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Exploring genetic relationships in *Artemisia* species growing in trans-Himalayan cold arid desert using RAPD markers

Jitendra Kumar, Gyan P. Mishra*, A.A. Murkute, G. Phanikumar***, Pradeep K. Naik**** and R.B. Srivastava**

Defence Institute of High Altitude Research, DRDO, C/o 56 APO, Leh 901 205, Jammu & Kashmir

ABSTRACT

Genetic relationship of five *Artemisia* species from Trans-Himalayas of Ladakh was assessed using random amplified polymorphic DNA (RAPD) marker. Twenty RAPD primers generated a total of 134 bands, with an average of 6.7 bands per primer. Out of 134 bands scored, 97% were found polymorphic while only 3% were monomorphic. The resolving power of primers ranged from 5.44 to 10.64. The ranges of similarity between species varied from 0.480 to 0.775. According to the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) derived dendrogram, at a similarity level of 0.25, the populations were divided into two main groups. With regard to the genetic diversity parameters calculated for different species of origin, maximum and minimum number of effective alleles was observed in *A. annua* (1.601) and *A. gmelinii* (1.049) species, respectively. The percentage of polymorphic loci (PPL) Nei's gene diversity (H) and Shannon's information index (I) for different species, indicated that diversity was highest in *A. annua* (PPL = 75.56%, H = 0.3283 and I = 0.472) and lowest in *A. gmelinii* (PPL = 9.63%, H = 0.032 and I = 0.049). The degree of genetic diversity observed using RAPD markers revealed that this approach is very effective in studying not only the phylogeny but also for the future conservation of medicinal wealth of various *Artemisia species* from this fragile environment of trans-Himalayas.

Key words: *Artemisia*, genetic diversity, RAPD, trans-Himalayas.

INTRODUCTION

Artemisia (family Asteraceae) is a rich source of highly valuable phytochemicals and essential oils, commonly found in the cold arid region of Ladakh, India. It is commercially exploited for Artemisinin compound which is being extracted from leaves, roots and stem. It acts as an antimalarial agent against *Plasmodium falciparum* and *Plasmodium vivax*, including multidrug resistant strains (Chaurasia *et al*., 5). There are several species known to have found in this region, however; very few of them are identified yet. Among known species, *Artemisia annua* is used for the production of antimalarial and possible antibacterial agents and natural pesticides. *Artemisia tournefortiana* has strong aromatic oil which could be used as medicine against worms. Leaf and flower extracts of *Artemisia gmelinii* is used for treating headache, cold, cough and abdominal upsets. *Artemisia sieversiana* leaves contain sieversinin and siersin that have antimicrobial properties. *Artemisia dracunculus* is commonly known as tarragon and its leaf and flower extract is used against toothache, urinary problems and roots are useful in treating pharyngitis, lung diseases and also used to control menstrual cycle. These five species have been selected for the present study. Characterization of accessions or species is an important step for germplasm conservation, maintenance and breeding studies (Balasaravanan *et al*., 4). Species identification based on morphological characteristics is often difficult, since most of these characteristics are under the influence of environmental factors. Overlapping morphological features between species of *Artemisia* have made them unreliable for phylogenetic analysis. Knowing the degree of genetic variations is of fundamental importance for species conservation. Measurement of genetic variation within and between plant species is important for several reasons including delimitation of species, conservation of endangered species and construction of phylogenetic relationships among species (Balasaravanan *et al*., 4). Due to the remoteness of the Trans-Himalayan region most of the *Artemisia* germplasm in Ladakh is still underutilized. Furthermore, the scarcity of literature about the distribution and genetic diversity in this region still hinders the standardization of its agro-practices and limits the commercial exploitation. Therefore, there is an urgent need to identify a more reliable tool for this purpose.

*Corresponding author's present address: Directorate of Groundnut Research, ICAR, Junagadh; E-mail: gyan.gene@gmail.com
**Directorate of Onion and Garlic Research, ICAR, Pune, Maharashtra
***Defence Food Research Laboratory, DRDO, Mysore, Karnataka
****Jaypee University of Information Technology, Solan, Himachal Pradesh

Advantage of RAPD markers is that a set of arbitrary primers can be utilized for any organism. Polymorphism detected by RAPD markers has proven to be useful for unraveling of genetic diversity and relationship in several medicinal plant species, *viz*. *Podophyllum* (Alam *et al*., 1-3), *Artemisia annua* (Kumar *et al*., 10) and *Rhodiola* and *Jatropha* (Gupta *et al*., 7 & 8). Owing to the facts the present investigation was conducted to examine the genetic relationships within and between 25 accessions belonging to five species using twenty RAPD markers.

MATERIALS AND METHODS

Fresh leaves of five *Artemisia* species, *viz*. *A. annua*, *A. sieversiana*, *A. dracunculus, A. gmelinii*, and *A. tourenefortiana* (Fig. 1) were collected from medicinal and aromatic plants fields in Defence Institute of High Altitude Research, Leh, Ladakh, India. The leaves were kept at -20°C until further analysis in laboratory. *Artemisia* contains high amount of secondary metabolites that interfere with DNA isolation. Hence, CTAB method with minor modifications was used to obtain good yield of DNA for PCR based assays and other applications (Doyle and Doyle, 6; Kumar *et al*., 9). The young leaves were used to get 20-50 mg of powdered material for DNA extraction. To the extraction buffer 5% (w/v) polyvinylpyrrolidone and 2% (v/v) 2-mercaptoethanol was added to remove polysaccharides, high essential oils and polyphenolic contents. DNA concentration was determined by electrophoresis on 1% agarose gel stained with ethidium bromide in comparison with standard DNA ladders and also by spectrophotometer readings at 260/280 nm. DNA samples were diluted to 25 ng/µl and kept at -20°C until used. A total of 20 random decamer oligonucleotides (IDT Tech, USA, Table 1) were used for RAPD analysis. PCR was conducted in 25 µl of a mixture containing 50 ng of genomic DNA, 2.5 µl of 10 × PCR buffer, 0.2 mM dNTPs, 2 µM primer and 1U of *Taq* DNA

Table 1. List of primers used for RAPD analysis and the number of scored bands in five *Artemisia* species.

Primer	Primer sequence	GC	Tm	Total No. of loci	No. of polymorphic loci	Polymorphic loci (%)	Total No. of fragments amplified	Resolving power
S21	CAGGCCCTT C	70	36.4	16	16	100	124	9.92
S22	TGCCGAGCT G	70	40.7	8	7	87.5	101	8.08
S23	AGTCAGCCA C	60	34.3	6	6	100	70	5.6
S24	AATCAGCCA C	50	30.1	6	6	100	87	6.96
S25	AGGGGTCTT G	60	32.6	8	7	87.5	109	8.72
S26	GGTCCCTGA C	70	35.2	7	7	100	95	7.6
S27	GAAACGGGT G	60	33.2	6	6	100	96	7.68
S28	GTGACGTAG G	60	31.1	7	7	100	80	6.4
S29	GGGTAACGC C	70	37.4	6	6	100	92	7.36
S30	GTGATCGCA G	60	33.1	7	6	85.7	91	7.28
S31	CAATCGCCG T	60	36.7	5	5	100	87	6.96
S32	TCGGCGATA G	60	34.0	6	6	100	88	7.04
S33	CAGCACCCA C	70	37.7	4	4	100	69	5.52
S34	TCTGTGCTG G	60	34.3	5	5	100	74	5.92
S35	TTCCGAACC C	60	34.2	5	5	100	73	5.84
S36	AGCCAGCGA A	60	38.3	5	5	100	68	5.44
S37	GACCGCTTG T	60	35.7	7	7	100	104	8.32
S38	AGGTGACCG T	60	36.2	6	6	100	80	6.4
S39	CAAACGTCG G	60	34.2	6	6	100	101	8.08
S40	GTTGCGATC C	60	33.5	9	8	88.8	133	10.64
Total				134	130	97.47	1822	-

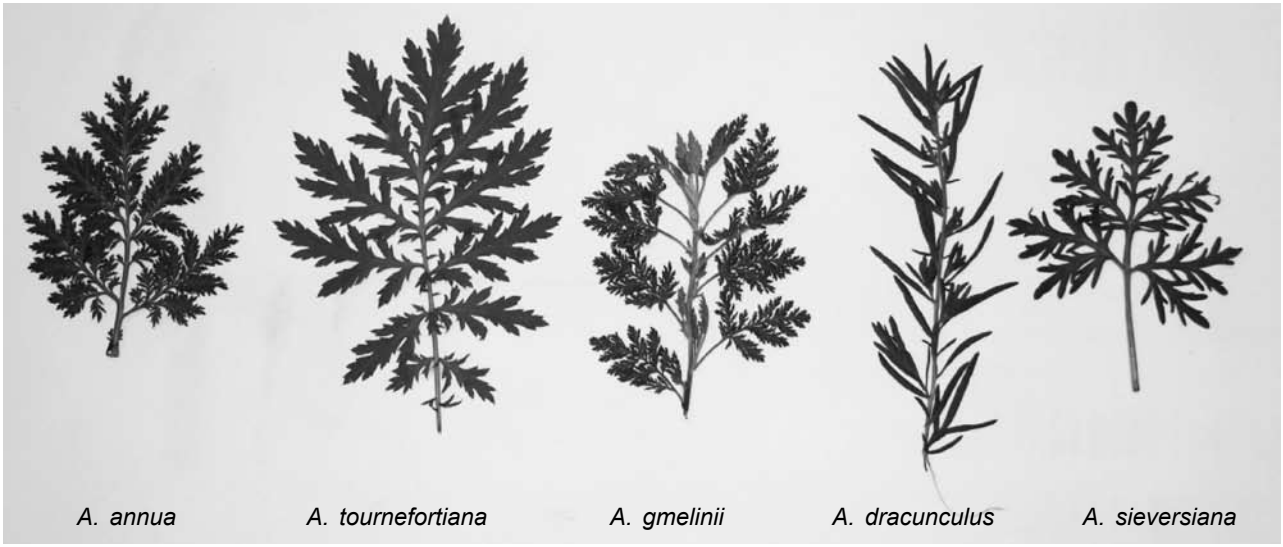

Fig. 1. Five *Artemisia* species selected for the present study.

polymerase (Sigma-Aldrich, USA). The Biometra Gradient Thermal Cycler was programmed as follows: an initial cycle of 3 min at 94°C, followed by 40 cycles each consisting of 45s at 94°C, 30 s at 35°C, 2 min. at 72°C; and final extension of 5 min. at 72°C. PCR products were stored at 4°C before analysis. Amplified PCR products were separated on 1.5% (w/v) agarose gel in TAE buffer containing ethidium bromide (0.5 µg/ml). The PCR products were visualized under UV light by gel doc system (Alfa Innotech). The 100 bp ladder (Bangalore Genei, India) was used as a molecular size marker (Fig. 2). The amplification for each primer was performed 2-3 times independently with the same procedure in order to ensure the reproducibility in amplifications.

The RAPD profile was subjected to estimate genetic similarity with the Jaccard's coefficient and cluster analyses, using the Numerical Taxonomy and Multivariate Analysis System program NTSYS-pc package (Exeter software, New York). Amplified bands on a gel were scored as 1 (present) or 0 (absent), while faintly stained bands or those which were not clearly resolved were not considered in the data collection. A band was assumed to be monomorphic if it appeared in all species. The matrix of generated similarities was analyzed by the Unweighted Pair Group Method with Arithmetic Average (UPGMA), using the Sequential Agglomerative, Hierarchical and Nested Cluster (SAHN) module. The cophenetic module was applied to compute a cophenetic value matrix using the UPGMA matrix. The MXCOMP module was used to

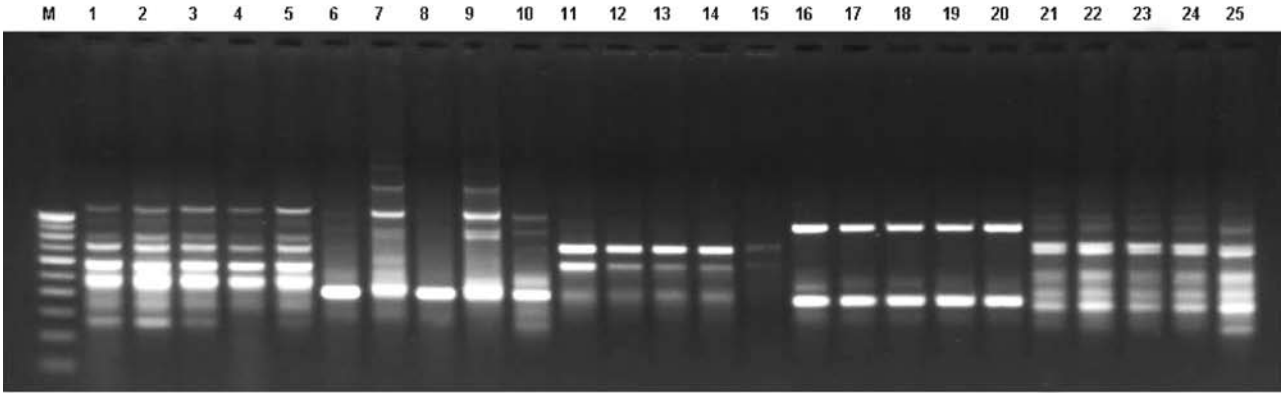

Fig. 2. RAPD fingerprint generated for different *Artemisia* species using S-21 primer. Amplification product was analyzed by electrophoresis on 1.5% agarose gel and detected by staining with ethidium bromide. Lane 1- 100 bp Marker, Lane 2 to 6 – *A. gmelinii genomic* DNA, Lane 7 to 11 – *A. annua*, Lane 12 to 16 – *A. dracunculus*, Lane 17 to 21 – *A. sieversiana*, Lane 22 to 26 – *A. tournefortiana*.

compute the cophenetic correlation. Percentage of polymorphic bands was defined as the percentage of the number of polymorphic bands amplified by a single primer to that of the total number of bands produced by the same primer. Resolving power of the primer was calculated using the formula: Resolving power (Rp) = *P* Ib (band informativeness). Band informativeness was calculated for each band scored by the primer individually. Ib = 1 – [2(0.5 – p)], p is the proportion of occurrence of bands in the species out of the total number of species screened. Genetic variation within species was estimated with Shannon's index using POPGENE ver. 1.31 (Yeh *et al*., 16). To examine patterns of genetic diversity, genetic variation statistics for all loci was performed. In addition, number of observed alleles (Na), number of effective alleles (Ne), Nei's gene diversity (H) and Shannon's information index (I) for each population was measured (Table 2). Nei's analysis of gene diversity in subdivided populations (Nei's, 13) was carried out by counting total Heterozygosity (Ht), Heterozygosity within population (Gst) and estimation of gene flow from Gst or Gcs (Nm) parameters. Fst index was measured as suggested by Wright (15). Multivariate cluster analysis and principal coordinate analysis (PCA) were used to analyze the data. Analysis of molecular variance (AMOVA) was performed with GENALEX V6.1 (Peakall *et al*., 14) to partition the total genotypic variance within and among species. The significance of the variance in components was tested by calculating their probabilities based on 999 random permutations.

RESULTS AND DISCUSSION

Five *Artemisia* species were analyzed using 20 random primers to produce reproducible banding patterns. Following amplification, a total of 134 bands with an average of 6.7 bands per primer were detected. The size of amplified product ranged from 100 to 1500 bp. S-33 primer gave the fewest bands (4 fragments) and S-21 the highest (16 fragments). There were 130 polymorphic bands and average polymorphism level was 97.47%. A dendrogram generated using the UPGMA for cluster analysis (Fig. 3) showed that individuals from the four species, *i.e. A. gmelinii, A. annua, A. sieversiana* and *A. tourenefortiana* formed one cluster. However, *A. dracunculus* was separated out indicating it as the most divergent species. The divergence of *A. dracunculus* could be attributed to its origin, which is quite different from other species (Chaurasia *et al*., 5).

Principal Coordinate Analysis was performed to provide spatial representation of the relative genetic distances among individuals and to determine the consistency of differentiation among species defined by the cluster analysis (Fig. 4). The first three principal coordinate axes obtained in the analysis of species accounted for 80.14, 7.64 and 4.84% of the total variation, respectively. The value of genetic identity between *A. sieversiana* and *A.*

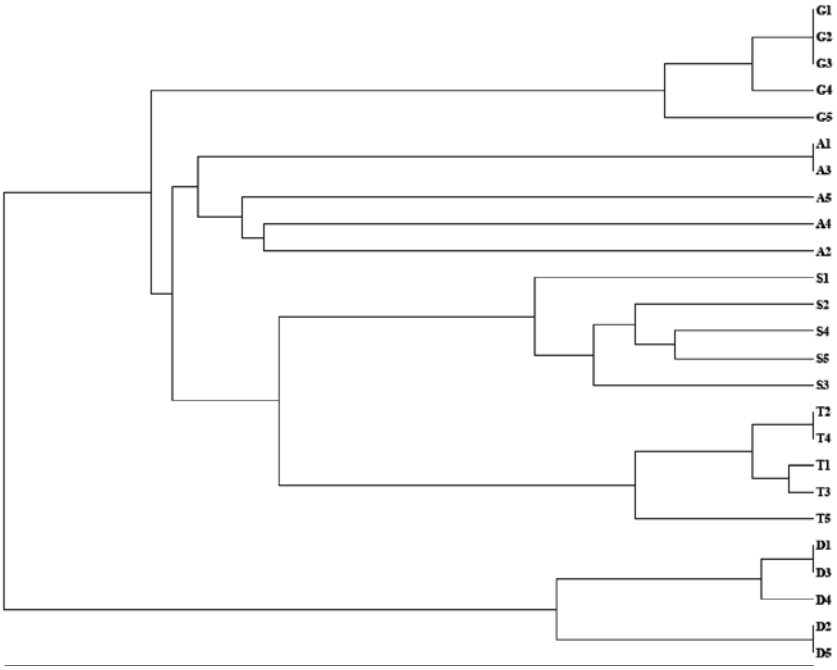

Fig. 3. Dendrogram of five *Artemisia* species generated using UPGMA.

Table 2. Summary of genetic variation statistics for all loci of RAPD.

Species	N	Na (mean±SD)	Ne (mean±SD)	H (mean±SD)	I (mean±SD)	Ht	NPL	PPL (%)
A. gmelinii	5	1.096 ± 0.296	1.049 ± 0.154	0.0321 ± 0.099	0.049 ± 0.153	0.032 ± 0.010	13	9.63
A. annua	5	1.756 ± 0.432	1.601 ± 0.386	0.3283 ± 0.198	0.472 ± 0.277	0.329 ± 0.039	102	75.56
A. dracunculus	5	1.474 ± 0.501	1.430 ± 0.459	0.2252 ± 0.239	0.316 ± 0.335	0.225 ± 0.057	64	47.41
A. sieversiana	5	1.340 ± 0.475	1.234 ± 0.353	0.1351 ± 0.194	0.198 ± 0.282	0.135 ± 0.038	46	34.07
A. tourenefortiana	5	1.140 ± 0.349	1.079 ± 0.209	0.0498 ± 0.126	0.075 ± 0.189	0.049 ± 0.016	19	14.07

N = Number of sample; Na = Observed number of alleles; Ne = Effective number of alleles; H = Nei's gene diversity; I = Shannon's Information index; Ht = Total genetic diversity; NPL = No. of polymorphic loci; PPL = % Polymorphic loci.

Table 3. Nei's genetic identity (above diagonal) genetic distance (below diagonal) between different *Artemisia* species based on RAPD data.

Species	*A. gmelinii*	*A. annua*	*A. dracunculus*	*A. sieversiana*	*A. tourenefortiana*
A. gmelinii	****	0.495	0.663	0.595	0.733
A. annua	0.610	****	0.466	0.574	0.574
A. dracunculus	0.516	0.628	****	0.786	0.718
A. sieversiana	0.551	0.563	0.456	****	0.574
A. tourenefortiana	0.480	0.563	0.488	0.563	****

Table 4. Summary of analysis of molecular variance (AMOVA) based on RAPD.

Source of variation	Among species	Within species	Total
Df	4	20	24
SS	10.16	10	20.16
MS	2.56	0.5	-
Est. Var.	0.408	0.5	0.908
Percentage	45	55	100
P-value	<0.001	<0.001	-

Where, df- degree of freedom; SS- sum of square; MS-mean squares; Est. Var.-Estimated variability; P-value: probability of null distribution.

dracunculus was highest with 0.786 (Table 3), thus revealing higher genetic identity, which reflects their high homology in genetic background. The least value (0.466) was found in *A. annua* and *A. dracunculus.* Genetic distance between *A. annua* and *A. dracunculus* was highest (0.628), while *A. sieversiana* and *A. dracunculus* had the lowest (0.456). AMOVA used to partition total genetic diversity among species and within species revealed highly significant variation ($p<0.001$) with 45% genetic variation among species and 55% within species (Table 4). Similar results have been reported earlier in *Eucalyptus* species (Balasaravanan *et al*., 4). Among the five species, higher genetic diversity in terms of percent polymorphic loci was observed in *A. annua* (75.56%), whereas *A. gmelinii* exhibited the lowest genetic diversity (9.63%). A similar trend was observed for mean gene diversity (Nei, 13), with minimum mean gene diversity (0.0321) in *A. gmelinii*, and maximum mean gene diversity in *A. annua* (0.3283). The higher genetic diversity helps in selecting the excellent cultivars with strong adaptation from the wild populations and thus proves to be beneficial for the breeding purposes (Kumar *et al*., 11 & 12; Balasaravanan *et al*., 4).

The percentage of polymorphism generated by the selected decamers indicated that RAPD could be the quite effective marker to access genetic diversity and is applicable to those cases where no or meager genomic information is available, which is true about

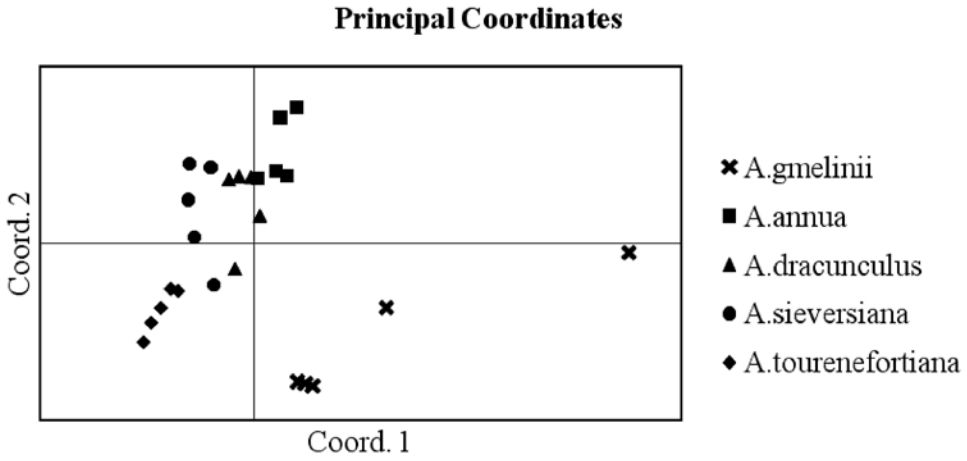

Fig. 4. Two-dimensional plot of principal coordinate analysis of five *Artemisia* species.

genus *Artemisia*. Thus, the present study on molecular analysis of these five important *Artemisia* species has opened new avenues for genetic improvement of these *Artemisia species.* Considering the high genetic differentiation among the wild populations of *Artemisia* species, conservation of only a few populations may not adequately protect the genetic variations among the species in the fragile Trans-Himalayan region of Ladakh. Since, no single or even a few plants, will represent the whole genetic variability in *Artemisia species*, there appears an urgent need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in *Artemisia* species.

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