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Full Length Research Paper

Congruence of inter simple sequence repeats (ISSR) and random amplification of polymorphic deoxyribonucleic acid (RAPD) markers in genetic characterization of *Artemisia annua* in the Trans-Himalayan region

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***Artemisia annua* is an important medicinal plant valued all over the world. Genetic characterization of 20 genotypes of *A. annua* collected from two valleys viz. Nubra (9,600 ft) and Leh (11,500 ft) of the Trans-Himalayan (Ladakh, India) region were analyzed using 37 polymerase chain reaction (PCR) markers (20 random amplification of polymorphic deoxyribonucleic acid. (RAPDs) and 17 inter simple sequence repeats) (ISSRs). RAPD analysis yielded 124 polymorphic fragments (96.9%), with an average of 6.2 polymorphic fragments per primer. ISSR analysis produced 85 bands, of which 78 were polymorphic (86.1%), with an average of 4.58 polymorphic fragments per primer. The primers based on (CT) n produced maximum number of bands (nine) while, (AT) n and many other motifs gave no amplification. The genetic diversity was high among the genotypes (Nei's genetic diversity = 0.336 and Shannon's information index = 0.495) as measured by combination of both RAPD and ISSR markers. The mean coefficient of gene differentiation (Gst) was 0.145, indicating 85.5% of the genetic diversity resided within the genotypes. RAPD markers were found more efficient with respect to polymorphism detection, as they detected 96.9% in comparison to 86.1% for ISSR markers. It was found that the genetic diversity among genotypes from Nubra valley was narrow than that of Leh valley, suggesting the importance and feasibility of introducing elite genotypes from different origins for *Artemisia* germplasm conservation and breeding programs.**

Key words: *Artemisia annua*, Ladakh, genetic diversity, random amplification of polymorphic deoxyribonucleic acid (RAPD), inter simple sequence repeats (ISSR), analysis of molecular variance (AMOVA).

INTRODUCTION

The herb *Artemisia annua* L. (Asteraceae) is one of the most important medicinal plants of the modern world for the production of antimalarial, possibly antibacterial agents and natural pesticides. It was originally collected by the Chinese as an herbal medicine and is currently processed by pharmaceutical firms for the production of artemisinin for artemisinin-based combination therapies (ACT) in the treatment of malaria. Artemisinins are also active against Schistosoma, Leishmania, Toxoplasma gondii and potential anticancer activity (Efferth et al., 2001). *A. annua* L. Thrives well in dry cold region of Ladakh having marginal rocky and sandy soils due to its aggressive fibrous root system. It is well adapted to

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survive at high altitudes of 2,500M to 5,000 m above mean sea level (MSL) and the temperature, nutrient and environmental stress that they are subjected to under the cold arid conditions. Chemical synthesis of the artemisinin, although available, is commercially non-viable, and efforts to produce it in cultured cells or in modified micro-organisms have so far not been very fruitful. Thus, the naturally growing as well as the cultivated plants is the sole source of the drug (Sangwan et al., 1993). This has generated worldwide interest in studying the genetic diversity of *A. annua* populations, clonal variants, chemotypes, ecotypes and in the synthesis of pure-line cultivars. Some attempt has been made to enhance the artemisinin content through different cycles of recurrent selection using selected genotypic and phenotypic traits. (Paul et al., 2010). Some primers and screening method for early identification of artemisinin producing plants has been identified. It will be helpful for next generation of plant population with further high content of artemisinin. (Khanuja et al., 2009). RAPD marker has been used for genetic variation in the Indian population that opens out a strong possibility of further genetic improvement for superior artemisinin content (Sangwan et al., 1999).

The genetic diversity of 11 *A. annua* types in the Westen Hubei has been investigated with the techniques of RAPD and ISSR (Shi Kai-ming et al., 2008). Over the years, the *Artemisia* populations in the Ladakh region have developed considerable variability pertaining to genetic characterization of this plant. No reports are available so far for the genetic characterization of this plant from Ladakh region and thus necessitate detail investigation. It is prerequisite towards effective utilization and protection of plant genetic resources (Weising et al., 1995), identification of molecular markers linked to agronomic traits and to achieve rational conservation. Unlike the morphological and biochemical markers which may be affected by environmental factors and growth practices (Xiao et al., 1996; Ovesna et al., 2002; Higgins, 1984), deoxyribonucleic acid (DNA) markers portray genome sequence composition, thus, enabling to detect differences in the genetic information carried by the different individuals. A wide variety of DNA-based markers have been developed in the past few decades. Restriction fragment length polymorphism (RFLP) was the first molecular marker (Bostein et al., 1980), developed for genome analysis and mapping. The development of the PCR technology has introduced a considerable number of useful molecular markers, for example RAPDs (Williams et al., 1990) and ISSR (Zeitkiewicz et al., 1994) which are independent of environmental factors and unaffected by developmental stages of the plant. These markers have been used both for DNA fingerprinting (Gupta et al., 2008) and population genetic studies (Alam et al., 2009). The objective of this study was to compare the effectiveness of both the PCR-based molecular approaches to determine the genetic relationships among several genotypes of *A. annua* from the Trans-Himalayan (Ladakh, India) region.

MATERIALS AND METHODS

Twenty genotypes of *A. annua* were collected from the two valleys with altitude ranging from 9,600 ft (Nubra) to 11,500 ft (Leh) from the cold arid desert of the trans-Himalayas (Ladakh, India). The young leaves were collected from 10 individual plants from each valley and stored in laboratory at 80°C until further analysis. The interval between samples was 100 to 200 m and the pair wise distance between valleys was 50 to 250 Km. *Artemisia* contains high amount of secondary metabolites that interfere with DNA isolation. Hence, Cetyl trimethylammonium bromide (CTAB) method with minor modifications was used to obtain good yield of DNA for PCR based assays. The young leaves were used to get 20 to 50 mg of powdered material for DNA extraction. To the extraction buffer 3% (w/v) polyvinylpyrrolidone and 0.5% (v/v) 2-mercaptoethanol was added to remove polysaccharides, high essential oils and polyphenolic contents. Twenty random decamer primers from IDT Tech, USA (Table 1) were used for RAPD amplification following the standard protocol (Williams et al., 1990).

Amplification reactions 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 Deoxynucleotide Triphosphates (dNTPs), 0.4 µM primer, 20 ng template DNA and 0.5 unit of Taq polymerase (Sigma-Aldrich, USA). The first cycle consisted of denaturation of template DNA at 94°C for 4 min, primer annealing at 37°C for 1 min, and primer extension at 72°C for 2 min. For the next 40 cycles the period of denaturation was reduced to 1 min at 92°C, while the primer annealing and primer extension time remained the same as in the first cycle. The last cycle consisted of only primer extension (72°C) for 5 min. Seventeen ISSR primers were obtained from Applied Biosciences, India (Table 2) and PCR amplification was performed in reaction cocktail and PCR cycle similar to RAPD. The amplification for each primer was performed twice independently with same procedure in order to ensure the fidelity of RAPD and ISSR markers. Amplification products were electrophoresed on 1.5% agarose gel at constant voltage (70 V) in 1X TAE for approximately 2 h, visualized by staining with ethidium bromide (0.5 µg ml^{-1}). A total of 2.5 µl loading buffer (6X) was added to each reaction before electrophoresis. After electrophoresis, the gels were documented on a gel documentation system (Alpha Innotech, Alpha Imager and USA). Molecular sizes of amplicons were estimated using a 100 bp DNA ladders (Bangalore Genei and India).

Data collection and analysis

The banding patterns obtained from RAPD and ISSR were scored as present (1) or absent (0), each of which was treated as an independent character. Jaccard's similarity coefficient (J) was used to calculate similarity between pairs of genotypes. The similarity matrix was subjected to cluster analysis by unweighted pair group method with arithmetic means (UPGMA) and a dendrogram was generated using the program NTSYSpc (Rohlf, 1992). POPGENE software was used to calculate Nei's unbiased genetic distance among genotypes. 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) were also analyzed (Zhao et al., 2006). Within species diversity (Hs) and total genetic diversity (Ht) (Nei, 1978) were calculated within the species and within two major groups (as per their collection site) using POPGENE software.

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

Primer	Primer sequence (5' – 3')	GC (%)	Tm (°C)	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Total number of fragments amplified	Resolving power
S21	CAGGCCCTT C	70	36.4	6	6	100	81	4.5
S22	TGCCGAGCT G	70	40.7	7	7	100	93	5.17
S23	AGTCAGCCA C	60	34.3	7	7	100	81	4.5
S24	AATCAGCCA C	50	30.1	4	4	100	46	2.56
S25	AGGGGTCTT G	60	32.6	9	8	88.9	89	4.94
S26	GGTCCCTGA C	70	35.2	7	7	100	64	3.56
S27	GAAACGGGT G	60	33.2	6	6	100	73	4.06
S28	GTGACGTAG G	60	31.1	7	7	100	88	4.89
S29	GGGTAACGC C	70	37.4	5	5	100	72	4.0
S30	GTGATCGCA G	60	33.1	8	7	87.5	100	5.56
S31	CAATCGCCG T	60	36.7	5	5	100	75	4.17
S32	TCGGCGATA G	60	34.0	6	5	83.3	72	4.0
S33	CAGCACCCA C	70	37.7	4	4	100	55	3.06
S34	TCTGTGCTG G	60	34.3	5	5	100	69	3.83
S35	TTCCGAACC C	60	34.2	5	5	100	65	3.61
S36	AGCCAGCGA A	60	38.3	5	5	100	58	3.22
S37	GACCGCTTG T	60	35.7	7	7	100	83	4.61
S38	AGGTGACCG T	60	36.2	6	6	100	55	3.06
S39	CAAACGTCG G	60	34.2	6	6	100	77	4.28
S40	GTTGCGATC C	60	33.5	9	7	77.8	86	4.78
	Total	-	-	124	119	96.9	1482	-

The RAPD and ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992), using two hierarchical levels; among valleys and among genotypes within each valley. Correlation between both the marker types used in the study was obtained by regression (R) analysis between similarities matrices obtained with two marker types. In this instance, the matrix regression corresponds to two independently derived dendrogram. The resolving power of the RAPD and ISSR primers was calculated according to Prevost and Wilkinson (1999). The resolving power (Rp) of a primer is: Rp = Σ IB where IB (band informativeness) takes the value of: 1–[2* (0.5–P)], P being the proportion of the 20 genotypes containing the band.

RESULTS AND DISCUSSION

RAPD analysis

Twenty RAPD primers that generated reproducible, informative and easily scoreable RAPD profiles were preselected. These primers produced multiple band profiles with a number of amplified DNA fragments varying from 4 to 9, with a mean of 6.2 markers per primer. All the amplified fragments varied in size from 200 to 1000 bp. Out of 124 amplified bands, 119 were found polymorphic (Table 1). The observed high proportion of polymorphic loci suggests that there is a high degree of genetic variation in the Artemisia genotypes. The resolving power of the 20 RAPD primers ranged from 2.56 for primer S24 to a maximum of 5.56 for primer S30. A dendrogram analysis based on UPGMA method grouped all the 20 genotypes into two main clusters (Figure 1) (with reference to their site of collection) with Jaccard's similarity coefficient ranging from 0.55 to 0.70. Genetic diversity analysis in terms of Na, Ne, H, I, Ht, Hs and PPL for both the valleys (Leh and Nubra) revealed higher values for Leh, indicating more variability among the genotypes in comparison to Nubra valley (Table 3). The respective values for overall genetic variability for Na, Ne, H, I, Ht, Hs, Gene flow (Nm), DI, EMR and MI across all the 20 genotypes are given in Table 4. The Gst value of 0.108 indicated that 89.2% of the genetic diversity resided within the population. Analysis of molecular variance among valley (10%) and among genotypes within valley (90%) (Table 5) revealed higher variations within the population. All the components of molecular variations were significant ($P < 0.001$). This is helpful in making strategy for germplasm collection and evaluation. The rate of gene flow estimated using Gst value was found to be 2.065. The present study and similar studies on ginger (Nayak et al., 2005), Podophyllum hexandrum (Alam et al., 2009) and Andrographic paniculata (Padmesh et al., 1999) suggested that RAPD is more appropriate for analysis of genetic variability in closely related genotypes. It indicates that *A. annua* populations in the northwestern Himalayan region are genetically highly diverse.

Table 2. List of primers used for ISSR amplification, sequence, GC content, total number of loci, the level of polymorphism, size range of fragments and resolving power, where, (Y = C,T; R = A,G).

Primer	Primer sequence (5' – 3')	GC (%)	Tm (°C)	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Total number of fragment s amplified	Resolving power
ISSR 1	(AG)8 T	47	47.0	4	4	100	67	3.72
ISSR 2	(GA)8 T	47	45.4	5	5	100	64	3.56
ISSR 3	(AC)8 T	47	51.4	3	2	66.7	49	2.72
ISSR 4	(TG)8 A	47	51.3	4	2	50	67	3.72
ISSR 5	(AG)8YT	47.2	49.2	6	4	66.7	87	4.83
ISSR 6	(GA)8YT	47.2	47.4	7	5	71.4	102	5.67
ISSR 7	(CT)8 RA	47.2	47.1	5	4	80	57	3.17
ISSR 8	(GT)8 YC	52.7	52.7	5	5	100	57	3.17
ISSR 9	(ACC)6	66.6	60.6	4	4	100	49	2.72
ISSR 10	CCG)6	10	76.8	4	4	100	42	2.33
ISSR 11	(GGC)6	10	77.3	11	8	72.7	97	5.39
ISSR 12	(AT)8 T	0	23.1	4	4	100	39	2.17
ISSR 13	(TA)8 RT	2.7	25.6	5	5	100	60	3.33
ISSR 14	(AT)8 YA	2.7	26.0	5	4	80	67	3.72
ISSR 15	(CT)8 T	47	45.7	4	4	100	51	2.83
ISSR 16	(TC)8 A	47	47.0	4	3	75	67	3.72
ISSR 17	(GT)8 A	47	49.4	5	5	100	65	3.61
	Total	-	-	85	72	86.02	1087	-

ISSR analysis

The 17 ISSR primers selected in the study generated a total of 85 ISSR bands (an average of 5 bands per primer), out of which 72 were polymorphic (86.02%). Number of bands varied from 3 to 11 with size ranges between 200 to 1000 bp. Average numbers of bands and polymorphic bands per primer were 5 and 4.23, respectively. Other primer amplification details are shown in Table 2. Amplification result of 17 primers seems to indicate that microsatellites more frequent in Artemisia contain the repeated di-nucleotides: (AG)n, (GA)n, (TG)n, (CT)n, (AT)n, (GT)nYA, and tri-nucleotides: (ACC)n, (CCG)n and (GGC)n. The number of bands produced with different repeat nucleotide were more with the (GT)n, (GA)n, (CT)n and (AC)n primers. The primers that were based on the (GA)n, (CT)n and (GT)n motif produced more polymorphism (on average 7 bands per primer) than the primers based on any other motifs used in the present investigation. We obtained good amplification products from primers based on (CT)n and (GT)n repeats while (AT)n and some other primers gave no amplification, despite the fact that (AT)n di-nucleotide repeats are thought to be the most abundant motifs in plant species (Martín and Sánchez-Yélamo, 2000). Similar results were obtained in grapevine (Moreno et al., 1998), rice (Blair et al., 1999), Vigna (Ajibade et al., 2000) and wheat (Nagaoka and Ogihara, 1997). A possible explanation of these results is that ISSR primers based on AT motifs are self-annealing, due to sequence complementarity, and would form dimers during PCR amplification (Blair et al., 1999) or it may be because of its-non annealing with template DNA due to low Tm. The primers with poly (GC)n and poly (GA)n motifs

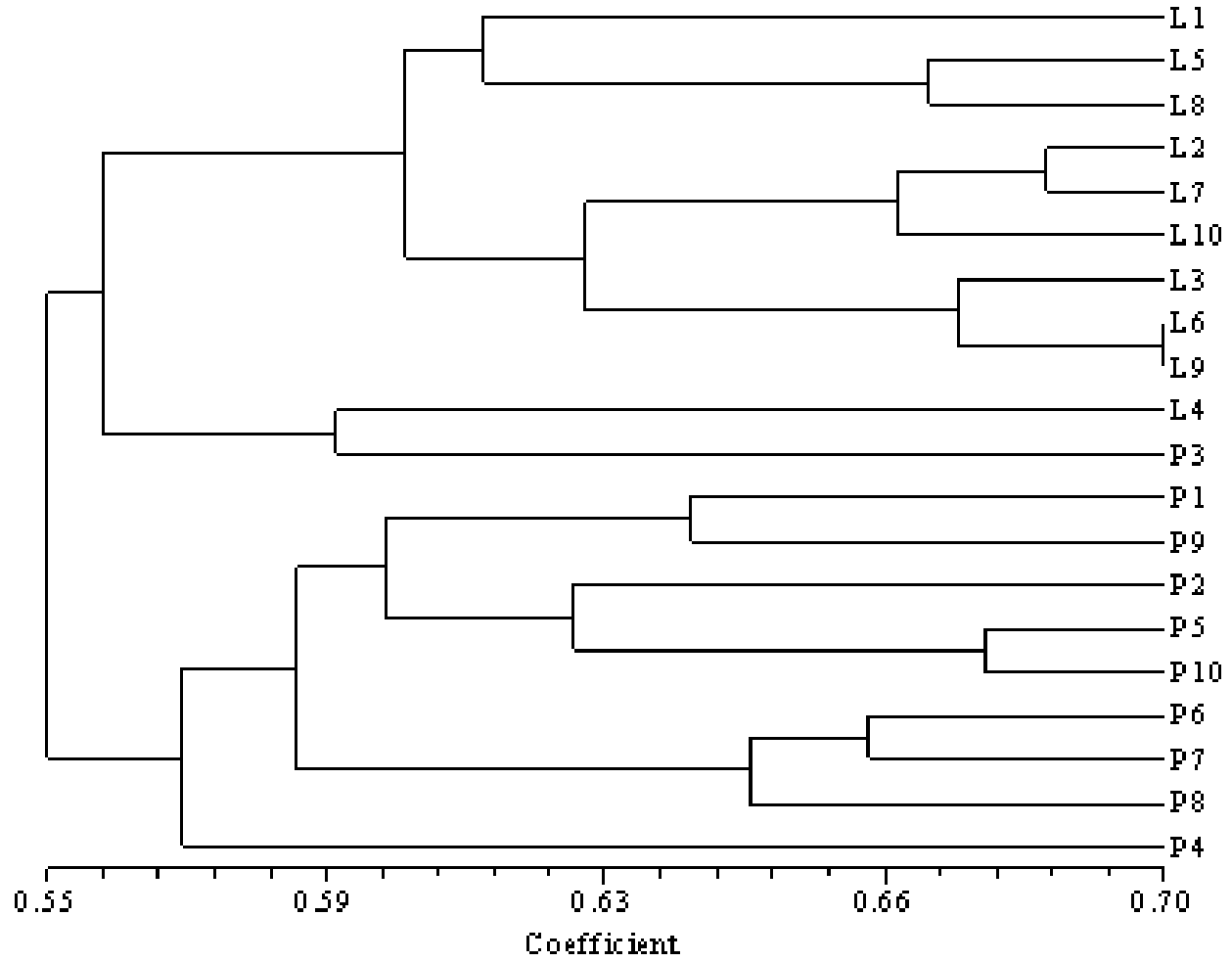

Figure 1. Dendrogram illustrating genetic relationships among 20 genotypes of *A. annua* collected from 2 different valleys: Leh (L1-L10) and Nubra (P1-P10). The dendrogram was generated by UPGMA cluster analysis calculated from 1482 RAPD bands produced by 20 primers.

Table 3. Summary of genetic variation statistics for all loci of RAPD, ISSR and RAPD + ISSR among the *A.annua* populations with respect to their distributions among two valleys.

Valley	Sample size	Na	Ne	H	I	Ht	PPL
(a) RAPD							
Leh	10	1.952 (0.215)	1.662 (0.300)	0.375 (0.134)	0.549 (0.172)	0.375 (0.018)	98.9
Nubra	10	1.871 (0.337)	1.620 (0.327)	0.351 (0.162)	0.512 (0.222)	0.351 (0.026)	94.9
Mean		1.911	1.641	0.363	0.530	0.363	96.9
(b) ISSR							
Leh	10	1.823 (0.383)	1.608 (0.370)	0.337 (0.184)	0.488 (0.253)	0.337 (0.034)	87.8
Nubra	10	1.812 (0.393)	1.558 (0.358)	0.318 (0.180)	0.467 (0.250)	0.318 (0.032)	84.3
Mean		1.817	1.583	0.327	0.477	0.327	86.1
(c) RAPD+ISSR							
Leh	10	1.871 (0.347)	1.604 (0.341)	0.341 (0.169)	0.500 (0.230)	0.342 (0.028)	96.1
Nubra	10	1.861 (0.336)	1.577 (0.337)	0.332 (0.164)	0.490 (0.223)	0.332 (0.027)	85.8
Mean		1.866	1.591	0.336	0.495	0.337	91.0

Na = observed number of alleles; Ne = effective number of alleles; H = Nei's gene diversity; I = Shannon's information index; Ht = total genetic diversity; PPL = percentage of polymorphic loci.

Table 4. Overall genetic variability across the 20 genotypes of *A. annua* based on RAPD, ISSR and RAPD+ISSR analysis.

Marker type	Na	Ne	H	I	Ht	Hs	Gst	Nm	DI	EMR	MI
RAPD	1.984 (0.125)	1.730 (0.258)	0.407 (0.106)	0.591 (0.124)	0.407 (0.011)	0.363 (0.012)	0.108	2.065	0.817	6.2	0.851
ISSR	1.906 (0.294)	1.658 0.325)	0.367 (0.154)	0.535 (0.207)	0.367 (0.234)	0.327 (0.021)	0.109	2.044	0.767	4.588	0.876
RAPD+ISSR	1.962 (0.192)	1.706 (0.283)	0.394 (0.125)	0.572 (0.160)	0.394 (0.016)	0.337 (0.015)	0.145	1.474	-	-	-

Hs = genetic diversity in population; Gst = genetic diversity between population; Nm= estimate of gene flow, Nm = 0.25 (1-Gst)/Gst; DI = diversity index; EMR = effective multiplex ratio; MI = marker index.

Table 5. Summary of nested analysis of molecular variance (AMOVA) based on RAPD, ISSR individually and in combination, among the populations of *A. annua.* Levels of significance are based on 1000 iteration steps.

Source of variation	Among valley			Among genotypes/valley		
df	1			18		
Marker	RAPD	ISSR	RAPD+ISSR	RAPD	ISSR	RAPD+ISSR
Variance component	2.962	1.859	6.168	24.994	15.461	23.622
Percentage	10	11	21	90	89	79
P-value	<0.001	<0.002	<0.001	<0.001	<0.002	<0.001

P-value = probability of null distribution.

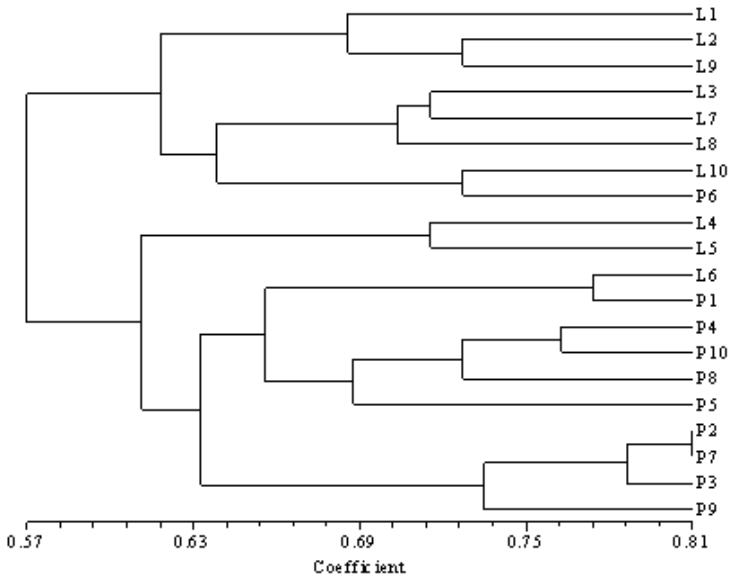

Figure 2. Dendrogram illustrating genetic relationships among 20 genotypes of *A. annua* collected from 2 different valleys: Leh (L1-L10) and Nubra (P1-P10). The dendrogram was generated by UPGMA cluster analysis calculated from 1087 ISSR bands produced by 17 primers.

produced more polymorphism than any other motif. Similar result was also reported by Ajibade et al. (2000) where they found that the primer containing the CT repeats was one of those, which did not gave interpretable phenotype when analyzed, while primers with GA and CA repeats revealed polymorphism in the genus Vigna. The Rp of the 17 ISSR primers ranged from 2.17 to 5.67 (Table 2). The complete data set of 1087

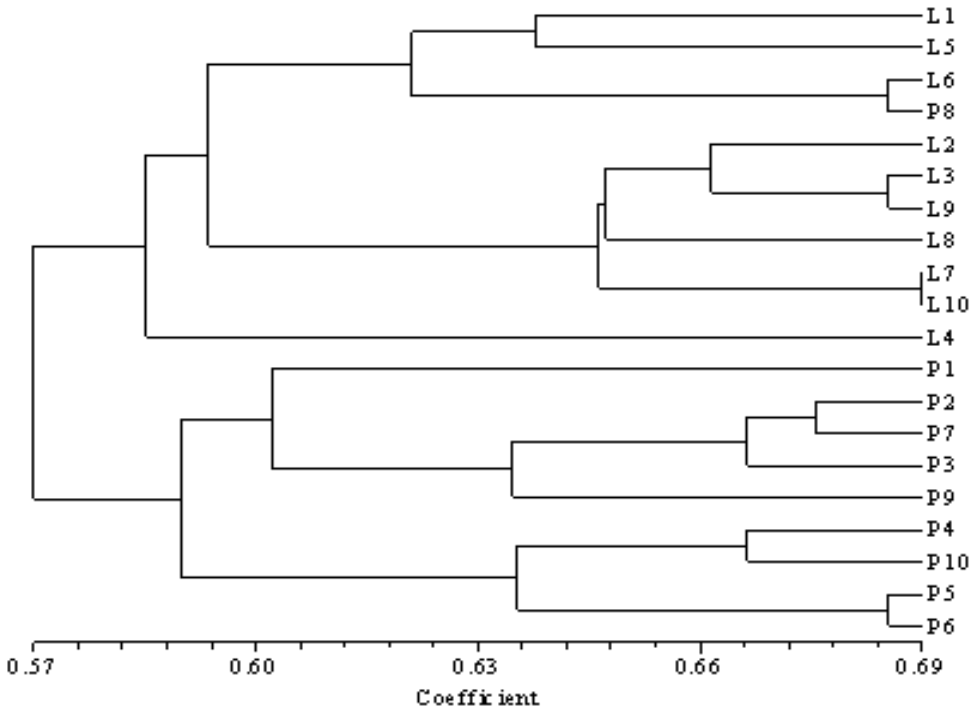

Figure 3. Dendrogram illustrating genetic relationships among 20 genotypes of *A. annua* collected from 2 different valleys: Leh (L1-L10) and Nubra (P1-P10). The dendrogram was generated by UPGMA cluster analysis calculated from 1482 RAPD bands+1087 ISSR bands.

bands was used for genetic characterization; Jaccard's similarity coefficient was found to be in the range of 0.57 to 0.81. The genotypes were clustered into two clusters (with respect to their site of collection) where, cluster I represents all the genotypes from Leh valley while cluster II contains all the genotypes from Nubra valley (Figure 2). The respective values of Na, Ne, H, I, Ht, Hs and PPL among the genotypes were found higher for Leh indicating that there is more variability in comparison to Nubra valley (Table 3). Gst value of 0.109 indicated that 89.1% of the genetic diversity resided within the population. The details of overall genetic variability across 20 genotypes were given in Table 4. AMOVA for among valley (11%) and among genotypes within the valley (89%) indicated that there are more variations within the population (Table 5).

RAPD and ISSR combined data for cluster analysis

Based on combined data set of RAPD and ISSR markers, the dendrogram obtained gave similar clustering pattern with Jaccard's similarity coefficient ranging from 0.57 to 0.69 (Figure 3). Cluster I represents all the genotypes from Leh valley whereas cluster II represents all the genotypes from Nubra valley. Other genetic variation studies were also performed on RAPD and ISSR combined data which are represented in different tables (Tables 3 and 4). The differences found among the dendrogram generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed (1482 for RAPDs and 1087 for ISSRs) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships as observed by Loarce et al. (1996) in barley. Another explanation could be the low reproducibility of RAPDs (Karp et al., 1997). The genetic closeness among the Leh valley and Nubra valley genotypes can be explained by the high degree of commonness in their genomes. Similar result has been obtained by Gaffor et al. (2001) in blackgram. In all the dendrograms, genotypes from both the valleys were found clustered distinctly. The genetic similarity of these genotypes is probably associated with their similarity in the genomic and amplified region.

Comparative analysis of RAPD with ISSR markers

RAPD markers were observed to be more efficient with respect to polymorphism detection, as they detected 96.9% polymorphism as compared to 86.02% for ISSR markers. Also the diversity index, effective multiplex ratio and marker index are more for RAPD than for ISSR markers (Table 4). This is in contrast to the results obtained for several other plant species like wheat (Nagaoka and Ogihara, 1997) and Vigna (Ajibade et al., 2000). More polymorphism in case of RAPD than ISSR

markers might be due to the fact that 17 ISSR primers used in the study only amplified 1087 number of fragments (Table 2). While in case of RAPD, all the 20 primers which were used in the investigation amplified 1482 number of fragments (Table 1). Same polymorphism pattern was also observed in case of Jatropha (Gupta et al., 2008) and Podophyllum (Alam et al., 2009). The regression test between the Jaccard's similarity matrix resulted in low regression between RAPD and ISSR based similarities (R = 0.014), moderate for ISSR and RAPD+ISSR (R= 0.699), while it is maximum for RAPD and RAPD+ISSR based similarities (R = 0.725). This shows that RAPD data is more close to RAPD+ISSR combined data. A possible explanation for the difference in resolution of RAPDs and ISSRs is that the two-marker techniques target different portions of the genome. With this study, we can conclude that the molecular analyses by both RAPD and ISSR markers were extremely useful for studying the genetic relationships of local *Artemisia* genotypes from the Trans-Himalayan region (Ladakh, India). The results indicates the presence of high genetic variability, which should be exploited for the future conservation and breeding of *Artemisia* from this region. Since no single, or even few plants, will represent the whole genetic variability in *A. annua*, there appears to be a need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in *A. annua* to avoid genetic erosion. Based on polymorphic feature, genetic diversity, genetic similarity, and gene flow among the populations of Artemisia based on RAPD and ISSR 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.

Abbreviations: PCR, Polymerase chain reaction; **RAPD,** random amplification of polymorphic deoxyribonucleic acid; **ACT,** artemisinin-based combination therapiesl; **MSL,** mean sea level; **DNA,** deoxyribonucleic acid; **RFLP,** restriction fragment length polymorphism; **ISSR,** inter simple sequence repeats; **CTAB,** cetyl trimethylammonium bromide; **NPL,** number of polymorphic loci; **dNTPs,** deoxynucleotide triphosphates; **UPGMA,** unweighted pair group method with arithmetic means; **PPL,** percentage polymorphic loci; **Rp,** resolving power; **DRDO,** defense research and development organization.

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