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Using Inter-simple Sequence Repeat Markers to Analyze the Genetic Structure of Natural *Rhodiola imbricata* Populations from the Trans-Himalayan Region and Implications for Species Conservation

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Inter-simple sequence repeat markers (ISSR) were used to estimate genetic diversity and structure within and among populations of *Rhodiola imbricata* a rare perennial herbaceous phytoadaptogen distributed in Trans-Himalayan region of India, especially the high mountain passes of Ladakh. The plants are harvested for rhizomes which lead to destruction of the whole individual. Analysis of molecular variance (AMOVA) showed that the genetic variation was found mainly within populations (62%) and the variance between populations was only 38%. Nei's genetic distance and unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis does not show a consistent pattern of spatial distribution to the genetic differentiation among the accessions which can be a indication for genetic drift within populations which was observed in our study. The size of the natural populations of *R. imbricata* noted in field observations were very small, chiefly owing to habitat destruction and overexploitation. Optimum harvesting and *in situ* conservation strategies, domestication and plant tissue culture should be developed to guarantee the sustainable use and protection of this rare species.

Key words: Genetic variation, Genetic structure, ISSR, *Rhodiola imbricata*, Trans-Himalayan region.

R. imbricata Edgew (Family: Crassulaceae) is a perennial, herbaceous, rare phytoadaptogen mainly distributed in the higher latitudes and elevations (2800-5600m) of the Northern hemisphere mainly in Asia and Europe (Furmanova et al. 1995). In India the plant grows in the Trans-Himalayan range above 5,420 msl. Salidroside, a phenolic glycoside is the main bioactive agent responsible for adaptogenic action of *R.imbricata* (Kurkin and Zapesochnayan, 1986).Adaptogens, are biologically active substances found in medicinal plants which are non-toxic and safe to be ingested by animals and humans and help the body to adapt to the changes and stress of life by increasing the nonspecific resistance (Brekhman and Dardymov, 1968). It has been used by astronauts, divers and mountaineers to improve their stress resistance (Yan and Wu, 2004) and known to exhibit a wide range of properties such as anti-fatigue, anti-stress, anti-hypoxic, anti-cancer, anti-oxidant, immune enhancing and anti-radiation sickness (Brown et al. 2002). The young leaves are used as vegetable while in traditional medicine the roots are used against lung problems, cold, cough, fever, loss of energy and pulmonary complaints (Chaurasia et al. 2007).

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The Indian-Trans Himalayas spans over 186,000 km^2 and is known for sparsely distributed vegetation and relatively low species diversity. Nevertheless, the area harbours many rare and endangered plant and animal species. The high potential instability and inherent vulnerability of the mountain ecosystems (Skeldon, 1985) renders the Trans-Himalaya one of the ecologically fragile biogeographic zones of India (Rodgers and Panwar, 1988). Opening up the previously inaccessible terrain due to road building and deteriorating pasture quality, threatens the large number of plants (Fox et al. 1994). The natural resource of *R. imbricata* has become rare due to overgathering and studies on the artificial cultivation have made little progress due to the temperature-sensitive nature of the plant (Xu *et al.* 1998).

Genetic variation in a plant species is determined by its mating system and dispersal ability of pollen and seed, which affect the genetic structure and dynamics of populations within the species (Muona,1990). Outcrossing rates in plant populations have traditionally been estimated from polymorphism data at isozyme loci. In recent years, the use of molecular markers is preferred over conventional morphological and biochemical markers for genetic diversity studies, since they are

not influenced by environmental effects and the developmental stages of the experimental material.

A number of molecular markers such as random amplified polymorphic DNA (RAPD) (Munthali et al. 1992), amplified fragment length polymorphism (AFLP) (Vos et al. 1995), simple sequence repeats (SSR) and inter simple sequence repeats (ISSR) have been widely used to detect genetic diversity in plants (Tang et al. 2003). Inter-simple sequence repeat (ISSR) is a technique that uses repeat-anchored primers to amplify DNA sequences between two-inverted SSR and have advantage over RAPD markers as they use longer primers that allow more stringent annealing temperatures and reveal many more polymorphic fragments (Zietkiewicz et al. 1994).

In the present investigation, ISSR marker techniques were used to determine the genetic variability and genetic structure of natural *R.imbricata* populations for the first time from Trans-Himalayan region, Ladakh, India. Due to the undulated topography of the area and the inaccessibility, diversity studies in this region are limited and mostly focused on taxonomy and botanical inventory. The main objective of this study was to explore the levels of genetic variability of *R.imbricata*, growing in the high mountain passess, Khardung-La and Chang-La, to identify levels of genetic differentiation and provide baseline information for establishment of conservation management strategies for this region.

Materials and Methods

Sample collection

The plant material used in the study of genetic diversity 35 accessions were obtained from 2 wild populations of Khardung-La (5,606 above msl) and Chang-La (above 5,360 MSL) from the Trans – Himalayan region, Ladakh, India. The accessions were designated with accession codes 'K' for Khardung-La and 'C' for Chang-La. The interval between accessions was 0.5- 5.0 km, whereas pair wise distance between populations was 200-300 km. About 10 g of young leaves from each representative sample were obtained and placed in a zip-lock plastic bag and placed in ice bucket. Each sample does not necessarily denote a genetic individual. The samples were stored at -20º C until use.

DNA extraction and ISSR assay

Total genomic DNA was extracted from frozen leaves (5 g) by the CTAB method (Saghai- Maroof et al. 1984) with minor modifications, which included the use of 200 mg per sample polyvinyl pyrollidone.

Evaluation of primers

Thirty eight ISSR primers (Applied Biosciences, India) were initially screened with 10 plant samples. After assessing the effects of template DNA concentration and temperature during annealing stage of amplification, seven primers which produced well amplified, reproducible and polymorphic bands among the ecotypes were selected for further analysis (Table 1).

ISSR amplification

Amplification reaction were performed in volumes of 25 µl containing 10 mM Tris- HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTPs, 0.4 µM primer, 20 ng template DNA and 0.5 unit of *Taq* polymerase (Sigma-Aldrich, USA). Amplification was done using a PTC Thermal Cycler (MJ Research Inc.,) programmed for an initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 1 min, annealing at X °C for 1 min., extension at 72°C for 2 min. the final extension at 72°C for 5 min. and then storage at 4°C where X°C refers to the annealing temperatures specific for each primer (Table 1). The amplification for each primer was performed twice independently with same procedure in order to ensure the fidelity of the ISSR markers. Amplification products were electrophoresed on 1.5 % agarose gel (Life Science Technologies, USA) and run at constant voltage (50
V) in 1X TBE for approximately 2 h, visualized by staining with ethidium bromide (0.5 µg ml-1) and a total of 2.5 µl loading buffer (6X) was added to each reaction before electrophoresis. After electro phoresis, the gels were documented on a gel documentation system (Alphaimager, Alpha Innotech, USA). Molecular size of amplicons was estimated using a 100 bp and 1 Kb DNA ladders ('Bangalore Genei, India').

Scoring and Data analysis

ISSR bands were scored as present (1) or absent (0) .Only those bands showing consistent amplification were scored. The resolving power (Rp) of a primer is $Rp=S^{IB}$ where IB is band informativeness takes the value of : 1-[2X(0.5-P)] P being the proportion of the accessions containing the *i*th amplicon (Prevost and Wilkinson, 1999). From the banding pattern the number of polymorphic loci
(p) and percentage of polymorphic loci at population level were calculated. Percentage of polymorphic loci (PPL), Shanon's Index (I) were calculated to estimate genetic variation level. A dendrogram based on Nei's (1978) unbiased genetic distances and the unweighted pair-group method with arithmetic averages (UPGMA) was constructed using the NTSYS program version 2.1 (Rohlf, 1998). Marker frequencies were estimated based on Lynch and Milligan's (1994) frequency correction for dominant markers. Analyses of molecular variance (AMOVA) based on the pairwise squared Euclidean distances between molecular phenotypes were carried out to partition the genetic diversity between populations using the WINAMOVA program version 1.55 (Excoffier et al. 1992). Statistical significance was determined using 1000 random permutations.

Results and Discussion

ISSR polymorphism

For the 35 *R. imbricata* accessions tested the seven primers produced bands of which six were polymorphic. The size of the amplified DNA fragments ranged from 200 bp to 1000 bp. The high reproducibility of ISSR primers may be due to the use of longer primers and higher annealing temperatures which increased the stringency. The annealing temperature ranged from 44^0 C to 52^0 C. The primers used, GC content, annealing temperature (Tm), total number of loci, level of polymorphism and resolving power are shown (Table 1). The resolving power of the 7 primers ranged from 8.74 for primer ISSR 4 to a maximum of 22.74 for primer ISSR 7. There were no population specific markers i.e markers present in one population but absent in others.

Table 1. List of primers used for ISSR amplification, GC content, total number of loci, the level of polymorphism and resolving power.

Primer	Primer Sequence (5'~3')	GC (%)	Tm (°C)	Total no. of loci	No.of polymorphic loci	% of polymorphic loci	Total no.of fragments amplified	Resolving power
ISSR 1	GAA AGA GAG AGA GAG AT	47	45.3	10	10	100	171	9.77
ISSR 2	GTG TGT GTG TGT GTG TA	47	49.4	10	10	100	221	12.62
ISSR 3	AGA GAG AGA GAG AGA GYT	47.2	49.2	14	14	100	283	16.17
ISSR 4	ACA CAC ACA CAC ACA CYG	52.7	49.3	8	8	100	153	8.74
ISSR 5	TGT GTG TGT GTG TGT GA	47	51.3	12	12	100	188	10.74
ISSR 6	GAG AGA GAG AGA GAG AYC	52.7	43.5	13	13	100	377	21.54
ISSR 7	AGA GAG AGA GAG AGA GYA	47.2	43.9	14	13	92.85	398	22.74
Total		-	-	81	80		1791	-

Single letter abbreviation for mixed base positions : Y=C,T.

Genetic diversity and differentiation

The percentage of polymorphic loci (PPL) among populations ranged from 81.48 % to 97.53 % with an average of about 89.51 % (Table 3). The percentage of polymorphic bands (PPB) for each primer combinations ranged from about 92.85 % to 100%. The mean observed number of alleles (Na) varied from 1.802 (Khardungla Pass) to 1.951 (Changla Pass) whereas the effective number of alleles (Ne) were less than those of mean observed number of alleles (Na) of both the regions and ranged from 1.543 to 1.639. Estimates of Shanon's Index (I) were 0.448 and 0.537 for the two populations studied.

Cluster analysis

Nei's genetic distance and unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis was carried out and a dendrogram was generated that represented the genetic relationship among 35 accessions from two populations (Fig.1). The similarity coefficient ranged from 0.27 to 0.94 across the accessions. The first major cluster contained the 34 accessions in several sub-clusters. The second major cluster contained one accession from Khardung-La (K 11). In the dendrogram all the individuals from two populations does not cluster distinctly.

AMOVA analysis

AMOVA showed significant ($p < 0.001$) genetic variation among populations. Partitioning of genetic variability by analysis of molecular variance revealed that most of the ISSR diversity was distributed between the individual plants within population (62%) with the remaining diversity distributed among population (38%) of the total genetic diversity (Table 2).

Table 2. Summary of genetic variation statistics for all ISSR loci among the *Rhodiola imbricata* populations

Populations	Sample size	Na	Ne	H	I	Percentage of polymorphic loci (%)
Chang-La	18.000±0.000	1.802±0.048	1.543± 0.042	0.306± 0.021	0.448± 0.029	81.48%
Khardung-La	17.000±0.000	1.951±0.035	1.639±0.033	0.335±0.016	0.537± 0.020	97.53%
Mean	17.50±0.039	1.877±0.030	1.591±0.027	0.318±0.018	0.493±0.018	89.51%± 8.02%

Na = observed no of alleles ; Ne = effective no of alleles ; H = Expected heterozygosity I = Shannon's Information Index

Mountain habitats experience special climatic conditions that often differ tremendously from the surrounding lowlands and valleys. Steep topographic and therefore climatic gradients lead to heavily fragmented habitats, characterized by barriers to migration and genetic exchange. Levels of natural fragmentation are thus generally high and several studies have demonstrated strong genetic effects and isolation by distance (Schonswetter *et al.* 2002). Patterns are similar in mountain areas

where several species are known to survive unfavourable conditions by extended clonal growth over dozens or hundreds of years (Wesche *et al.* 2005).

The ability of a population to respond adaptively to environmental changes depends on the level of genetic variability it contains and a species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors or parasites (Schaal *et al.*1998). Moreover, if species are composed of distinct populations, then it is particularly important to gather data on the genetic structure of extant populations since knowledge of the genetic diversity within and among populations of rare species has become a common prerequisite in developing any strategic plans for conservation programs. Genetic diversity studies aid in devising pragmatic conservation strategies and in formulating core collections for rare plant species as one is able to identify regions representing maximum diversity and hence can conserve the same with least population size.

ISSR-PCR has been successfully employed to reveal genetic variation in endemic plants (Kothera et al. 2007), endangered medicinal plants (Zhou et al. 2010), to characterize genomic diversity. Based on its unique characters ISSR technique can detect more genetic loci than isozyme and has higher stability than RAPD. The ISSR markers used for genetic diversity analysis grouped the 35 accessions of *R.imbricata* into two major clusters of 34 and 1. Population from two mountain regions Khardung-La and Chang-La were to a large extent intermingled in the dendrogram. Hence the UPGMA dendrogram showed no clear geographic grouping of the population studied implying the genetic differentiation of *R.imbricata* is lesser extent across locations (Fig 1). The observed diversity pattern suggests that gene flow among the populations is limited and this may be attributed to the patchy habitats of *R.imbricata* and the specific topology of the region such as huge mountain barrier formed by the Himalayan range separated by deep valleys. The populations of the same ecological zone inbred among them therefore diversity within the populations was higher rather between them.

Genetic exchange relies on movement of pollen or seeds. However, climatic conditions in Trans-Himalaya are generally harsh and seedling establishment is exceedingly difficult. The genetic variation within Chang-La and Khardung-La populations of *R.imbricata* were high with Shannon's Information Index (I) values of 0.448 and 0.537 (Table 2) respectively and it may be credited to the clonal propagation through rhizomes and root slits as reported earlier in alpine plants with high levels of clonal diversity (Pluess and Stocklin, 2004).

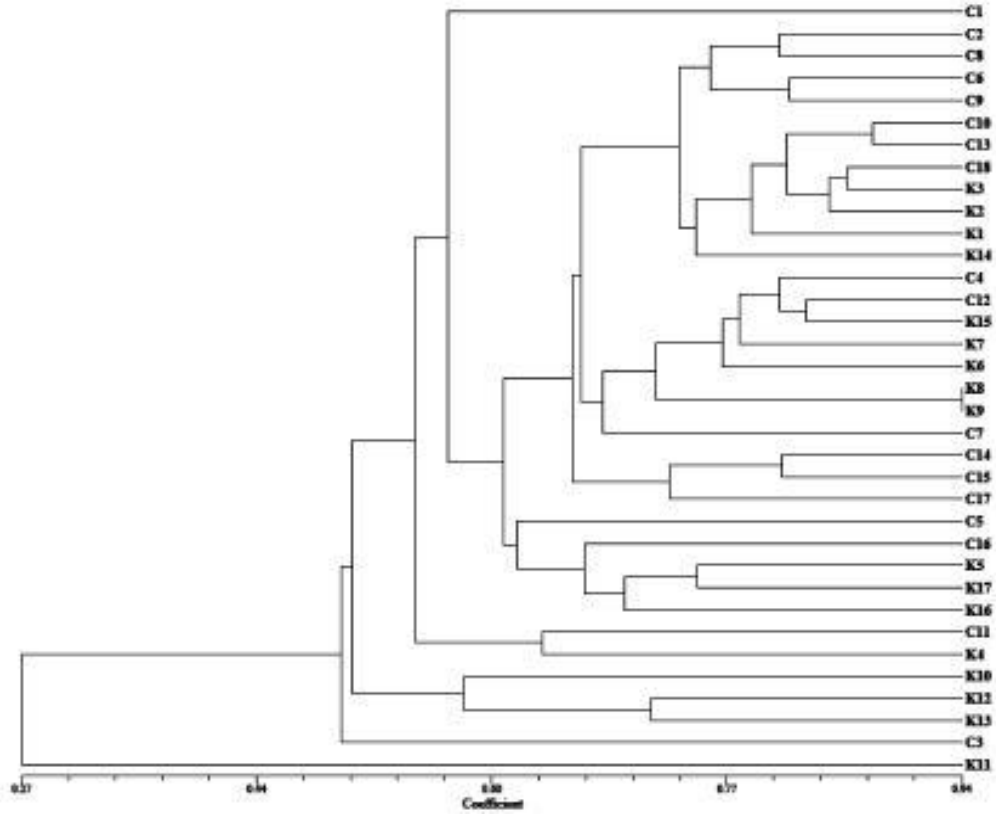

Fig 1. UPGMA clustering based on Jaccard's coeffiecient from ISSR data of *Rhodiola imbricata* ecotypes from the Trans-Himalayan region, Ladakh of India (K represents Khardung-La and C represents Chang-La).

If an appropriate and efficient protection strategy is employed, the natural resources of this rare plant can be restored sooner. However, the fact those *Rhodiola* natural resources have decreased remarkably recently, owning to over-exploitation for medicine and shrinkage of their natural habitat, indicates that the threats to the survival of species of this genera mainly come from human activity and not genetic variation.

The results of AMOVA were highly significant ($p < 0.001$) and it partitioned most of the variation among individuals within populations (62%) and the remainder among populations (38%) (Table 3), as reported in our study is a pattern often described for mountain plants (Cotrim et al. 2003) and Central Asian deserts plants (Sheng et al. 2005). Similar report was made by Lei et al. (2006) in *R. crenulata* using ISSR markers from Hengduan Mountains Region, China. Higher levels of genetic variation among populations were also reported for *R. chrysanthemifolia* and *R.asia* from Central Asia (Ge et al. 2003) and Tibet (Xia et al. 2005; Xia et al. 2007).

Principal coordinate analysis (PCA) clustered the populations in two major groups along a geographic gradient. A graph of the first two principal coordinates was generated, which plotted each *R. imbricata* accession as a point. The high levels of population differentiation imply that isolation in *R.imbricata*, may be more severe or has occurred earlier in evolution. This is indicated by the distinct grouping of populations in the PCA (Fig. 2) and by the results of the AMOVA analysis (Table 3).

Table 3. Summary of nested Analysis of molecular variance (AMOVA) of *Rhodiola imbricata* based on inter-simple sequence repeats. Levels of significance are based on 1000 iteration steps.

Source of variation	Degrees of freedom	Sum of squared	Mean of squared	Variance component	Percentage	p
Among populations	1	7.200	7.200	0.377	38%	<0.001
Within populations	33	20.114	0.610	0.610	62%	<0.001
Total	34	27.314	7.810	0.986	100%	<0.001

Implication of the genetic information for conservation

The *R.imbricata* population structure revealed by the present investigation has apparent conservation and management implications for this region.

Firstly, the populations are found only in patches and in reduced numbers. These small-sized populations are likely subjected to strong genetic drift, especially after a long-time isolation from one another as populations with continually small effective population sizes are especially susceptible to the loss and reorganization of variation by genetic drift. Dodd and Helenurm (2002) reported that if there is no significant correlation between genetic distance and geographic distance as reported in the present study there is clear evidence of genetic drift.

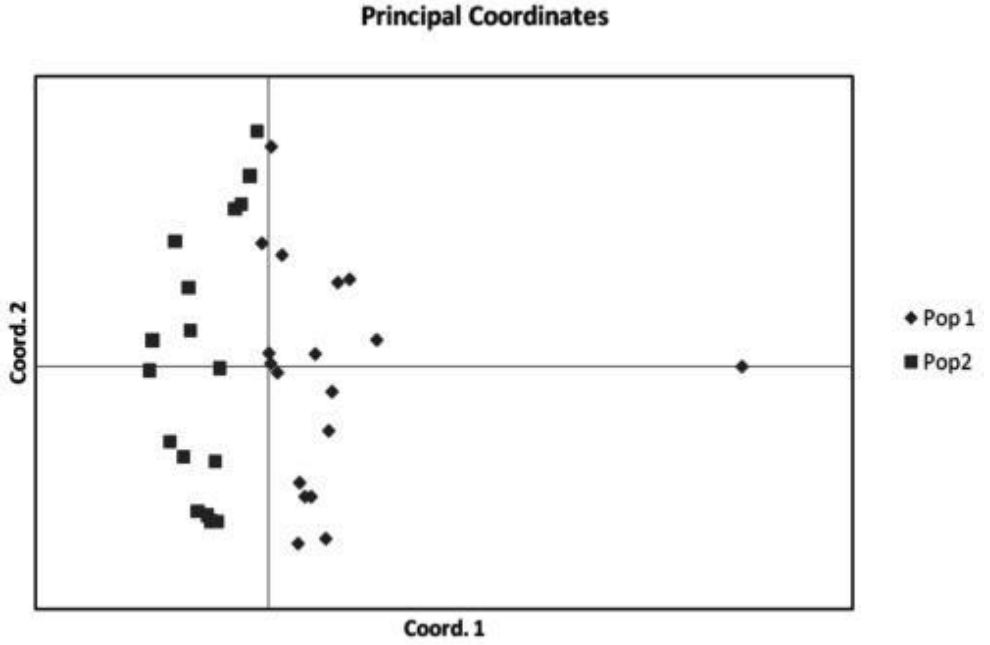

Fig 2. Two-dimensional plot of principal component analysis of thirty-five ecotypes of *Rhodiola imbricata* using ISSR analysis. The numbers plotted represents individual cultivars (Where, Pop 1 = Chang-La, Pop 2 = Khardung-La).

Secondly, the greater part of genetic variability is captured within populations and sampling for conservation *ex situ* should concentrate on representing a high number of individuals rather than representing more populations; a strategy which has already been proposed for other Central Asian endemics (Young *et al*. 2002). This may be an adequate strategy to protect the genetic variation found in the high mountain passes of the Trans-Himalayan region to avoid genetic erosion in the near future owing to its importance and consequent exploitation as a medicinal plant.

Thirdly, genetic structure of a species is dramatically influenced by its breeding system, and selfing can result in low genetic diversity within populations. During the *ex situ* conservation measures, the efficient breeding strategies should be carefully designed and performed to fill up the gap between the common and rare alleles so that they will mate and get preserved in the environment. Conservation goals may be achieved by introducing individuals to new sites, in order to increase the number of conserved populations as translocation and establishment of new populations will curtail the extinction risk of rare plant species.

Future prospects

We are currently developing a conservation plan in collaboration with local authorities, in addition to further field searches and experimental work. DIHAR is establishing a Gene Bank at Chang-La (above 5,360 msl) where the individuals noted as rare ones from this study will be preserved as vegetative propagules and seeds for conservation and reintroduction. Additionally, in some regions where the exploitation of rhizomes for traditional medicine is high, it is necessary to establish sustainable management plans and adequate collection of the rhizomes. We have also established an *in vitro* regeneration protocol for the rare species through shoot tips and cotyledonary explants which will be useful in mass propagation of this rare wild species.

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