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ISSR analysis of genetic diversity of the Qinghai-Tibet Plateau endemic *Rhodiola chrysanthemifolia* (Crassulaceae)

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Abstract

Inter-simple sequence repeat markers (ISSR) were used to estimate genetic diversity within and among 10 populations of *Rhodiola chrysanthemifolia* along Nianqingtangula Mountains and Brahmaputra, a species endemic to the Qinghai-Tibet Plateau and an endangered medicinal plant. Of the 100 primers screened, 13 produced highly polymorphic DNA fragments. Using these primers, 116 discernible DNA fragments were generated of which 104 (89.7%) were polymorphic, indicating substantial genetic diversity at the species level. Genetic diversity measured by the percentage of polymorphic bands (PPB) at the population level ranged from 21.97% to 48.8%. Analysis of molecular variance (AMOVA) showed that the genetic variation was found mainly among populations (77.3%), but no regional differentiation was discernible. Variance within populations was only 22.7%. The main factor responsible for this high level of differentiation among populations is probably the historical geographical and genetic isolation of populations in a harsh mountainous environment. Concerning the management of *R. chrysanthemifolia*, the high genetic differentiation of populations indicates the necessity of conserving the maximum possible number of populations.

Keywords: *Rhodiola chrysanthemifolia*; ISSRs; Genetic diversity; Qinghai-Tibet Plateau

1. Introduction

Rhodiola chrysanthemifolia (H. Léveillé) S. H. Fu (Crassulaceae) is an endemic, perennial herbaceous plant of the Qinghai-Tibet Plateau. It is mainly distributed in eastern Tibet, China, at altitudes between 3200 and 4200 m above sea level and can be found in *Rhododendron* forests, rocky slopes and caves (Fu and Ohba, 2001). In addition, the plants of *Rhodiola* have been used in traditional Tibetan medicine. *R. chrysanthemifolia* is also used as an herbal remedy by Tibetan people as a substitute for popular species of *Rhodiola* in the Qinghai-Tibet Plateau. Earlier, the medicine

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of *Rhodiola* was used only in small quantities by local people, but recently commerce and demand have increased. Heavy extraction from the wild, loss of habitat by deforestation and excessive grazing at high altitude pastures in the entire Qinghai-Tibet region now threaten its survival.

Although adaptive genetic diversity, rather than neutral genetic diversity, controls the adaptability of individuals or populations, neutral genetic markers are extensively used in conservation genetics. Neutral markers can give insight not only into genetic processes in populations (e.g. mating system, gene flow), but also into past demographic events (e.g. bottlenecks, founder events), both kinds of information being necessary to understand population history and to propose conservation measures (Milligan et al., 1994). The molecular markers best suited for detecting genetic diversity should be relatively easy and inexpensive to use and should evolve rapidly enough to be variable within populations. In the past decade, inter-simple sequence repeat polymorphism (ISSR) markers have been developed to investigate the genetic diversity of natural populations (Zietkiewicz et al., 1994). ISSR fingerprinting has demonstrated a hypervariable nature of the markers and its potential power for population studies (Culley and Wolfe, 2001; Ge et al., 2005). Here, we report the genetic structure of *R. chrysanthemifolia* populations throughout its known distribution in Tibet. The following questions were asked. (i) What is the level of genetic diversity in populations of *R. chrysanthemifolia*? (ii) How is genetic diversity distributed within and among populations? (iii) How are populations related each other? We tried to interpret the results in order to understand dominant evolutionary forces and propose conservation measures for *R. chrysanthemifolia.*

2. Materials and methods

Populations were collected along the Nianqingtangula Mountains and the Brahmaputra, representing most of the range of *R. chrysanthemifolia* in Tibet. In total, 164 individuals of *R. chrysanthemifolia*, representing 10 populations, were included in the analyses (Fig. 1; Table 1). Young leaf tissue was collected from each sampled individuals and dried in silica gel for subsequent DNA extraction.

Total DNA was extracted using the protocol of Doyle and Doyle (1987). One hundred ISSR primers from the University of British Columbia (The Michael Smith Laboratories, University of British Columbia, primer set # 9, Vancouver, BC, Canada: http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/Primers.pdf) were initially screened to identify well amplified, polymorphic bands among populations. Thirteen primers from the initial screening process (UBC # 807, 809, 812, 826, 836, 841, 857, 859, 861, 881, 885, 888 and 889) that had a high level of polymorphism and the best readability were used for polymerase chain reaction (PCR) amplification. PCR and gel electrophoresis were carried out as described in Chen et al. (2005). Only those bands that showed consistent amplification were scored. Smeared and weak bands were excluded. ISSR bands were scored as present (1) or absent (0) for each sample, and the Jaccard coefficient was employed to calculate pairwise band similarities for all 164 individuals using the NTSYS program (Rohlf, 1994). Genetic diversity was measured by the percentage of polymorphic bands (PPB), calculated by dividing the number of polymorphic bands at population and species levels by the total number of bands surveyed. The Shannon index of diversity (I) was also calculated using the POPGENE program (Yeh et al., 1997). The nonparametric analysis of molecular variance (AMOVA program version 1.5) was used to describe the genetic structure and variability among populations as described by Excoffier et al. (1992). The matrices containing Jaccard distances between all pairs of phenotypes were used as input distance matrices constructed by the AMOVA-PREP program (Huff et al., 1993). A dendrogram was also constructed by an unweighted paired group method of cluster analysis using arithmetic averages (UPGMA).

3. Results

The 13 polymorphic primers generated 116 bands ranging in sizes from 200 to 2100 bp, corresponding to an average of 8.9 bands per primer. Of these bands, 89.7% (104 in total) were polymorphic among 164 individuals, i.e, the percentage of polymorphic bands (PPB) for this species was 89.7%. Every primer produced polymorphic bands when all 10 populations were considered. Genetic diversity varied among populations with PPB values ranging from 22.0% (P_9) to 48.8% (P_1). The Shannon index (I) showed a similar trend (Table 1).

AMOVA showed significant ($p < 0.001$) genetic variation among populations (Table 2). Most of the total variation (77.3%) was found among populations, whereas only 22.7% occurred among individuals within populations. When the studies were restricted to populations based on their geographical regions, genetic variation among populations on

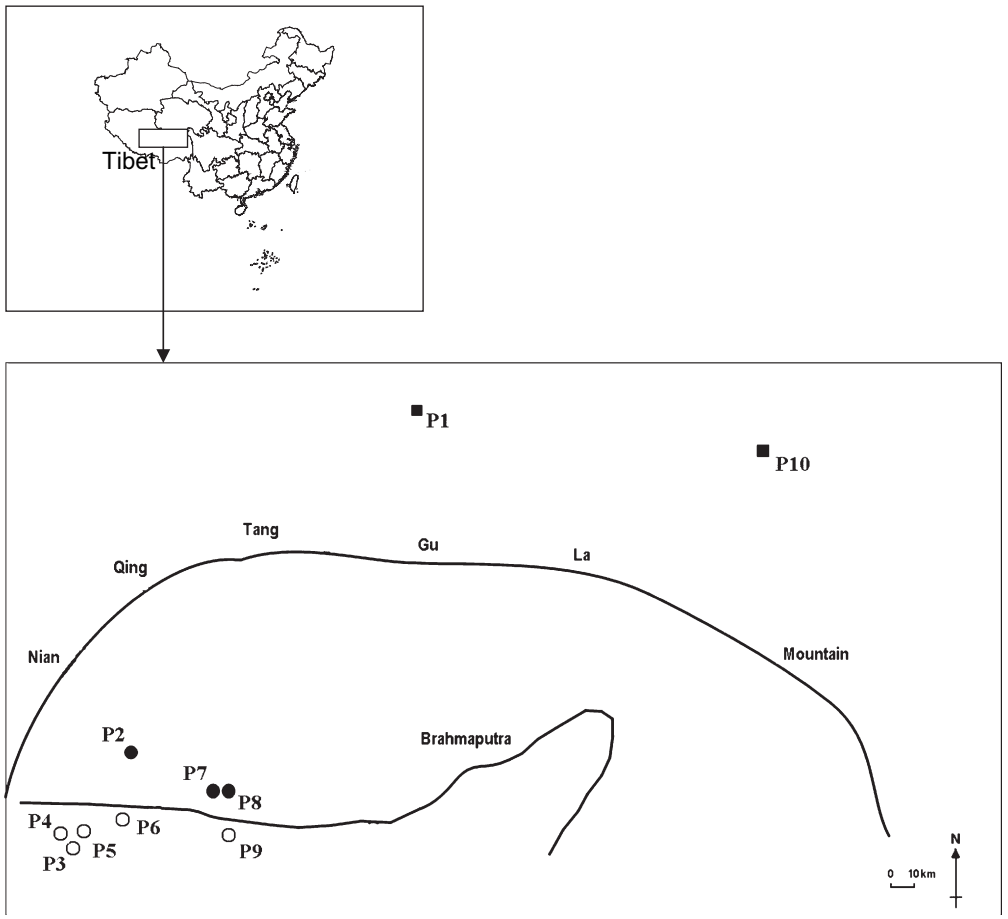

Fig. 1. Location of the 10 *Rhodiola chrysanthemifolia* populations sampled from the Tibet of China. ■: northern side of Nianqingtangula Mountains; ●, ○: southern side of Nianqingtangula Mountains; ●: northern side of Brahmaputra; ○: southern side of Brahmaputra. For population abbreviations see Table 1.

the northern and southern side of Nianqingtangula was 6.5%, the variation among population was 71.8% and 21.6% within populations, whereas genetic variation among populations on the northern and southern side of Brahmaputra was only 4.6%, the variation among population was 73.1% and 22.1% within populations. This indicates that the genetic variation mainly occurred among populations rather than between geographical regions.

The UPGMA analysis was performed based on genetic similarity (Jaccard coefficient). There were two clusters (Fig. 2), one containing populations P_2, P_3, P_4, P_5, P_6 from the southern side of Nianqingtangula except for population P_1 from its northern side. Populations P_7, P_8, P_9 from the southern side of Nianqingtangula, along with population P_{10} from its northern side grouped into a second cluster. Populations from two geographical regions (northern and southern side of Nianqingtangula) were to a large extent intermingled in the UPGMA. Hence, both the AMOVA analysis and the UPGMA dendrogram showed no clear geographic grouping of the populations studied (northern and southern side of Nianqingtangula or Brahmaputra). A dendrogram based on genetic similarity between individuals showed that all individuals from the same population formed a group (data not shown).

4. Discussion

By using ISSR primer, we demonstrated that there was considerable genetic diversity within populations of *R. chrysanthemifolia*, 89.66% of bands were polymorphic in 10 populations. The percentage of polymorphic bands (PPB) in each population ranged from 21.97% to 48.8%. The genetic diversity in all populations was a little higher,

Table 1
Location, region, abbreviation, the number of plants, percentage of polymorphic bands (PPB) and Shannon index (*I*) of the 10 populations of *Rhodiola chrysanthemifolia*

Population	No of plants	Region	Latitude (°N)	Longitude (°E)	No. of bands	No. of polymorphic bands	PPB	*I*
P_1	16	Baqing	31°48′06″	94°30′95″	84	41	48.80	0.2415 (0.1634)
P_2	20	Lasha	29°39′43″	91°07′03″	93	32	34.40	0.1390 (0.2401)
P_3	17	Langkazi	28°58′52″	90°28′19″	82	28	34.15	0.1108 (0.2251)
P_4	12	Langkazi	29°06′23″	90°24′29″	80	24	30.00	0.1231 (0.2432)
P_5	8	Langkazi	29°09′35″	90°30′30″	81	25	30.86	0.1198 (0.2297)
P_6	20	Gongga	29°15′07″	90°37′15″	89	25	28.09	0.0833 (0.1888)
P_7	22	Shangri	29°17′32″	91°58′13″	79	21	26.58	0.1128 (0.2236)
P_8	20	Shangri	29°14′35″	92°05′16″	74	24	32.43	0.1513 (0.2453)
P_9	18	Qushong	29°02′24″	92°18′14″	91	20	21.97	0.1162 (0.2293)
P_{10}	11	Changdu	31°09′24″	97°15′27″	74	25	33.78	0.1530 (0.2605)
Species					116	104	89.66	

but the genetic diversity in each population was a little bit lower compared with other species in the same genus. Using the same primers, we detected that 80% of the bands were polymorphic in 10 populations of *Rhodiola alsia* (Xia et al., 2005), ranging from 63.37% to 88.57% in each population. By using 16 RAPD primers, Yan et al. (1999a) estimated the genetic diversity of *Rhodiola sachalinensis* in 12 populations between 69.6% and 78.8%. Using isozymes, Zu et al. (1998) revealed low genetic diversity in four populations of *R. sachalinensis* with the percentage of polymorphic loci (*P*) ranged from 22.2 to 44.4 within populations. Based on 17 allozyme loci encoded by 10 enzymes, Yan et al. (1999b) found that genetic diversity within populations of *R. sachalinensis* at high altitude was greater than that of populations at low altitude, but we did not draw the similar conclusion in this study. In comparison, *R. chrysanthemifolia* presents lower genetic diversity in each population and higher genetic diversity in all populations.

As in the present study on *R. chrysanthemifolia*, pronounced genetic differentiation among populations has been found in several rare plant species and attributed to low or absent interpopulation gene flow (Fischer and Matthies, 1998; Fischer et al., 2000; Kreivi et al., 2005). Giles and Goudet (1997) studied 52 populations of *Silene dioica* using allozyme analysis and found that the turnover of local populations along with environmental heterogeneity and spatial restriction increases genetic variation among populations. Observed genetic differentiation among populations of *R. chrysanthemifolia* suggests low historical gene flow, which is in accordance with the geographical isolation of its populations. Generally, distances among populations of *R. chrysanthemifolia* are large, the median distance to the

Table 2
Analysis of molecular variance (AMOVA) for 164 plants from 10 populations of *Rhodiola chrysanthemifolia* using ISSRs

Source of variation	df	SSD	MSD	Variance components	% of total variance
Among N and S side of Nianqingtangula group	1	327.7	327.7	1.6	6.5
Among populations within groups	8	2633.2	292.6	17.2	71.8
Among individuals within populations	154	796.8	5.2	5.1	21.6
Among populations	9	2305.5	288.1	17.6	77.3
Among individuals	154	796.8	5.1	5.1	22.7
Among groups	9	327.7	327.7	6.8	26.4
Within groups	154	3102.3	19.1	19.1	73.6
Among N and S side of Brahmaputra group	1	389.3	389.3	1.1	4.6
Among populations within groups	8	2633.1	292.6	17.0	73.1
Among individuals within populations	154	796.7	5.1	5.1	22.1
Among populations	9	2243.8	280.4	17.6	77.3
Among individuals	154	796.7	5.1	5.1	22.7
Among groups	9	389.3	389.3	4.5	19.5
Within groups	154	3040.6	18.7	18.7	80.5

All *P* values obtained were < 0.001. Levels of significance were based on 3000 iteration steps. Abbreviation: df, degrees of freedom; SSD, sum of squared deviations; MSD, mean squared deviations.

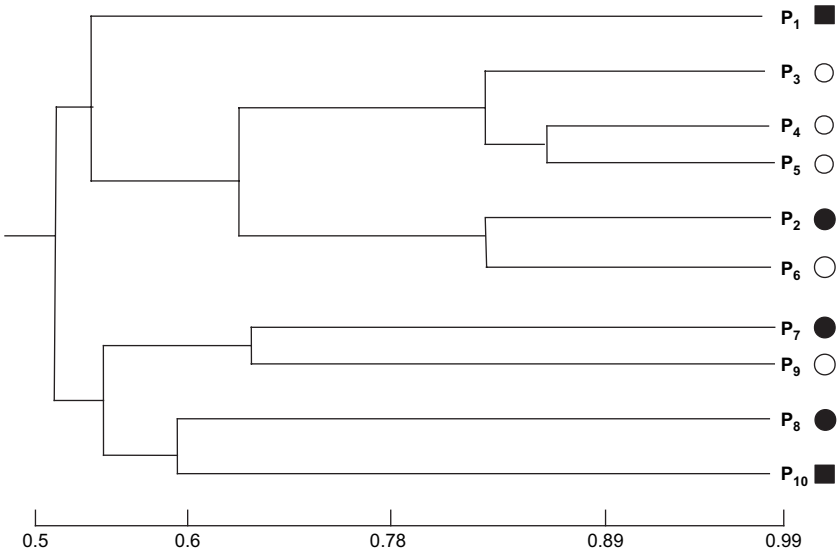

Fig. 2. UPGMA clustering based on Jaccard coefficient from ISSR data of 10 *Rhodiola chrysanthemifolia* populations from Tibet of China. For population abbreviation see Table 1, for regional grouping of populations see Fig. 1.

closest population was more than 10 km in our sample, and the harsh climate and mountainous environments are probable barrier to pollinator movement and seed dispersal. Therefore, populations of *R. chrysanthemifolia* appear to be acting as independent evolutionary units.

Observed small-scale differentiation in ISSR variation within populations of *R. chrysanthemifolia* may be caused by founder effects, restricted seed and pollen dispersal, genetic drift or habitat isolation. This is consistent with habitats and biological characteristics of *R. chrysanthemifolia*. In an tundra area at more than 4000 m above sea level, there is an array of factors which leads to expectations on the partitioning of total genetic variation of a plant species which deviates from the general pattern, such as the short vegetation-period (about 120 days), the low temperature, the insufficient content of oxygen and the common snowstorm, so the chance of seeding recruitment may be a rather rare event. Zu et al. (1998) found that one of the internal factors of endangered species of *R. sachalinensis* in the Changbai Mountains of China is pollen infertility and the external factors are the severe environment and over-collection by local people. Tang et al. (2002) revealed that geographical conditions were one of the most important factors for dispersal of seeds based on seed dispersal pattern and germination of *R. sachalinensis* from Changbai Mountains and no regular dispersal pattern was found because of the effects of wind and habitats. The seeds could not be spread because the plants usually grow on rocky caves or in leeward areas. The germination rates are very low under natural conditions and only 5–10%, although under experimental conditions the rates are up to 80% (Tang et al., 2002). Plants of *R. chrysanthemifolia* grow in more severe habitats (higher altitude) compared to *R. sachalinensis*, suggesting that germination, vegetation and propagation will be rare events even the seeds can disperse. In this context, the genetic diversity within population is mostly depended on the first coloning plants. The low level of genetic variation within populations and high genetic variation among populations in *R. chrysanthemifolia* may be attributed to the clonal propagation of this species.

We also found limited genetic differentiation among groups. Populations from different side of Nianqingtangula Mountains and Brahmaputra are poorly differentiated. The most reasonable interpretation of these data is that a very short time has been available for genetic divergence of the species *R. chrysanthemifolia* after the uplift of the Qinghai-Tibet Plateau. The lack of genetic divergence among different geographical regions of *R. chrysanthemifolia* is consistent with an in situ origin of the endemics of the Qinghai-Tibet Plateau at the time of uplift (Wulff, 1944; Wu, 1987).

5. Conservation implications

Information about the genetic structure and the diversity of populations in an endangered species are essential for the conservation of the species. Hamrick et al. (1991) suggested that as >50% of the total genetic variation resides

among populations, six strategically placed populations should maintain 95% of their genetic diversity. In our study, 77.3% of the total genetic variation was observed among populations of *R. chrysanthemifolia*, which implied the need to conserve more populations to maintain within this species. In addition, it would be wise to save populations in different regions in order to limit population declines caused by large-scale environmental catastrophes. Nevertheless, a demographic study is necessary to monitor the current and future decline of the species. If populations become very small, they will be more sensitive to stochastic events and potential inbreeding depression could be expressed due to mate limitation (Innes and Hermanutz, 1988).

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