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Genetic diversity in sewan grass (*Lasiurus sindicus* Henr.) in the hot arid ecosystem of Thar Desert of Rajasthan, India

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Abstract

Aim : Sewan grass (*Lasiurus sindicus* Henr.), considered as the "King of Desert Grasses", is a dominant grass species of hot arid ecosystem of Great Indian Desert, covering Western Rajasthan and parts of Pakistan. This grass is extremely drought resistant and thrives even in areas receiving very low rainfall (100 to 150 mm) annually under extreme temperatures ranging from -3 to 50 ºC. The present study was undertaken to analyze the extent of genetic variability existing among the *L. sindicus* germplasm collected from Bikaner, Barmer and Jaisalmer, the diversity rich districts of hyper-arid Rajasthan, using ISSR and RAPD markers.

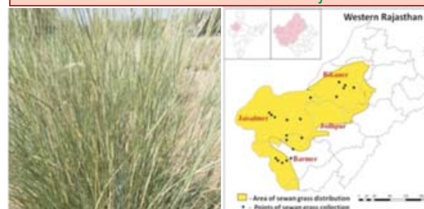
Methodology : Twenty seven genotypes of *L. sindicus* were collected from Jaisalmer (10 accessions), Barmer (9 accessions) and Bikaner (7 accessions) and 1 old collection maintained at CAZRI, Jodhpur, Rajasthan was used in this study. A total of 18 RAPD and 14 ISSR markers were screened of which 12 RAPD and 10 ISSR primers amplified distinct and scorable fragments. Data analysis was performed using NTSYS-pc, SIMQUAL, Genalex 6.5 and POPGENE version 1.32 programs, and dendrograms were generated using unweighted pair group method for arithmetic mean (UPGMA).

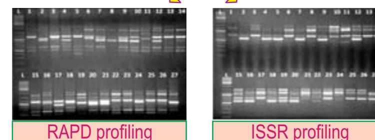
Results : The comparative analysis of data showed that RAPD markers were better than ISSR with regard to polymorphism detection, as they detected 90% polymorphism in comparison to 74% for ISSR markers. The values of average number of polymorphic fragments per assay, polymorphism information content (PIC) and discriminatory power (Dj) were more for RAPD (5.83, 0.222 and 0.78 respectively) than for ISSR (5.7, 0.138 and 0.605 respectively) markers. The UPGMA clustering was not conspicuous under the influence of high within region diversity, however, accessions collected from same region tended to cluster together. Genetic similarity values obtained from Jaccard's coefficient using combined data of both the marker systems were between 0.58 and 0.74.

Interpretation : The results indicated the existence of wide genetic variability within and among regions in this species which can be used for germplasm conservation and improvement.

Genetic diversity analysis of sewan grass

Germplasm collected from Jaisalmer, Barmer and Bikaner districts of Western Rajasthan





RAPD profiling

ISSR profiling

Data analysis and interpretation

RAPD markers more efficient in detecting polymorphism

Wide genetic variability exists within and among regions

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Introduction

Lasiurus sindicus, locally known as “Sewan” is one of the dominant grass species of *Dichanthium-Cenchrus-Lasiurus* type grass cover (Shankarnarayan and Dabadghao, 1973) of hot arid ecosystem of Great Indian Desert, covering western Rajasthan and parts of Pakistan. It thrives well in dry climate, receiving annual rainfall below 250 mm, prevailing between 25-27°N latitude on well aerated alluvial soils or light sandy soils with 8.5 pH, rocky ground and gravel soil (Quattrocchi, 2006; FAO 2010). The grass is extremely drought resistant and grows even in areas receiving very low rainfall (<150 mm annually) under extreme temperatures ranging from -3 to 50 ºC. Until the last decade, about 80% of the total geographical area of Jaisalmer district covering Nachna, West Puggal, Mohangarh, Sultana and Binjewala with 100-150 mm annual rainfall supported sewan grasslands (Mertia *et al.,* 2006). Though this grass tolerates prolonged droughts, but has not been found growing in higher rainfall zones and faces a serious threat of becoming an endangered species due to changes in the land use pattern, increase in soil moisture regime and overgrazing.

Sewan, a member of family poaceae, is a tufted perennial, forming a more or less oblique and woody rhizomatous rootstock with several shoots arising from the base, often appearing almost bushy. Leaves are thick and amphistomatic (Pearson *et al.,* 1995). The leaf blade is narrow culminating in a sheath enclosing the stem with distinct mid vein and denser networks of small longitudinal and transverse veins supporting a superior photosynthetic translocation and water distribution system (Ueno *et al.,* 2006). The Sewan grass, considered as the “King of Desert Grasses”, is quite palatable and nutritious for the livestock. Crude protein in young leaves varies from 7 to 14% and remains high even at maturity leading to its better suitability for efficient utilization in the animal based agri-horti-pastoral production system prevalent in hyper arid regions of Western Rajasthan (Gupta and Saxena, 1970). In three districts of Western Rajasthan *viz.* Bikaner, Barmer and Jaisalmer the sustainability and productivity of livestock mainly depends on the sewan based pasture system. Though, productivity of range land remains low in this fragile zone (estimated fodder yield ~300 kg ha^{-1} $year^{-1}$), the capacity of sewan to regenerate at a faster rate with less than 40 mm rain is crucial in supporting the livestock (Mertia *et al.,* 2006).

The area under Sewan is continuously shrinking due to modernization of agriculture, alternative land uses, frequent droughts, expanding canal irrigation, overgrazing and increased human activities which have created threat to this species. Hence, it is important to collect and conserve the diversity of this species to fulfil the future needs under changing climate regime. Effective conservation of vulnerable species depends largely on the knowledge of extent and patterns of genetic variation. For example, the spatial structure of genetic variation can provide information for sampling strategies for *ex situ* or *in situ* conservation (Torre *et al.,* 2008). Several methods viz. morphological, biochemical and molecular have been used for the detection of genetic variability in various species, however, lack of polymorphism in morphological and biochemical traits that are highly influenced by environment limits variability studies in this species (Kulhari *et al.,* 2015). A molecular marker technique remains a rapid, sensitive and reliable method for assessing the genetic diversity and relatedness among plant germplasm in comparison to other techniques available. Among the various types of DNA markers available, Random Amplified Polymorphic DNAs (RAPDs) and Inter Simple Sequence Repeats (ISSRs) have remained the marker of choice in assessment of genetic variability and relationships amongst different accessions of a species. Since, RAPD and ISSR primers are random in targeting DNA, cover a wider range of genome, are more informative for diversity analysis, while being simple, fast and cheaper compared to other sequence based markers (Singh *et al.,* 2013). The present study for the first time reports the genetic diversity available in *L. sindicus* species using RAPD and ISSR markers .

Materials and Methods

Twenty seven genotypes of *L. sindicus* Henr. collected from sewan growing area of western arid Rajasthan covering three districts, Jaisalmer (10 accessions), Barmer (9 accessions) and Bikaner (7 accessions) and 1-old-collection maintained at CAZRI, Jodhpur, Rajasthan were used in this study. All these genotypes were maintained under field conditions at the Central Research Farm of Central Arid Zone Research Institute, Jodhpur (India). The region under study experiences low and erratic annual rainfall (150-300 mm). Climate of Jaisalmer is comparatively harsh with 185 mm annual rainfall compared to Barmer and Bikaner with about 250 mm annual rainfall. Extreme temperature (-3.0 to 50.0°C), long sunshine duration (6.6-10 hours), low relative humidity (20-60%), high wind velocity (9-13 kmh^{-1}) and high evapo-transpiration (1600-1800 mm) are common climatic features of the region (Tanwar *et al.,* 2014). The soil is poor in nutrients, wind erosion occurs on a mammoth scale and paucity of water is a perennial bottleneck in these regions.

Genomic DNA extracted from approximately one gram of tender leaves using cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1990) was treated with RNAase, assessed on 0.8% agarose gel and quantified using UV/VIS spectrophotometer (Thermo scientific UV-VIS). The quantified DNA was diluted to 25 ng μl^{-1} concentration for PCR amplification. Eighteen RAPD primers of OPA, OPC, OPN and OPZ series (Operon Technologies) and 14 ISSR primers (ISSR-1 to ISSR-14) synthesized from Life Technologies India Pvt Ltd. were used for diversity analysis. The PCR was carried out in 25µl reaction volume consisting of 1x assay buffer, one unit *Taq* DNA polymerase (Banglore Genei Pvt. Ltd., India), 200 µM of each dNTPs (Banglore Genei Pvt. Ltd., India), 10 pM primers and 50 ng

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template DNA. PCR amplifications were performed in a CGI-96 thermal cycler (Corbett Research, Australia) with following cycling conditions - initial denaturing at 94°C for 6 min; 44 cycles x [94 ºC for 1 min, 37 ºC (RAPD) / 42 ºC (ISSR) for 1 min, 72 ºC for 2 min] followed by final extension at 72 ºC for 7 min. The PCR product was electrophoretically separated on 1.2% agarose gel in 0.5x TBE (Tris-borate-EDTA) buffer, containing ethidium bromide (10 mM). GeneRuler™ DNA ladder mix (Fermentas International Inc.) was used as size marker.

All PCR reactions were run in duplicate to ensure the consistency of results and only consistently produced; well-resolved fragments obtained through amplification were considered and scored manually. The scoring of fragments was done on the basis of their presence ('1') or absence ('0') in the gel and missing data was denoted by '9'. Data analysis was performed using NTSYS-pc (Numerical Taxonomy System, version 2.02 software (Rohlf, 1998), while SIMQUAL program was used to calculate the Jaccard's similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The dendrogram was generated from similarity matrix data by cluster analysis using unweighted pair group method for arithmetic mean (UPGMA). Multivariate Principal Component Analysis (PCA) was carried out using Genalex 6.5 (Peakall and Smouse, 2012). The discriminatory power of included primers was analyzed according to Tessier *et al.* (1999). Polymorphic information content (PIC) that provides a measure of the degree of polymorphism was calculated following Botstein *et al.* (1980) using Powermarker Version 3.25 (Liu and Muse, 2005) software.

Pairwise population comparisons were performed with analysis of molecular variance (AMOVA) using Genalex 6.5 (Peakall and Smouse, 2012). Nei's genetic diversity among natural population were also calculated, including total genetic diversity (HT, expected Heterozygosity), mean genetic diversity within populations (HS), and proportion of genetic diversity occurring among populations, GST (inter-population genetic differentiation) = (HT-HS)/HT. All of these genetic diversity parameters were estimated using POPGENE version 1.32 (Yeh *et al.,* 1999) assuming all loci to be dominant and in Hardy–Weinberg equilibrium.

Results and Discussion

DNA profiles developed for twenty seven *L. sindicus* genotypes using RAPD and ISSR primers revealed considerable genetic diversity. All the eighteen decamer primers screened generated RAPD profiles; however, twelve primers that generated sharp and distinct DNA fragments were considered for profiling of all the 27 genotypes (Fig. 1). A total of 71 amplicons (5.83 per primer) were obtained of which 64 (90 %) were polymorphic (Table 1). The size of amplicons generated varied from 250 to 2000 bp. Likewise, 10 ISSR primers, out of total 14 screened, produced well resolved amplicons (Fig. 2) with lesser bands per primer (5.7) of which only 74% were polymorphic (Table 1). The fragment size amplified was similar to RAPD amplicons ranging between 230-2000 bp.

Primer efficiency in terms of resolving polymorphism is an important parameter for genetic diversity analysis. Polymorphism information content (PIC) shows the primer efficiency in detecting polymorphism based on number of alleles identified and their frequency (Anderson *et al*., 1993). To distinguish genotypes PIC is expressed as discriminatory power (Dj) introduced by Tessier *et al*. (1999). PIC value for RAPD markers, with an average of 0.222, ranged from 0.00 for OPC 04 having 2 monomorphic fragments to 0.295 for OPN 04 with all 10 polymorphic fragments. With a mean value of 0.780, discriminatory power (Dj) was also maximum for OPN 04 with highest resolution power or patterns generated. The PIC and discriminatory power for RAPD markers were highly

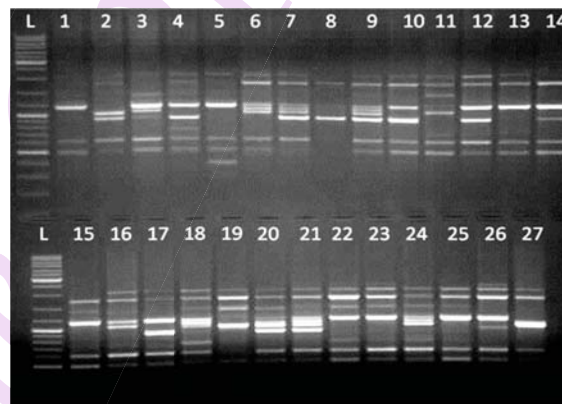

Fig. 1 : RAPD profile of *Lasiurus sindicus* generated using primer OPA-20 (L= Ladder, GeneRuler™ DNA ladder mix, Fermentas). 1-27 are 27 genotypes

Fig. 2 : ISSR profile of *Lasiurus sindicus* generated using primer ISSR 2 (L= Ladder, GeneRulerTM DNA ladder mix, Fermentas). 1-27 are 27 genotypes

Table 1 : Summary of PCR amplification of RAPD and ISSR primers used for genetic diversity analysis of 27 *Lasiurus sindicus* genotypes

	Marker	Sequence	Total number of fragments	Polymorphic fragments	% Polymor-phism	Size range (bp)	PIC	Dj
1	OPA-04	AATCGGGCTG	6	5	83.33	800-1700	0.222	0.903
2	OPA-10	GTGATCGCAG	3	2	66.67	700-1300	0.128	0.427
3	OPA-11	CAATCGCCGT	7	7	100.00	600-1500	0.247	0.903
4	OPA-17	GACCGCTTGT	7	7	100.00	250-200	0.294	0.95
5	OPA-20	GTTGCGATCC	9	8	88.89	500-2000	0.237	0.969
6	OPC-04	CCGCATCTAC	2	0	0.00	500-1031	0.00	0.00
7	OPN-11	TCGCCGCAAA	6	5	83.33	300-1800	0.282	0.960
8	OPN-13	AGCGTCACTC	4	4	100.00	450-2000	0.234	0.789
9	OPN-19	GTCCGTACTG	7	7	100.00	400-1700	0.266	0.926
10	OPN-20	GGTGCTCCGT	4	3	75.00	500-1500	0.186	0.632
11	OPN-04	GACCGACCCA	10	10	100.00	300-1700	0.295	0.986
12	OPZ-18	AGGGTCTGTG	6	6	100.00	400-1100	0.272	0.917
	Total		**71**	**64**				
	Mean		**5.92**	**5.33**	**90.00**	**250-2000**	**0.222**	**0.780**
1	ISSR-1	GC+(AG)7	8	6	75	850-1800	0.226	0.93
2	ISSR-2	CAC+(CA)6	6	6	100	550 -1500	0.201	0.87
3	ISSR-4	T+(GA)8	4	4	100	250-2000	0.248	0.89
4	ISSR-5	CCA+(GTG)4	5	5	100	400-1500	0.069	0.27
5	ISSR-6	GCGA+(CA)6	2	0	0	800-830	0.00	0.00
6	ISSR-7	CGC+(GA)6	6	3	50	230-830	0.124	0.64
7	ISSR-8	CCC+(GA)6	8	6	75	350-850	0.187	0.91
8	ISSR-9	GCG+(GT)6	2	0	0	700-800	0.00	0.00
9	ISSR-11	CCC+(GT)6	8	6	75	450-1200	0.145	0.78
10	ISSR-12	GCAA+(GACA)3	8	6	75	600-1700	0.184	0.76
	Total		**57**	**42**				
	Mean		**5.7**	**4.2**	**74**	**230-2000**	**0.138**	**0.605**

PIC - Polymorphic information content; Dj - Discriminatory power

Table 2 : Average Jaccard's similarity coefficients based on RAPD, ISSR and combined analysis in *Lasiurus sindicus*

Population	RAPD	ISSR	Combined
Over all 0.58	0.74	0.66	
Bikaner 0.57	0.74	0.65	
Jaisalmer	0.62	0.78	0.70
Barmer 0.65	0.80	0.71	
Among Bikaner - Jaisalmer	0.54	0.73	0.63
Among Bikaner - Barmer	0.54	0.70	0.61
Among Jaisalmer - Barmer	0.60	0.75	0.67

correlated (R^2 = 0.975). As compared to RAPD, ISSRs with a mean Dj value of 0.605 were less efficient in identification of genotypes and also in detecting polymorphism with average PIC value of only 0.138. Two ISSR primers (ISSR-6 and ISSR-9) amplifying two monomorphic fragments each had null PIC and Dj value. Whereas, ISSR-1 with 6 polymorphic fragments showed high PIC of 0.226 and also generated highest distinguishing patterns with Dj of 0.93 (Table 1).

Though none of the RAPD primers produced unique patterns for any of the 27 genotypes, they could resolve all the genotypes collectively with an average diversity of 42%. Two genotypes from Barmer region (Barmer 43 and 44) were most closely related (83 % similarity) while LSGP 2363 from Bikaner and JSM-8 from Jaisalmer region had least similarity index (0.37). Unlike RAPD markers, ISSRs could reveal less diversity (29 %) among genotypes. The most similar genotypes, based on ISSR data, were again from Barmer region (Barmer 45 and 49) with similarity index of 0.93 while LSGP 2364 from Bikaner and Barmer-47 were most diverse (54% similarity). Barmer 43 and 44 (84.8 % similarity) remained the most related genotypes based on collective analysis of the two marker systems (similar to RAPD results), however the most distant genotypes were LSGP 2365 and Barmer 47 (50 %), unlike RAPD results.

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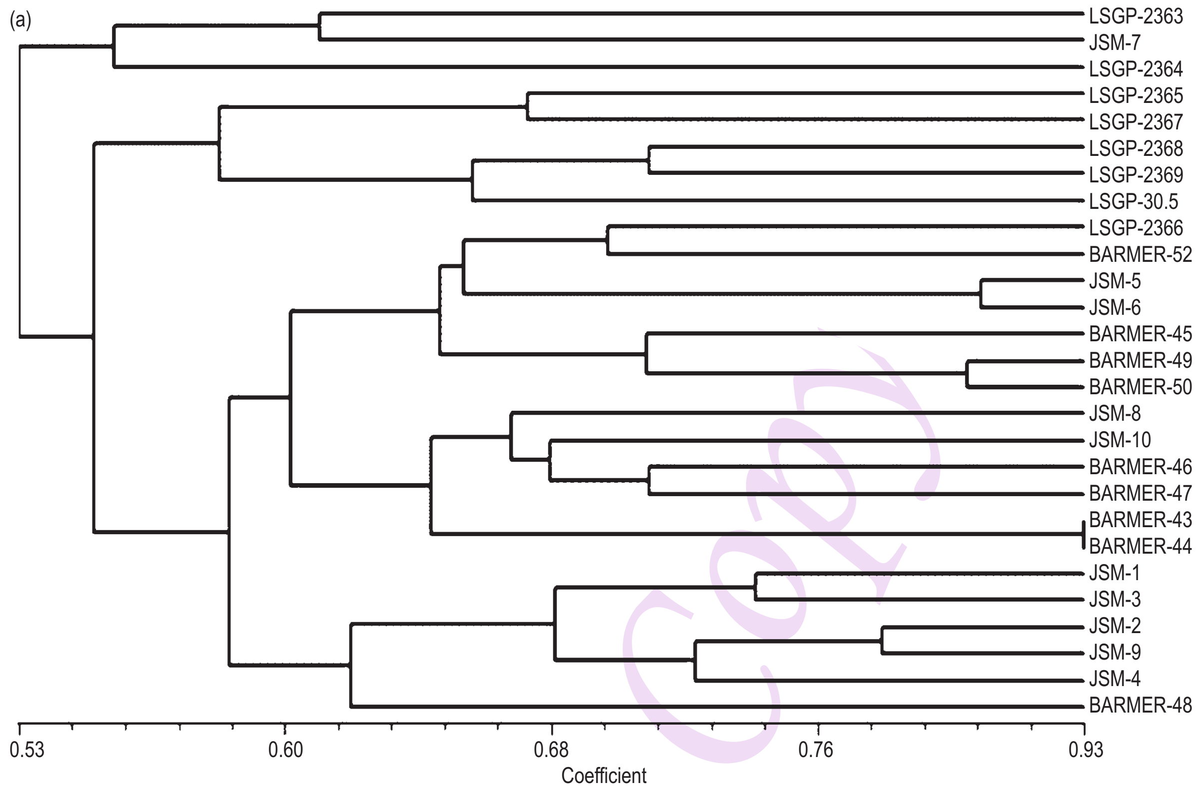

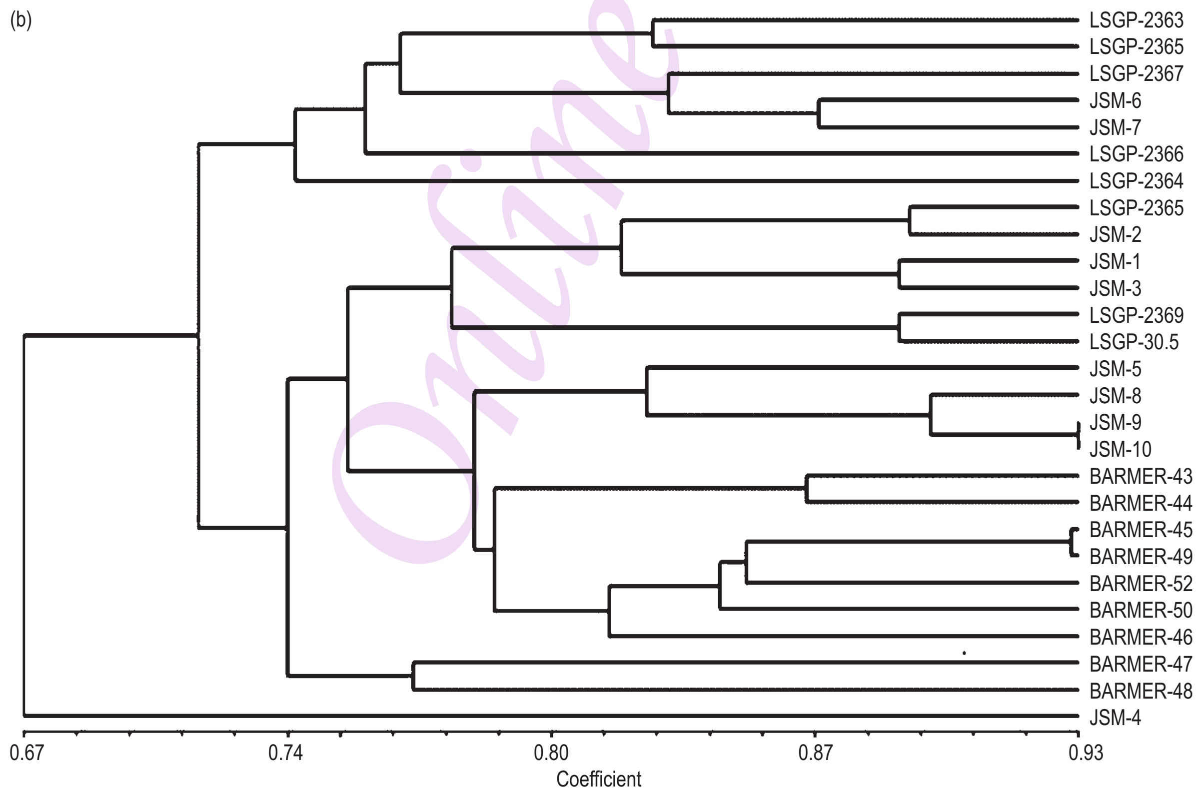

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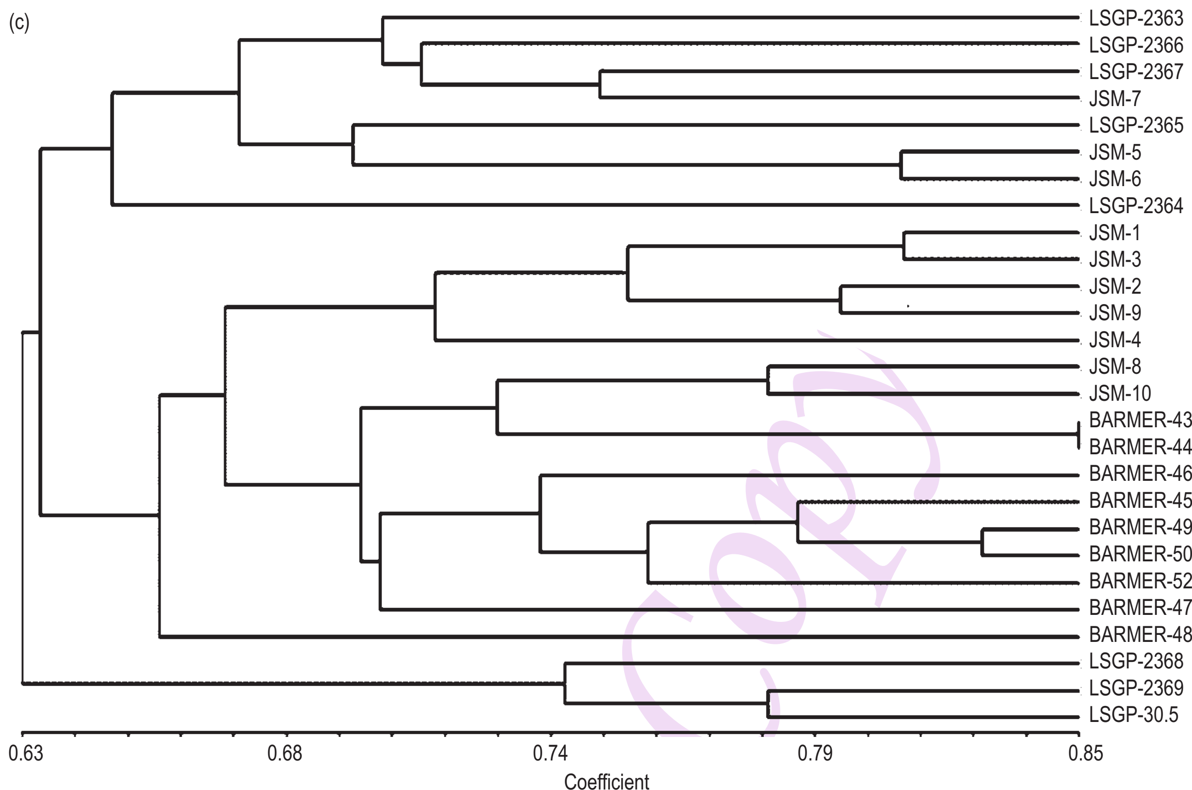

Fig. 3 : UPGMA dendrogram based on (a) RAPD similarity matrix data, (b) ISSR similarity matrix data and (c) combined similarity matrix data of RAPD and ISSR markers

RAPD based AMOVA revealed that genotypes belonging to regions of Jaisalmer and Barmer had more within region similarity, 62 and 65% respectively, compared to Bikaner (43%). ISSR markers also confirmed higher similarity within regions for Jaisalmer and Barmer collections (75%). More similarity and close relationship between collections from Barmer and Jaisalmer can be explained based on the fact that the collection sites from Barmer and Jaisalmer have similar climatic conditions and occur in continuity covering broader region while collection sites from Bikaner were geographically separated and more localized.

Similar associations were revealed by both the markers for the three regions, however, ISSR markers had lesser resolution. Jointly two markers systems retained the similar trend revealing highest similarity in Barmer (71%) and least in Bikaner (66%) populations. Polpulations from Jaisalmer and Barmer were most similar while Bikaner remained most distant.

Both RAPD and ISSR markers resolved all the genotypes independently and grouped them according to their similarity through UPGMA based dendrogram (Fig. 3). The groups made were not conspicuous under the influence of high within population diversity. However, in both RAPD and ISSR dendrograms, most genotypes were clustered on the basis of region of their collection. At 61 % thresh hold cut off value, RAPD markers generated five groups leaving aside three genotypes, namely LSGP-2363, LSGP 2364 and JSM-7. Five of the Jaisalmer collections grouped together in a cluster with one accession from Barmer (Barmer-48). Other two groups had collections from Barmer and Jaisalmer, while most of the Bikaner collections were grouped separately at a higher level of diversity. At 74 % similarity most of the Bikaner collections clustered together with two accessions from Jaisalmer, whereas, another larger group contained maximum accessions from Barmer and Jaisalmer regions with three accessions from Bikaner region. Clustering of accession LSGP 30-5 an old collection with accessions from Bikaner indicates its origin from that region. All the Barmer genotypes were grouped together, except Barmer-48 at about 21.5% diversity. ISSR based clustering also placed the Bikaner collections at a higher diversity level compared to Jaisalmer and Barmer collections. Though, both RAPD and ISSR markers grouped the genotypes on the basis of geographical distributions, but similarity matrices were not highly correlated with Mental test (Z- 0.18). Three coordinates of PCA could explain only about 35 % of the total diversity exhibited by RAPD and ISSR

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markers, hence groupings were not expected to be representative of total diversity.

RAPD markers have been efficient over ISSR markers in resolving polymorphism leading to higher PIC and discriminatory power. Better efficiency of RAPD markers in resolving polymorphism was also evident by better clustering at a lower level of similarity. Similar results were obtained by Bhattacharya *et al.* (2010) in *Cymbopogon winterianus*. Cuesta *et al.* (2010) also reported that only RAPD markers from among RAPD, ISSR, AFLP and SAMPL were effective in detecting interclonal variation in micropropagated plants of *Pinus pinea* L. However, ISSR markers have been reported to be more efficient in *Cyamopsis tetragonoloba* (L.) Taub (Sharma *et al.,* 2014). The differential resolution of RAPD and ISSR markers may be due to their origin from different genomic regions, as well as the primer length, shorter the length more are the probabilities of finding complimentary sequence. The greater ability of RAPD over ISSRs may be attributable to their arbitrary nature virtually exploring wider genomic region. On the contrary Zietkiewicz *et al*. (1994) visualized that ISSRs exhibit a higher capacity to reveal polymorphism and have a greater potential to determine intra and intergenomic diversity than other arbitrary primer methods such as RAPD. However, the number of potential ISSR markers is expected to depend on the frequency of microsatellites, which changes with species (Depeiger *et al.,* 1995).

Shannon index for diversity was higher for Bikaner region (0.3570) followed by Jaisalmer (0.3304) and Barmer (0.3047); this is in agreement with average similarities calculated on the basis of Jaccard's similarity matrix, 0.65, 0.70 and 0.71 respectively (Table 2). On the basis of RAPD marker polymorphism, diversity among Barmer-Jaisalmer region was less (40%) as compared to Bikaner-Jaisalmer (46%) and Bikaner-Barmer (46%). Similar trend was revealed by ISSR markers, however, with lower levels of diversity *viz*., 25, 27 and 30% for Barmer-Jaisalmer, Bikaner-Barmer and Bikaner-Jaisalmer. The higher diversity for allele content and its frequency in Bikaner collections could be due to less harsh conditions and availability of irrigation facilities, whereas adaptive pressures might be limiting diversity in harsher climate of Jaisalmer and Barmer region. Moreover, eradication of natural rangelands leaving only some protected fields further limit the diversity.

Both AMOVA and Nei's gene diversity index were used to partition the diversity within and between populations. Considerable genetic diversity among populations was detected by both the methods; however, diversity within population was higher. Both RAPD and ISSR markers independently and collectively detected diversity among populations in same range viz., 14, 17 and 15%, respectively. The Nei's genetic diversity based on combined RAPD-ISSR analysis, among population was also in the same range (18%). Genetic diversity is of considerable importance to the sustainability of plant populations (Wang *et al.*, 2007). Moreover, the geographically isolated individuals tend to accumulate genetic variations during the course of environmental adaptations (Sarwat *et al.*, 2008). Higher level of within population diversity is important for the survival of *L. sindicus* under unpredictable fragile ecosystem and diversity among populations might have been important in niche specific adaptations.

The present molecular investigation has disclosed a great deal of genetic variability in *L. sindicus* that was largely unrevealed through morphological observations. Being a perennial rhizomatous crop limited variations are depicted for most traits. Leaf, stem and spike morphology tend to vary with phenology, season and age of clone. The present study clearly suggest a need to cover a large number of areas with good representation from each area for conservation of diverse germplasm for future needs. It is also suggested to develop sequence specific markers like SSRs for more extensive and specific information.

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