Genetic Characterisation of *Rhodiola rosea* Using Gene Specific SSR and CAPS Molecular Markers

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Abstract

The phylogenetic relationships of 30 *Rhodiola rosea* genotypes collected from three valleys *viz*. Changla (17,800 ft), Khardungla (17,582 ft) and Khalse (18,000 ft) of the Ladakh region were analyzed using 10 PCR markers (5 gene specific SSRs and 5 CAPS). This is the first report of molecular genetic diversity studies in *Rhodiola rosea* from this region of the world. The gene specific SSR analysis yielded 12 polymorphic loci, with an average of 1.8 polymorphic bands per primer. The primers based on (CTC)n produced maximum number of bands (3) while, (GA)n, (CG)n and (CCA)n motifs gave lesser number of bands. The CAPS marker did not reveal any polymorphism. Clustering results showed the distribution of 30 *Rhodiola rosea* genotypes into 3 main clusters. While Changla genotypes formed a separate cluster (Cluster C1), there was an overlap between the clusters representing Khardungla (Cluster C2) and Khalse (Cluster C3) genotypes. The results of PCA analysis were comparable to the cluster analysis using dendogram. The genetic differentiation observed was considerable low, indicating a high level of gene flow, which had a strong influence on the genetic structure. Our results indicate considerable gene flow among *Rhodiola rosea* population that might be a result of seed dispersal rather than cross-pollination. More studies are required to understand the different patterns of gene flow and compare the level of genetic diversity which will be useful for characterization of *R.rosea*.

Keywords: *Rhodiola rosea*; Ladakh; Genetic diversity; Gene specific SSRs; CAPS; AMOVA.

1. Introduction

Rhodiola rosea, also known as golden root, belongs to the Crassulaceae family of plants. *Rhodiola rosea* use as a medicinal plant can be traced back to 77 A.D. - when Greek Physician Dioscorides documented *Rhodiola rosea* (called rodia riza back then) in his medical text - *De Materia Medica.* It grows in areas up to 2280 meters elevation. In India, this plant is mainly distributed in the harsh cold arid conditions of Ladakh region. *Rhodiola rosea* thrives under harsh conditions presented by bitter cold climates and high altitude areas. *Rhodiola rosea* is a perennial plant with red, pink, or yellowish flowers. Several shoots grow from the same thick root. The root of this plant is used for medicinal purposes. *Rhodiola rosea* is a popular plant in traditional medical systems in Eastern Europe and Asia with a reputation for stimulating the nervous system, decreasing depression, enhancing work performance, eliminating fatigue, and preventing high altitude sickness. *Rhodiola rosea* has been categorized as an adaptogen by Russian researchers due to its observed ability to increase resistance to a variety of chemical, biological, and physical stressors. *Rhodiola rosea* claimed benefits include antidepressant, anticancer [1,2,3], central nervous system enhancer and also reduces the chances of heart attack by reducing the level of creatinine kinase.

Recent studies have found that this plant contains many secondary metabolites such as Rosavin, Rosarin, Rosin (Cinnamyl alcohol glycosides) and Salidroside which are responsible for the high medicinal value of this plant and demonstrate high adaptogenic activity [4,5,6]. With DNA being the only basis of genetic differences between distinct organisms, DNA fingerprinting is presently the ultimate method of biological individualization. In principle, genetic uniqueness is brought about by two factors, inheritance and mutations and since all genetic differences between individuals are laid down in the primary sequence of their genomic DNA, the most straightforward method is identifying an individual sequence for genomes under comparison [7].

The accelerated and uncontrolled use of *R. rosea* in Ladakh region has been leading to deforestation, with a number of *Rhodiola* species, including *R. rosea*, considered for inclusion in the checklist for conservation purpose. Little is known about the genetic background of *R. rosea* and there is a need for more information on genetic variability and the population structure of natural populations to support *R. rosea* conservation and management programs. Moreover, the analysis of genetic diversity and

relatedness between or within different genotypes is a prerequisite towards the effective utilization and protection of plant genetic resources [8,9]. It will then be possible to achieve rational conservation, and the identification of diagnostic or agronomic traits linked to molecular markers. A wide variety of DNA-based markers has been developed in the past few years. Restriction fragment length polymorphism (RFLP) was the first molecular marker [10] generated for genome analysis and mapping. However, the development of the polymerase chain reaction (PCR) technology has introduced a considerable number of useful molecular markers, e.g., random amplified polymorphic DNA (RAPDs) [11], inter simple sequence repeats (ISSR) [12], amplified fragment length polymorphism (AFLP) [13], simple sequence repeats (SSR) [12] which are in contrast to morphological and biochemical markers are independent of environmental factors and unaffected by developmental stages of the plant. These markers have been used both for DNA fingerprinting [14] and population genetic studies [15]. We focused on the simple sequence repeats (SSRs) and cleavage amplified polymorphism sequence (CAPS) method, which needs neither expensive equipment nor complicated procedures and displays simple and stable results from plants [16,17]. In particular, simple sequence repeats can be exploited as genetic markers. The SSRs have been widely used in plant germplasm evaluation [18,19] and genetic dissection of quantitative trait loci. The use of SSRs to interpret population structure provides much greater resolution than other types of markers because of co dominance and high level of polymorphism at individual SSR loci [20, 21].

Since ages, this plant has been of high medicinal value but till date no research has been conducted on this plant with conservation point of view. In the present investigation, gene specific markers like CAPs and SSR were used to study the genetic variability between and among the *Rhodiola rosea* population from the 3 valleys of Ladakh region of India, i.e. Changla, Khardungla and Khalse. To our knowledge, no report has been published on genetic characterization among the *Rhodiola rosea* populations from Ladakh region using gene specific SSR and CAPS markers and its implications on future *Rhodiola* conservation and management programs.

2. Methods

2.1 Plant Materials

30 locally grown genotypes from 3 valleys (10 from each) were obtained with altitude ranging 17,582 feet (Khardungla) to 18,000 feet (Khalse) from the cold arid desert region of Ladakh (Figure 1). Young leaves were frozen in liquid nitrogen and stored at -80 ^{0}C prior to DNA isolation.

Figure 1: Collection sites of 30 *Rhodiola rosea* genotypes from three valleys viz. khalatse, khardungla and changla (encircled in map) located in Ladakh region of Jammu and Kashmir, India.

2.2 In silico analysis

The ortholog gene sequences involved in the biosynthesis of rosavin, rosin and salidroside of *Arabidopsis thaliana, Oryza sativa, P. patens* and *Zea mays* were retrieved from KEGG database [22] (Figure 2).

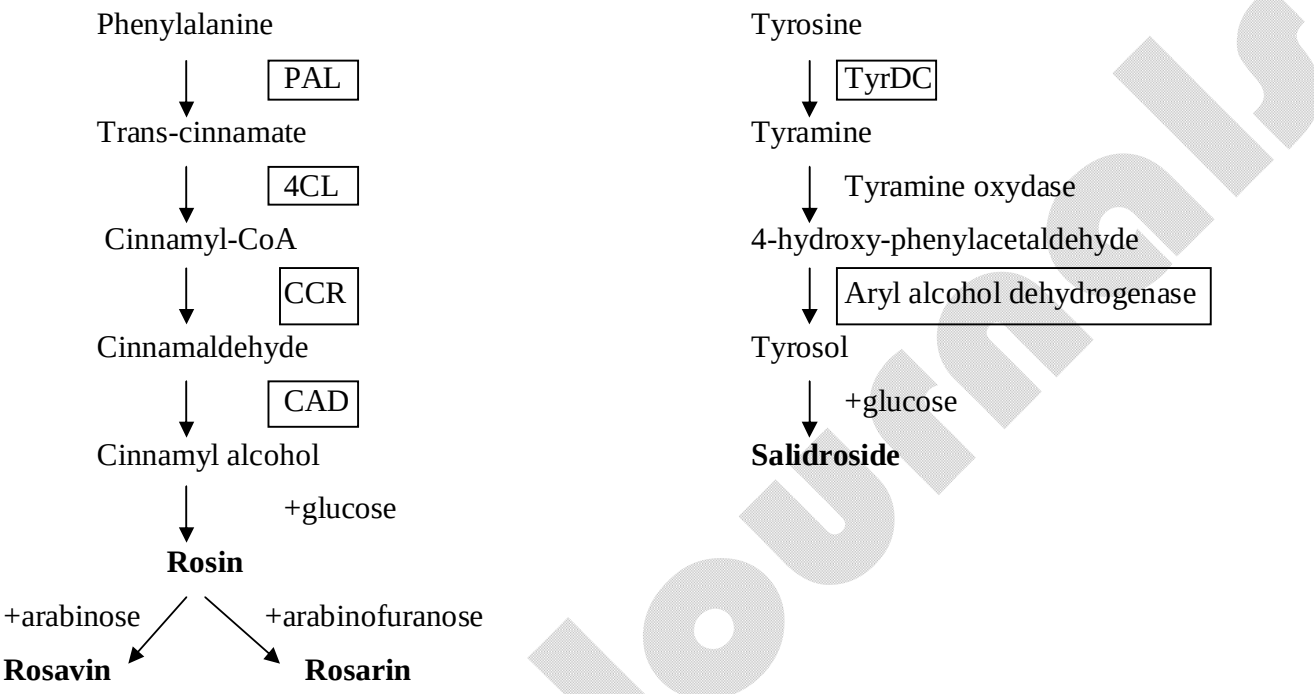

Figure 2: Biosynthetic pathway of rosavin, rosarin and salidroside synthesis in *Rhodiola rosea,* enzymes in boxes were targeted for primer designing.

Meg-align module of DNASTAR [23] was used for multiple sequence alignment of retrieved sequences with available *Rhodiola rosea* EST database to identify the conserved domains for primer designing. BatchPrimer3 [24] was used for the designing of 11 gene specific primers which were custom synthesized (Table 1).

Table 1. List of 5 polymorphic primers used for Simple Sequence Repeat analysis, GC content, total number of loci and level of polymorphism.

Primer ID	Primer sequence (5'->3')	SSR motif	GC (%)	Annealing temperature (^{0}C)	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Total number of fragments amplified
CCR_di_f	GTTGTTGAGATTCTGGCTAAG	CT	42.8	50	3	3	100	31
CCR_di_r	ATTGCTTGATGGATGTGAAC		40.0	47				
CCR_tri_f	GAGATTCTGGCTAAGCTCTTC	CCA	47.6	52	2	2	100	43
CCR_tri_r	ATTGCTTGATGGATGTGAAC		40.0	47				
PAL_f	ATGCTCGTCCGTGTCAAC	CTC	55.5	50	3	3	100	39
PAL_r	GCGATGTAGGAGAGAGGAA		52.6	50				
CAD_di_f	ATAATGGGGGACCTTGAA	GA	44.4	45	2	2	100	27
CAD_di_r	TGTAAGTCAGAGTGGCAGATT		42.8	50				
ARL_di_f	CTGGACACTGCTGCTTTT	CG	50.0	47	2	2	100	32
ARL_di_r	GCGCCCACTACTACTGAC		61.1	52				
	Total				12	12		172

2.3 DNA extraction and Simple Sequence Repeat analysis

Total genomic DNA was extracted from frozen leaves (1g) by CTAB method [25]. 11 primer pairs were used for the study of genetic diversity among the 30 genotypes of *Rhodiola rosea*. 5 primer pairs were polymorphic (Table 1). Amplification reaction was performed in volumes of 15µl containing 0.83pM of each primer, 0.2mM of dNTP mix, 1mM $MgCl_2$, 1.3X PCR buffer and 0.05 units of Taq polymerase (Sigma-Aldrich, U.S.A.). The amplification was carried out using Touchdown PCR (Veriti thermal cycler, ABI). The first cycle consisted of denaturation of template DNA at 94 ^{0}C for 5 min. The denaturation was reduced to 1 min for the next 20 cycles during which the primer annealing was done at a fall of 0.5 ^{0}C for 1 min. followed by primer extension at 72 ^{0}C for 1 min. Now, when the template at an appropriate temperature was observed, the denaturation of template DNA was carried out at 94 ^{0}C for 1 min, primer annealing at Tm ± 2.5^{0}C for 1.50 min and the primer extension at 72 ^{0}C for 2 min. for 42 cycles followed by final extension at 72 ^{0}C for 7 min and storage of the PCR product at 4 ^{0}C.

2.4 Cleaved Amplified Polymorphic Sequence analysis

Five additional primers were synthesized for the target genes and DNA amplifications were carried out in 50 µL reaction mixtures, containing 50 ng DNA, 200 µM dNTPs, 1 µM of each primer, 0.5 units of *Taq* DNA polymerase and 1× *Taq* polymerase buffer. Samples were initially denatured at to 94°C for 7 min and then subjected to 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 74°C and a final extension at 74°C for 7 min. A 5 µL sample of PCR products was individually digested with *Alu*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RV, *Hind*III, *Hinc*II, *Hinf*I, *Mbo*I, *Pst*I, *Rsa*I, *Sac*I, *Sau*3AI, *Sph*I, *Taq*I and *Xba*I, separated by 4.0% NuSieve GTG agarose gel (BioWhittaker Molecular Applications, Rockland, ME, USA) electrophoresis and visualized with ethidium bromide.

2.5 Agarose Gel Electrophoresis

Amplification products were electrophoresed on 2% agarose gel and at constant voltage (70V) in 1X TAE for approximately 1.5 hour, visualized by staining with ethidium bromide (0.5µg/ml) and a total of 2µl loading buffer (6X) was added to each reaction before electrophoresis. After electrophoresis, the gels were documented on a gel documentation system (Biorad). Molecular size of amplicons was estimated using a 100 bp DNA ladder.

2.6 Data Collection and Analysis

The banding patterns obtained from gene specific primers were scored as present (1) or absent (0), each of which was treated as an independent character. Dice coefficient was used to calculate the similarity between pairs of accessions. The similarity matrix was subjected to cluster analysis by unweighted pair group method with arithmetic means (UPGMA) and a dendogram was generated using the program NTSYS pc [26]. POPGENE software was used to calculate Nei's unbiased genetic distance among different genotypes with all markers. Data for observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) across all the three populations were also analyzed [27]. Within species diversity (Hs) and total genetic diversity (Ht) [28] were calculated within the species and within three major groups (as per their collection site) using POPGENE software. The data was subjected to a hierarchical analysis of molecular variance (AMOVA) [29]. GenAlEx software was used to do principal coordinates analysis (PCA) that plots the relationship between distance matrix elements based on their first two principal coordinates.

3. Results and Discussion

3.1 Analysis of genetic variability

This study is the first application of SSR and CAPS markers to the assessment of genetic diversity in *R. rosea*. The partition of genetic diversity using AMOVA indicated that genetic diversity in *R. rosea* is distributed equally within and between populations. Since the population genetic structure of a species is affected by multiple evolutionary factors including the mating system, gene flow, mode of reproduction and natural selection [30], it could be speculated that *R. rosea* might have a mixed mating system, partial out crossing by pollen and seed dispersal and partial selfing by sprouting prostrate rhizomes. In general, the detection of high levels of polymorphism makes SSR analysis a powerful tool for assessing genetic diversity in *R. rosea*.

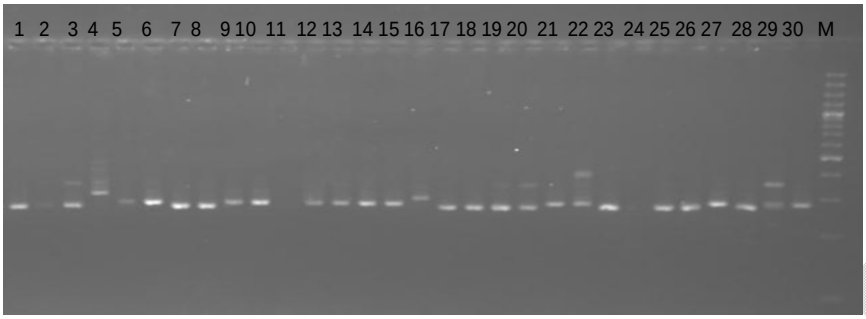

Figure 3: Representative agarose gel showing polymorphism for coding regions in thirty genotypes of *R.rosea* for CCR_di SSR loci. The numerals represent the genotypes from 3 valleys (where Pop 1- Changla(1-10), Pop 2- Khardungla(11-20) and Pop 3- Khalse(21-30), M: Lambda 100bp+ ladder.

Ortholog primers were tested on 30 genotypes from 3 valleys of Ladakh region, for all the polymorphic primers the number of amplified fragments ranged from 31 (CCR_di) to 43(CCR_tri) and the size of the amplified product ranged between 119-200bp which yielded 172 fragments. The 5 polymorphic gene specific primers flanking SSR generated a total of 12 polymorphic loci which ranged from 2-3 loci. The observed high proportion of polymorphic loci (100%) suggests that there is high genetic variation in *Rhodiola* genotypes. In contrast to the level of polymorphism observed with SSR marker, the 5 primer tested for CAPS analysis on the 30 *R.rosea* genotypes showed no polymorphism which may be attributed to high selection pressure and presence of conserved regions in the exonic regions of the gene as compared to the intergenic regions [31,32].

Table 2. Summary of genetic variation statistics of gene specific markers among the *Rhodiola rosea* population with respect to their distributions in three valleys.

Forest divisions	Sample size	Na	Ne	H	I	Ht	Hs	Gst	Number of polymorphic loci	Percentage of ploymorphic loci (%)
Changla	10	1.500 (0.5270)	1.2863 (0.3372)	0.1760 (0.1984)	0.2658 (0.2923)	0.1760 (0.0394)	0.0	1.0	5	50.00
Khardungla	10	1.500 (0.5270)	1.3842 (0.4277)	0.2140 (0.2304)	0.3088 (0.3294)	0.2140 (0.0531)	0.0	1.0	5	50.00
Khalse	10	1.400 (0.5164)	1.2389 (0.3198)	0.1480 (0.1940)	0.2223 (0.2893)	0.1480 (0.0376)	0.0	1.0	4	40.00

Na = Observed number of alleles; Ne = Effective number of alleles; H = Nei's gene diversity; I = Shannon's Information index; Ht = Total genetic diversity; Hs = Genetic diversity in population; Gst = Genetic diversity between populations; NPL = Number of Polymorphic Loci; PPL = percentage of Polymorphic Loci.

The respective details of Na, Ne, H, I, Ht, Hs, NPL and PPL were studied for valley divisions (i.e., Khardungla, Khalse and Changla) and were observed to be higher for Khardungla valley genotypes indicating more variability in Khardungla valley than in other two valleys (Table 2).

Table 3. Summary of analysis of molecular variance (AMOVA) based on genotypes of *Rhodiola rosea* (levels of significance are based on 1000 iteration steps, d.f.: degree of freedom; S.S.D.: sum of square deviation; P-value: probability of null distribution).

Source of variance	d.f	S.S.D.	Variance component	Percentage	P-value
Among valleys	2	21.80	0.99	50	< 0.001
Among populations within valleys	27	26.9	0.996	50	< 0.001
Total	**29**	**48.7**	**1.986**		

AMOVA helps in partitioning of the overall gene specific variations among groups and populations within the group. Molecular variance among valley and among population within valleys was observed to be 50% (Table 3) the similarity in the values of molecular variance may be attributed to mixed mating system, partial out crossing by pollen and seed dispersal in *R.rosea*. The Gst value 0.4476 indicated that 55.24% of the genetic diversity resided within the population (Table 4).

Table 4. Overall genetic variability across all the populations of *Rhodiola rosea*.

Sample size	Observed number of alleles	Effective number of alleles	Nei's gene diversity	Shannon's information index	Ht	Hs	Gst	Estimate of gene flow	Percentage of polymorphic loci (%)
30	1.9 (0.3162)	1.5710 (0.3656)	0.3247 (0.1868)	0.4786 (0.2505)	0.3247 (0.0349)	0.1793 (0.0229)	0.4476	0.6171	90.00

Ht = Total genetic diversity; Hs = Genetic diversity in population; Gst = Genetic diversity between populations.

The overall percentage of polymorphic loci was 90 percent with the rate of gene flow estimated using Gst value was observed to be 0.6171 (Table 4) In general, dispersal resulting in colonization and gene flow into existing population is very important for both the persistence and genetic success of a species [33]. In population genetics, a value of a gene flow (Nm) <1.0 (less than one migrant per generation into a population) or, equivalently, a value of gene differentiation (Gst) >0.25 is generally regarded as the threshold quantity beyond which significant population differentiation occurs [34]. Although the genus *Rhodiola* originates in the mountainous regions of Southwest China and the Himalayas, botanists have established that various species of this genus naturally display a circumpolar distribution in the mountainous regions at the very high latitudes and elevations of the Northern Hemisphere [35,36].

3.2 Dendrogram analysis

A dendrogram based on UPGMA analysis grouped the 30 genotypes into three main clusters with DICE similarity coefficient ranging from 0.59-1.0 (Fig. 2). Cluster 1 represents all genotypes from Changla valley while both cluster 2 and cluster 3 include genotypes from Khardungla as well as Khalse valley, implying that the genetic differentiation of *R. rosea* is correlated to its geographic distribution. The results of the PCA analysis were comparable to the cluster analysis. The first three most informative PC components explained 34.82% of the total variation (Fig. 3). Although the genotypic differences within the valley was observed but the genotypes from different valleys did not fall into a distinct clustering pattern especially for Khardungla and Khalse valleys. However, the genotypes from Changla valley represented a distinct cluster.

4. Conclusion

Considering the high genetic differentiation among the wild populations of *Rhodiola rosea*, conservation of only a few populations may not adequately protect the genetic variations within the species in the Himalayan region. This species, or at least a large part of its genetic diversity, may be lost in near future, owing to its importance and consequent exploitations as a medicinal plant, if appropriate conservation measures are not adopted. Since no single, or even a few plants, will represent the whole genetic variability in *Rhodiola rosea*, there appears to be a need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in *Rhodiola rosea* and avoid genetic erosion. Based on polymorphic feature, genetic diversity, genetic similarity, and gene flow among the populations of *Rhodiola* sp. based on SSR study, we recommend that any future conservation plans for this species should be specifically designed to include representative populations with the highest genetic variation for both in situ conservation and germplasm collection expeditions.

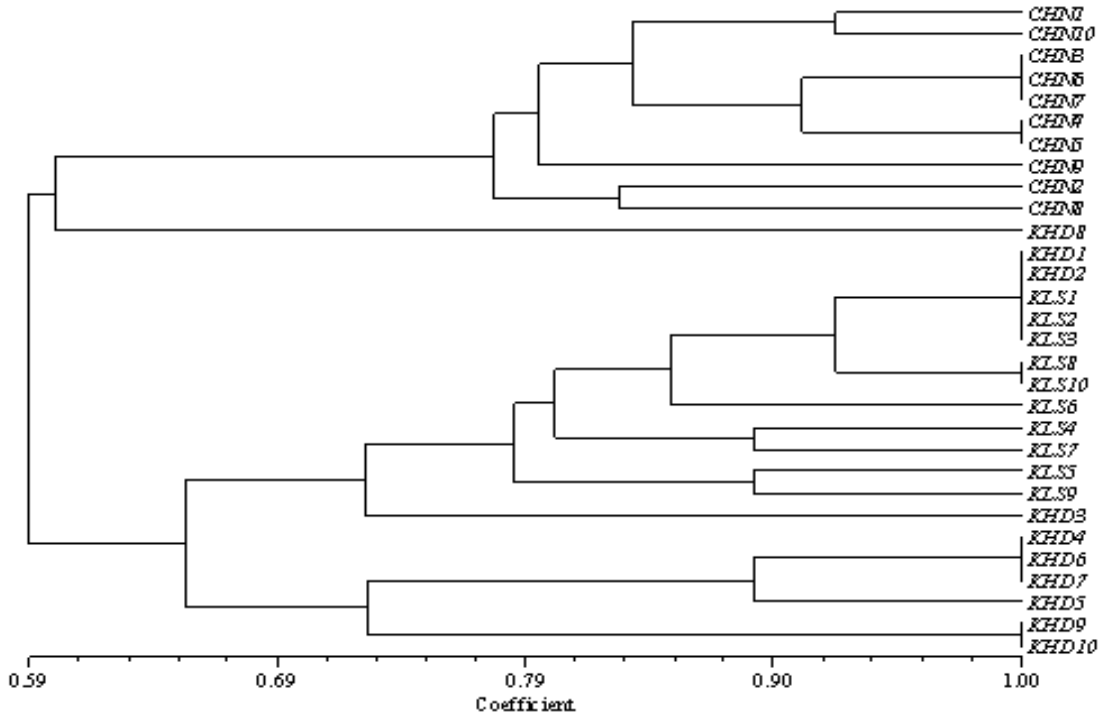

Figure 4: Dendrogram generated using unweighted pair group method with arithmetic average analysis, showing relationships among 30 *Rhodiola rosea* genotypes.

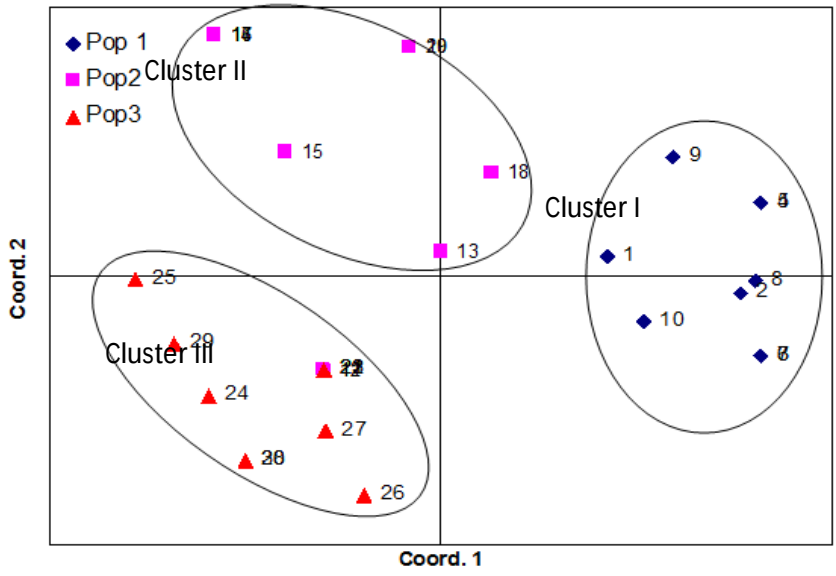

Figure 5: Two-dimensional plot of principal component analysis of 30 genotypes. The numbers plotted represent individual cultivars (where Pop 1- Changla(1-10), Pop 2- Khardungla(11-20) and Pop 3- Khalse(21-30).

List of Abbreviations

PAL - Phenylalanine ammonia lyase

4CL - CoA ligase

CCR - Cinnamyl-CoA reductase

CAD - Cinnamyl alcohol dehydrogenase

TyrDC - Tyrosine decarboxylase

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

KS carried out the gene-specific Sequence Specific Repeats analysis of *Rhodiola rosea*. SR carried out the gene-specific Sequence Specific Repeats analysis. AG carried out the Cleaved Amplified Polymorphic Sequence analysis of *Rhodiola rosea*. KY carried out the Cleaved Amplified Polymorphic Sequence analysis. SP and PKN carried out the statistical analysis of the profiling data. HS helped in compilation of the data and drafted the manuscript.

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