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Full Length Research Paper

Characterization of genetic structure of alfalfa (*Medicago* sp.) from trans-Himalaya using RAPD and ISSR markers

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Twenty five (25) accessions of Lucerne (*Medicago* sp.) collected from Leh valley of trans-Himalayan region of Ladakh (Jammu and Kashmir) were analyzed using inter simple sequence repeats (ISSRs) and random amplified polymorphic DNA (RAPD). The results of this study revealed that the level of genetic variation in the collected *Medicago* ecotypes were relatively high (*P*=96.54%, *I*=0.430, *Ht*=0.285). RAPD fingerprinting detected more polymorphic loci (97.96%) than ISSR fingerprinting (95.12%). Clustering of genotypes within groups was not similar when RAPD and ISSR derived dendrogram were compared, whereas the pattern of clustering of the genotypes remained more or less the same in RAPD and combined data of RAPD + ISSR. The mean coefficient of differentiation (Gst) was 0.0584 indicating 30.23% of the genetic diversity within the populations. The overall value of mean estimated number of gene flow (Nm = 8.0682) revealed large gene exchanges among populations. Analysis of molecular variance (AMOVA) indicated that the distribution of genetic diversity was 49% among populations and 51% within populations. The plant is capable of reproducing by self-sowing, thus can influence population genetic structure. The pronounced genetic variation tells us that *Medicago* species is a proper plant for genetic research and that there is great potential of breeding this species for improved forage varieties.

Key words: Genetic variation, ISSR, RAPD, *Medicago* species, population structure.

INTRODUCTION

The genus *Medicago* is distributed worldwide and consists of approximately 83 species (Small and Jomphe, 1989). The cultivated alfalfa (*Medicago sativa* L.) is autotetraploid (2*n* = 4*x* = 32), cross-pollinated (allogamous) and seed propagated (McCoy and Bingham, 1988). It is one of the most important forage crops, with about 32 million hectares globally and is believed to have originated in the Caucasus region of northeastern Turkey, Turkmenistan and northwestern Iran (Michaud et al., 1988). Ladakh is designated as one of the centres of origin of *Medicago* by De Candolle (1967). It has been the oldest cultivated as well as wild crop of this area and most of the alfalfa fields have not been re-sown due to self-seeding. The original alfalfa of Ladakh *Medicago falcata* was yellow flowered. The traders of the old silk route introduced *M. sativa* from Yarkand (Central Asia) to enhance the availability of forage for their horses and locally *M. falcata* is known as *Ole* while *M. sativa* as Yarkandi *Ole.* A lot of natural hybridization has taken place and now it is rare to find a true stand of either *M. sativa* or *M. falcata* and resulted in a wide range of variability in habit, leaf size, height, colour of the flower, shape of pods and the resistance of plants to cold and aridity. The American scientists named it variety Ladakh and was introduced in U.S.A during 1910 (Bolton, 1962). Patel and Wanchu (1964) observed the diversity in Ladakh alfalfa and proposed a basic grouping according to the habit of the plants. The present populations of Ladakh *Medicago* may be assigned to the variegated group of *Medicago* classification proposed by Whyte et al. (1953).

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Three species of *Medicago* such as *M. sativa, M. falcata and Medicago media* adapted to cold arid habitats above 3500 m above the sea level are found in Ladakh. Life form, mating systems and seed dispersal are important adaptive traits shaping genetic structure and geographical distribution of plant populations (Levin, 1981; Loveless and Hamrick, 1984; Ennos, 1994; Hamrick and Godt, 1996; Bohonak, 1999; Clauss and Mitchell-Olds, 2006; Song et al., 2006; Mable and Adam, 2007). These species hold a rich source of natural variation and are valuable genetic resources for developing better grazing legumes especially for drier and colder regions where green fodder availability is very insufficient. Genetic diversity details of initial selection materials are essential for successful breeding and creation of new cultivars. Analyses of population genetic variation together with phenotypic variation will provide insights into the evolutionary history and processes of plant species (Barrett et al., 1996; Juan et al., 2004). Though, morphology, physiology, and phenology of the three species had been studied previously (Misri, 1986); their population genetic structures had not been characterized. Molecular markers are useful in identifying the maximally diverse parental genotypes through an evaluation of genetic diversity which is useful in cultivar identification, seed purity analysis, breeding and germplasm management. Different molecular marker types have been used to assess genetic diversity in alfalfa: Random amplified polymorphic DNA (RAPD) (Musial et al., 2002), amplified fragment length polymorphism (AFLP) (Segovia-Lerma et al. 2003), restriction fragment length polymorphism (RFLP) (Maureira et al. 2004), simple sequence repeat (SSR) (Flajoulot et al., 2005) and sequence related amplified polymorphisms (SRAP) (Vandemark et al., 2005).

Among the various molecular markers, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are simple and quick techniques and have become popular as their application does not need any prior information about the target sequences in the genome. These markers have been used for DNA fingerprinting, conservation biology (Li et al., 2005), phylogenetic studies (Kochieva et al., 2006) and population genetics (Zhang et al., 2010). The present study characterizes the genetic variability found in the *Medicago* species using RAPD and ISSR as the combined results would be more credible to analyze the genetic structure of *Medicago* populations of Ladakh.

MATERIALS AND METHODS

Sample collection

The plant material used in this study of genetic diversity was obtained from 25 wild populations (25 sites) from the trans–Himalayan region, Ladakh, India. Each accession consisted of five plants and for genetic diversity analysis, representative samples of 3 plants were used. The accessions were designated with accession codes 'D' for DIHAR accessions and 'P' for Phyang Village accessions. The interval between samples was 0.5- 5.0 km, whereas distance between populations was 20-30 km. About 10 g of young leaves from each representative sample were obtained and placed in a zip-lock plastic bag and placed in ice bucket. Each Sample does not necessarily denote a genetic individual. The samples were stored at -20˚C until use.

DNA extraction and PCR amplification

Total genomic DNA was extracted from frozen leaves (5 g) by the CTAB method (Saghai- Maroof et al., 1984) with minor modifications, which included the use of 200 mg per sample polyvinyl pyrollidone. Twenty random decamer primers from IDT Tech, USA were used for RAPD amplification following the protocol of Williams et al. (1990). Amplification reaction 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 dNTPs, 0.4 µM primer, 20 ng template DNA and 0.5 unit of *Taq* polymerase ('Sigma-Aldrich, USA'). Amplification was done using a PTC Thermal Cycler (MJb Research Inc.,) programmed for an initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min., extension at 72°C for 2 min. The final extension at 72°C for 5 min and then storage at 4°C for each primer (Table 1). In case of ISSR, the primers were obtained from 'Applied Biosciences, India' and PCR amplification was performed in reaction cocktail similar to RAPD. Amplification reaction 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 dNTPs, 0.4 µM primer, 20 ng template DNA and 0.5 unit of *Taq* polymerase ('Sigma-Aldrich, USA'). Amplification was done using a PTC Thermal Cycler (MJb Research Inc.,) programmed for an initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 1 min, annealing at X °C for 1 min; extension at 72°C for 2 min the final extension at 72°C for 5 min and then storage at 4°C where X°C refers to the annealing temperatures specific for each primer (Table 2). The amplification for each primer was performed twice independently with the same procedure in order to ensure the fidelity of RAPD and ISSR markers.

Amplification products were electrophoresed on 1.5% agarose gel (Life Science Technologies, USA) and run at constant voltage (50 V) in 1X TBE for approximately 2 h, visualized by staining with ethidium bromide (0.5 µg ml-1) and 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, Alphaimager, USA). Molecular size of amplicons was estimated using a 100 bp and 1 Kb DNA ladders ('Bangalore Genei, India').

Scoring and data analysis

RAPD and ISSR bands were scored as present (1) or absent (0). Only those bands showing consistent amplification were scored. The resolving power (Rp) of a primer is $Rp= \Sigma IB$ where, IB is the band informativeness which takes the value of: 1-[2 X (0.5-P)] P being the proportion of the accessions containing the *i*th amplicon (Prevost and Wilkinson, 1999). Data analysis were performed using the NTSYS pc version 2.2 computer package program (Rohlf, 2005) and a dendrogram based on Nei's (1978) unbiased genetic distances and the unweighted pair-group method with arithmetic averages (UPGMA) was constructed. Genetic diversity within and among populations was measured by the percentage of polymorphic bands (PPB). The data matrix of RAPD and ISSR was also used for assessment of genetic structure, genetic differentiation, gene flow and diversity. Measurement of diversity

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 (℃)	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Total number of fragment amplified	Resolving power
S 21	CAGGCCCTT C	70	36.4	7	7	100	41	3.28
S 22	TGCCGAGCT G	70	40.7	5	5	100	29	2.32
S 23	AGTCAGCCA C	60	34.3	6	6	100	60	4.80
S 24	AATCAGCCA C	50	30.1	8	8	100	44	3.52
S 25	AGGGGTCTT G	60	32.6	4	4	100	25	2.00
S 26	GGTCCCTGA C	70	35.2	5	5	100	51	4.08
S 27	GAAACGGGT G	60	33.2	5	5	100	31	2.48
S 28	GTGACGTAG G	60	31.1	9	9	100	54	4.32
S 29	GGGTAACGC C	70	37.4	8	8	100	68	5.44
S 30	GTGATCGCA G	60	33.1	7	7	100	32	2.56
S 31	CAATCGCCG T	60	36.7	5	5	100	43	3.44
S 32	TCGGCGATA G	60	34.0	3	3	100	10	0.80
S 33	CAGCACCCA C	70	37.7	5	4	80	80	6.40
S 34	TCTGTGCTG G	60	34.3	8	8	100	56	4.48
S 35	TTCCGAACC C	60	34.2	5	5	100	62	4.96
S 36	AGCCAGCGA A	60	38.3	4	4	100	12	0.96
S 37	GACCGCTTG T	60	35.7	5	5	100	32	2.56
S 38	AGGTGACCG T	60	36.2	8	7	87.5	124	9.92
S 39	CAAACGTCG G	60	34.2	8	8	100	39	3.12
S 40	GTTGCGATC C	60	33.5	12	11	91.66	148	11.84
	Total	--	--	127	124	97.96	1041	--

Table 2. List of primers used for ISSR amplification, GC content, total number of loci, the level of polymorphism and resolving power

Primer	Primer Sequence (5'~3')	GC (%)	Tm (℃)	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Total number of fragment amplified	Resolving power
ISSR 12	(AT)8 T	23.1	47.4	10	10	100	102	8.16
ISSR 13	(TA)8 RT	25.6	47.1	10	10	100	55	4.4
ISSR 21	$(GA)_8$ C	52.9	46.8	8	8	100	38	3.04
ISSR 22	$(TC)_8$ C	52.9	48.1	10	10	100	96	7.68
ISSR 24	$(CT)_8$AG	52.6	49.4	10	9	90	69	5.52
ISSR 26	$(GACA)_4$	50	47.4	9	8	88.8	47	3.76
ISSR 31	$(AG)_8$ YA	47.2	48.9	4	4	100	18	1.44
ISSR 32	$(GA)_8$ YC	52.7	48.5	4	4	100	26	2.08
ISSR 33	$(AC)_8$ YG	52.7	54.3	5	4	80	50	4
ISSR 34	$(TG)_8$RC	80	54.5	8	7	87.5	84	6.72
ISSR 36	BHB$(GA)_7$	50.9	48.3	7	7	100	28	2.24
	Total	--	--	85	81	95.12	613	--

Table 3. Summary of genetic variation statistics for all loci of RAPD, ISSR and RAPD + ISSR among the *Medicago* populations.

Population	Sample size	Na	Ne	H	I	Ht	NPL	PPL
RAPD								
Population 1	12	1.8740 (0.3331)	1.5164 (0.3398)	0.3044 (0.1658)	0.4575 (0.2229)	0.3044 (0.0275)	111	87.40
Population 2	13	1.8976 (0.3043)	1.5208 (0.3356)	0.3075 (0.1623)	0.4633 (0.2150)	0.3075 (0.0263)	114	89.76
Mean		1.8858	1.5186	0.30595	0.4604	0.30595	--	--
ISSR								
Population 1	12	1.7647 (0.4267)	1.4223 (0.3650)	0.2508 (0.1849)	0.3811 (0.2558)	0.2508 (0.0342)	65	76.47
Population 2	13	1.7765 (0.4191)	1.4209 (0.3441)	0.2545 (0.1775)	0.3880 (0.2480)	0.2545 (0.0315)	66	77.65
Mean		1.7706	1.4216	0.25265	0.38455	0.25265	--	--
RAPD+ISSR								
Population 1	12	1.8302 (0.3764)	1.4787 (0.3523)	0.2829 (0.1752)	0.4269 (0.2390)	0.2829 (0.0307)	176	83.02
Population 2	13	1.8491 (0.3588)	1.4807 (0.3417)	0.2863 (0.1701)	0.4331 (0.2312)	0.2863 (0.0290)	180	84.91
Mean		1.83965	1.4797	0.2846	0.4300	0.2846	--	--

Na, Observed number of alleles; Ne, effective number of alleles; H, Nei's gene diversity; I, Shannon's Information index; Ht, total genetic diversity; Hs, genetic diversity in population; Gst, genetic diversity between population; NPL, number` of polymorphic Loci; PPL, percentage of polymorphic loci

including gene diversity (H), observed number of alleles (Ne), gene flow and Shanon's information index (I) were estimated by POPGENE 1.31 software (Yeh, 1999). Analyses of molecular variance (AMOVA) based on the pair wise squared Euclidean distances between molecular phenotypes were carried out to partition the genetic diversity between populations using the WINAMOVA program version 1.55 (Excoffier et al. 1992).The input files for AMOVA were prepared by using AMOVA-PREP version 1.01 (Miller,1998)

RESULTS

Genetic variability details from RAPD markers

Of the 25 *Medicago* accessions tested with 20 random decamer primers, seventeen polymorphic bands were produced. The size of the amplified DNA fragments ranged from 201 to 2000 bp. The numbers of bands and polymorphic bands produced by each primer varied, the highest number of bands (148) was produced by the primer S 40 and the lowest number of bands (10) by primer S 32. The annealing temperature ranged from 28.0 to 38.0˚C. The resolving power of the 20 primers ranged from 0.8 for primer S 32 to a maximum of 11.84 for primer S 40. The total number of polymorphic markers and the percentage of polymorphism were 124 and 97.96 respectively. The primers used, GC content, annealing temperature (Tm), total number of loci, level of polymorphism and resolving power are shown (Table 1).

The respective values of Na, Ne, H, I, Ht, Hs, NPL and PPL were found higher for population from Phyang village (P) ecotypes indicating that, there is more variability in Phyang village than in DIHAR ecotypes (D) (Table 3). AMOVA for among population (49 %) and within the population (51 %) indicated that there are more variations within the population (Table 4). The details of the overall genetic variability across 25 ecotypes are given (Table 5).

RAPD derived dendrogram analysis

The similarity coefficient based on 127 RAPD markers ranged from 0.17 to 0.66 across the accessions with a mean of 0.42. Of the pair wise combinations generated by *Medicago* accessions, D 12 and P 17 showed highest similarity index (0.66) and accessions D 1 and P 20 showed the lowest similarity index (0.17). Nei's genetic distance and unweighted pair-group method with arith-metic averages (UPGMA) cluster analysis was carried out and a dendrogram was generated that represented the genetic relationship among 25 accessions from Ladakh into 2 major clusters. The first major cluster consisted of 5 sub-clusters or groups (Group I, II , III, IV and V) comprising of most accessions and showing 100% similarity between D 12 and P 17 while the second major cluster consisted of accession P 20 alone which

Table 4. Summary of Analysis of molecular variance (AMOVA) based on RAPD, ISSR individually and in combination of *Medicago* species. Levels of significance are based on 1000 iteration steps.

Source of variation	Degrees of freedom	Sum of squared	Variance component	Percentage	p*
RAPD					
Among populations	1	6.472	0.479	49	<0.001
Within populations	23	11.288	0.491	51	<0.001
ISSR					
Among populations	1	7.300	0.549	55	<0.001
Within populations	23	10.340	0.450	45	<0.001
RAPD+ISSR					
Among populations	1	6.472	0.479	49	<0.001
Within populations	23	11.288	0.491	51	<0.001

Table 5. Overall genetic variability across all the 25 genotypes of *Medicago* based on RAPD, ISSR and RAPD+ISSR analysis.

Marker type	Na	Ne	H	I	Ht	Hs	Gst	Nm	NPL	PPL
RAPD	2.0000 (0.0000)	1.5429 (0.3188)	0.3228 (0.1436)	0.4903 (0.1741)	0.3228 (0.0206)	0.3060 (0.0191)	0.0520	9.1181	127	100
ISSR	1.9882 (0.1085)	1.4372 (0.3183)	0.2717 (0.1503)	0.4265 (0.1867)	0.2717 (0.0226)	0.2527 (0.0208)	0.0697	6.6773	84	98.82
RAPD+ISSR	1.9953 (0.0687)	1.5005 (0.3221)	0.3023 (0.1481)	0.4647 (0.1816)	0.3023 (0.0219)	0.2846 (0.0204)	0.0584	8.0682	211	99.53

Na, Observed number of alleles; Ne, effective number of alleles; H, Nei's gene diversity; I, Shannon's Information index; Ht, total genetic diversity; Hs, genetic diversity in population; Gst, genetic diversity between population; Nm, estimate of gene flow from Gst; NPL, number of polymorphic loci; PPL, percentage of polymorphic loci.

appeared to be distinct from all other accessions (Figure 1). The dendrogram was not in accordance with geographic distribution of *Medicago* species, but within each group the accessions collected from the same place clustered together.

Genetic variability details from ISSR markers

For the 25 *Medicago* accessions tested with 11 primers, seven polymorphic bands were produced. The size of the amplified DNA fragments ranged from 200 to 3500 bp. The number of bands and polymorphic bands produced by each primer varied, the highest number of bands (102) being produced by the primer ISSR 1 and the lowest number of bands (18) by primer ISSR 7. This study observed unique banding pattern that is, each specimen presented a unique ISSR phenotype indicating extensive genetic variation in the individual analyzed. The annealing temperature ranged from 45.1 to 52.4˚C. The total number of polymorphic markers and the percentage of polymorphism were 81 and 95.12 respectively. The percentage of polymorphic loci (PPL) among accessions ranged from 80.00 to 100% with an average of about 95.12%. The resolving power of the 11 primers ranged from 1.44 for primer ISSR 7 to a maximum of 8.16 for primer ISSR 1. The primers used, GC content, annealing temperature (Tm), total number of loci, level of polymorphism and resolving power are shown (Table 2). The respective values for the overall genetic variability for Na, Ne, H, I, Ht, Hs, NPL and PPL across all the 25 ecotypes were given (Table 3). The values for all the parameters were higher for the populations from Phyang village (P) than DIHAR collection (D). AMOVA helps in partitioning of the overall ISSR variations among and within populations. The molecular variance among populations (55%) and within the population (45%) indicated more variation among the populations (Table 4). The details of the overall genetic variability across 25 ecotypes are given (Table 5).

ISSR derived dendrogram analysis

The similarity coefficient based on 85 ISSR markers ranged from 0.26 to 0.73 across the accessions with a

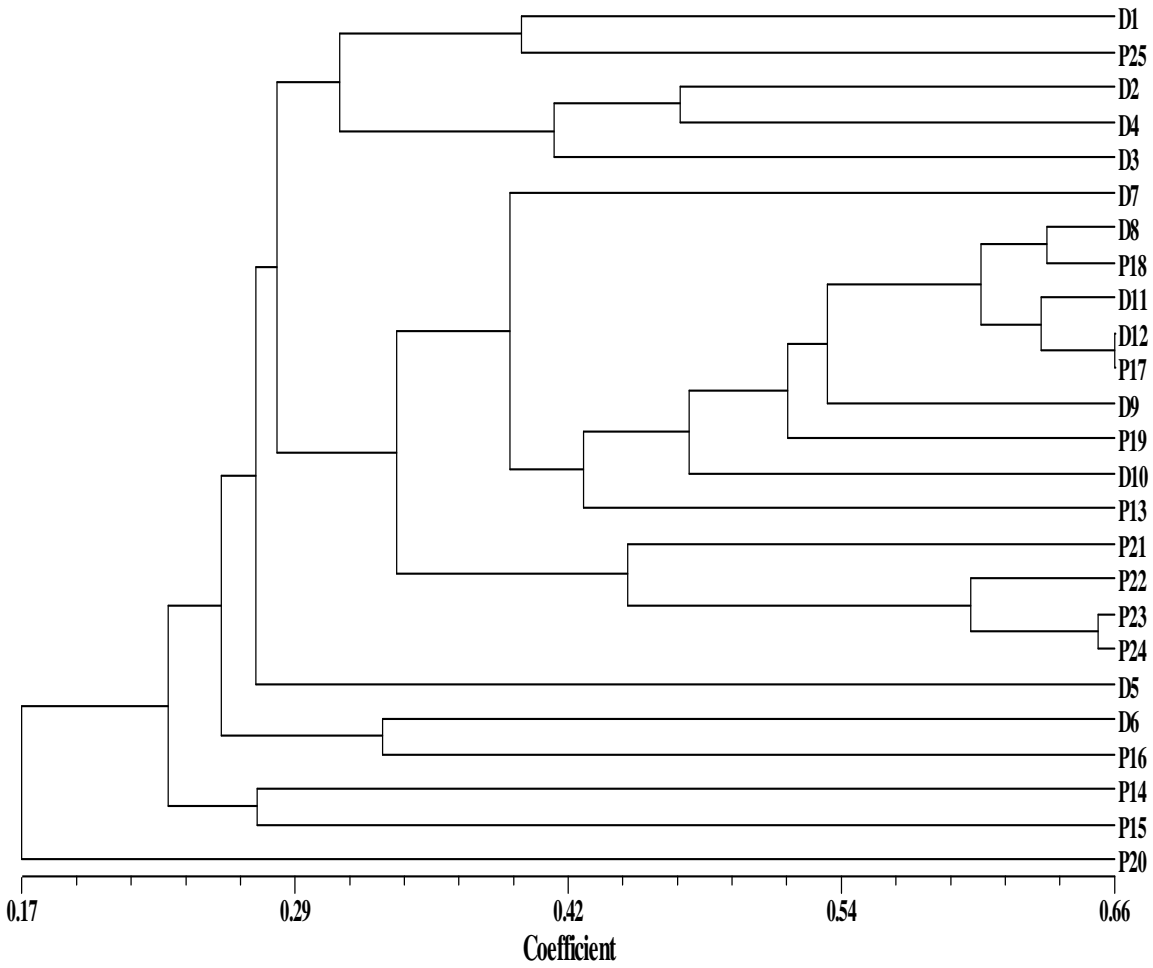

Figure 1. Dendrogram generated using unweighted pair of group method with arithmetic average analysis, showing relationships between 25 *Medicago* genotypes, using RAPD data.

mean of 0.49. Of the pair wise combinations generated by *Medicago* accessions, D 9 and D 10 showed the highest similarity index (0.73) and accessions D 1 and P 19 showed the lowest similarity index (0.26). Nei's genetic distance and unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis was carried out and a dendrogram was generated that represented the genetic relationship among 25 accessions from Ladakh (Figure 2). The 25 accessions clustered into 2 major clusters. The first major cluster had 2 sub-clusters or groups; these comprised of most of the accessions showing 100% similarity between D 9 and D 10 while the second major cluster contained the accessions P 18 and P 19 showing least similarity with other accessions studied (Figure 2).

Genetic variability details from RAPD + ISSR combined data

When Na, Ne, H, I, Ht, Hs, NPL and PPL parameters were analyzed for populations from DIHAR (D) and Phyang village (P), then the values were found higher in Phyang village ecotypes (Table 3). AMOVA for among population (49%) and within the population (51%) indicated that, there were more variations within the population (Table 4). The details of the overall genetic variability across the 25 ecotypes are given (Table 5).

RAPD and ISSR combined data derived cluster analysis

The similarity coefficient based on 127 RAPD markers and 85 ISSR markers ranged from 0.24 to 0.62 across the accessions with a mean similarity index of 0.43. Of the pair wise combinations generated by *Medicago* accessions, P 23 and P 24 showed the highest similarity index (0.62) and accessions D 1 and P 20 showed the lowest similarity index (0.24). Nei's genetic distance and unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis was carried out and a

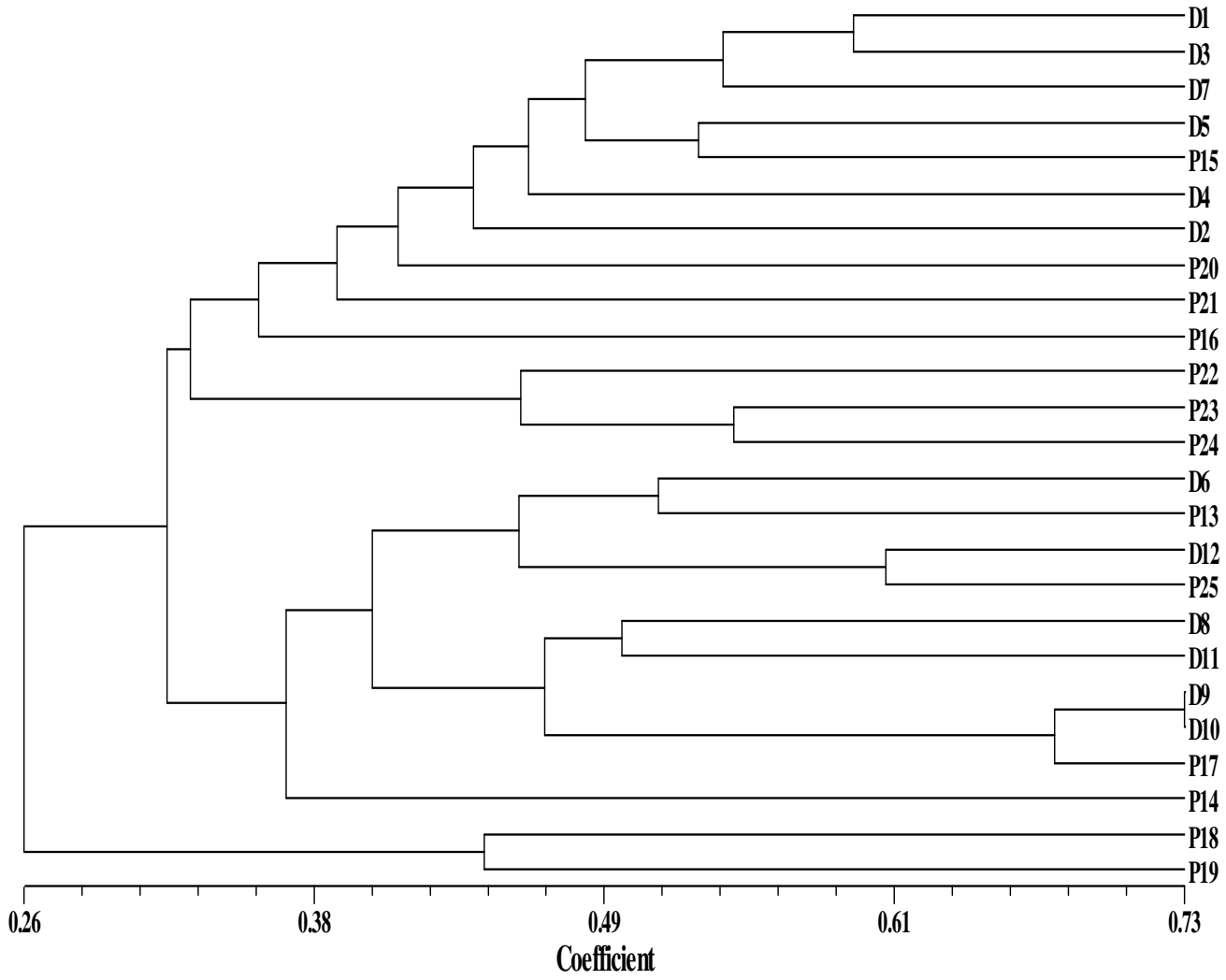

Figure 2. Dendrogram generated using unweighted pair of group method with arithmetic average analysis, showing relationships between 25 *Medicago* genotypes, using ISSR data.

dendrogram was generated that represented the genetic relationship among the 25 *Medicago* accessions from Ladakh. The accessions clustered into 2 major clusters with four sub-clusters; in the first major cluster with 100 percent similarity between P 23 and P 24 and the accession P 20 alone in the second major cluster indicating higher variability from other accessions (Figure 3). The results of PCA analysis were comparable to the cluster analysis (Figure 4).

DISCUSSION

Genetic diversity of *Medicago* species

The *Medicago* accessions of Ladakh constitute a rich source of biodiversity and utilization and conservation of these genotypes requires their genetic structure well characterized and understood. DNA fingerprinting is a regular method employed to study the extent of genetic diversity across a set of germplasm or accessions to group them into specific categories. RAPD markers (Williams et al., 1990) and ISSR markers (Zietkiewicz et al., 1994) are two molecular typing approaches used extensively to identify and determine relationships at the species and cultivar levels (Raina et al. 2001; Martins et al. 2003). RAPD provides a useful tool to evaluate the genetic diversity and the structure of alfalfa and other forage crops such as white clover (Gustine et al., 2002), *Dactylis glomerata* (Tuna et al., 2004), red clover (Grlju et al. 2005), and perennial ryegrass (Bolari et al., 2005). It has been widely applied to assess the genetic structure of diploid and polyploid plant populations (Guldahl et al., 2005; Pe´rez-Collazos and Catala´n, 2006). ISSR analysis has been used for cultivar identification in numerous plant species, including rice (Joshi et al.,

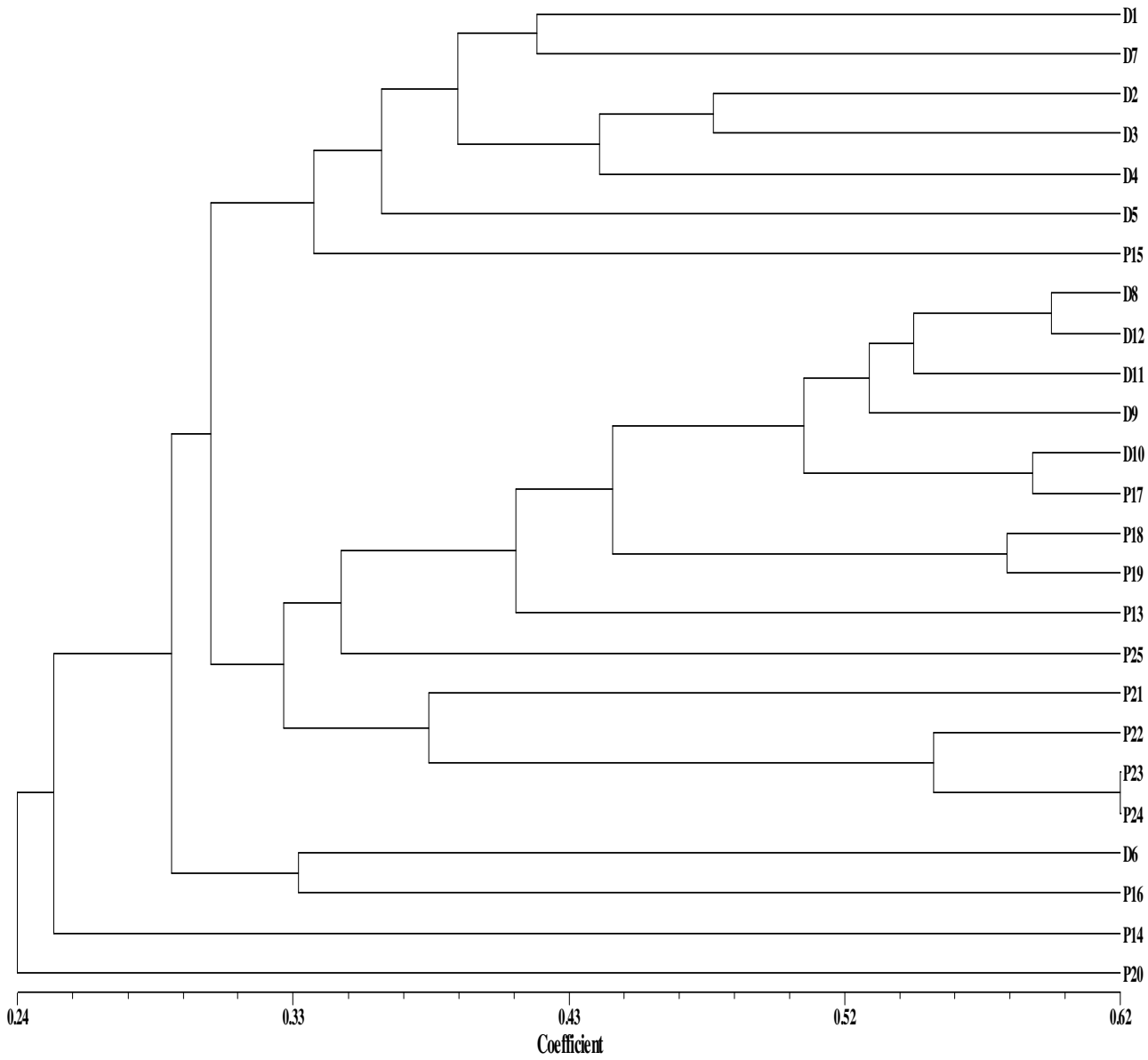

Figure 3. Dendrogram generated using unweighted pair of group method with arithmetic average analysis, showing relationships between 25 *Medicago* genotypes, using RAPD+ ISSR data.

2000), apple (Goula˜o and Oliveira, 2001) and strawberry (Arnau et al., 2003). The major limitation of these markers is their dominant nature and this problem can be overcome by the use of efficient statistical methods such as analysis of molecular variance (AMOVA) (Excoffier et al., 1992; Holsinger and Wallace, 2004).

The present investigation found that high level of genetic diversity at molecular level among the *Medicago* accessions from Ladakh 20 random decamer primers and 11 ISSR primers produced 97.63 and 95.29% polymorphic bands respectively. Several studies have been conducted using molecular markers to assess the level of variation among perennial *Medicago* species and populations (Yu and Pauls, 1993; Kidwell et al., 1994). Brummer et al. (1995) used RAPD (random amplified polymorphic DNA) markers to study variation among annual *Medicago* sp. and found higher level of genetic diversity among and within Iranian genotypes than Italian alfalfa. Higher levels of genetic diversity in alfalfa are reported using different marker systems, such as RAPD (Mengoni et al., 2000a), SSR (Dhiwan et al., 2000) nuclear microsatellite (Mengoni et al., 2000b) markers and mitochondrial DNA (Muller et al., 2001). Field observations showed that, plants of *Medicago* sp. are perennials pollinated by bumble bees which largely promotes out crossing. In addition, floral structure of this species facilitates the loading of pollen on the body of the pollinator by flower opening on landing of the insect over

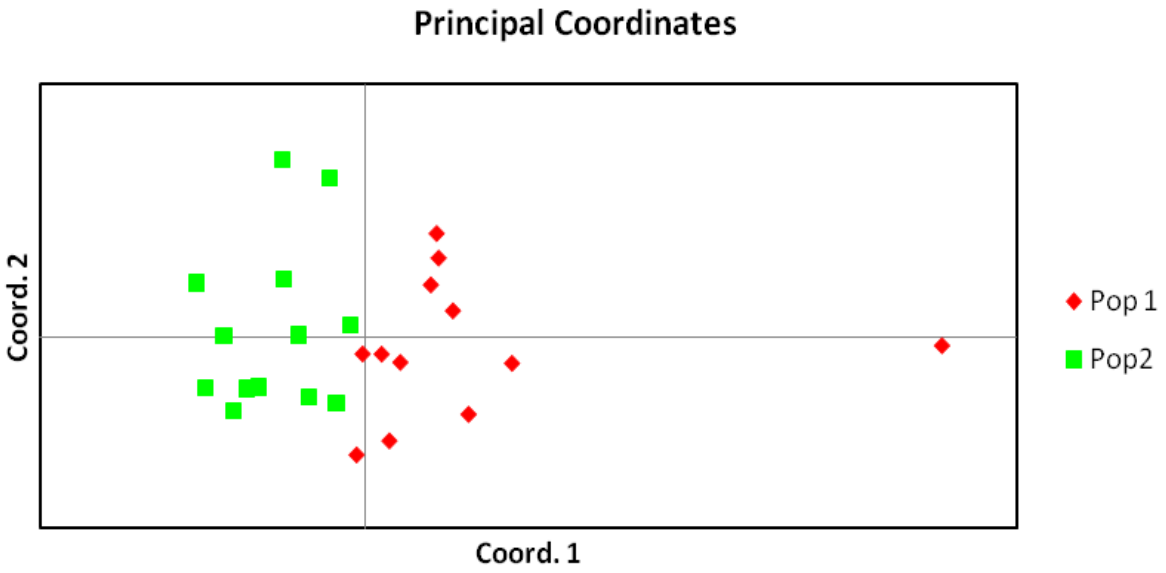

Figure 4. Two-dimensional plot of principal component analysis of twenty-five ecotypes of *Medicago* sp using RAPD+ISSR analysis. The numbers plotted represents individual cultivars (Where, Pop 1 = DIHAR, Pop 2 = Phyang Village).

the corolla. Both mechanisms help to maintain genetic polymorphisms across populations.

Alloploids are typically expected to have potentially or more genetic variation than their diploid progenitors and are known for their great ability to adapt to different ecological habitats and to buffer against genetic erosion (Mahy et al., 2000; Soltis et al., 2003; Pe´rez-Collazos and Catala´n, 2006). The high genetic variability among population in the plant may be a consequence of sexual reproduction, mutations of somatic cells, selection, gene flow, genetic drift and changing environment (Gao and Yang, 2006). The cross-pollination mechanism, sexual reproduction, high seed ratio and self sown ability to produce offspring of the *Medicago* species could have resulted in the accumulation of abundant genetic variation during the long evolution history.

RAPD markers were found more efficient with regards to polymorphism detection, as they detected 97.84% as compared to 96.5% for ISSR markers in this study. The results were similar in the case of Jatropha and *Arachis hypogea* (Raina et al., 2001) and are contrasting with other plant species like wheat (Nagaoka and Ogihara, 1997) and *Vigna* (Ajibade et al., 2000). More polymorphism in the case of RAPD than ISSR markers might be due to the fact that out of 20 ISSR primers used in the study, only 11 primers amplified 1367 number of fragments (Table 2) while in case of RAPD, all the 20 primers which were used in the investigation amplified 3004 number of fragments (Table 1). The differences found among the dendrograms generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed (1041 for RAPDs and 613 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 among Lucerne accessions as reported in Rye (Loarce et al., 1996).

Population genetic structure

In population genetics, a value of gene flow Nm < 1.0 (less than one migrant per generation into a population) or equivalently, a value of gene differentiation (Gst) > 0.25 is generally regarded as threshold quantity beyond which significant population differentiation occurs (Slatkin, 1987). Nm > 1 means that gene flow among populations is more and sufficient to encounter the effects of random drifts. The lower Gst values (RAPD) 0.0520, (ISSR) 0.0697and (RAPD+ISSR) 0.0584 with higher Nm values of (RAPD) 9.1181, (ISSR) 6.6773 and (RAPD+ISSR) 8.0682 respectively suggest more pollen and seed dispersal among populations. High levels of gene flow indicate from the study that there is a chance of adaptive gene flow from landraces to cultivars. Phenotypically similar individuals of cross-pollinated landrace are highly heterozygous and genotypically different. Therefore, breeding approaches aiming to develop new synthetic cultivars have to collect a number of superior selected single plants with high levels of gene diversity (Falahati-Anbaran et al., 2007).

According to Hamrick and Godt (1989), outcrossing plant species tend to exhibit between 10 and 20% genetic variation among populations while selfing species exhibit on average, 50% variation between populations. Studies on the biology of flowering and pollination indicate that

Medicago sp. is an out crosser. It is also supported by the genetic differentiation (G_{ST} = 0.0584) among populations of *Medicago* sp which was very close to the average genetic differentiation (G_{ST} = 0.144) in outbred populations (Bussell, 1999).The variation among individuals within populations was the main source of variation of *Medicago* sp. The reasons for this population genetic structure are as follows:

1. Medicago sp is widely distributed in Ladakh in various kinds of environments. The strong adaptability and wide distribution results in little differentiation between populations.
2. Medicago sp exchanged its genes mainly by seeds and pollen. The spread of seeds and pollen is the main way of gene flow in natural populations of plants (Li and Chen, 2004). Seeds and pollen are both small and dispersed by wind. Due to the high frequency of strong winds, the effects of long distance dispersal of seeds and pollen by wind are similar.
3. Gene flow is the most important factor in making population genetic structure homogeneous. The greater the amount of gene flow among populations, the less gene differentiation occurs (Slatkin, 1985). The large gene flow (Nm = 8.0682) of *Medicago* sp could counteract most of the gene differentiation which is caused by genetic drift within populations. We also found little genetic differentiation among three groups, which suggests that the populations from different sides are poorly differentiated. This small vitiation might be due to the continuous distribution in Ladakh.

AMOVA partitioned the genetic variation as 51% within populations and 49% among populations. The more or less same values of genetic variation within and among populations and high gene flow detected in this study point towards the fact that, there is less instance of single isolated populations possessing unique genotypes not found in other populations. It is therefore imperative in conservation point of view that it is adequate to target few selected populations for conservation of genetic variation within the *Medicago* species in trans - Himalayan region as reported in *Pinus tabulaeformis* (Wang et al., 2009) and *Dendrobium officinale* (Ding et al., 2009).

Implications for development

Traditional agriculture is practiced in Ladakh and most of the farmers do not purchase or exchange seeds; instead a part of saved produce is used as seed (Misri, 1987). This has resulted in the conservation of many old land races of cultivated plants (Misri, 1989). It is obvious from the variations scored that, the alfalfa of Ladakh has undergone a very high level of natural hybridization and a number of characters have evolved which are quite different and more useful than the characters of *M. sativa* or *M. falcata*. The high genetic diversity maintained in populations of *Medicago* sp. is encouraging. As is concluded, it probably will be sufficient for conservation purposes to maintain a few populations placed across the whole distribution range to ensure that the total genetic diversity is represented and populations from Phyang village with high genetic diversity should firstly protected and used in breeding programmes (Marshall et al., 1975). Moreover, highly diverse populations should be conserved separately because the mixing of populations may give rise to out breeding depression, loss of adaptation or breakup of co-adapted gene complexes (Waser et al., 1993) which should be avoided during conservation of these alfalfa genotypes.

Conclusion

A successful management program of *Medicago* sp cannot be based only on RAPDs and ISSRs as markers might not be correlated to adaptive traits (Volis et al., 2005). A continuous monitoring of the species, including more populations, co-dominant genetic markers and the assessment of 'adaptive' genetic variation (that is by means of genes related to freezing tolerance) will be useful to design effective conservation strategies and develop high yielding and cold resistant cultivars for different ecological niches of the cold desert.

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