Genetic diversity assessment of *Valeriana jatamansi* Jones using microsatellites markers

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***Valeriana jatamans*i (family Valerianaceae) is a high-value medicinal plant used in traditional and modern medicine. In the present study, 25 populations (151 genotypes) of *V. jatamansi* were collected from Uttarakhand, India and investigated using nuclear and chloroplast SSR markers. Six nuclear and seven chloroplast polymorphic SSR primer pairs were used to evaluate genetic variability and population relatedness. These primer pairs have generated 59 fragments (26 nuclear, 33 chloroplast). The number of alleles per locus ranged from 3 to 7. Expected heterozygosity of the 25 *V. jatamansi* populations was 0.108–0.222 with a mean of 0.165 for nuclear SSR markers and 0.147–0.265 with a mean of 0.215 for chloroplast SSR markers. Based on AMOVA analysis, 6.0% (P = <0.001) of total genetic variation was found among populations. Nuclear SSR markers exhibited highest genetic diversity in samples collected from 1501 to 1800 m amsl altitudinal range and from pine forest habitat. In case of cpSSR, samples collected from 2101 to 2400 m amsl and grassland habitat exhibited highest diversity. These markers could be helpful in the identification and prioritization of genetically diverse populations/individuals for conservation and utilizing them in genetic improvement of *V. jatamansi*.**

Keywords: Conservation, gene flow, genetic diversity, microsatellites, *Valeriana jatamansi*.

VALERIANA jatamansi Jones is a perennial, dioecious and tetraploid ($2n = 4x = 32$) herb, with a genome size of 3.01 Mbp (ref. 1). The species is a native of the Himalaya, but distributed widely in the tropical and subtropical regions of the world[2]. It reproduces sexually and asexually, and stylar movement plays an important role in adverse conditions for species survival and reproductive assurance[3,4]. The species is used in various traditional and modern medicines. Essential oil and extract from the species are used in flavour, pharmaceutical and fragrance industries with over 39 commercial products[5]. The roots and rhizomes of *Valeriana* contain valepotriates[6], dihydrovaltrate[7], linarin-isovalerianate[8], sesquiterpenoids[9], 6-methylapigenin and hesperidin[10] as major ingredients. Antioxidant activity in leaves and roots of the species has also been reported[11,12]. Earlier studies on *V. jatamansi* reported variations in morphological, phytochemical and genetic attributes among the growing localities[4,11,12], which might be one of the factors affecting the activity of raw materials as well as final products. Further, this is more important when raw materials are largely collected from the wild to meet the increasing demand of pharmaceutical industries. Thus, it has put huge pressure on its natural population and led to genetic erosion[4]. Therefore, there is a need to identify and prioritize individuals/populations with higher genetic diversity, which can further be utilized in breeding programmes of the species and in developing new high-yielding varieties.

Classical breeding techniques such as hybridizing high-yielding clones and generation of synthetic variants can also be used for genetic improvement of this species. However, conventional breeding programmes of *V. jatamansi* might not be effective because of its tetraploid nature. Therefore, molecular marker-based breeding strategies are preferred for improvement of the species. As such, the genetic map of *V. jatamansi* is not available. Although studies have been performed on the genetic diversity analysis of *V. jatamansi* using ISSR[4,12], RAPD[13] and AFLP[14] markers, studies using microsatellites (SSR markers) are not available. The traditional methods of microsatellites development are time-consuming and labour intensive[15]. Therefore, an alternative strategy (known as cross-transferability) has been undertaken to identify SSR markers. This technique has been successfully used in agriculture crops like barley, wheat, rice[16], brassica[17], pea[18], cucurbit species[19], *Carthamus* sp.[20] and medicinal plants like *Salvia miltiorrhiza*[21], *Epimedium sagittatum*[22], *Acanthopanax sessiliflorus* and *A. gracilistylus*[23]. However, expressed sequence tags (ESTs) and nuclear sequence information on *V. jatamansi* are not available on the public domain, but sequences of few related species of the *Valeriana* genus are now available. Keeping the above in view, the present study attempts to (i) screen, design and validate nuclear (nuSSR) and chloroplast (cpSSR) markers for *V. jatamansi*, and (ii) analyse the genetic diversity across habitat types and altitudinal range. The findings of the present study will be helpful in genetic improvement programmes and conservation of the target species.

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Table 1. Details of geographic location, accession number and habitat condition of the 25 studied populations of *Valeriana jatamansi* in Uttarakhand (western Himalaya)

Population	District	Accession number	Altitude (m amsl)	Latitude (°N)	Longitude (°E)	Habitat
Daulaghat	Almora	113261	1215	29°46′00″	79°36′00″	Grassland
Katarmal	Almora	BSD-112793	1250	29°38′25″	79°37′20″	Oak forest
Tharali	Chamoli	113206	1330	30°04′06″	79°30′08″	Oak forest
Dolti	Chamoli	GBPI-3201-2	1626	30°02′56″	79°29′50″	Oak forest
Berinag	Pithoragarh	113257	1672	29°43′17″	80°02′14″	Pine forest
Majkhali	Almora	113209	1702	29°40′20″	79°31′47″	Pine forest
Talwari	Chamoli	113205	1785	30°01′46″	79°30′59″	Oak forest
Didihat	Pithoragarh	113253	1850	29°46′16″	80°17′59″	Oak forest
Kausani	Bagheshwar	113210	1869	29°50′30″	79°36′12″	Grassland
Pithoragarh	Pithoragarh	113214	1872	29°36′14″	80°11′40″	Pine forest
Khirshu	Pauri	113212	1880	30°10′05″	78°42′45″	Oak forest
Sitlakhet	Almora	GBPI-3201-2	1900	29°35′40″	79°32′42″	Pine forest
Gwaldam	Chamoli	113213	1923	30°00′24″	79°33′30″	Oak forest
Ukhimath	Rudraprayag	113258	1985	30°31′00″	80°05′00″	Pine forest
Camel back	Dehradun	113200	2000	30°27′43″	78°04′26″	Grassland
Jaberkhet	Dehradun	113260	2080	30°27′18″	78°06′50″	Grassland
Joshimath	Chamoli	113211	2100	29°47′37″	79°27′38″	Mixed forest
Doonagiri	Almora	113254	2125	29°47′37″	79°27′40″	Grassland
Buranskhanda	Tehri	113207	2150	30°27′22″	78°05′59″	Oak forest
Nainital	Nainital	113208	2176	29°23′34″	79°27′45″	Mixed forest
Makku band	Rudraprayag	113262	2240	30°34′00″	79°13′00″	Grassland
Munsyari	Pithoragarh	113202	2241	30°03′39”	80°14′36″	Mixed forest
Malyadaur	Bagheshwar	113255	2350	30°08′16″	79°57′56″	Mixed forest
Dwali	Bagheshwar	113204	2730	30°10′38″	79°59′46″	Mixed forest
Surkunda	Tehri	113259	2775	30°24′21″	78°17′21″	Mixed forest

Materials and methods

Sample collection

The individuals of *V. jatamansi* were collected from 25 distantly located populations in Uttarakhand, India during February 2009. Table 1 presents the site characteristics of the identified populations. The individuals collected from each population were planted in the Herbal garden (1150 m; 29°38″28′N; 79°37″22′E) of the G.B. Pant Institute of Himalayan Environment and Development (GBPIHED), Almora. Botanical identity of the species was authenticated from the Botanical Survey of India (BSI), Dehradun, and the specimens were deposited at BSI and GBPIHED Herbarium. Voucher specimen numbers are included in Table 1.

DNA isolation and primer designing

Total genomic DNA was extracted from the mature leaves of selected plants following standard protocols[4–12]. Sequence information (accessions belonging to different species) on the nuclear region of *V. jatamansi*, *V. officinalis*, *V. hardwickii*, *Plectritis congesta* and *Nardostachys jatamansi* was retrieved from the NCBI (http://www.ncbi.nlm.nih.gov) database for designing nuSSRs. An alternative strategy for designing SSR markers is given in Figure S1 (see Supplementary Information online). However, sequences of chloroplast DNA of *V. jatamansi* were used to design cpSSR markers. The SSR primer pairs were designed from unique sequences flanking SSR loci identified for the development of genomic SSR markers and designated as SSR 1 to SSR 25. Primer pairs could not be designed from the remaining 30 SSR-motif containing sequences because their flanking sequences were either too short or the nature of sequences did not fulfil the criteria for primer design using BATCH PRIMER 3 version 1.0 (ref. 24). All the sequences were processed and screened for microsatellite motifs and primer pairs were designed following standard condition [product length 150–500 bp (optimum 300 bp), primer size 18–25 bp (optimum 20 bp) and primer melting temperature of 55–60°C (optimum 57°C)]. A total of 25 SSR primer pairs were designed for the present study. However, 10 universal microsatellite primer pairs of chloroplast origin were also selected for screening and validation within the target species[25].

Microsatellite amplification

Twenty-five SSR primer pairs (20 nuclear and 5 chloroplast SSR markers) have been developed in the present study. However, 10 universal chloroplast SSR primer pairs[24] were also used for initial screening. On the basis of initial screening for polymorphism, 13 primer pairs (12 developed in this study and 1 universal) were selected

and used for PCR amplification of all the 25 *V. jatamansi* populations. PCR amplification was carried out in 20 µl volume containing 0.2 µM of each primer, 1 unit *Taq* DNA polymerase, 1× reaction buffer, 0.2 µM of each dNTP and 10–20 ng DNA template. Negative controls were included to check for the reliability of each DNA extraction. DNA concentration and purity were assessed using a spectrophotometer (Hitachi, Japan, model U-2001). PCR amplification was performed using three different touchdown PCR programs. An initial denaturation step at 94°/95°C for 3/5 min; ten touchdown cycles followed with 94°/95°C for 30/45 sec, 55°/60°C (@ –0.5°C per cycle) for 30 sec and 72°C for 45 sec. The remaining 30 cycles were carried out as follows: denaturation at 94°C for 30 sec, annealing at 55°/60°C and extension at 72°C for 45 sec followed by final extension at 72°C for 10 min. Annealing temperature varied from 50°C to 60°C, to amplify specific microsatellite markers (Tables S1–S3; see Supplementary Information online). The PCR product size was separated using 3% metaphor agarose gel electrophoresis.

Data analysis

The amplification profiles for each microsatellite were scored visually and independently twice using the 100 bp size standard DNA ladder (Fermantas, India). Ambiguous data were re-examined in a new electrophoresis run. The amplification products were scored as 1 (presence) and 0 (absence). For better understanding of genetic structure of the species, populations were grouped into four different habitat types (grassland, oak forest, pine forest and mixed forest) and five altitude ranges (1201–1500, 1501–1800, 1801–2100, 2101–2400 and >2400 m amsl). The generated population genetic data were analysed using GenAlEx 6.5 for binary data[26]. Different parameters of genetic diversity like percentage polymorphic loci (Pp%), number of alleles (Na), number of effective alleles (Ne), Shannon's information index (I), expected heterozygosity (He) and unbiased expected heterozygosity (uHe) were calculated with same software. Polymorphism information content (PIC) of individual markers was estimated using the following formula[27]

$$\mathrm{PIC} = 1 - \sum xi^2,$$

where xi is the relative frequency of the ith allele of the SSR loci. Principle coordinate analysis (PCoA) was performed to visualize genetic relationship between populations based on Nei genetic distance[28] using a covariance-standardized PCoA method in GenAlEx 6.5. The significance of the differences between nuSSRs and chloroplast-based SSRs was tested using Student's t test. A dendrogram was constructed using PHYLIP, version 3.68. The binary matrix data were converted into PHYLIP format and distance matrices were produced using the GENEDIST program of the PHYLIP package[29]. Unrooted phylogenetic trees were constructed by the neighbour-joining method using NEIGHBOR component of PHYLIP package. The statistical significance of the groups obtained was assessed by bootstrapping (1000 replicates), using the SEQBOOT, GENEDIST, NEIGHBOR and CONSENSE programs[28]. Genetic differentiation among populations (ϕPT) and among individuals was calculated with AMOVA module in GenAlEx 6.5. The number of populations of *V. jatamansi* (n) which was necessary to represent 99.99% of the total genetic diversity among populations (P) was calculated as follows[30]

$$P = 1 - (\phi PT)n.$$

Results

Genetic diversity

Transferability of interspecific and intergeneric SSR markers was tested for PCR amplification and genetic diversity of 151 individuals belonging to 25 *V. jatamansi* populations. Among the 21 nuSSR primer pairs (two pairs designed from *V. jatamansi* sequences and 19 from other species), 18 (85.71%) yielded PCR amplicons of expected size and were designated as validated markers. However, only six (28.57%) nuSSR primer pairs were polymorphic and were further used for detailed study (Table 2). Among the 15 cpSSR primer pairs, 7 exhibited clear, reproducible and polymorphic fragments and were used for further study (Table 3). Analysis of the nucleotide sequences of EST-SSRs revealed 33.33% trinucleotides repeats, 50% dinucleotide repeats and 16.66% pentanucleotides repeats in *V. jatamansi*. A total of 59 loci (33 CpSSR + 26 nuSSR) were identified ranging from 75 to 600 bp. PIC of the markers used was relatively low (mean 0.2721), ranging between 0.1825 and 0.3596 for chloroplast SSR markers (Table 2). Similarly, PIC value for nuclear SSR markers ranged from 0.1463 to 0.3615 with a mean of 0.2521 (Table 3).

Genetic variability across populations

Analysis of data using SSR markers revealed the presence of 26 and 33 loci using nuSSR and cpSSR markers respectively, in all the populations of *V. jatamansi* (Figures S2 and S3; see Supplementary Information online). Across populations, the highest percentage of polymorphic loci (69.23), number of alleles (1.538 ± 0.15), Shanon's information index (0.341 ± 0.05) and unbiased expected heterozygosity (0.241 ± 0.04) were recorded in the Majkhali population, while highest effective number of alleles (1.39 ± 0.08) and genetic diversity (0.222 ± 0.04) were recorded in the Jaberkhet population using nuSSRs

Table 2. List of nuSSR (nuclear) primers designed and amplified for genetic diversity analysis in the 25 *V. jatamansi* populations

Species	Accession ID	Region	Length (bp)	GC%	Sequences (5′–3′)	Product size (bp)	Code	Number of fragments	Repeat motif	PIC
V. jatamansi	JF317627.1	Genomic	21	47.62	TCTTGAGTACTTCCACGTAGC	133	SSR1	4	CGTAC	0.1463
			18	66.67	CAGACTAGCAGCGCTCGC					
Plectritis congesta	JF714696.1	Con228	21	42.86	GACAATCTCAGACAAGGACAA	150	SSR4	4	GT	0.2384
			21	38.10	TCTCCGAATAAGTATGGTAGT					
P. congesta	JF714691.1	Pcon151	21	33.33	CCCTTTCTCTCAAATCAAAAT	150	SSR 12	4	GAT	0.2306
			21	42.86	TACACGATCGACAACGTCATA					
P. congesta	JF714688.1	Microsatellite	21	42.86	ATGATTGCCTAACACACACAC	150	SSR 15	5	GA	0.3424
			22	45.45	CTCATCAAGCAACAACACACAG					
P. congesta	JF714688.1	Microsatellite	21	52.38	ACGGAGAGAGAGAGAGAGAGA	144	SSR 16	4	AG	0.3615
			21	47.62	AGCAGCAGCAATGCATGATGA					
P. congesta	JF714688.1	Microsatellite	21	33.33	AGTTTGCCGATGTATTGATTA	158	SSR 17	5	TGA	0.1933
			21	42.86	TTCCATTGCATTTCCACTTCG					
							Total	26		

Table 3. List of cpSSR (chloroplast) primers designed and successfully amplified for genetic diversity analysis in the 25 *V. jatamansi* populations

Code	Orientation	Sequence (5′–3′)	Length (bp)	Melting temperature (°C)	No. of amplified fragments	Polymorphic information content (PIC)
SSR22	F	GATCCCGGACGTAATCCTG	19	53	3	0.3040
	R	ATCGTACCGAGGGTTCGAAT	20	52		
SSR23	F	AATGCTGAATCGAYGACCTA	20	48	6	0.2384
	R	AATGCTGAATCGAYGACCTA	20	48		
SSR25	F	CGATGCATATGTAGAAAGCC	20	50	3	0.2561
	R	CATTACGTGCGACTATCTCC	20	52		
SSR32	F	GCTAGTTCCTTTATTCCAAAGA	22	49	3	
	R	CGGTTGATTTTAAATGAGAAG	21	47		
SSR33	F	GATTCTTTCTCCACGACTGTA	21	50	3	0.2920
	R	AGAAAGACTAAGATGGATTCGT	22	49		
SSR34	F	CTTTAGGAACCTGTGAATGTG	21	50	7	0.3596
	R	ACTTGAGGGTATCCTATGCTT	21	50		
SSR35	F	CAAATTGCAAAACTTCAGAGT	21	47	8	0.1825
	R	GTCTATGGAGAGAGACCCTGT	21	54		
				Total	33	

F, Forward; R, Reverse.

(Table 4). Percentage of polymorphic loci and number of alleles were found lowest in the Gwaldam population. Similarly, the Surkunda population showed lowest value for other genetic variability parameters using nuSSRs (Table 4).

Across populations, the highest percentage of polymorphic loci (72.73) and number of alleles (1.576 ± 0.06) were recorded in the Pithoragarh population. While effective number of alleles (1.467 ± 0.07), Shannon's information index (0.388 ± 0.05), expected heterozygosity (0.265 ± 0.04) and unbiased expected heterozygosity (0.294 ± 0.04) were recorded highest in the Nainital population using cpSSR. Effective number of alleles was found to be lowest (1.25 ± 0.06) in the Dolti population. However, other parameters of genetic variability were lowest in the Ukhimath population (Table 4). No significant difference was recorded in the number of amplified fragments by cpSSR and nuSSR markers ($t = 0.764$; $P < 0.474$) using paired *t*-test.

Genetic diversity across altitudinal range

Across the five altitudinal ranges, the highest value for genetic variability parameters was recorded at 1501–1800 m amsl (Pp – 55.77%; Na – 1.31; He – 0.18; I – 0.28) using nuSSR markers. Similarly, all genetic variability parameters showed the lowest value in the range >2400 m amsl in case of nuSSR markers (Table 5).

Table 4. Genetic diversity of the 25 *V. jatamansi* populations using nuSSR and cpSSR markers

	Pp (%)		Na		Ne		I		He		uHe	
Population	nuSSR	cpSSR	nuSSR	cpSSR	nuSSR	cpSSR	nuSSR	cpSSR	nuSSR	cpSSR	nuSSR	cpSSR
Daulaghat	46.15	66.67	1.077	1.485	1.238	1.399	0.229	0.345	0.149	0.230	0.158	0.246
Katarmal	57.69	60.61	1.346	1.394	1.346	1.357	0.302	0.319	0.201	0.211	0.224	0.235
Tharali	57.69	51.52	1.346	1.212	1.296	1.336	0.276	0.282	0.180	0.191	0.192	0.208
Dolti	46.15	42.42	1.154	1.152	1.294	1.250	0.252	0.223	0.169	0.147	0.194	0.169
Berinag	46.15	48.48	1.115	1.182	1.278	1.368	0.244	0.293	0.162	0.202	0.186	0.231
Majkhali	69.23	48.48	1.538	1.182	1.355	1.382	0.341	0.300	0.221	0.209	0.241	0.228
Talwari	61.54	60.61	1.423	1.364	1.312	1.382	0.288	0.326	0.187	0.219	0.204	0.239
Didihat	46.15	60.61	1.154	1.333	1.215	1.436	0.213	0.353	0.136	0.243	0.149	0.265
Kausani	65.38	69.70	1.500	1.545	1.332	1.426	0.312	0.366	0.203	0.245	0.217	0.262
Pithoragarh	65.38	72.73	1.500	1.576	1.257	1.438	0.268	0.387	0.167	0.258	0.175	0.270
Khirshu	57.69	60.61	1.308	1.303	1.323	1.465	0.287	0.373	0.190	0.258	0.204	0.278
Sitlakhet	65.38	66.67	1.500	1.455	1.340	1.454	0.318	0.377	0.208	0.256	0.224	0.276
Gwaldam	26.92	51.52	0.769	1.212	1.188	1.336	0.159	0.282	0.108	0.191	0.118	0.208
Ukhimath	34.62	39.39	0.885	0.970	1.205	1.265	0.185	0.219	0.123	0.149	0.137	0.165
Camel Back	50.00	60.61	1.231	1.364	1.249	1.410	0.236	0.342	0.152	0.233	0.164	0.250
Jaberkhet	61.54	57.58	1.462	1.333	1.390	1.425	0.329	0.336	0.222	0.232	0.239	0.249
Joshimath	42.31	51.52	1.077	1.212	1.248	1.348	0.221	0.287	0.146	0.195	0.163	0.217
Doonagiri	38.46	42.42	1.000	1.091	1.228	1.304	0.202	0.247	0.134	0.169	0.153	0.193
Buranskhanda	53.85	57.58	1.269	1.333	1.239	1.393	0.240	0.324	0.152	0.221	0.164	0.238
Nainital	53.85	66.67	1.269	1.455	1.344	1.467	0.294	0.388	0.198	0.265	0.220	0.294
Makku band	57.69	63.64	1.385	1.455	1.275	1.397	0.269	0.344	0.173	0.231	0.188	0.252
Munsyari	50.00	63.64	1.269	1.455	1.309	1.448	0.274	0.370	0.183	0.253	0.204	0.281
Malyadaur	30.77	48.48	0.846	1.182	1.196	1.395	0.172	0.304	0.116	0.213	0.129	0.236
Dwali	38.46	42.42	0.962	1.061	1.237	1.281	0.212	0.243	0.142	0.164	0.162	0.188
Surkunda	34.62	60.61	0.923	1.424	1.152	1.331	0.159	0.307	0.101	0.201	0.112	0.224
Mean	50.30	56.60	1.212	1.309	1.274	1.380	0.251	0.317	0.165	0.215	0.181	0.236

Table 5. Genetic diversity of *V. jatamansi* across the altitudinal range using nuSSR and cpSSR markers

Marker	Altitude (m amsl)	Pp (%)	Na	Ne	I	He	uHe
nuSSR	1201–1500	53.84	1.26	1.29	0.27	0.18	0.19
	1501–1800	55.77	1.31	1.31	0.28	0.18	0.21
	1801–2100	51.54	1.24	1.27	0.25	0.17	0.18
	2101–2400	47.44	1.17	1.27	0.24	0.16	0.18
	>2400	36.54	0.94	1.19	0.19	0.12	0.14
cpSSR	1201–1500	59.60	1.36	1.36	0.32	0.21	0.23
	1501–1800	52.52	1.24	1.38	0.31	0.21	0.23
	1801–2100	56.57	1.30	1.39	0.32	0.22	0.24
	2101–2400	58.59	1.36	1.41	0.34	0.23	0.26
	>2400	53.87	1.28	1.34	0.30	0.20	0.22

cpSSR revealed the highest percentage of polymorphic loci (59.60) and number of alleles (1.36) at 1201–1500 m amsl, but the highest effective number of alleles (Ne – 1.41), expected heterozygosity (He – 0.23), Shannon's information index (I – 0.34) and unbiased expected heterozygosity (uHe – 0.26) were noticed at 2101–2400 m amsl. The lowest Shannon's information index (I – 0.30), expected heterozygosity (He – 0.20) and unbiased expected heterozygosity (uHe – 0.22) were measured in the range >2400 m amsl (Table 5). The lowest percentage of polymorphic loci (Pp – 52.52%) and number of alleles (Na – 1.24) were found in the range 1501–1800 m amsl.

Genetic diversity among habitat types

Among habitat types, highest genetic diversity parameters were recorded in pine forest habitat (Pp – 56.152; Na – 1.308; Ne – 1.287; I – 0.271; He – 0.176; uHe – 0.193) and lowest in mixed forest (Pp – 41.668; Na – 1.058; Ne – 1.248; I – 0.222; He – 0.148; uHe – 0.165) using nuSSR markers (Table 6). However, in the case of cpSSR markers, highest genetic parameters were recorded in grassland habitat (Pp – 57.690%; Na – 1.385; I – 0.269; He – 0.173; uHe – 0.188) and lowest (Pp – 41.668; Na – 1.058; Ne – 1.248; I – 0.222; He – 0.148; uHe – 0.165) in mixed forest habitat (Table 6).

Table 6. Genetic diversity in *V. jatamansi* across habitat types using nuSSR and cpSSR markers

Marker	Habitat type	Pp (%)	Na	Ne	I	He	uHe
nuSSR	Grassland	53.203	1.276	1.285	0.263	0.172	0.187
	Mixed forest	41.668	1.058	1.248	0.222	0.148	0.165
	Pine forest	56.152	1.308	1.287	0.271	0.176	0.193
	Oak forest	50.960	1.221	1.277	0.252	0.165	0.181
cpSSR	Grassland	57.690	1.385	1.275	0.269	0.173	0.188
	Mixed forest	41.668	1.058	1.248	0.222	0.148	0.165
	Oak forest	50.960	1.221	1.277	0.252	0.165	0.181
	Pine forest	50.106	1.221	1.266	0.248	0.162	0.178

Table 7. Analysis of molecular variance (AMOVA) based on nuclear SSR markers of the 25 *V. jatamansi* populations from four types of habitats in Uttarakhand (western Himalaya)

Source	Degree of freedom	Sum of square	Variance component	Variation (%)
Among populations	24	102.94	0.193	6 [*P*(rand> = data) = 0.001]
Within populations	126	394.24	3.129	94
Total	150	497.18	3.322	100

Genetic structure

Analysis of molecular variance revealed significant variations (6.0%; $P = <0.001$) among populations and 94% within populations using nuclear SSR markers (Table 7). Among the 25 populations, Pithoragarh, Kausani, Majkhali and Jaberkhet populations had maximum genetic variation within population, while Doonagiri had the lowest. No significant genetic variation among populations of *V. jatamansi* was detected using cpSSRs. Dendrogram based on neighbour-joining method using cpSSR and nuSSR markers separated the 25 populations by showing similar types of topology and two major groups, namely A and B. Group B was further subdivided into two subgroups in case of both markers (Figures 1 and 2). Genetic distance measurement based on Nei's genetic distance (pair wise) of cpSSR revealed the lowest (0.026) genetic distance between Didihat (1850 m amsl; oak forest) and Jaberkhet (2080 m amsl; grassland) populations, and the highest distance (0.192) between Dolti (1626 m amsl; oak forest) and Camelback (2000 m amsl; grassland) populations. Similarly, Nei's genetic distance (pairwise) of nuSSR revealed the lowest genetic distance (0.011) between Makku band (2240 m amsl; grassland) and Malyadaur (2350 m amsl; mixed forest) populations, and the highest distance (0.187) between Berinag (1672 m amsl; pine forest) and Munsyari (2241 m amsl; mixed forest) populations.

Nei genetic distance matrix from nuSSR data was further used for PCoA to illustrate the genetic diversity and/or relationship among populations based on two major principal axes that explained 43.73% of the total variation. PCoA of nuSSRs depicted clear spatial separation of each population in the PCoA diagram (Figure 3 *a*). In the diagram, the Munsyari population was the farthest apart from the Berinag population as they had the highest Nei genetic distance among them. Besides, populations like Dolti, Jaberkhet, Didihat and Ukhimath were clearly isolated from the rest. Although cpSSRs data did not reveal any significant variation among populations in AMOVA analysis, the PCoA analysis of cpSSR data revealed that Dolti, Ukhimath and Malyadaur are well separated from the rest (Figure 3 *b*).

The nuSSRs analysis revealed significant variation among *Valeriana* populations and also detected pairwise population genetic distances that clearly separated most of the populations in the PCoA diagram. According to the calculation of genetic differentiation (ϕ_{PT} value – 0.06) in nuSSR AMOVA analysis, the conservation of 3–4 populations (actual calculated number is 3.23) is the minimum necessary condition to protect 99.99% of the total genetic diversity of these 25 *V. jatamansi* populations.

Discussion

The present study reports on designing and testing cross-transferability of nuSSR and cpSSR markers among different populations of *V. jatamansi*. Here, five microsatellite markers were found cross-transferable and polymorphic in *V. jatamansi*. These molecular markers can be used for both basic and applied research, including germplasm characterization and evaluation, breeding application and phylogentic study of *Valeriana* species. Studying genetic diversity of a natural plant species is an important attribute for its adaptation to future environmental changes and developing strategies for its conservation and/or maintenance. In this context, study of SSR markers (nuSSR and cpSSR) is important due to their role in genetic diversity analysis, breeding programmes, phylo-

genetic studies, understanding the past and present gene flow, etc.[22,31]. Genetic diversity analysed using SSR markers revealed low genetic base in the present study compared to earlier studies where ISSR markers revealed higher genetic diversity[4–12]. The higher genetic diversity in *V. jatamansi* using ISSR markers might be due to amplification of the samples with more number of markers, which cover maximum variation within the whole genome compared to the present SSR diversity. However, SSR markers have an advantage over ISSR marker to detect better genetic polymorphism due to their multi-allelic and co-dominance nature. The lower expected heterozygosity using nuSSR markers might be due to amplification of limited number of SSR loci. However, in the case of cpSSRs, the presence of conserved gene order, uniparental inheritance, non-recombination, low mutation rate and low polymorphism in chloroplast genome are the additional factors responsible for the observed low genetic diversity[31]. As *V. jatamansi* reproduces by sexual and asexual means, the chloroplast genome can only be disseminated by seeds or rhizome, and chloroplast DNA markers provide information on past changes in species distribution that are mostly unaffected by subsequent pollen exchange or dispersal. The chloroplast genome shows length polymorphisms associated especially with mononucleotide repeats. Noncoding intron and intergenic spacers are particularly variable and contain microsatellite and non-microsatellite polymorphisms even between closely related individuals and taxa in a range of plant groups[31].

Further, the case of cpSSR, a large proportion of the loci is monomorphic but some of these loci are likely to show polymorphism on analysis of larger set of populations/genotypes. Use of more sensitive techniques for DNA fragment size analysis, e.g. polyacrylamide gel electrophoresis or capillary electrophoresis might be expected to show higher rate of polymorphism. However, low numbers of nuSSR and cpSSR markers were not able to cover enough variation within the whole genome of *V. jatamansi* and exhibit low genetic diversity.

While comparing genetic diversity among populations, highest genetic diversity in Pithoragarh (1872 m amsl) and lowest in Surkunda (2775 m amsl) population were recorded. Similarly, high genetic diversity at low altitude range (nuSSRs: 1501–1800 and cpSSRs: 1201–1500 m amsl) supports the results of an earlier study[4]. At lower altitudinal range, the species shows profuse flowering that attracts a large number of pollinators and thereby facilitates better gene flow among individuals[4]. On the contrary, steep valleys and high mountain areas in the high-altitude region demarcate the plant habitats which possibly limit gene flow among plant populations and

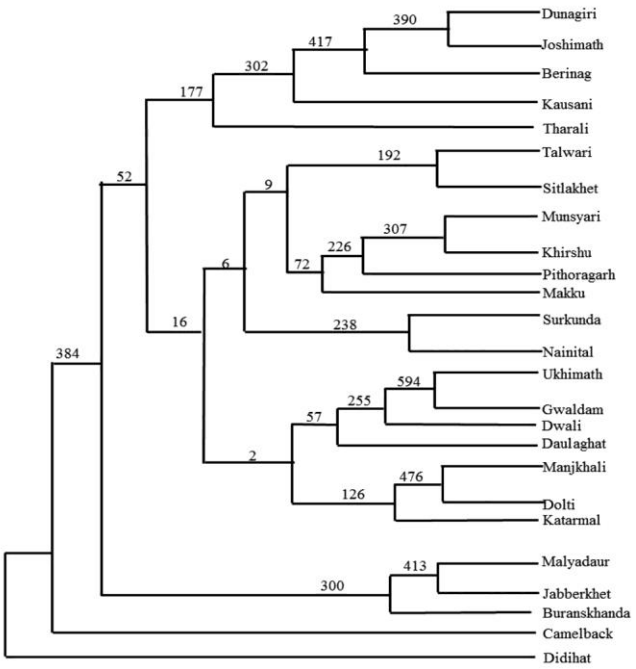

Figure 1. Relationship among the 25 *Valeriana jatamansi* populations based on cpSSR markers analysis.

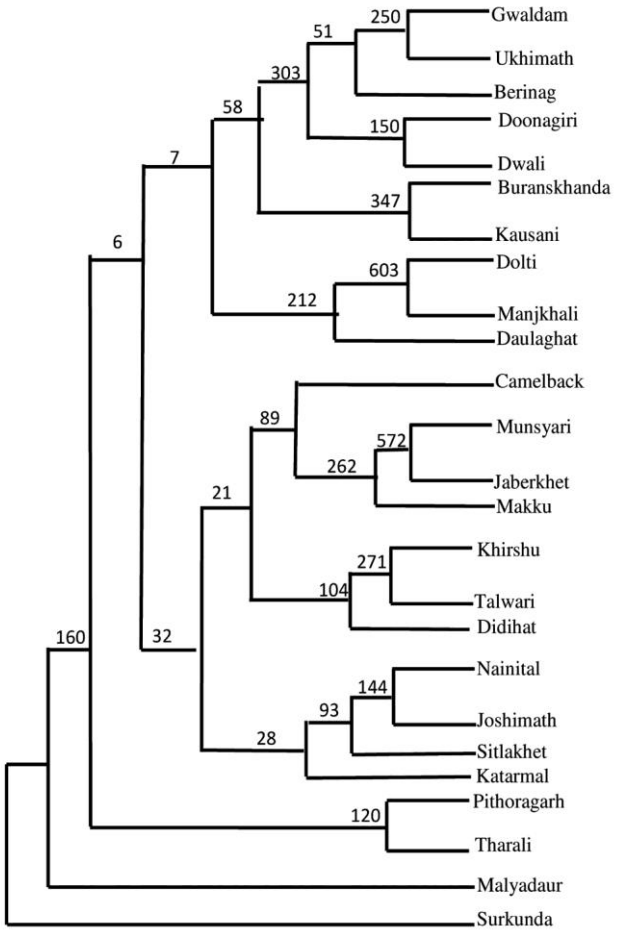

Figure 2. Relationship among the 25 *V. jatamansi* populations based on nuSSR markers analysis.

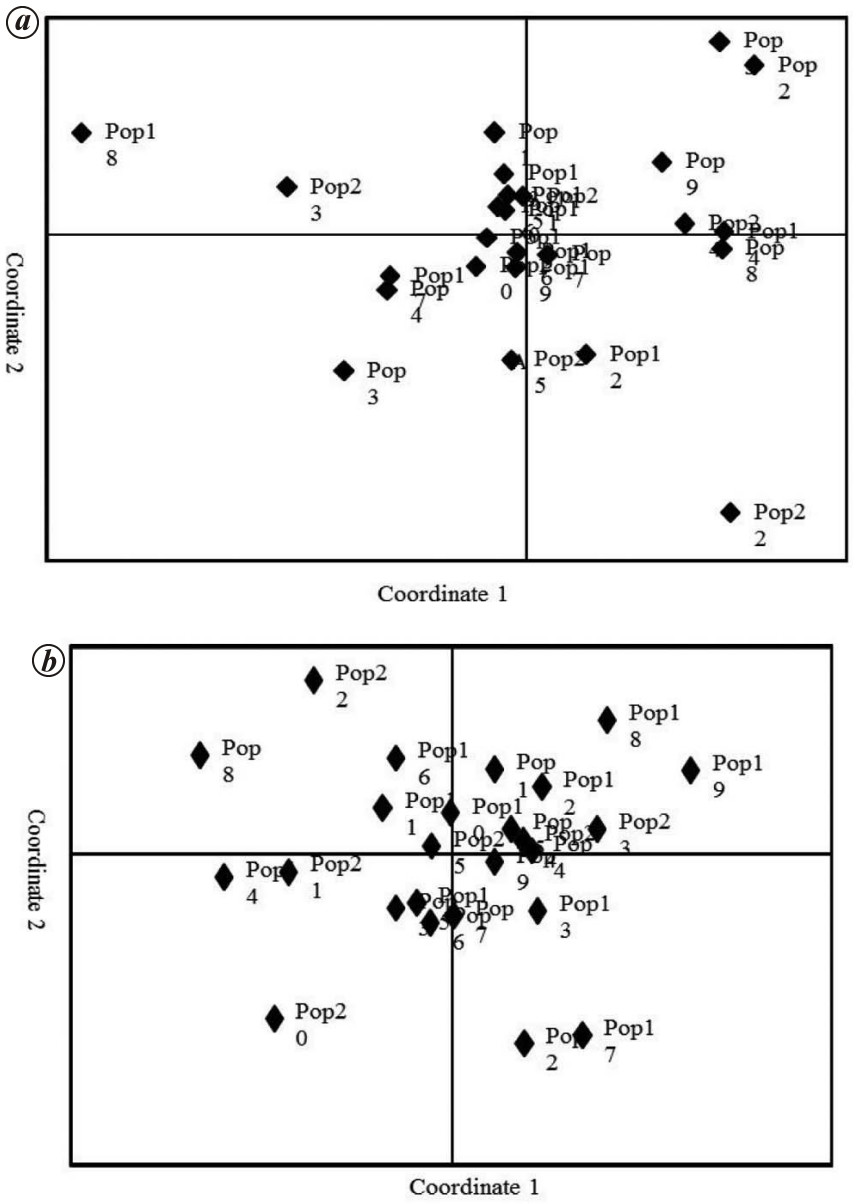

Figure 3. Principal coordinate analysis showing relationship among 25 studied populations of *V. jatamansi*: ***a***, cpSSR markers; ***b***, nuSSR markers.

colonization opportunities of new sites[32]. It is known that limited gene flow due to habitat isolation could lead to stronger genetic differentiation and/or low genetic diversity[33]. The genetic differentiation of Munsyari (Figure 3 *a*) from other populations might be due to its presence in higher altitude. Adverse climatic conditions at high altitudes limit the diversity of pollinators, which in turn affects gene flow and seed setting[34]. Further, prominent effect of temperature was recorded on anthesis and breeding behaviour of *V. jatamansi*[35]. Generally, abundance and efficiency of pollinators and their diversity generally decrease with altitude, probably because of constraints in pollinator visit and pollinator density[36]. In addition, decrease in pollinator density and diversity was observed in *V. jatamansi* populations growing at higher elevation, which would ultimately lead to reduction in the genetic diversity of *V. jatamansi* in those geographical locations[4].

While considering habitat types, a clear pattern of increasing genetic diversity from mixed forest to grassland habitats was observed with increase in the value of Pp%, Ne, Na, He, and I (mixed forest < oak forest < pine forest < grassland) using cpSSR markers; however, deviation was found in the case of nuSSR markers. The reason for high genetic diversity in the grassland habitat (as detected by both cpSSR and nuSSR) might be attributed to better exposure of the plants to sunlight, which in turn attracts more pollinators compared to the plants growing in other habitat types (especially under forest canopy).

Similarly, studies considering habitat as an important attribute imparting genetic diversity in *Ranunculus acris*[37] and *Hedychium spicatum* are available[38].

The reported trend in variation in *V. jatamansi* (94% within and 6% among populations) corresponds more or less with the earlier report for this species[13]. The high within population diversity of this species could be explained on account of its life-history traits and breeding system, which have been shown to influence distribution and magnitude of genetic diversity in plant populations. Further, it has been reported that out-crossing plant species have higher genetic variations within populations, compared to self-pollinating species or species with mixed mating system[33]. Moreover, the loss of intraspecific genetic diversity from populations helps to increase inbreeding and genetic drift[39]. The high level of population diversity is also evident in PCoA, where most of the populations are well separated and their distribution is not affected by their geographic nature.

Conclusion

The present study shows that higher genetic diversity in the Pithoragarh population may serve an important reservoir of potentially useful genes and thus deserves high priority of management, conservation and genetic improvement. In addition, protection of a particular population with high genetic variation is unlikely to protect all the variations. Therefore, multiple genetically diverse populations, at diverse locations from the entire range, must be brought under conservation. This would require attempts to bring as many populations as possible under *in situ* conservation, especially extant populations with high level of genetic variations in different regions (e.g. Pithoragarh, Majkhali, Kausani and Jaberkhet). It is also suggested that a sustainable harvesting system for industrial needs should also be employed to protect habitats of such populations. Towards *ex situ* conservation, it is imperative to collect and maintain live accessions from as many populations as possible, including whole distribution range; the populations with small size and low genetic diversity should receive special attention. At least the populations with low expected heterozygosity, which are genetically diverse from other populations should be strictly brought under the *ex situ* conservation strategy.

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