Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 701975, 9 pages
doi:10.1155/2010/701975

Research Article

Analysis of Genetic Diversity in *Astragalus rhizanthus* Benth. ssp. *rhizanthus* var. *rhizanthus* (Fabaceae) Using Molecular Markers from India

Kumar Kamal Anand, Ravi Kumar Srivastava, and Lal Babu Chaudhary

Plant Biodiversity & Conservation Biology Division, National Botanical Research Institute (Council of Scientific and Industrial Research), Lucknow 226 001, India

Correspondence should be addressed to Lal Babu Chaudhary, chaudharylb@nbri.res.in

Received 1 September 2009; Accepted 23 February 2010

Academic Editor: Takashi Hashimoto

Astragalus rhizanthus with three infraspecific taxa (i.e., *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus*, A. *rhizanthus* ssp. *rhizanthus* var. *pindreensis,* and *A. rhizanthus* ssp. *candolleanus*) is widely scattered in the Himalaya from Jammu and Kashmir to Uttarakhand provinces in India. Among them, *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* exhibits enormous morphological diversity throughout its range of distribution. An assessment of genetic diversity studies was undertaken to understand the level and pattern of diversity, using Inter simple sequence repeats (ISSR), Random amplified polymorphic DNA (RAPD) and Directed amplification of minisatellite DNA (DAMD) profiles. Fifteen ISSR, 18 RAPD and 6 DAMD primers were used to unravel the diversity among 20 genotypes collected from the known localities in the Indian Himalaya. A total of 242 bands from ISSR, 352 from RAPD and 142 from DAMD were obtained with an average of 92.23% polymorphism in the species. Pair-wise genetic similarity for the cumulative data was determined using Jaccard's similarity coefficient which varied from 0.19 to 0.84. A combined UPGMA dendrogram was generated which revealed that different genotypes exhibited their affinity according to their geographical distribution. Tree topology suggests the existence of two distinct groups of the genotypes. Gene diversity (H) and Shannon's information index (I) were estimated and these values were found higher in the genotypes collected from Jammu and Kashmir than Himachal Pradesh.

1. Introduction

Astragalus L., with about 2500–3000 species, is the largest genus of flowering plants in the world [1–3]. In India, it has about 80–90 species, chiefly distributed in the temperate to alpine regions of the Himalaya with the main center of diversity in the cold deserts of Lahul-Spiti (Himachal Pradesh) and Leh and Ladakh (Jammu and Kashmir) [4, 5] (Figure 1). *A. rhizanthus* Benth. with three infraspecific taxa, *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus*, *A. rhizanthus* ssp. *rhizanthus* var. *pindreensis* (Baker) Podl., and *A. rhizanthus* ssp. *candolleanus* (Benth.) Podl. occurs in the Himalaya from Afghanistan to Nepal and in India from Jammu and Kashmir to Uttarakhand [6], out of which *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* exhibits enormous amount of morphological variations among different populations as observed during the examination of Indian *Astragalus* by one of us (L. B. Chaudhary) [7]. The taxon *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* grows on the mountain slopes and near agriculture fields in dry, stony, or sandy soils in sparsely scattered and fragmented populations. The Indian women chewed the roots of the taxon to stimulate lactation. *Astragalus* roots are also used in herbal medicines in China and USA for the treatment of diabetes, nephritis, HIV infection, back pain, tumors of eyes, liver, throat, and chest and to enhance the immune system of the body after cancer therapy treatment [7]. In addition, due to overgrazing by animals like goats, sheep, and horses the populations of the taxon are also decreasing day by day. The taxon is mostly caespitose perennial herbs with thick woody rootstock and generally without distinct stem. The extensive survey in the Himalaya revealed that the species

Figure 1: Distribution sites of *Astragalus rhizanthus* ssp. *rhizanthus* var. *rhizanthus* in India.

exhibits considerable amount of morphological variations especially in the length of stems and leaves, number, size, and shape of leaflets, length of bracts, flower size, length of calyx lobes, size of pods, density and orientations of hairs, and so forth on different parts of the plants within and between populations in different regions. Sometimes variations are so prominent either in the same population or among different populations or sometimes even on the same plant that creates doubt whether different individuals belong to the same or different taxon. Therefore, In view of the above, the genetic diversity of *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* which has not been studied so far has been examined to understand the level and pattern of diversity for knowing the relationship of morphological variations in respect to their geographical distribution as well as to know the more diverse populations to suggest the conservation strategies using PCR-based methods in India.

In recent years a large number of molecular taxonomic works have been carried out to explain the different kinds of taxonomic problems in *Astragalus* [8–13]. The PCR-based methods (ISSR, RAPD, and DAMD) have also been used extensively for the comparison of the genetic materials of two individual plants. The Intersimple Sequence Repeats (ISSRS) markers generated from single-primer PCR reactions are scattered evenly throughout the genome and inherited in a dominant or codominant Mendelian fashion, and they circumvents the challenge of characterizing SSR loci that microsatellite requires [14–18]. The Random Amplification of Polymorphic DNA (RAPD) technique provides a rapid assessment of the differences in the genetic composition of related individuals to access the genetic diversity [19–21]. Similarly, Directed Amplification of Minisatellite DNA (DAMD) reveals polymorphism due to minisatellites [22]. Since DAMD is carried out in higher PCR stringencies the patterns produced have greater reproducibility. Therefore, the techniques like ISSR, RAPD, and DAMD, which provide many polymorphic bands, are efficient to unravel the intraspecific relationships amongst different genotypes of *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus*.

2. Materials and Methods

2.1. Collection of Plant Materials. The leaf samples were collected in the field in silica gel following Chase and Hills [23] for DNA extraction. A large number of samples were collected from all possible and known localities of the cold desert of Lahul-Spiti (Himachal Pradesh) and Leh and Ladakh (Jammu and Kashmir) in India out of which some 20 genotypes have been selected for ISSR, RAPD, and DAMD analyses (Table 1). The number of accessions collected from Jammu and Kashmir is lesser than Himachal Pradesh because the area is not congenial for frequent visit due to terrorist activities prevailing there. A closely related Fabaceae taxon, *Oxytropis tatarica* Bunge, was used as the out-group for comparison. The voucher specimens were prepared, labeled, and deposited in the herbarium of National Botanical Research Institute, Lucknow, India (LWG) and all were critically examined to record the range of morphological variations available within the taxon.

2.2. DNA Isolation. Silica gel dried leaf tissues (100 mg) were frozen in liquid nitrogen and grounded in mortar and pestle. The genomic DNA was extracted from the powdered leaf material using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) as per the instructions of the manufacturer with minor modifications. Extracted DNA was quantified using ND-1000 spectrophotometer (Nanodrop, USA) and its quality was evaluated by gel electrophoresis on 0.8% agarose stained with ethidium bromide.

2.3. PCR Amplification with ISSR, RAPD, and DAMD Primers. A set of 100 anchored microsatellite primers procured from University of British Columbia, Canada were screened, out of which 15 primers generated reproducible polymorphic profiles were selected (Table 2). PCR amplification of 50 ng DNA was performed in 10 mM Tris-HCl pH 7.5, 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primer, and 0.9 U Taq DNA (Bangalore Genei, India) in a 25 μl reaction using PTC 200 thermocycler (MJ Research, Inc., USA). After initial denaturation at 94°C for 4 minutes, each cycle consisted of 1-minute denaturation at 94°C, 1 minute of annealing at 52°C, 2-minute extension at 72°C, along with 7-minute extension at 72°C at the end of 35 cycles.

The RAPD primers were procured from Operon Tech. Inc. Alameda, CA, USA. In total, 80 decamer primers (B, C, N and U kits) were screened for PCR amplification. Out of which 18 primers that generated polymorphic profiles were selected for screening data for all the accessions in the present study (Table 2). The final RAPD reactions were carried out in 25 μl volumes which contain 25 ng of template DNA, 10-pmole primer, 200 μM of each dNTP, 2.5 mM Mg^{2+} ion concentration in suitable 1X assay buffer supplied, along

Table 1: List of collected accessions of *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* with collection details.

Sample no.	Locality	Longitude/latitude	Altitude	Collection number
AR1	Before Rohtang, Lahul-Spiti	N 38°24.427′ E 77°14.806′	3814 m	229407
AR2	Before Rohtang, Lahul-Spiti	N 38°24.427′ E 77°14.806′	3814 m	229408
AR3	Before Rohtang, Lahul-Spiti	N 38°24.427′ E 77°14.806′	3814 m	229409
AR4	Koksar, Lahul-Spiti	N 32°24.755′ E 77°14.180′	3136 m	229416
AR5	Koksar, Lahul-Spiti	N 32°24.755′ E 77°14.180′	3136 m	229417
AR6	Koksar, Lahul-Spiti	N 38°24.809′ E 77°14.225′	3134 m	229421
AR7	Koksar, Lahul-Spiti	N 38°24.809′ E 77°14.225′	3134 m	229422
AR8	Gondla, Lahul-Spiti	N 32°36.286′ E 76°55.996′	2905 m	229463
AR9	Gondla, Lahul-Spiti	N 32°36.286′ E 76°55.996′	2905 m	229464
AR10	Gondla, Lahul-Spiti	N 32°36.286′ E 76°55.996′	2905 m	229466
AR11	Gondla, Lahul-Spiti	N 32°36.286′ E 76°55.996′	2905 m	229468
AR12	Triloki Nath, Lahul-Spiti	N 32°47.265′ E 77°17.099′	3845 m	229476
AR13	Gondla and Sissu, Lahul-Spiti	N 30°30.842′ E 76°58.986′	2996 m	229480
AR14	Chota Dara, Lahul-Spiti	N 32°18.035′ E 77°27.704′	3475 m	229485
AR15	Chatru and Chota Dara, Lahul-Spiti	N 32°20.079′ E 77°20.413′	3027 m	229493
AR16	Chatru, Lahul-Spiti	N 32°21.654′ E 77°18.247′	3034 m	229498
AR17	Gramphu, Lahul-Spiti	N 32°30.842′ E 77°58.987′	2996 m	229500
AR18	Panikhar, Leh and Ladakh	N 34°30.000′ E 76°13.987′	4200 m	PANI-05
AR19	Penzilla, Leh and Ladakh	N 33°30.000′ E 77°10.987′	4800 m	PANI-05
AR20	Khardungla, Leh and Ladakh	N 34°16.440′ E 77°36.170′	5000 m	225052
Og	Tanglang La, Leh and Ladakh	N 33°30.275′ E 77°46.126′	5200 m	225038

AR: *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus*; Og: *Oxytropis tatarica.*

with the enzyme and 0.5 units of the thermostable *Taq* DNA polymerase (Bangalore Genei, India). The amplification of DNA was performed on a PTC-200 thermocycler (MJ Research, Inc. USA), which was programmed to include predenaturation at 94°C for 1 minute, followed by 45 cycles of denaturation at 94°C for 1 minute, annealing at 35°C for 1 minute, and extension at 72°C for 1 minute. The final cycle allowed an additional 5 minute period of extension at 72°C.

Table 2: ISSR, RAPD, and DAMD primers used for PCR profiling of DNA of *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* and the extent of polymorphism detected with these primers.

Primer	Sequence (5′–3′)	No. of loci amplified	Polymorphic bands	% Polymorphism
		ISSR		
808	(AG)8C	18	17	94.44
809	(AG)8G	20	19	95
810	(GA)8T	19	18	94.73
811	(GA)8C	18	17	94.44
812	(GA)8A	18	17	94.44
816	(CA)8T	21	20	95.23
817	(CA)8A	21	20	95.23
818	(CA)8G	13	12	92.30
823	(TC)8C	14	13	92.85
824	(TC)8G	11	7	63.63
825	(AC)8T	15	14	93.33
830	(TG)8G	23	22	95.65
840	(GA)8CT	17	15	88.23
848	(CA)8AG	10	10	100
855	(AC)8CT	22	21	95.45
		RAPD		
OP-B06	TGCTCTGCCC	22	20	90.91
OP-B07	GGTGACGCAG	21	19	90.47
OP-B08	GTCCACACAG	18	15	83.33
OP-B09	TGGGGGACTC	18	17	94.44
OP-C05	GATGACCGCC	17	16	94.12
OP-C15	GACGGATCAG	17	17	100
OP-N06	GAGACGCACA	23	23	100
OP-N08	ACCTCAGCTC	25	20	80
OP-N10	ACAACTGGGG	14	12	85.71
OP-N17	CATTGGGGAG	21	20	95.23
OP-N18	GGTGAGGTCA	24	22	91.66
OP-N19	GTCCGTACTG	23	21	91.30
OP-N20	GGTGCTCCGT	20	19	95
OP-U01	ACGGACGTCA	25	24	96
OP-U07	CCTGCTCATC	26	25	96.15
OP-U08	GGCGAAGGTT	24	23	95.83
OP-U10	ACCTCGGCAC	21	18	85.71
OP-U12	TCACCAGCCA	22	21	95.45
		DAMD		
M13	GAGGGTGGCGGTTCCT	25	23	92
33.6	GGAGGTTTTTCA	25	21	88
HVR	CCTCCTCCCTCCT	22	20	90.90
HVA	AGGATGGAAAGGAGGC	32	29	90.62
HVY	GCCTTTCCCGAG	26	25	96.15
HVV	GGTGTAGAGAGGGGT	26	24	92.30

Similarly, DAMD-PCR was carried out according to Zhou and Gustafson [24]. This technique involves the use of minisatellite core sequence as a primer, singly, in the amplification reactions. Six minisatellite core sequence primers (custom synthesized from Bangalore Genei, India) were analyzed (Table 2). The reaction mixture (25 μl) contained 10 mM Tris-HCl (pH8.3), 50 mM KCl, 2 mM Mg^{2+} ion concentration, 200 μM each dNTP, 20-pmole

primer, 1 unit of *Taq* polymerase (Bangalore Genei, India) and approximately 60 ng genomic DNA. Optimal DNA amplification was obtained through 40 cycles at different temperature and time duration (92°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes). The amplification of DNA was performed on a PTC-200 thermocycler (MJ Research, Inc. USA).

Amplified PCR products were separated on 1.5% (w/v) agarose gel in 0.5 Tris-Borate EDTA (TBE) buffer at 100 V for 3 hours, stained with ethidium bromide and then visualized and photographed on a UV Transilluminator using a Gel Documentation System (UV Tech, UK). Amplified products were estimated on the gel by comparison with molecular weight-marker low-range DNA ruler. The reproducibility of the amplification products was checked twice for each polymorphic primer and only reproducible fragments were counted. The representative gels for each marker system have been provided in Figure 2.

2.4. Data Analysis. Data generated by ISSR, RAPD, and DAMD (amplified products estimated on the gel by comparison with molecular weight-marker low-range DNA ruler) were scored "1" to indicate presence and "0" to indicate absence of a band. A pairwise matrix of distances between genotypes was determined for the band data from each method using Jaccard's similarity coefficient in the Free Tree program [25]. From the pairwise distance data, the UPGMA trees were computed after allowing a 1000-replicate bootstrap test using the same program. The trees were viewed, annotated, and printed using Tree View (version 1.6.5; page 2001) [26]. Group analysis, gene diversity (H) [27], and Shannon information index (I) [28] were calculated using POPGENE program [29]. For this analysis, genotypes were considered in two groups consisting of genotypes of Lahul-Spiti and Leh and Ladakh.

3. Results

3.1. Morphological Diversity. A. rhizanthus ssp. *rhizanthus* var. *rhizanthus* exhibits enormous amount of variations especially in the size of plants, length of leaves, shape and size of leaflets, nature of inflorescence, size of bracts, length of calyx and pods, and so forth. Usually, the plants are almost stemless or with very short stem which hardly reaches up to 10 cm in length. The plants growing in very hard or rocky soils become stunted in comparison to the plant growing in moist and sandy soils. They grow singly or in tufts from a thick and woody perennial rootstock which also varies in length depending upon the nature of soils. The leaves considerably vary from 3 to 22 cm in length among different plants even within the same population. The plants with the longest leaves have been encountered in some pockets of Sissu-Gondla region of Lahul-Spiti in Himachal Pradesh; however, at higher altitudes in Ladakh region, the plants are of very dwarf size. Similarly, the leaflets also express great variations in number, shape, and size and density of hairs from plant to plant irrespective to the populations. The number of leaflets in each leaf varies from 29 to 45 depending upon the length of leaf rachis. The size of the leaflets ranges from 3 to 15 mm in length and from 3 to 7 mm in width. The shape also drastically varies within population and it may be orbicular, obovate, elliptic, or lanceolate with different types of apices (rounded, obtuse, acute, or emarginated). The leaflets are usually hairy on both sides but the density of hairs differs among plants chiefly on the upper surface. However, the plants collected from Jammu and Kashmir have been found comparatively more hairy than others. Generally, the inflorescence/flowers are aggregated towards the tip of stem without distinct peduncles; however, occasionally, especially when stems get slightly elongated, the peduncle length reaches up to 10 mm. Bract, which is considered an important taxonomic character in the genus *Astragalus,* remarkably varies in its length within the taxon. It may be equal or distinctly longer than pedicel. Like bract, calyx is also an another useful character in the whole genus to discriminate the taxa; however, here it distinctly varies. Apart from the variation in the length (12–17 mm), the calyx also varies in the proportion of tube and lobes length. Usually, the tube is longer than lobes; however, sometimes lobes become more or less equal to tube. Variations have also been noticed in flower size (18–23 mm) and shape of standard petal (broadly obovate to oblong-elliptic). Although, the wing petals are usually shorter than standard, in some cases they reach up to the length of standard petal. The styles are glabrous throughout but quite rarely hairs have been observed in the lower portion. Pods also show some variations in length (12–20 mm) and density of hairs (moderately to densely hairy). The above variations are quite irregular and cannot be correlated with any particular population.

3.2. Genetic Diversity. In the present study, three independent methods such as ISSR, RAPD, and DAMD were used on different genotypes of *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus*. The three methods revealed polymorphism independent of each other from several regions of genome. Therefore, a combined data analysis of all three methods which consists of more numbers of primers and more coverage of genome considered together revealed a comprehensive pattern of genetic diversity amongst the genotypes.

In ISSR, 15 discrete primers were used that resulted in 260 bands, out of which 242 bands were polymorphic revealing 93.07% polymorphism. Number of bands per primer varied from 11 (824) to 23 (830) with 17.3 bands per primer. The size of fragments ranged from 450 bp to 2800 bp. In case of RAPD, 352 bands were polymorphic out of the 381 bands obtained with 18 primers revealing 92.38% polymorphism. The number of bands per primer ranged from 14 (OPN-10) to 26 (OPU-07) with 21.16 bands per primer. The size of the fragments ranged from 250 bp to 3000 bp. Six DAMD primers resulted in 142 polymorphic bands out of total 156 bands revealing 91.02% polymorphism. Size of bands ranges from 200 bp to 3000 bp. The number of bands per primer varied from 22 (HVR) to 32 (HVA) with an average of 23.5 bands per primer. The cumulative band data generated through the above three Single Primer Amplification Reaction (SPAR) methods resulted is 92.34% polymorphism (Table 2) across all genotypes and this level of

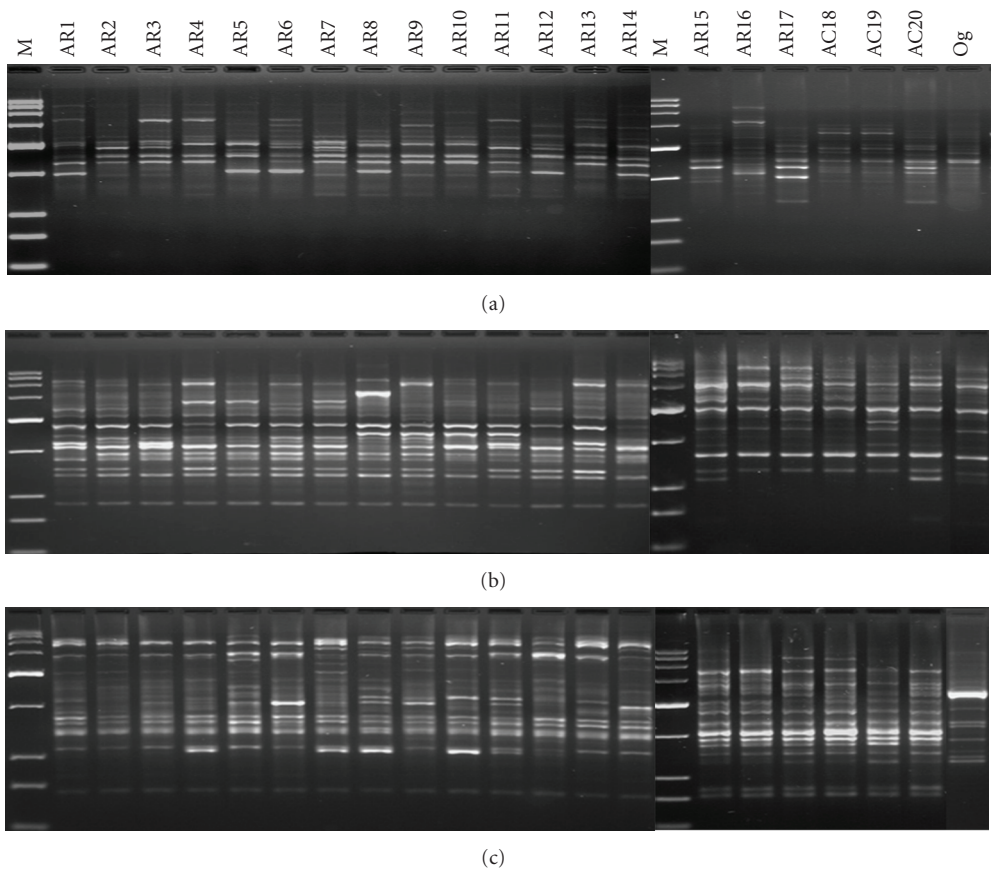

Figure 2: Gel profiles obtained typically with (a) ISSR primer (810), (b) RAPD primer (OP-B06), and (c) DAMD primer (33.6). All profiles were resolved in 1.5% agarose gels in 0.5 X TBE. The lanes marked as Marker (M) contain the Low Range Ruler (Bangalore Genei, India) as DNA fragment size marker. The other lanes are marked with taxon abbreviation in Table 1.

intraspecific polymorphism suggests that *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* was significantly diverse in India.

A cumulative data was used to generate the similarity index for ISSR, RAPD, and DAMD methods based on Jaccard's similarity coefficient. The similarity values for the combined data varied from 0.19 to 0.84 between pairs of genotypes (Table 3). Cluster analysis was used to generate UPGMA dendrogram after 1000-replicate bootstrap test. All the genotypes of candidate taxon had been grouped into two major clusters (with 99% bootstrap) marked as A and B in Figure 3. The genotypes of Lahul-Spiti region had been clubbed in cluster A, while cluster B contains the genotypes of Leh and Ladakh region of *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus*. The *Oxytropis tatarica* Bunge was used as an out-group (Og) in the study which has been clearly separated from *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus*. In the subcluster A1, all accessions (AR1-AR7) collected from Koksar region clubbed together. The individuals of this group exhibited 80% of similarity. Similarly, all accessions (AR8-AR10) except AR11 collected from Gondla were grouped together in subcluster A2 with 77% similarity. Further subclustering of accessions AR12-17 was neither according to geographical affiliation nor based on any morphological affinities. In subcluster A3, the accessions of two quite different localities and altitudes (AR11 from Gondla, 2905 m and AR12 from Triloki Nath, 3812 m) had been grouped together with 71% similarity. The elements AR13 of Gondla and Sissu and AR14 of Chota Dara with 60% similarity coefficient value had been grouped into subcluster A4. These populations were geographically quite distant from each other and distinctly differ in climatic and edaphic conditions. The accessions AR15 (from between Chatru and Chota Dara) and AR17 (from Gramphu) again were grouped together in subcluster A5 with only 47% similarity. Likewise, the accession AR16 collected from Chota Dara has not clubbed with the other individuals of the same locality and distinctly separated out in the dendrogram. Cluster B includes all the accessions of *Astragalus* ssp. *rhizanthus* var. *rhizanthus* of Leh and Ladakh region (4800 m–5200 m). The UPGMA dendrogram thus depicts apparent correlation between geographical and genetic diversity amongst Lahul-Spiti and Leh and Ladakh genotypes (Figure 3). Estimates of Shannon Index (I) and gene diversity (H) showed that the target genotypes of Leh and Ladakh were more heterogeneous and had higher H and I values (0.2216 and 0.3826,. resp.) compared to the corresponding values (0.2106 and 0.3512,. resp.) in the genotype of Lahul-Spiti (Table 4).

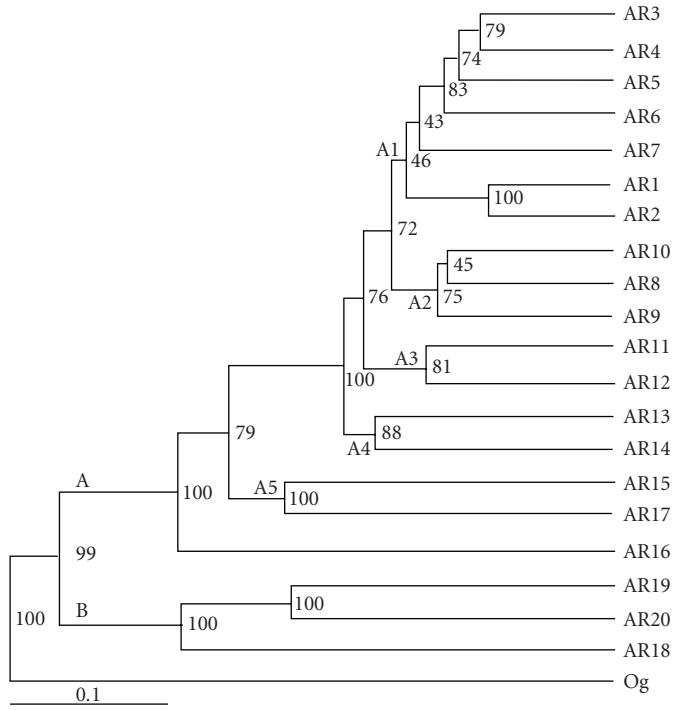

Figure 3: UPGMA tree generated from band data by the three methods are considered cumulatively after a 1000-replicate bootstrap test. Accession abbreviations are as in Table 1 and are to the right of the tree branches. The small numbers at the nodes are the bootstrap percent values. Nodes with oval labels indicate specific subgroups within the broad groups.

Table 3: Pairwise Jaccard's similarity coefficients matrix from the cumulative band data for *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus.*

	AR1	AR2	AR3	AR4	AR5	AR6	AR7	AR8	AR9	AR10	AR11	AR12	AR13	AR14	AR15	AR16	AR17	AR18	AR19	AR20	Og
ARl																					
AR2	0.84																				
AR3	0.75	0.78																			
AR4	0.76	077	083																		
AR5	072	0.74	0.79	0.81																	
AR6	0.68	072	077	0.79	077																
AR7	0.69	072	074	075	074	0.76															
AR8	0.7	0.7	0.7	072	0.71	073	0.76														
AR9	0.66	0.69	0.69	0.74	0.71	073	072	0.78													
AR10	0.7	0.7	0.71	0.74	0.71	073	073	0.78	0.76												
AR11	0.65	0.68	0.68	0.71	0.67	0.69	0.69	0.7	0.75	0.75											
AR12	0.61	0.63	0.64	0.66	0.65	0.66	0.64	0.66	0.66	0.69	0.76										
AR13	063	062	062	0.66	063	0.65	0.65	0.62	063	0.67	0.66	0.67									
AR14	0.66	0.66	0.64	0.67	0.64	0.67	0.66	0.63	0.67	0.67	0.65	0.64	0.69								
AR15	0.53	0.54	0.5	0.51	0.51	0.5	0.52	0.5	0.5	0.52	0.51	0.49	0.5	0.52							
AR16	0.44	0.42	0.44	0.44	0.42	0.42	0.43	0.42	0.43	0.43	0.43	0.41	0.42	0.43	0.47						
AR17	0.49	0.49	0.47	0.48	0.46	0.46	0.5	0.49	0.49	0.49	0.49	0.5	0.49	0.49	0.57	0.49					
AR18	0.35	0.36	0.33	0.36	0.34	0.35	0.36	0.36	0.36	0.34	0.35	0.35	0.34	0.32	0.36	0.31	0.35				
AR19	0.27	0.29	0.27	0.28	0.28	0.27	0.29	0.28	0.28	0.29	0.29	0.3	0.29	0.26	0.32	0.29	0.31	0.48			
AR20	0.19	0.2	0.19	0.2	0.19	0.19	0.2	0.21	0.2	0.2	0.19	0.21	0.2	0.2	023	0.21	023	0.4	0.58		
Og	0.21	0.22	0.22	0.22	023	0.22	023	0.22	0.21	023	0.22	0.21	023	023	023	0.26	0.22	0.2	0.19	0.13	

Table 4: Group analysis of gene diversity and Shannon information index for cumulative band data for the three methods.

Genotype group	Mean H^* (Std. Dev.)	Mean I^* (Std. Dev.)
Lahul-Spiti	0.2106 (0.1839)	0.3512 (0.2532)
Leh and Ladakh	0.2216 (0.1938)	0.3826 (0.2697)

H^* is Nei's measure of genetic diversity [27].
I^* is Shannon's information index [28].

4. Discussion

In recent years, PCR-based methods like ISSR, RAPD, and DAMD have been extensively used to assess the intraspecific genetic diversity as well as to establish the interspecific relationships in several plant groups like *Cicer* [30], *Astragalus* [31–36], *Phyllanthus* [37], and *Trigonella* [38]. In the present study, 20 genotypes of the *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* and one of *Oxytropis tatarica* as the out-group were considered to assess the genetic diversity by using ISSR, RAPD, and DAMD analyses as no such type of study has been carried out on this species in the past.

The ISSR, RAPD, and DAMD data analysis revealed the genetic similarity coefficients among the genotypes and that varied from 0.19 to 0.84. In all three methods the average percentage of polymorphism has been marked at very high level between 19% and 84% amongst the genotypes. The maximum (84%) and minimum (19%) polymorphism has been observed in the accessions AR1 and AR3 (collected from Koksar) and AR19 and AR20 (collected from Jammu Kashmir), respectively. At the population level, the genotypes collected from Leh and Ladakh were more heterogeneous as estimated by Shannon Index (I) and gene diversity (H) and may be conserved (Table 4). Large number of bands obtained per primer in the present study indicated the efficiency of the selected primers to characterize the genotypes of the candidate species. Amongst all the three methods, viz., ISSR, RAPD, and DAMD, ISSR has shown maximum (93.07%) polymorphism across the different genotypes of *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* in comparison to RAPD (92.38%) and DAMD (91.02%). This is indicative of the fact that ISSR is the most suitable method for unraveling the genetic diversity in *Astragalus* species [28].

The UPGMA tree generated for all the genotypes resulted in a discrete clustering of the genotypes which have been grouped more or less according to their geographical distribution and not in accordance with any morphological characters. Furthermore, the out-group accession of *Oxytropis tatarica* has clearly been separated out from the genotypes analyzed. The high bootstrap percentages, at the nodes, support the robustness of the clustering of the genotypes and the genetic diversity amongst them. The genotypes from similar geographical regions showed closer genetic similarity than those from the geographically distant regions. All genotypes of the candidate taxon of Lahul-Spiti (Himachal Pradesh) region resulted in a single large cluster marked as A at the node of the tree and the genotypes collected from Leh and Ladakh (Jammu and Kashmir) were clustered together in a separate cluster B. Within cluster A of Lahul-Spiti region again the genotypes collected from Koksar and Gondla regions have clubbed together except one accession of Gondla. The individual accession collected from other localities (AR12–AR16) has been grouped separately, it further indicated that genotypes have more affinity to their local climate /microclimate than any morphological similarities. It appears that different morphological characters have evolved independently respective to their geographical location and climatic factors to adapt in particular climate. This may be due to adaptation of the species in different extreme ecological conditions in the Himalaya which vary from region to region.

Acknowledgments

The authors are grateful to Dr. Rakesh Tuli, Director of National Botanical Research Institute, Lucknow for facilities and encouragements and to the Department of Biotechnology, government of India, New Delhi for financial support.

References

[1] D. Podlech, "Taxonomic and phytogeographical problems in *Astragalus* of old world and South–West Asia," *Proceedings of the Royal Society of Edinburgh*, vol. 89B, pp. 37–43, 1986.
[2] S. H. Zarre and D. Podlech, "Problems in the taxonomy of tragacanthic Astragali," *Sendtnera*, vol. 4, pp. 243–250, 1997.
[3] A. A. Maassoumi, *Astragalus L. in the World*, Research Institute of Forests and Rangelands, Tehran, Iran, 1998.
[4] L. B. Chuadhary, K. K. Anand, and R. K. Srivastava, "Taxonomic study of endemic species of *Astragalus* L. (Fabaceae) of India," *Taiwania*, vol. 52, no. 3, pp. 216–237, 2007.
[5] L. B. Chaudhary and S. K. Srivastava, "Taxonomic and distributional notes on some *Astragalus* L. (Fabaceae) in India," *Taiwania*, vol. 52, no. 1, pp. 25–48, 2007.
[6] D. Podlech, "Revision von *Astragalus* L. sect. *Caprini* DC. (Leguminosae)," *Mitteilungen Botanische Staatssam München*, vol. 25, pp. 1–294, 1988.
[7] L. B. Chaudhary, T. S. Rana, and K. K. Anand, "Current status of the systematics of *Astragalus* L. (Fabaceae) with special reference to the Himalayan species in India," *Taiwania*, vol. 53, no. 4, pp. 338–355, 2008.
[8] M. J. Sanderson, "Phylogenetic relationship within North American *Astragalus* L. (Fabaceae)," *Systematic Botany*, vol. 16, no. 3, pp. 414–430, 1991.
[9] A. Liston, "Variation in the chloroplast genes *rpo*C1 and *rpo*C2 of the genus *Astragalus* (Fabaceae): evidence from restriction site mapping of a PCR amplified fragment," *American Journal of Botany*, vol. 79, no. 8, pp. 953–961, 1992.
[10] M. F. Wojciechowski, M. J. Sanderson, and J.-M. Hu, "Evidence on the monophyly of *Astragalus* (Fabaceae) and its major subgroups based on nuclear ribosomal DNA ITS and chloroplast DNA trnL intron data," *Systematic Botany*, vol. 24, no. 3, pp. 409–437, 1999.
[11] M. F. Wojciechowski, "*Astragalus* (Fabaceae): a molecular phylogenetic perspective," *Brittonia*, vol. 57, no. 4, pp. 382–396, 2005.
[12] S. Kazempour Osaloo, A. A. Maassoumi, and N. Murakami, "Molecular systematics of the genus *Astragalus* L. (Fabaceae): phylogenetic analysis of nuclear ribosomal DNA internal transcribed spacers and chloroplast gene *ndh*F sequences," *Plant Systematics and Evolution*, vol. 242, no. 1–4, pp. 1–32, 2003.

[13] S. Kazempour Osaloo, A. A. Maassoumi, and N. Murakami, "Molecular systematics of the old world *Astragalus* (Fabaceae) as inferred from nrDNA ITS sequence data," *Brittonia*, vol. 57, no. 4, pp. 367–381, 2005.

[14] H. Hamada and T. Kakunaga, "Potential Z-DNA forming sequences are highly dispersed in the human genome," *Nature*, vol. 298, no. 5872, pp. 396–398, 1982.

[15] D. Tautz and M. Renz, "Simple sequences are ubiquitous repetitive components of eukaryotic genomes," *Nucleic Acids Research*, vol. 12, no. 10, pp. 4127–4138, 1984.

[16] R. Condit and S. P. Hubbell, "Abundance and DNA sequence of two-base repeat regions in tropical tree genomes," *Genome*, vol. 34, no. 1, pp. 66–71, 1991.

[17] M. Gupta, Y. S. Chyi, J. Romero-Severson, and J. L. Owen, "Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats," *Theoretical and Applied Genetics*, vol. 89, no. 7-8, pp. 998–1006, 1994.

[18] Y. Tsumura, K. Ohba, and S. H. Strauss, "Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*)," *Theoretical and Applied Genetics*, vol. 92, no. 1, pp. 40–45, 1996.

[19] J. G. K. Williams, A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey, "DNA polymorphisms amplified by arbitrary primers are useful as genetic markers," *Nucleic Acids Research*, vol. 18, pp. 6531–6535, 1990.

[20] A. D. Wolfe and A. Liston, "Contributions of PCR based methods to plant systematics and evolutionary biology," in *Molecular Systematics of Plants II: DNA Sequencing*, D. E. Soltis, P. S. Soltis, and J. J. Doyle, Eds., pp. 43–86, Kluwer Academic Publishers, Boston, Mass, USA, 1998.

[21] J. Harris, "RAPDs in systematics-a useful methodology?" in *Molecular Systematics and Plant Evolution*, P. M. Hollingsworth, R. M. Bateman, and R. J. Gornall, Eds., pp. 221–228, Taylor & Francis, London, UK, 1999.

[22] D. D. Heath, G. K. Iwama, and R. H. Devlin, "PCR primed with VNTR core sequences yields species specific patterns and hypervariable probes," *Nucleic Acids Research*, vol. 21, no. 24, pp. 5782–5785, 1993.

[23] M. W. Chase and H. H. Hills, "Silica gel: an ideal material for field preservation of leaf samples for DNA studies," *Taxon*, vol. 40, pp. 215–220, 1991.

[24] Z. Zhou and J. P. Gustafson, "Genetic variation detected by DNA fingerprinting with a rice minisatellite probe in *Oryza sativa* L.," *Theoretical and Applied Genetics*, vol. 91, no. 3, pp. 481–488, 1995.

[25] A. Pavlicek, S. Hrda, and J. Flegr, "FreeTree-freeware program for construction of phylogenetic trees on the basis of distance data and Bootstrap/Jackknife analysis of the tree robustness. Application in the RAPD analysis of genus *Frenkelia*," *Folia Biologica*, vol. 45, no. 3, pp. 97–99, 1999.

[26] R. D. M. Page, "Tree view (win 32)," ver. 1.6.5., 2001, http://taxonomy.zoology.gla.ac.uk/rod/treeview.html.

[27] M. Nei, "Genetic distance between populations," *The American Naturalist*, vol. 106, pp. 283–292, 1972.

[28] C. E. Shannon and W. Weaver, *The Mathematical Theory of Communication*, University of Illinois press, Urbana, Ill, USA, 1949.

[29] F. C. Yeh, R. Yang, and T. Boyle, "POPGENE- a Microsoft Windows based freeware for population genetic analysis," ver. 1.32 (32 bit), 1997, http://www.ualberta.ca/~fyeh/.

[30] V. J. Sant, A. G. Patankar, N. D. Sarode, et al., "Potential of DNA markers in detecting divergence and in analysing heterosis in Indian elite chickpea cultivars," *Theoretical and Applied Genetics*, vol. 98, no. 8, pp. 1217–1225, 1999.

[31] M.-C. Luo, K.-K. Hwu, and T.-C. Huang, "Taxonomic study of Taiwan *Astragalus* based on genetic variation," *Taxon*, vol. 49, no. 1, pp. 35–46, 2000.

[32] J. A. Alexander, A. Liston, and S. J. Popovich, "Genetic diversity of the narrow endemic *Astragalus* oniciformis (Fabaceae)," *American Journal of Botany*, vol. 91, no. 12, pp. 2004–2012, 2004.

[33] H.-J. Na, J.-Y. Um, S.-C. Kim, et al., "Molecular discrimination of medicinal *Astragali* radix by RAPD analysis," *Immunopharmacology and Immunotoxicology*, vol. 26, no. 2, pp. 265–272, 2004.

[34] M. Mehrnia, S. Zarre, and A. Sokhan-Sanj, "Intra- and interspecific relationships within the *Astragalus* microcephalus complex (Fabaceae) using RAPD," *Biochemical Systematics and Ecology*, vol. 33, no. 2, pp. 149–158, 2005.

[35] L. B. Chaudhary, T. S. Rana, D. Narzary, and S. Verma, "A new species of *Astragalus* L. (Leguminosae) from India based on morphological and molecular markers," *Botanical Journal of the Linnean Society*, vol. 154, pp. 27–34, 2007.

[36] S. Zarre, Z. Khodaei, Z. Karamali, V. Niknam, and M. Mirmasoumi, "Isoenzyme variation patterns and species concept in *Astragalus gossypinus* and *Astragalus persicus* complexes (Fabaceae) in Iran," *Biochemical Systematics and Ecology*, vol. 35, no. 11, pp. 757–763, 2007.

[37] N. Jain, A. K. Shasany, V. Sundaresan, et al., "Molecular diversity in *Phyllanthus amarus* assessed through RAPD analysis," *Current Science*, vol. 85, no. 10, pp. 1454–1458, 2003.

[38] R. S. Dangi, M. D. Lagu, L. B. Choudhary, P. K. Ranjekar, and V. S. Gupta, "Assessment of genetic diversity in *Trigonella foenum-graecum* and *Trigonella caerulea* using ISSR and RAPD markers," *BMC Plant Biology*, vol. 4, article 13, 2004.