the relationship between genetic diversity and geographic distance. The Pearson correlation was used to evaluate the correlation between genetic diversity and altitude, latitude and longitude. These analyses were conducted using the SPSS statistics program (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL, USA).

**Results**

**Genetic diversity**

A total of 212 ISSR bands were generated with the eight selected primers, 205 (96.7%) of which were polymorphic in \( S. \) purpurea populations. With the aid of these primers, among 475 individuals, 466 (98.1%) genets were identified. The number of DNA fragments amplified per ISSR primer varied from 23 to 29, with a length ranging from 300 to 2600 bp. The PPB generated by each of these primers ranged from 93.1% to 100% (Table 2). The parameters of genetic diversity showed that PPB of \( S. \) purpurea populations ranged from 68.2% to 90.6%, with a mean of 75.7%. The mean number of effective alleles \( (A_e) \) was 1.217 at the population level. The gene diversity values \( (H_e) \) varied from 0.089 to 0.222, with a mean of 0.135. Shannon indices \( (I) \) ranged from 0.147 to 0.345, with a mean of 0.215. Among the eight populations, P1 had the lowest genetic diversity levels, while P8 showed the highest genetic diversity. Multilocus linkage disequilibrium indicated a degree of association among loci \( (r_d) \) higher for P6 than other populations (Table 3).

**Genetic differentiation**

The coefficient of genetic differentiation between populations \( (G_{ad}) \) was 0.391, indicating approximately 39%
genetic variation between *S. purpurea* populations. AMOVA analysis further revealed highly significant (*P* < 0.001) genetic differences among the eight populations. Of the total genetic diversity, 42.02% was attributable to among-population diversities and the rest (50.64%) to differences within populations. No significant genetic differentiation was found between populations located on different sides of Tanggula Mountains (*P* = 0.087) (Table 4). The level of gene flow (*N*$_{em}$) was 0.780 individuals per generation. Population genetic differentiation was significantly correlated with physical distance of the populations (Mantel test, *r* = 0.447, *P* = 0.008) that suggested that neighbouring populations would have high genetic similarity, although this pattern was not completely confirmed by the UPGMA tree based on Nei’s (1978) unbiased genetic distance in which geographically distant populations P6 and P1/P2 showed relatively close genetic relationship, as well as, the populations P4 and P7 (Fig. 2). In addition, the two populations (P3 and P5) of contrasting habitats (steppe and meadow) clustered together by themselves (Fig. 2).

### Correlation of geographical factor and genetic diversity

Pearson’s correlation analyses showed that the genetic diversity (*H*$_{e}$) of *S. purpurea* populations in the hinterland of Qinghai–Tibet Plateau significantly decreased with latitude (*R*$_{2}$ = 0.616, *P* = 0.021) and longitude (*R*$_{2}$ = 0.771, *P* = 0.004) (Fig. 3). Genetic variation expressed as PPB and Shannon index (*I*) presented the same pattern (*R*$_{2}$ = 0.81, *P* = 0.002 and *R*$_{2}$ = 0.646, *P* = 0.016 with latitude; *R*$_{2}$ = 0.846, *P* = 0.001 and *R*$_{2}$ = 0.790, *P* = 0.003 with longitude, respectively). No significant correlation was found between elevation and genetic diversity (*R*$_{2}$ = 0.061, *P* = 0.556 with *H*$_{e}$; *R*$_{2}$ = 0.127, *P* = 0.385 with PPB; *R*$_{2}$ = 0.064, *P* = 0.547 with *I*).

### Discussion

**Genetic diversity in *S. purpurea***

*S. purpurea* showed relatively low levels of genetic diversity within populations sampled from the hinterland of Qinghai–Tibet Plateau. For example, the mean value of genetic diversity within populations of *S. purpurea* (*H*$_{e}$ = 0.14) was lower compared with the reported average value of long-lived perennial species (*H*$_{e}$ = 0.25) and widespread species (*H*$_{e}$ = 0.22) (Nybom, 2004) and also lower than several other *Stipa* species, e.g. *Stipa grandis* (*H*$_{e}$ = 0.17) (Zhang et al., 2003), *S. grandis* (*H*$_{e}$ = 0.23) (Zhao et al., 2006b), and *Stipa krylovii* (*H*$_{e}$ = 0.16) (Wang et al., 2006).

We assume the main reason of low genetic diversity in *S. purpurea* is the fragmentation of the environment. The heterogeneous and fragmented environment by natural and human disturbance may block gene flow between different populations. And low gene flow among small, isolated populations is proposed to result in inbreeding, loss of heterozygosity because of genetic drift and genetic differentiation among populations. This would contribute to the low genetic diversity (Lewis & Crawford, 1995; Wang et al., 2006). Many alpine plants are characterised by the ability to reproduce asexually through clonal growth, which are thought to be the result of adaptation to the harsh conditions where pollinators are sparse and seedling survival is low (Billings, 1973). In this study, however, 98.1% of sampled individuals had different multilocus genotypes, suggesting that sexual reproduction may play an important role in retaining genetic variability in *S. purpurea*, although it has the ability to survive by clonal growth. In the hinterland of Qinghai–Tibet Plateau, the environmental conditions are very steep and hard for seedling establishing. Seed germination rate in *S. purpurea* was relatively low (0.67–6.0%) in laboratory tests (Bu et al., 2006; Liu et al., 2007).
Limited seedling recruitment contributed to the maintenance of relatively low within-population genetic variation (Vellend & Waterway, 1999), which seems to be one reason for low genetic diversity in *S. purpurea*. Nevertheless, studies also show that repeated seedling recruitment even with low rate is sufficient to maintain the levels of genotypic variation within plant populations (Eriksson, 1993; Song et al., 2006).

**Population genetic structure**

In *S. purpurea*, approximately 39% genetic variability retained interpopulations, suggesting significant genetic differentiations between populations. Such a pattern was also found in *S. grandis* (G\textsubscript{st} = 38%, Zhang et al., 2003) and *S. krylovii* (G\textsubscript{st} = 32%, Wang et al., 2006), which both suffered from heavy habitat destruction. First, the significant population differentiation in *S. purpurea* can be explained by the geographic isolation between populations that can limit gene flow and result in significant differentiation between populations. For clonal plants, clonality and spatial isolation of populations may decrease genetic diversity within and increase genetic differentiation between populations (Pluess & Stocklin, 2004). The closest geographic distance between the sampled *S. purpurea* populations in this study was over 46 km, and the populations were separated by hills on the plateau that should be beyond the ranges of both seed and pollen dispersals of this species. Therefore, it is reasonable that a greater proportion of genetic diversity resides among populations of *S. purpurea*.

Second, significant differentiation is expected from the heterogeneous environment on the plateau. As the highest plateau in the world, the heterogeneity of environmental factors, such as the pattern of snow melting and the microclimate, which may influence flowering time, probably block the genetic exchange between populations. And the absence of seedling recruitment under harsh conditions could hold back the survival of new genetic recombinations and enable the flourishing of clonal growth.

The third rational explanation of the high differentiation is the fragmentation of habitats on the plateau (Young et al., 1996; Zhang et al., 2003; Wang et al., 2006). Habitat fragmentation under human disturbance may be an important factor affecting genetic structure. Evidence showed that there was habitat destruction and degradation because of decades of human activities in *S. purpurea* distribution. Habitat fragmentation and degradation would decrease population size and increase the selfing ratio of *S. purpurea*, which both might enhance the effects of genetic drift and result in low genetic diversity and significant population genetic differentiation. The test of multilocus linkage disequilibrium also showed that a high selfing rate existed.

However, the clustering analysis showed that some geographically distant *S. purpurea* populations had a relatively close genetic relationship (e.g. P4 and P7). The population in Erdagou (P4) and the one in Guluzhen (P7) were from contrasted habitats of steppe and meadow. This phenomenon could probably be caused from similar intensive
low genetic variation at the population level and the nature conservation and the structure of genetic diversity established. Genetic diversity is one primary base for global climate changes (Liu & Chen, 2000), has been espoused here, whereas the Qinghai–Tibet Plateau, which is an important area to conservation implication

Correlation of geographical factor and genetic diversity

The genetic variation within populations of *S. purpurea* decreased with the latitude and longitude; the possible reasons might be the superior environmental conditions (e.g. humidity and temperature) in the south than in the north, which could facilitate the emergence and survival of seedlings. Raised temperature may increase fruit set and fruit weight (Dorken & Eckert, 2001), which may help to increase plants’ genetic diversity. In *S. purpurea*, laboratory tests showed that under higher temperature conditions (with temperature fluctuating from 10°C to 20°C, Liu et al., 2006), the germination rate (6% vs 0.67%) is higher than under relatively lower temperature conditions (with temperature fluctuating from 5°C to 25°C) (Bu et al., 2006). Higher precipitation might also help in seedling survival and have great influence on genetic diversity, for example, genetic diversity of *S. grandis* was found positively correlated with precipitation (Zhao et al., 2006b). Genetic diversity of *Caragana* spp. (Wei et al., 1999) and *Kobresia humilis* (Zhao et al., 2006a) was also found to be higher in moist sites than xeric ones. In contrast, there is no significant correlation between genetic diversity of *S. purpurea* populations and altitude. It might be because of a relatively limited altitude range (4110–5044 m) sampled here, whereas *S. purpurea* has a wide distribution at a broad range of elevation (QPIST, 1988).

Conservation implication

The importance of protecting the fragile habitats on the Qinghai–Tibet Plateau, which is an important area to global climate changes (Liu & Chen, 2000), has been established. Genetic diversity is one primary base for nature conservation and the structure of genetic diversity is valuable for proposing conservation strategies. The low genetic variation at the population level and the high amount of genetic differentiation among the studied populations of *S. purpurea* indicate that more efforts should be undertaken to preserve the populations in a wide distribution. According to the high fragmentation of *S. purpurea* populations in the hinterland of the Qinghai–Tibet Plateau, we suggest the first measure we should take is to reduce disturbance by humans and animals. For in situ conservation, the results of the present study appear to justify that preserving only one or two populations is not enough for conservation purposes. However, the populations in Dangxiong (P8) and Kaixinling (P5) harbour the highest level of genetic diversity and should be given priority in conservation activities. Understanding the diversity pattern of the dominant grass and its relation with geographic factors could also be helpful in vegetation restoration along the long and newly constructed railway on the plateau. Further information on breeding systems, which will affect the maintenance of variation in the population, is necessary for making detailed and efficient conservation strategies.

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References


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