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Genetic Diversity of Moringa (*Moringa oleifera.* Lam) Using SSR Markers

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ABSTRACT: *The present investigation was conducted at the lab of the Horticulture College and Research Institute, Tamil Nadu Agricultural University, Coimbatore during 2011-12 to study the magnitude of genetic diversity of 34 moringa ecotypes collected from Southern districts of Tamil Nadu. Study on genetic diversity of 34 moringa ecotypes of on the basis of molecular parameters. In the present study, 20 SSR markers produced 33 alleles and the number of alleles ranged from 2 to 3 with an average of 2.2 alleles per primer. The maximum number of amplified products was generated by primer MO 41 followed by MO 64. Allele sizes varied from 110-400 bp. PIC content of the SSR primers ranged from 0.34 to 0.76 with an average of 0.57. Hence, the primer pairs MO 6, MO 8 and MO 64 are considered to be worth in future studies in the field of taxonomical and genetic resource management.*

Keywords: *Moringa oleifera, molecular diversity, SSR Markers, PIC value.*

INTRODUCTION

Moringa oleifera Lam. (synonym: *M.ptreygosperma* Gaertn.), an economically important multipurpose tree indigenous to North West India, is the most widely cultivated and well known one of all 13 species in the monogeneric family Moringaceae (Olson, 2002). Popularly known as ''Drumstick'' tree, horseradish tree, or Ben tree, *M. oleifera* is a deciduous to evergreen shrub or small tree with a height of 5 to 10 m (Morton, 1991). Its seedlings are fast growing with early sexual maturity and a height up to 4.5 m in 9 months and flowering in half a year (Von Maydell, 1986). *M. oleifera* used to distribute wildly in the forests of Western Himalaya, and then throughout India by cultivation (Selvam, 2005).

Despite limited knowledge of the levels of genetic diversity and relatedness of introduced populations, their utilization as a source of seed for planting is widespread. Knowledge of population genetic diversity is one of the prerequisites for development of plant species conservation strategies.

When the variation existing between and within the population is not detectable through visual or morphological means, there is a need for a highly reliable and precise method to detect the variation without any environmental effects. The advent of molecular techniques, an analysis based on the polymorphisms found in protein or DNA has greatly facilitated research activities in the fields of phylogeny, taxonomy, genetics and plant breeding. At present, there are number of molecular markers available for such detection of variability, and they are not influenced by environmental effects. Simple Sequence Repeats (SSR), is a polymerase chain reaction (PCR) based DNA marker system, which is simple, cost effective and capable of revealing variation even at single nucleotide level. Based on co-dominant features and high allelic polymorphism, microsatellites [simple sequence repeats (SSRs)] have become a useful marker system in genetic diversity studies (Walter and Epperson, 2001); (Chaix *et al.*, 2003).

MATERIALS AND METHODS

This experiment was done in laboratory of Horticulture College and Research Institute, Coimbatore to find the diversity within the species of moringa by using Simple Sequence Repeat (SSR) Marker. List of primers used in the analysis is enclosed in Table 1.

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3 g of young leaf tissue was ground with liquid nitrogen and to this powder 15 ml of preheated CTAB buffer (65°C) was added. It was then incubated at 65°C in a water bath for one hour. After bringing the tubes to room temperature equal volume (15 ml) of chloroform: isoamyl alcohol (24:1) was added and the contents were mixed well for 10 minutes to form an emulsion. It was then centrifuged at 10,000 rpm for 15 minutes at 15°C. The supernatant was transferred to a fresh tube and the chloroform : isoamyl alcohol step was again repeated. The aqueous phase was transferred to a new tube and equal volume of ice cold isopropanol was added and incubated in a freezer for overnight. The contents were then centrifuged at 10,000 rpm for 20 minutes at 16°C.

The pellet was now saved by discarding the solution. The pellet was washed with 70% ethanol by centrifuging the contents at 10,000 rpm for 10 minutes. The alcohol was discarded and the pellet was air dried. The pellet was then dissolved in 3 ml of double distilled water. Then 1 µl of RNase was added and incubated at 37°C for 30 minutes. DNA was precipitated by adding 50 µl of 3M sodium acetate and 7.5 ml of 100% ethanol and the contents were again centrifuged at 10,000 rpm for 10 minutes. Supernatant was discarded. The pellet was washed with 70 % ethanol and air dried. It was finally dissolved in TE buffer (150 µl) and stored at - 20°C.

The quality and quantity of the extracted genomic DNA was determined by using gel electrophoresis (0.8% agarose gel). DNA concentration for PCR amplification was estimated by comparing the band intensity of a sample with the band intensities of known dilutions of Lambda DNA/EcoRI+HindIII Marker (Fermentas, Germany). Based on the band intensity the DNA was further fractionized to the required concentration (25-50 ng) using double distilled water.

Two µl of template DNA, 1.2 µl of dNTP were mixed, 2 µl of 10 µM primer, 1.5 µl of 10X assay buffer, 0.20 µl of 1U Taq DNA polymerase and sterile water (9.5 µl) were used as reaction mixture. Annealing temperature was calculated based on melting temperature of primer. Denaturation (95°C) and extension (72°C) temperatures were set. After running 35 cycles in PCR (Make:MJ Research), the products were run in 3% agarose gel containing 5 µl of ethidium bromide dye. Gels were documented using Alpha imager TM 1200-Documentation and analysis System of Alpha Innotech Corporation, USA. The primers that produced amplification were used for analyzing diversity in all the selected genotypes. Allele sizes of PCR products were determined by referring to the 100bp ladders (Bangalore Genei Cat. No. 105656). PCR and Gel electrophoresis have been done with these markers. Scoring, Polymorphic Information Content (PIC) and cluster analysis have been calculated based on the marker data.

Markers were scored for the presence and absence of the corresponding band among the different accessions. The scores '1' and '0' were given for the presence and absence of bands, respectively. Polymorphism information content (PIC) for each SSR

Table 1
List of primers used for SSR analysis

S. No.	*Primer Name (Max. 15 Characters)*	*Primer Sequence 5' to 3'*	*Anealing temperature*
1	MO1 F	TTGTCTGCCTCCTTTTGTCA	58
	MO1 R	AACTGTCACCCTCCTATCCA	
2	MO6 F	GCATAGCCACCTTTACTCCT	61
	MO6 R	GACTTTTGAACTCCACCACC	
3	MO8 F	GTAGATGGTGCAGCTACTCA	58
	MO8 R	TGGGGTTCTTGTTCTTTATT	
4	MO10 F	CTTTACACCTCAGTATCCCT	58
	MO10 R	GTTCGGCTTATGTTCTCGTT	
5	MO12 F	ACCGAAGATGATAAGGTGGG	59
	MO12 R	CAAAAGGAAGAACGCAAGAG	
6	MO13 F	TTTCGGGTTTTCTTTCACGC	58
	MO13 R	AGCTCACTTTCCATCTCCAT	
7	MO15 F	CCCCTCTATTTCCATTTTCC	59
	MO15 R	GCTCCATAAACCCTCTTGCT	
8	MO18 F	TTTTCCTCCCTTATTGTGCC	58
	MO18 R	CCGTTGCCCTTTGTGGTTCA	
9	MO41 F	TGGGATTAGGGCATTAGAAA	55
	MO41 R	TAGTGGGTCCAAGACAAAGC	
10	MO44 F	GGCACATAGGCACGCAATAC	59
	MO44 R	CAACAAACCCATCCAGAAA	
11	MO45 F	CCTTTGAAGTTGAAAATCTC	58
	MO45 R	TTCTAGGGTAGTTGAATCCA	
12	MO46 F	ACCAAGGGTTTCAACTGCTG	61
	MO46 R	CATTTTGCGACGGTCTCACG	
13	MO48 F	AGAAGAACCCAACAGAGGAT	58
	MO48 R	CTTTTCACTAACCACCACCC	
14	MO55 F	ATTACAGAACGATGAAACCA	56
	MO55 R	CTCTTTCCCTCCATTCAACC	
15	MO56 F	TCAATACGCCAAGTAAGCAA	60
	MO56 R	AAGCACTTCACGCATAAAAC	
16	MO58 F	TGGATTTCTTCTCCTGCTAT	58
	MO58 R	CACAGTTCTTATTGTATTGG	
17	MO61 F	TGTGGGTCCTGCCTTTTCTC	60
	MO61 R	CTTCTGTCTTTCTTCCTGCT	
18	MO62 F	AAACATAGCAACTGTGAGAT	55
	MO62 R	CTCCAACAACATACAAAATC	
19	MO64 F	TCGGCACCTTCTTCCTCTTT	58
	MO64 R	AATCCCTTGACGGACACCAG	
20	MO68 F	TGCTTCGCTTCCTCTATTCT	56
	MO68 R	ACCACAGGCTTGCTTCAGTA	

marker was calculated based on the formula $PIC_i = 2f_i (1-f_i)$, where PIC_i is the polymorphic information content of the marker i, f_i is the frequency of marker bands present and $(1-f_i)$ is the frequency of absent marker bands (Roldan-Ruiz *et al*., 2000).

The data obtained by scoring the SSR profiles of different primers were subject to cluster analysis. Similarity matrix was constructed using Jaccard's coefficient (Jaccard, 1908) and the similarity values were used for cluster analysis and dendrogram was constructed by Unweighted Pair-Group Method using Arithmetic averages (UPGMA) with the Sequential Agglomerative Hierarchical and Nested (SHAN) function (Sneath and Sokal, 1973). Data analysis was done using **NTSYS-pc version 2.02i** (Rohlf, 1998).

RESULTS AND DISCUSSION

SSR analysis

Thirty four accessions of moringa were also analyzed using twenty SSR markers (Table 1). Twenty primer pairs were screened out of which fifteen pairs showed amplification. Out of fifteen primer pairs eleven were found to be polymorphic and four of them were monomorphic (Table 2).

The fifteen primers generated a total of 28 amplicons and 21 of them show polymorphism (61.54). The number of amplicons per primer ranged from one to three and polymorphic amplicons ranged from one to two. The highest number of polymorphic amplicons (2) was generated by primer MO 1, MO 6, MO 8, MO 41, MO 46, MO 48, MO 61, MO 62, MO 64 and MO 68. Primers MO 1, MO 6, MO 8, MO 41, MO 46, MO 48, MO 62, MO 64 showed the highest per cent of polymorphism (100%) while the least per cent of polymorphism was recorded in primer MO 12 with 50 %.

Polymorphism Information Content

Polymorphism Information Content (PIC) value was calculated for 15 polymorphic primers and given in the Table 3. PIC value was highest for the primer MO 6 (0.76) followed by primer MO 8 (0.70) while, the lowest PIC value recorded by the primer MO 61 (0.34). The mean PIC value for 15 polymorphic primers was 0.575. In the present study, 15 SSR markers produced 33 alleles and the number of alleles ranged from 2 to 3 with an average of 2.2 alleles per primer. The number of alleles amplified ranged from 2 to 4 in cassava (Moyib *et al*., 2007). The maximum number of amplified products was generated by primer MO 41 followed by MO 64. Allele sizes varied from 110-400 bp which were in close agreement with allelic size reported by Asare *et al*. (2011) in cassava.

Polymorphic Information Content (PIC) reveals the quantity of information that can be obtained from a particular primer. In the present study, PIC content of the SSR primers ranged from 0.34 to 0.76 with an average of 0.57. Similar PIC values were obtained in earlier studies using SSR markers. PIC values ranged from 0.07 to 0.77 with an average of 0.52 (Asare *et al*., 2011) in cassava. The higher PIC value indicated the in formativeness of the primer pairs. Hence, the primer pairs MO 6, MO 8 and MO 64 are considered

Table 2
List of SSR primers showing total and polymorphic amplicons generated pattern for 34 genotypes of moringa accessions

S.No.	Primer Name	Total no. of bands (a)	Total no. of polymorphic bands (b)	% Polymorphism (b/a x 100)
1.	MO 1	2	2	100
2.	MO 6	2	2	100
3.	MO 8	2	2	100
4.	MO 10	1	0	0
5.	MO 12	2	1	50
6.	MO 41	2	2	100
7.	MO 46	2	2	100
8.	MO 48	2	2	100
9.	MO 55	1	0	0
10.	MO 56	1	0	0
11.	MO 58	1	0	0
12.	MO 61	3	2	66.66
13.	MO 62	2	2	100
14.	MO 64	2	2	100
15.	MO 68	3	2	66.66
	Total	28	21	75.00

Table 3
List of SSR primers used and the level of polymorphism detected

S. No	primer	PIC Value	Allele size range (bp)	Number of alleles
1.	MO 1	0.64	150-160	2
2.	MO 6	0.76	350-400	2
3.	MO 8	0.70	150-200	2
4.	MO 10	0.66	200-250	2
5.	MO 12	0.56	150-250	2
6.	MO 41	0.39	150-170	3
7.	MO 46	0.58	150-250	2
8.	MO 48	0.60	120-130	2
9.	MO 55	0.60	150-250	2
10.	MO 56	0.58	250-300	2
11.	MO 58	0.57	250-280	2
12.	MO 61	0.34	280-310	3
13.	MO 62	0.65	110-120	2
14.	MO 64	0.62	200-300	2
15.	MO 68	0.37	200-300	3
	Total	8.62		33
	Mean	0.575		2.2

to be worth in future studies in the field of taxonomical and genetic resource management particularly in moringa.

Cluster analysis

The cluster analysis of 34 moringa accessions based on UPGMA suggested the formation of six clusters (Fig. 1). Among the six clusters, cluster I was the largest with 9 accessions followed by cluster II (7 accessions). Cluster III comprised of 6 accessions, cluster IV comprised of 5 accessions, cluster V consisted of 5 accessions and Cluster VI comprises of 2 accessions. Cluster I and II has many sub clusters. It is inferred from the cluster that Cluster 1 and VI have more differences in molecular level and it may be used in molecular breeding.

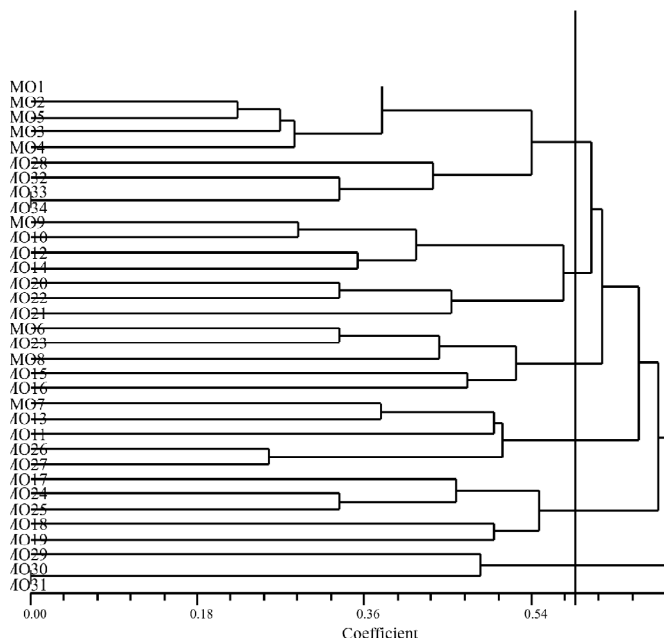

Figure 1: Dendrogram showing the distribution of the thirty four moringa accessions using SSR markers

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