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Assessment of Genetic Diversity among the population of *Moringa oleifera* in Rajasthan using ISSR markers .

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Abstract
Moringa oleifera Lam. Family moringaceae, locally known as Segna, Horseradish tree and Drumstick is indigenous of Himalayas of North-Western India. This plant has earned its name as 'the miracle tree' due to its amazing healing abilities for various ailments and even some chronic diseases. Several investigations were carried out to isolate bioactive compounds from various parts of the plant due to its various applications. Present investigation is mainly focused on feasible research on assessment of genetic diversity within and among the variants of Moringa oleifera in different parts of Rajasthan using ISSR markers. Genomic DNA of plant samples was extracted and purified using (CTAB) Cetyl Trimethyl Ammonium bromide method. Jaccard's pair-wise similarity coefficient values among all the populations of Moringa Oleifera using all the ISSR primer profiles generated vary between 0. 55 and 1.00. A phenetic representation of genetic relationships as revealed by the similarity coefficient for all the ISSR profiles that have been generated for M. oleifera. Out of the 09 ISSR markers utilized, 07 produced reproducible polymorphic banding patterns. In the 800 P series of ISSR primers, 811 P produced 02 polymorphic loci, while primers 814P, 818P and 826P generated 01 polymorphic locus each.

Keywords : *Moringa Oleifera, ISSR Marker,Genetic diversity*

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I. Introduction

Moringa oleifera Lam. locally known as Segna, Horseradish tree and Drumstick is indigenous of Himalayas of North-Western India. This species is also found in the sub-Hamalayan tract from the river Chenad to the Eastwards Sarda and at tract of Uttar Pradesh in India. Later, this species has also been cultivated to other parts of the continent such as Malaysia, Philippines, Singapore, Sri-lanka, Cuba, Burma and Cuba.The tree have been distributed in Nigeria, Egypt and Sudan; also spread to Central and South America, Peru to Mexico, Paraguay and other cities of the world (Ramachandran *et al*., 1980; Mishra *et al*., 2011). This plant is a type of local medicinal Indian herb which has turn out to be familiar in the tropical and subtropical countries. The botanical classification of *Moringa oleifera* is Kingdom: Plantae, Division: Magnoliphyta, Class: Magnoliopsida, Order: Brassicales, Family: Moringaceae, Genus: Moringa, Species: *M.oleifera* (Fahey, 2005).

This plant has earned its name as 'the miracle tree' due to its amazing healing abilities for various ailments and even some chronic diseases. Several investigations were carried out to isolate bioactive compounds from various parts of the plant due to its various applications (Guevara et al.,1999). *M. oleifera* is one of the best known medicinal plant. The *Moringa* plant has been consumed by humans (Iqbal *et al*., 2006). It is one of the richest plant sources of Vitamins A, B, C, D, E and K (Anwar and Bhanger, 2003; Babu 2000; Caceres *et al.,* 1992; Dayrit *et al*., 1990; Delisle *et al*., 1997).

It contains a huge amount of nutritional content Such as amino acids, vitamins and minerals which are critical for good health. It is loaded with potassium, protein, iron, calcium, vitamin A and C, and as many more properties which promote a healthy body that has the tools to fight cancer. It is known to have anti-inflammatory, pain relief, antioxidant, anti-viral and anti-allergenic uses. It has also been put to use to fight a variety of infections.

Genetic diversity can be defined as any quantitative measure of the variability of a population, which reflects the equilibrium between mutation and the loss of genetic variation The development of molecular markers for plants, initially isoenzymes provided access to the genetic variability found in the accessions, which was useful for characterizing the germoplasm and for genetic improvement, based on specific markers . Given their genetic link, DNA markers can be used to detect allelic variation in the genes underlying the target characteristics**.** According to Namkoong 1998) .

There have been a lot of biochemical studies conducted on *Moringa* due to its therapeutic and nutritional properties; however few studies have been focused on elucidating genetic variation within *Moringa* population. Genetic variation offers insurance against genetic erosion and maintenance of biodiversity. High

levels of genetic variation can aid against co-evolving biotic factors . It is thus necessary to elucidate the genetic diversity and relatedness of an introduced population that has been established for future conservation and breeding strategies, as suggested by Wu *et al.*, 2010 on the study of *Moringa* trees.

Present investigation is mainly focused on feasible research on assessment of genetic diversity within and among the variants of *Moringa oleifera* in different parts of Rajasthan using ISSR markers to offer genetic resource information for future meaningful implementation of conservation programs applicable for the plant.

II. Materials And Methods

Extensive survey was conducted to identify the plant gender in each population. Plant materials were collected from different eco-climatic regions in Rajasthan where *Moringa oleifera* thrives in its natural habitat. The sample size for each area varied due to the fact that these plant samples were collected from their natural habitats and the population size of each area was starkly different from the other.

Genomic DNA Extraction and Purification

Genomic DNA of plant samples was extracted and purified using (CTAB) Cetyl Trimethyl Ammonium bromide method. Absorbance of the solution was measured at wavelengths 260 nm and 280 nm. Genomic DNA from leaves of regenerants and mother plant (P) was extracted using CTAB method31. The total genomic DNA was quantified spectro-photometrically (UV-Vis CTAB) protocol described by Saghai- Maroof et al., (1984). DNA concentration was estimated using Spectrophotometer, Pharmaspec UV-1700, Shimadzu, Japan) and aliquots were diluted to the final concentration of 10-15 ng µL−1.

PCR reaction for ISSR was performed in a programmable thermal cycler (Master cycle epgradient S, Eppendroff, Germany).A total of 25 ISSR primers of 800P and UBC series (Eurofins Genomics, India) were initially screened for their applicability in PCR amplification of total genomic DNA of *M. koenigii*. Out of 25 ISSR, only 10 produced clear and reproducible amplified products.

ISSR profiles were produced through PCR amplification using the protocol described by Verma & Rana28. PCR amplification was carried out in 25 µL volume using 10 different decamer primers. The reaction buffer consisted of 2.5 µL of 10X PCR buffer, 2.0 µL MgCl2 (2.5 mM), 0.50 µL dNTPs (10 mM each of dATP, dGTP, dTTP and dCTP) (Bangalore Genei, India), 2 µL primer, 0.2 µL DNA Taq polymerase (*In vitrogen* platinum), 5 µLDNA sample and 12.80 µL water. The primers showing polymorphic bands were then used for analysing the clonal fidelity of micro-propagated plants.

For all the samples, PCR programme involved an initial denaturation at 94°C for 04 min followed by 35 cycles of 1.0 min denaturation at 94°C, 1.0 min primer annealing at 52°C, 2 min primer extension at 72°C and final extension for 7 min at 72°C. The amplified PCR-ISSR products were electrophoresed in 1.5% agarose in 1X TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer. The gels were stained with ethidium bromide and documented using gel documentation system (Expert Vision, Mumbai, India).

Data analysis

For *in vitro* plantlet regeneration, all the experiments were set up in a Randomized Block Design (RBD). Each experiment was repeated thrice with minimum 10 replicates per treatment. The data were analyzed statistically using SPSS ver. 7.5 (SPSS Inc., Chicago, USA) and the results are express as means ± SD32.For genetic fidelity analysis, the fingerprints were scored considering fragment size at a locus as bi-allelic (present = 1, absent = 0). Reactions with each primer were repeated at least thrice and only those fragments that were well resolved and reproduced in each instance were scored and included in the analysis ignoring the bands intensity.

III. Results

Cluster Analysis of combined ISSR primers

As mentioned earlier a dendrogram is a branching diagram that represents the relationships of similarity among a group of entities, and enables to compute the scale of the relationships among these entities or sample populations. The row dendrogram in Fig. 4.24 has been constructed using un-weighted pair group method with arithmetic averages or UPGMA with a SAHN module of the NTSYS software. It shows a phenetic representation of genetic relationships as revealed by the similarity coefficient for all the ISSR profiles that have been generated for *M. oleifera*. The dendrogram reveals three clusters with outliers. Cluster I comprises of A3, KG2, PB1 and BH. Cluster II is composed of JOB6, NM1 and M423; with H2 being an outlier for these 2 clusters. KVT2, TH2 and J6 form cluster III and SN2 is outlier in the picture.

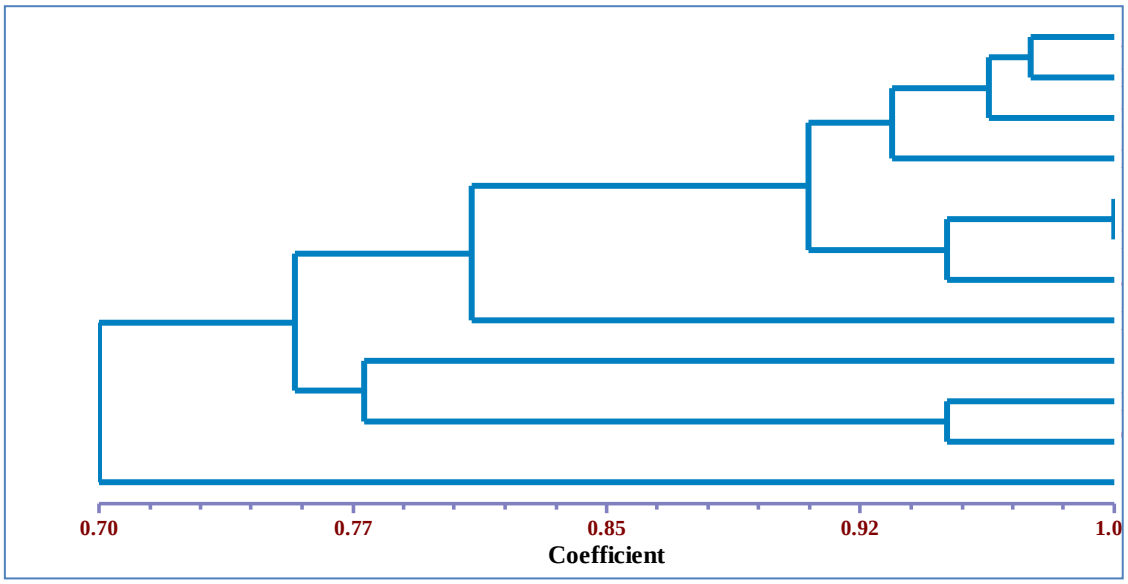

Fig.4.24: Dendrogram generated using un-weighted pair of group method with arithmetic average analysis (UPGMA), showing relationships between different populations of *M.oleifera* using the data obtained from all the ISSR primers used.

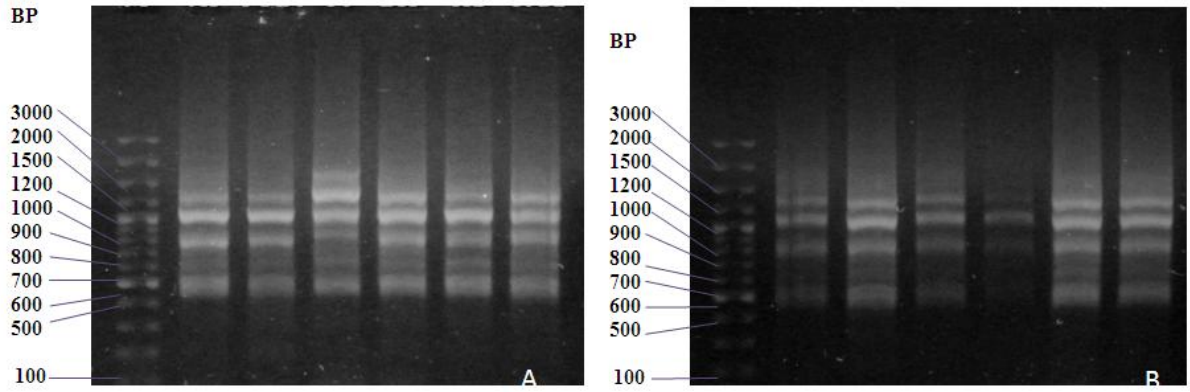

Fig. 4.14 (A & B) Fingerprinting profile of amplified genomic DNA of *M. oleifera* samples generated by **ISSR primer 826P**. **M -** Represents 100bp ladder.

Similarity Coefficient Analysis of the combined ISSR Profiles

Jaccard's pair-wise similarity coefficient values among all the populations of *Moringa Oleifera* using all the ISSR primer profiles generated vary between 0. 55 and 1.00 as shown in table 4.16. 55% similarity which happens to be the least in terms of similarity coefficient data for all ISSR profiles has been observed between S3 and S10.

S5-S7 and S9-S5 show 60% similarities while 62% similarity is observed between: S4-S10 & S5-S10. A similarity of 65% has been recorded between 3 sample populations' pairs namely: S3-S5, S10-S6 and S9-S10. 77% similarity has been seen in many sample populations like S1-S5, S7-S1, S2-S3, S3-S7, S8-S3, S9-S3 and S3-S12. 85% is observed in S1-S11¸S2-S5 and S5-S8. 92% similarity is observed in S1-S2, S4-S1 S2-S11, S1-S12 and S8-S11. A similarity of 97% is observed between S1 and S6 of the sample populations. The highest similarity coefficient of 1.00 or 100% similarity is observed between S8 and S2.

Table 4.16 Jaccard's similarity coefficient of *M. oleifera* by all the ISSR primer profiles combined.

Sample	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
S1	1.00											
S2	0.92	1.00										
S3	0.80	0.77	1.00									
S4	0.92	0.90	0.82	1.00								
S5	0.77	0.85	0.67	0.75	1.00							
S6	0.97	0.95	0.82	0.95	0.80	1.00						
S7	0.77	0.70	0.77	0.70	0.60	0.75	1.00					
S8	0.92	1.00	0.77	0.90	0.85	0.95	0.70	1.00				
S9	0.82	0.75	0.77	0.75	0.60	0.80	0.95	0.75	1.00			
S10	0.65	0.72	0.55	0.62	0.62	0.67	0.72	0.72	0.67	1.00		
S11	0.85	0.92	0.75	0.82	0.82	0.87	0.77	0.92	0.72	0.80	1.00	
S12	0.92	0.90	0.77	0.90	0.75	0.95	0.75	0.90	0.80	0.72	0.87	1.00

Determination of DNA Polymorphism among populations of *M. oleifera* by ISSR primers

Moringa Oleifera populations were analyzed using 25 ISSR primers, out of which 09 generated amplified products. Out of the 09 ISSR markers utilized, 07 produced reproducible polymorphic banding patterns. In the 800 P series of ISSR primers, 811 P produced 02 polymorphic loci, while primers 814P, 818P and 826P generated 01 polymorphic locus each.
A total of 23 loci with band range size between 200bp to 1,500bp were generated from these primers of series 800P. 811P and 814P both generated 33.33% polymorphism, while primer 818P and 826P generated 20% and 16.66% polymorphism respectively.

A total four primers from the UBC series that were tested out and three of them generated polymorphic bands. In this series of ISSR primers, the highest percentage of polymorphism i.e. 50% with 03 polymorphic loci was generated by UBC 881; the band size for these loci ranged between 500bp to 1300bp. UBC 842 generated 07 loci while UBC 857 and UBC 880 generated 03 and 08 loci respectively; also the latter two generated 01 polymorphic locus each. UBC 857 generated 33.33% of polymorphism while UBC 880 generated 12.5%, with 01 polymorphic locus each. A total of 24 loci within a band range of 100bp-1500 bp have been amplified by the primers of UBC series.

Table 4.30 Polymorphic loci generated from all the ISSR primers in *M.oleifera*

ISSR Primer	Sequence 5'–3'	Total Loci amplified	Polymorphic loci	Percentage polymorphism (%)	Range of amplicons (bp)
811P	$(GA)_8C$	06	02	33.33	800-200
814P	$(CT)_8$ A	03	01	33.33	1200-500
815P	$(CT)_8G$	03	00	00	1200-600
818P	$(CA)_8G$	05	01	20	1000- < 300
826P	(AC)8C	06	01	16.66	1500-500
UBC 842	(GA)8YG	07	00	00	1100-200
UBC 857	(AC)8YG	03	01	33.33	100-500
UBC 880	(GGAGA)3	08	01	12.50	1500-200
UBC 881	(GGGTG)3	06	03	50	1300-500
	Total	47	10	22.11	-

IV. Discussion

There have been a lot of biochemical studies conducted on *Moringa* due to its therapeutic and nutritional properties; however few studies have been focused on elucidating genetic variation within *Moringa.* Genetic variation offers insurance against genetic erosion and maintenance of biodiversity. High levels of genetic variation can aid against co-evolving biotic factors . It is thus necessary to elucidate the genetic diversity and relatedness of an introduced population that has been established for future conservation and breeding strategies, as suggested by Wu *et al.,* 2010 on the study of *Moringa* trees.

RAPD marker analysis of genetic diversity in *Moringa* has proven very successful lately and reaffirms the importance of elucidating genetic variation for management of *Moringa* (Abubakar *et al*., 2011). A genetic diversity study conducted on 161 accessions of *Moringa*, 131 wild accessions collected from the wild in Pakistan and 30 accessions obtained from ECHO (Florida), has identified the usefulness of 19 microsatellite markers in determining great genetic diversity within wild collections globally.

Very recently the genetic diversity of commercially grown *Moringa* cultivars from India was investigated in a comparative evaluation of three genetic marker systems, including RAPD‟s, ISSR‟s, cytochrome P450-based, and determined the cultivars to be highly diverse genetically based on all three markers (Saini *et al*., 2013). High genetic diversity was found among the cultivars and out of the three marker systems used, the ISSR marker system was found to be most effective for genetic diversity evaluation of *Moringa* trees within that study. Genetic diversity in *M. oleifera* accessions has been reported using AFLP (Muluvi et al. 1999) and RAPD markers (Muluvi et al. 2004; Mgendi et al. 2010). Muluvi et al. (1999) analysed the genetic diversity by AFLP among 140 accessions of *M. oleifera*, collected from south India, Southern Malawi and Kenya and found the highest levels of genetic diversity within the Indian Genotypes. Substantial genetic variability in existing natural population of *Moringa* trees from India, has been reported (Ramachandran et al. 1980).

In present study, the major issue was the isolation of genomic DNA because the *M. oleifera* contains ample gum resins and polyphenols that hinder the isolation of genomic DNA and inhibit amplification. Polysaccharides contamination is a common problem in the genomic DNA extracted in many plant species (Murray & Thompson, 1980).

The issue of high viscosity of genomic DNA in *M. oleifera* was resolved by adding 3M NaCl in the T.E. Buffer + DNA solution followed by precipitation. Marked improvement was observed when 3 M NaCl treatment was repeated twice, it resulted in the recovery of translucent and non sticky genomic DNA.

Several studies on genetic diversity of plants by molecular markers have established the correlation between geographical distances and genetic similarity between individuals. (Haque and Bandhopadhyay, 2009b; Suthar et al., 2008 and Harish et al., 2014; Khatik N. and Joshi R, 2020). In the present study the dendrogram for the all the 12 sample populations using ISSR markers showed an intriguing pattern formation of clusters. The major cluster comprises of 11 samples divided into smaller sub clusters and sample population SN2 from the Sri Nagar village, being an outlier for all these. This can be attributed to the change in edaphic factors, as the area where the plant population grows, is a low ditch, which caused accumulation of water and nutrients for the plants to thrive well, in comparison to the other zones.

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