Evaluation of the genetic diversity and population structure in drumstick (*Moringa oleifera* L.) using SSR markers

R. Rajalakshmi[1], S. Rajalakshmi[1] and Ajay Parida[1,2,*]

[1]M.S. Swaminathan Research Foundation, 3rd Cross Street, Institutional Area, Taramani, Chennai 600 113, India
[2]Present address: Institute of Life Sciences, Bhubaneswar 751 023, India

Moringa **belongs to the family Moringaceae comprising 13 species of which *Moringa oleifera* is more widely cultivated. It is an economically important multipurpose tree with immense nutritional value and has significant potential to address malnutrition. In the present study, a total of 97 accessions collected from different districts of Tamil Nadu, Andhra Pradesh and Odisha were genotyped using 20 simple sequence repeat (SSR) markers to assess the genetic diversity and population structure. A total of 140 alleles were detected with the polymorphic information content value of 0.6832 and gene diversity 0.7292. Population structure analysis through a model-based approach divided the accessions into two subgroups. Molecular variance analysis using principal coordinate analysis (PCoA) summarized a 18.32% variation in the first 3 axes and analysis of molecular variance analysis indicates a 2% variance among the population with the remaining 98% variance attributed to variation within individuals. Cluster analysis based on unweighted neighbour-joining showed a clear separation of samples into two subgroups. Further comparison of the cluster subgroup showed high consistency with the STRUCTURE pattern and PCoA plot. The findings reveal a high diversity in the analysed genotypes from which a few distinct accessions could be utilized for further exploration based on their nutritional content and for conservation of nutritionally superior germplasm.**

Keywords: Genetic diversity, *Moringa oleifera*, population structure, SSR.

MORINGA OLEIFERA Lam. is the most widely cultivated and well known of the 13 species in the monogeneric family Moringaceae[1]. Commonly known as the drumstick tree or horseradish tree, it is a short to medium-sized tree native to the sub-Himalayan tracts of India. It is an outcrossing diploid ($2n = 28$) species with distinct tri-pinnate leaves having yellow or white petiole streaks with some trees flowering throughout the year while some in two distinct seasons[2,3]. It is an economically important multipurpose tree indigenous to India[4] with almost every part of the plant used for human consumption. The leaves of *M. oleifera* are a rich source of vitamins and nutrients like calcium, iron, potassium, phosphorus and highly digestible proteins and have been suggested for addressing rampant malnutrition in several parts of the developing world; the seed powder is used to purify water[5,6]. The plant is naturally biofortified with immense nutritional, medicinal, culinary and phytochemical values and it is therefore important to identify genetically superior germplasm for the purpose of conservation. Since the genetic diversity and detailed gene flow patterns in *M. oleifera* are not well understood, studies have been carried out with molecular markers to evaluate the population diversity within the species. Among several methods currently available for analysis of diversity in germplasm, DNA-based molecular markers provide a reliable and detailed understanding of the diversity pattern.

Molecular markers are a useful tool for assaying genetic variation, and have greatly enhanced the genetic analysis of crop plants[7]. Several molecular markers like RFLP, RAPD, AFLP, ISSRs and SSRs are utilized for analysing genetic diversity of which SSRs have been the most commonly used in recent times. SSRs are highly popular genetic markers because of their codominant inheritance, high abundance, dispersal throughout the genome, multi-allelic variation, high level of polymorphism and high reproducibility[8]. The development of advanced automated sequencing systems and availability of fluorescent tagged primers allow for relatively quick marker development[8]. This study focusses on estimating the population structure and genetic diversity existing in *M. oleifera* accessions collected from three states of India (Tamil Nadu, Andhra Pradesh and Odisha) using SSR markers.

A total of 97 *Moringa* seed samples comprising hybrids and 95 naturally grown local varieties, were collected from different districts of Tamil Nadu, Andhra Pradesh and Odisha (Table 1). The seeds were germinated in triplicates under natural conditions containing 1 : 1 : 1 ratio of red soil, sand and manure.

Leaves were collected from 2-month-old plants for DNA isolation. Genomic DNA was extracted from 1 g leaf samples using CTAB buffer[9]. The samples were finely ground using liquid nitrogen and incubated at 60°C for 45 min, followed by a spin at 12,000 rpm for 15 min. The supernatant was transferred to fresh centrifuge tubes and washed with (25 : 24 : 1) phenol : chloroform : isoamyl alcohol followed by chloroform: isoamyl alcohol wash and mixed thoroughly and centrifuged. To the supernatant three-fourths volume of ice cold isopropyl alcohol was added gently, mixed and the tubes incubated at –20°C for 2 h, centrifuged at 12,000 rpm for 20 min, supernatant was discarded and the pellet washed with 70% ethanol, air-dried and dissolved with 1 ml HPLC grade water. The samples were RNase-treated at 37°C for 60 min and

*For correspondence. (e-mail: drajayparida@gmail.com)

Table 1. Details of 97 *Moringa oleifera* sample collection

Sample ID	State	District	Sample ID	State	District
NMK 3	TN	Namakkal	KCP 88	TN	Kanchipuram
NMK 4	TN	Namakkal	PDC 89	TN	Pondicherry
NMK 6	TN	Namakkal	ERD 90	TN	Erode
NMK 8	TN	Namakkal	PKM 1	TN	Coimbatore
NMK 9	TN	Namakkal	PAVM	TN	Dindugal
NMK 12	TN	Namakkal	AP 1	AP	Vishakhapatnam
NMK 15	TN	Namakkal	AP 3	AP	Vishakhapatnam
NMK 16	TN	Namakkal	AP 4	AP	East Godavari
NMK 17	TN	Namakkal	AP 5	AP	East Godavari
NMK 21	TN	Namakkal	AP 6	AP	East Godavari
NMK 23	TN	Namakkal	AP 7	AP	West Godavari
NMK 24	TN	Namakkal	AP 8	AP	West Godavari
NMK 26	TN	Namakkal	AP 9	AP	Guntur
NMK 32	TN	Namakkal	AP 10	AP	Guntur
NMK 35	TN	Namakkal	AP 11	AP	Krishna
NMK 36	TN	Namakkal	AP 12	AP	Krishna
NMK 41	TN	Namakkal	AP 13	AP	Krishna
NMK 42	TN	Namakkal	AP 14	AP	Krishna
NMK 43	TN	Namakkal	AP 15	AP	Krishna
DP 45	TN	Dharmapuri	AP16	AP	Vishakhapatnam
DP 48	TN	Dharmapuri	AP 17	AP	Chittoor
DP 49	TN	Dharmapuri	AP 18	AP	East Godavari
DP 52	TN	Dharmapuri	KDP 1A	AP	Kadapa
DP 53	TN	Dharmapuri	KDP 1B	AP	Kadapa
DP 54	TN	Dharmapuri	KDP 1C	AP	Kadapa
DP 55	TN	Dharmapuri	KDP 2	AP	Kadapa
SVG 61	TN	Sivagangai	KDP 4A	AP	Kadapa
NGC 62	TN	Kanyakumari	KDP 5	AP	Kadapa
MDU 63	TN	Madurai	KDP 6	AP	Kadapa
MDU 64	TN	Madurai	KDP 7	AP	Kadapa
MDU 65	TN	Madurai	KDP 8	AP	Kadapa
THN 66	TN	Theni	KDP 10	AP	Kadapa
DDG 67	TN	Dindugal	KDP 12A	AP	Kadapa
CBE 68	TN	Coimbatore	KDP 12B	AP	Kadapa
CBE 69	TN	Coimbatore	KDP 13	AP	Kadapa
CDL 70	TN	Cuddalore	KDP 14	AP	Kadapa
CDL 71	TN	Cuddalore	KK 1	OR	Koraput
CDL 72	TN	Cuddalore	NKK 2	OR	Koraput
CDL 73	TN	Cuddalore	KPK 3	OR	Koraput
CDL 74	TN	Cuddalore	UB 5	OR	Boudh
CDL 75	TN	Cuddalore	BK 6	OR	Kalahandi
NGP 76	TN	Nagapattinam	BK 7	OR	Kalahandi
NGP 77	TN	Nagapattinam	KGK 8	OR	Kalahandi
KRK 79	TN	Karaikal	NYG 9	OR	Nayagarh
KRK 80	TN	Karaikal	BB 10	OR	Bolangir
CHN 82	TN	Chennai	PB 11	OR	Bargarh
CHN 84	TN	Chennai	JG 12	OR	Jarsuguda
NMK 85	TN	Namakkal	KRP 13	OR	Koraput
KCP 87	TN	Kanchipuram			

TN, Tamil Nadu; AP, Andhra Pradesh; OR, Odisha.

purified for impurities with an initial phenol chloroform wash and a chloroform isoamyl alcohol wash, each at 10,000 rpm for 15 min. The supernatant was transferred to fresh eppendorf tubes and one-third volume of 3 M sodium acetate and double volume of cold absolute alcohol were used for precipitation. The tubes were incubated at –20°C for 1 h and centrifuged at 10,000 rpm for 15 min, the pellet was washed with 70% ethanol, air-dried and the pellet was dissolved in 500 µl HPLC grade water. The samples were quantified using spectrophotometer at 260/280 wavelength.

Twenty primers were used for analysing the samples[10]. PCR reaction was performed for a total volume of 10 µl using 25 ng of template DNA, 0.25 µM forward primers which were M-13 tailed[11], 0.25 µM rev primer, 0.15 µM FAM labelled M-13 primer, 0.7X conc. of Amplicon master Mix and water. Amplification was performed at an initial denaturation of 95°C for 5 min followed by 30

Table 2. Summary of genetic diversity parameters obtained for 20 SSR loci genotyped using 97 *Moringa oleifera* accessions

Marker	SSR motif	Genotype size based on CE (bp)	Size obtained (–) M13 tail (bp)		Major allele frequency	Allele no.	Gene diversity	Heterozygosity	PIC
			Minimum	Maximum					
MO1	$(TC)_{17}$	164–178	146	160	0.3962	6	0.715	1	0.6658
MO6	$(AG)_7$-$(AG)_6$	515–540	494	522	0.4766	5	0.6077	1	0.5305
MO8	$(CT)_{13}$	156–189	138	171	0.4015	9	0.7159	0.9545	0.6725
MO10	$(CT)_{20}$-$(CT)_5$	296–324	278	306	0.2685	8	0.8006	1	0.7717
MO12	$(CT)_{11}$	260–280	242	262	0.3661	7	0.6944	1	0.6381
MO13	$(CT)_{15}$	348–390	330	372	0.2394	11	0.8388	1	0.8189
MO15	$(TC)_{10}CCT(TC)_6$	155–170	137	152	0.3692	6	0.7052	1	0.6532
MO18	$(GA)_6A(AG)_{16}$	254–280	236	262	0.3304	8	0.8098	1	0.7876
MO41	$(GA)_{10}$	158–168	140	150	0.3358	6	0.7584	0.9851	0.7188
MO44	$(GTCT)_5(TC)_6$	372–380	354	362	0.3205	5	0.7610	0.9487	0.7222
MO45	$(TC)_5TT(TC)_{10}$	211–227	193	209	0.4700	6	0.6026	1	0.5218
MO46	$(AG)_5$-$(GA)_6$	416–460	398	442	0.3611	9	0.7948	0.9815	0.7712
MO48	$(TC)_8C(CT)_{15}A(AC)_7$	221–243	203	225	0.3358	10	0.7606	0.8955	0.7261
MO55	$(AG)_8AA(AG)_{14}$	110–155	92	137	0.4844	4	0.5444	0.875	0.4402
MO56	$(AG)_{13}$	357–367	339	349	0.3548	5	0.7134	1	0.6618
MO58	$(CT)_6T(TC)_9$	205–224	187	206	0.2986	9	0.7941	0.8194	0.7660
MO61	$(TC)_{11}$	318–334	300	316	0.3594	7	0.7396	1	0.6968
MO62	$(AG)_{11}$	197–214	179	196	0.4200	5	0.7038	1	0.6544
MO64	$(TC)_{14}G(CT)_9$	212–240	194	222	0.3883	4	0.7007	1	0.6460
MO68	$(GA)_{12}$	248–279	230	261	0.2713	10	0.8232	1	0.8003
Mean					0.3624	7	0.7292	0.973	0.6832

CE, Capillary electrophoresis; PIC, Polymorphic information content; bp, Base pair.

cycles of denaturation at 94°C for 30 sec, annealing at 52–56°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 7 min.

Amplified PCR product (0.5 µl) along with HI-DI and ROX 500 as size standard was denatured at 95°C for 5 min and snap-freezed. The denatured samples were fragment-sized using capillary electrophoresis in ABI 3130 sequencer (Applied Biosystems, Inc., CA, USA). The fragments obtained were analysed using GeneMapper software (see Supplementary Information 1 online).

The amplified fragment peaks were scored based on their allele size using GeneMapper software. SSR alleles were analysed using diversity parameters such as, major allele frequency, number of alleles, gene diversity and heterozygosity. PIC values were calculated using PowerMarker V3.25 (ref. 12) (Table 2). Principal coordinate analysis (PCoA) and analysis of molecular variance (AMOVA) were computed for the sample set using the Nei distance matrix[13] generated by GenAlExV6.5 software[14]. A distance-based unweighted neighbour-joining cluster tree was constructed based on pair-wise distance matrix and dissimilarity index. The dissimilarity matrix was computed using a shared allele index with DARwin software[15].

The software program STRUCTURE ver. 2.3.4 (ref. 16) uses model-based clustering in which a Bayesian approach is used to infer population structure and assign individuals to populations based on SSR genotypes. To determine the actual K value, the following structure parameter set with the possibility of admixture and allele frequency was correlated. The length of the burning period was 100,000 iterations followed by 200,000 Monte Carlo Markov Chain replicates. Each K value was run 5 times with values ranging from K 1 to K 10. To obtain the optimal K value, it was plotted against the mean estimate of log probability of the data $L(K)$. The actual number of sub-population was identified using the maximum $L(K)$ value. The final population structure was calculated with ΔK, based on the second-order rate of change of likelihood distribution mean $L''(K)$ and with respect to K estimated using STRUCTURE Harvester, it showed a clear peak at optimal K value[17,18].

The 97 *Moringa* accessions genotyped with 20 SSR markers produced a total of 140 alleles. The number of alleles per loci was 4 to 11 with an average of 7 alleles per locus. Major allele frequency ranged from 0.2394 (MO13) to 0.4844 (MO55). Highest number of alleles was detected for loci MO13 and lowest for MO55 and MO64. Gene diversity calculated according to Nei[13] varied from 0.8388 (MO 13) to 0.5444 (MO 55) with an average of 0.7292 and heterozygosity was 0.9730. PIC value ranged from 0.4402 (MO55) to 0.8189 (MO13) with an average of 0.6832.

A model based Bayseian approach was used to assign the 97 *Moringa* accessions with reference to their population structure. The estimated membership fraction ranged from K 1 to K 10 with the maximum log likelihood value obtained at $K = 2$ (Figure 1). However,

Figure 1. Pattern of variation of 97 accessions with 20 SSR markers, bar length representing the membership probability of accessions belonging to different subgroups at $K = 2$.

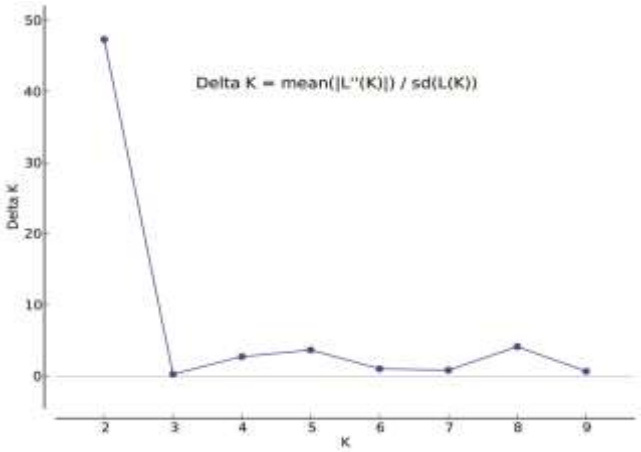

Figure 2. Graph of estimated membership fraction for $K = 2$.

due to the increasing function of K for all the K examined, an ad hoc delta K value was used to interpret the true K. The maximum ad hoc measure of delta K was observed at $K = 2$ (Figure 2) which indicates the division of entire population into two subgroups. Based on the membership probability threshold score of 0.80 (Figure 3), 97 accessions were individually assigned to each subgroup and fractions less than 0.20 score were considered to be admixed. Subgroup 1 consisted of 17 accessions all of which belonged to samples collected from Tamil Nadu. Subgroup 2 consisted the major number of 61 accessions with the remaining 19 accessions forming an admixture pattern (see Supplementary Information 2 online).

PCoA analysis using SSR markers showed equal distribution of accessions across the axes with few accessions from Tamil Nadu alone forming a separate cluster (Figure 4). The plot diagram was concordant with the STRUCTURE data and the cluster tree constructed exhibiting a similar clustering pattern. The two-dimensional PCoA plot containing the 97 accessions accounted for the maximum percent of genetic variation in the first 3 axes with (8.44%, 5.39% and 4.49%) among the populations.

Genetic variance analysis by AMOVA was used to determine molecular variance within and among the population. The results showed 2% variance among the population and 98% of variance within the population (Figure 5 and Table 3). Calculation of F-statistics revealed a negative F_{IS} (–0.30) and F_{IT} (–0.268) value. The mean F_{ST} value of 0.025 indicates a negligible level of genetic differentiation among the accessions.

Cluster analysis based on the unweighted neighbour-joining method separated the clusters into two major groups with each group having 2 sub-clusters each. Group 1 consisted of a total of 48 accessions with sub-cluster 1 (36 accessions) and sub-cluster 2 (12 accessions). Group 2 composed of a total of 49 accessions with sub-cluster 3 (45 accessions) and sub-cluster 4 with (4 accessions). The majority of the accessions falling in sub-cluster 1 was dominated by Tamil Nadu accession (32 accessions) and sub-cluster 3 with Andhra Pradesh and Odisha accessions (31 accessions), the remaining 34 accessions were found to be clustered across the groups (Figure 6). This sub-clustering of accessions espically sub-cluster 1 is similar to the results obtained from STRUCTURE and PCoA plot where a weak clustering of a few accessions could be observed separated from rest of the accessions.

In the present study we used 20 SSR markers to estimate the genetic diversity in 97 *M. oleifera* accessions collected from three different states (Tamil Nadu, Andhra Pradesh and Odisha). A total of 140 alleles were obtained across the 97 genotypes with an average of 7 alleles per loci, which compares with previous report where a total of 158 alleles were obtained from 161 accessions with an average of 6–13 alleles per loci. The PIC value obtained is slightly higher compared to earlier studies which might be due to the higher gene diversity observed in the population. Based on the PIC value the most informative markers were MO13, MO68, MO18, MO10 and MO46 with values ranging from 0.8189 to 0.7712 and MO13 had the maximum number of 11 alleles. Gene diversity was measured to be 0.7292 higher than the previous report of 0.64, which indicates the diversity present in the samples studied[19]. However, studies using RAPD, ISSR and cyt P_{450} markers have shown similar PIC values

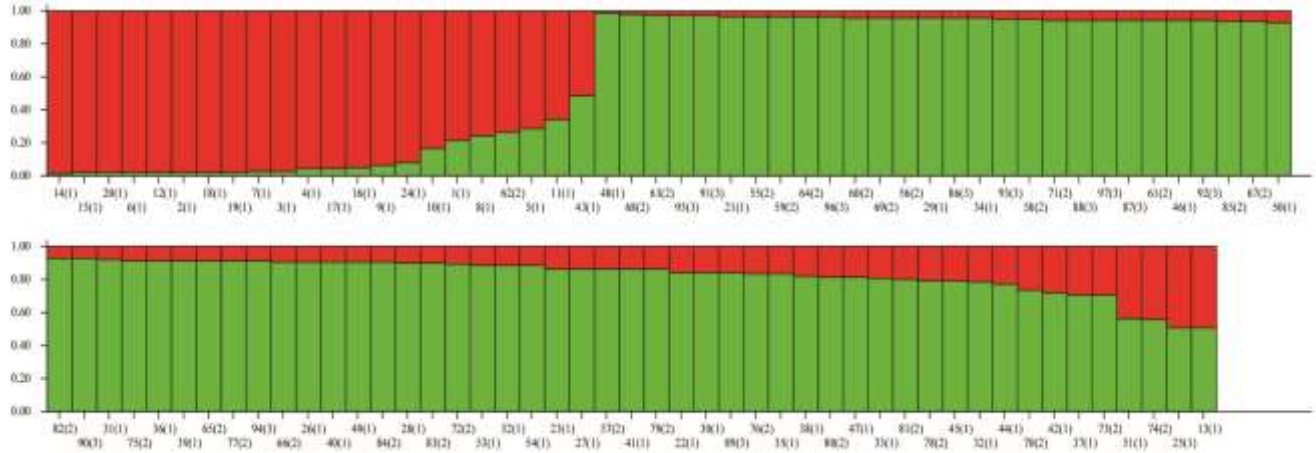

Figure 3. Population structure of 97 accessions arranged based on inferred ancestry.

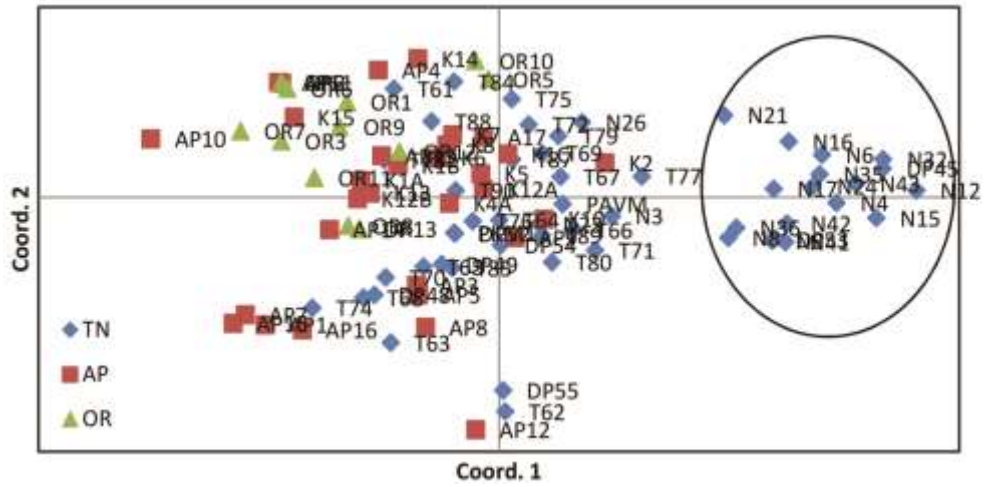

Figure 4. Principal coordinate analysis for 97 *Moringa oleifera* accessions based on 20 SSR loci.

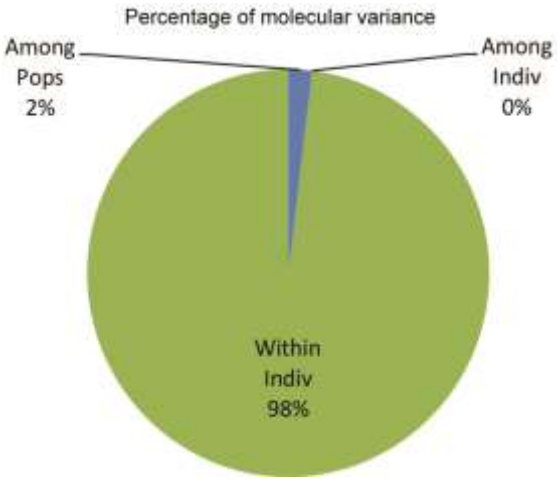

Figure 5. Pie chart for AMOVA.

which could be related to the band scoring pattern for these markers[20].

Model-based approach by STRUCTURE has detected 2 subgroups in the entire population with maximum peak obtained at delta $K = 2$. Previous studies have detected grouping of accessions to be more distinct using a threshold value of 0.08. Assigning genotypes to sub-groups based on inferred ancestry value varies between different groups; in this study a threshold value of 0.80 (80%) ancestry value leaves 19 genotypes as admixture and the rest of 78 accessions belonging to sub-groups 1 and 2 respectively[19,21].

Genetic variance study based on PCoA showed a maximum variance in the first 3 axes (8.44%, 5.39% and 4.49%). There was no location-specific grouping of accessions which might be attributed to the spread of planting material and gene flow between populations. Similar patterns were observed with a total of 31.69% variation in the first 3 axes and intermixing of genotypes not specific to their geographic origin[21].

Analysis of molecular variance in the entire population set showed a maximum of 98% variance within the

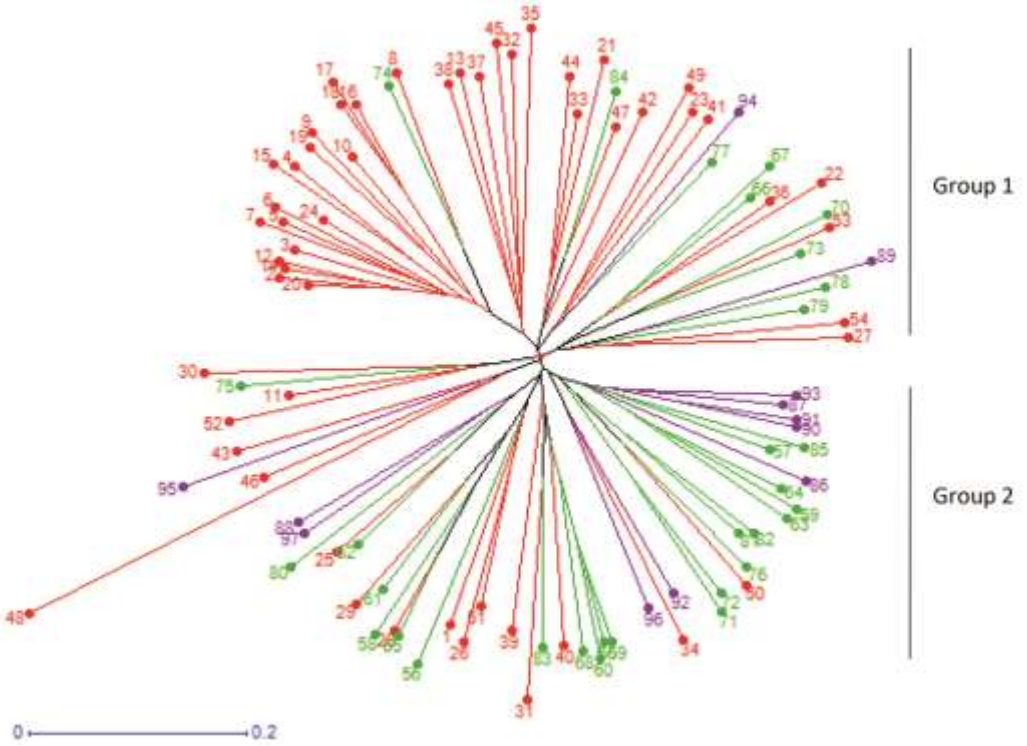

Figure 6. Unrooted neighbour joining tree of 97 *Moringa oleifera* accessions.

Table 3. Summary of genetic variance among accessions of *Moringa oleifera* by AMOVA

Source	Degrees of freedom	Sum of squares	Mean square	Estimated variance	Percent
Among population	2	31.778	15.889	0.191	2
Among individuals within population	94	498.135	5.299	0.000	0
Within individuals	97	954.000	9.835	9.835	98
Total	193	1483.912		10.026	100

Fixation indices: F_{ST} = 0.025; F_{IS} = –0.300; F_{IT} = –0.268.

population and 2% among the population which is similar to previous studies where 95% variance within population and 3% among population was reported[21,22]. However, a high level of genetic divergence was observed between populations, which is in contrast to the expectations for a woody, perennial, predominantly out-crossed species which maintain variation within population[3,23]. Calculation of *F*-statistics showed a mean F_{ST} value of 0.025 indicating a negligible amount of genetic differentiation among accessions, with a negative F_{IS} value (–0.30) representing a heterozygote excess or out-breeding of population which could be related to various factors such as genetic drift, gene flow, selection and mating system[22,24].

Distance-based cluster analysis is in accordance with the results obtained using STRUCTURE and PCoA plot, with no clear geographical isolation of the population studied, which is similar to previous reports using RAPD and SSR markers[21,22]. The type of clustering pattern observed in the present study can be related to the spread of planting material and rate of gene flow[20], which is associated with the location of sample collection in this study, that are in close proximity to each other. Similar observation had been reported in several other plant species[25,26]. On the other side, clustering of individuals from the same population in different clusters indicates high genetic variation within population which may be attributed to the use of seed sources, mutation or breeding system which is in agreement with the fact that it is predominantly an out-crossed plant[27]. However, such results are contrary to several other studies[3,28,29] where clustering of accession was based on their geographic origin.

In conclusion, the set of 20 SSR markers used in this study clearly explained the extent and nature of genetic diversity existing in the entire population set which con-

sists of accessions collected from natural population. The mean allele value, PIC value and gene diversity indicate the wide genetic base in the collection. Results obtained from population structure analysis are in accordance with the PCoA and cluster dendrogram based on neighbour-joining tree. These results can be utilized to predict diverse genotypes for exploiting breeding programmes focusing on traits related to nutritional aspect and identifying the natural genetic variation existing in the Indian *Moringa* population which will complement the Farming Systems for Nutrition (FSN) approach elaborated by M. S. Swaminathan to combat micronutrient deficiency by promoting *M. oleifera* for homestead gardens[30].

Conflict of interest: The authors declare that they have no conflict of interest.

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ACKNOWLEDGEMENTS. We express our sincere thanks to Pratiksha Trust for financial support. We are also thankful to Dr Oliver King and Dr Ramasubramanium, our colleagues at MSSRF, for their help in collection of seed materials.

Received 4 August 2016; revised accepted 9 November 2016

doi: 10.18520/cs/v112/i06/1250-1256