Research Journal of Agriculture and Forestry Sciences _____ISSN 2320– 6063
Vol. **11(3),** 15-28, November **(2023)** *Res. J. Agriculture and Forestry Sci.*

Evaluating genetic diversity and relationships among eastern Indian *Moringa oleifera* Lam. genotypes at molecular level

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Available online at: www.isca.in, www.isca.me
Received 10th July 2023, revised 23rd September 2023, accepted 27th October 2023

Abstract

Genetic diversity and relationships among 24 genotypes of Moringa oleifera collected from wide geographical range consisting of 11 agroclimatic zones in eastern India was studied employing Random Amplified Polymorphic DNA (RAPD) and microsatellite markers. Both the markers appeared as highly informative for assessment of intra-specific genetic diversity among populations of M. oleifera from different geographical areas. A total of 137 fragments ranging in size from 0.4–3.3 kb with 87.63% polymorphic loci were produced from the amplification profiles of total genomic DNA from the genotypes with 25 RAPD primers. The extent of genetic diversity across the genotypes was also evaluated employing 10 microsatellite markers dispersed across the genome. Total 87 alleles were identified with the help of 9 markers. The number of allele per locus varied from 5 to 15, with a mean of 9.66 allele per locus. 100 % polymorphism was exhibited by the makers. Polymorphism in the present study as well as Nei's mean estimates of diversity- H can be considered sufficiently high. Assemblage of genotypes irrespective of geographical locations in the cluster analysis through both the marker systems indicates high within population genetic variation possibly due to seed source variability, mutations and/or breeding systems. The study reveals that there exists sufficient genetic variation among genotypes from different areas of eastern India, despite the fact that the species has acquired status of a predominantly clonal propagated species in the region. As eastern India is the native range, genotypes collected from the region should be considered while selecting materials for improvement of cultivated individuals and conservation of M. oleifera genetic resources.

Keywords: Affinity, drumstick tree, molecular characterization, polymorphism, variability.

Introduction

Moringa oleifera Lam. (Drumstick tree) belonging to monogeneric family Moringaceae is a medium sized, fast growing tree. The Centre of origin is Indian sub-continent but the species has spread all over Asia, Africa, South America, southern part of North America and some pockets of Europe[1-4]. *M. oleifera* has originated from Himalayan tract in north western part of India[5-7] and has been reported to be distributed wildly in the forests of Western Himalaya[8]. However, it occurs now in semi-domesticated cultivated condition throughout India.

For its multifarious utilities *M. oleifera* is considered as a wonder tree. Every part of the trees is edible and have long consumed by humans for sustenance and medicine. Its parts and seed oil is used in treatment of inflammation, transmissible diseases, cardiac, abdominal, hematological and hepatorenal disorders in traditional medicine systems of south Asia[9,10]. Remarkable nutritional and medicinal qualities are present in the leaves of *M. oleifera*[11-13].

Indians employ them as hypocholesterolemic agent and regulation of thyroid hormone[14,15]. They have got global recognition of valuable nutritional supplement because they are rich source of vitamin C, protein, Iron and beta-carotene[16-18].

M. oleifera has a true diploid chromosome $2n = 28$. It has both selfing and out crossing behaviour but out crossing results in larger fruit-seed set and fecundity[16,19,20]. Mixed mating system with 74% out crossing and 26% selfing has been recorded[21]. The scenario indicates towards complex patterns of gene flow particularly when the species has a preferred clonal mode of propagation adapted traditionally by the farmers in Eastern India[13]. Information about the extent and distribution of intra-specific genetic diversity will be paramount for efficient management of this valuable species enabling estimation of genetic linkages, identification of suitable breeding genotypes and rational planning for breeding to enhance the yield and quality of its produce. Overall, scanty information on genetic base, genetic makeup and the patterns of genetic diversity of *M. oleifera* constitutes an impasse in development of varieties/ clones having superior traits of commerce.

Thus, genetic diversity and relationships among genotypes collected from wide geographical range in eastern India was studied employing Ramdom Amplified Polymorphic DNA (RAPD) and microsatellite (SSR) markers.

Material and Methods

Plant material: Germplasm of 24 genotypes of *M. oleifera* collected from different locations from 11 agroclimatic zones of Eastern India in the states of Bihar, Jharkhand, Odisha and West Bengal (Table-1, Figure-1) maintained as a germplasm garden at Institute of Forest Productivity, Ranchi (India) was utilized.

DNA isolation: Young expanded leaves were taken for extraction of DNA. The extraction procedure consisted of Doyle and Doyle[22] employing N-cetyl-N, N, N-trimethylammonium bromide (CTAB) with some modifications[23]. 250 mg of fresh leaf material were washed in Wash buffer [0.1 % (w/v) Polyvinyl pyrrolidon; 4% β-mercapto ethanol (v/v)] and then ground in liquid nitrogen. This was followed by addition of 1 ml of extraction buffer [2% CTAB (w/v), 0.2% β-mercaptoethanol (v/v), 100mM Tris-HCl (pH 8.0), 2mM EDTA, 1.4M NaCl] and 3µl RNase. DNA puffs were separated by using equal amount of Chloroform and isoamyal alcohol (24:1) followed by ice-cold isopropanol. 200–300 µl of Tris-EDTA was used to re-suspend the DNA pellet. For use in amplification reactions the re-suspended DNA was diluted in sterile distilled water to 5 ng/µl concentration. DNA was visualized under UV light, after electrophoresis on 0.8% agarose gel for quantifications.

Marker systems and PCR set up: RAPD markers: A set of 25 random decamer oligonucleotides (Bangalore Genei, India) were used as single primers to amplify RAPD fragments (Table-2). A final volume of 25 µl was maintained with 10 ng template DNA, 10µM each deoxynucleotide triphosphate, 20ng of decanucleotide primers, 25mM $MgCl_2$, 1 × taq buffer and 3 U taq DNA polymerase (Bangalore Genei, India) for carrying out Polymerase Chain Reactions (PCR). Thermocycler (Gene Amp. PCR System 9700) was set up for amplification with for a preliminary 5 min denaturation step at 95°C followed by 45 cycles of denaturation at 95°C for 1 min; annealing at 34°C for 1 min and extension at 72°C for 2 min, final extension at 72°C for 5 min.

Separation of amplification products was accomplished alongside a molecular weight marker (100 bp ladder, Bangalore Genei, India) by electrophoresis on 1.2% agarose gels run in 0.5X TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide. Gel was visualized under UV light and the photographs were scanned over Gel Doc System (Gel Doc. Syngene).

The images were examined to record presence (1) or absence (0) of amplified products. Only clear, unambiguous and reproducible bands were considered for scoring. POPGEN version 1.32 software was used to calculate population genetic diversity parameters like percentage of polymorphic loci, genetic diversity, Shannon's Information Index and Nei's genetic distance[24].

SSR markers: The sequences of *M. oleifera* available at NCBI database were screened for generating microsatellite primers using WEBSAT software. Ten SSR primer pairs were designed for the present study (Table-3). Initially, a gradient was performed to check for the best annealing temperature. PCR amplification reactions were done in 10 µl reaction mixtures, containing 0.5µl of template DNA (50 ng/ µl), 1 µl of 10 X Taqbuffer A, 0.8µl 0.2mM each deoxyribonucleotide triphosphate (Bangalore GeNei, India), 0.166µl of 3.0U/µl Taq DNA polymerase, 0.5µM of each SSR primer (Bangalore Sigma Aldrich, & Ahmedabad Xcelris, India), PCR grade water was used to make the final volume to 10 µL. All PCR reactions were performed in Palmcycler (GeneAmp* PCR9700Thermalcycler).

Programming for PCR had the sequence of initial denaturation at 94 °C for 4 min which followed 35 cycles at 92°C for 30 sec, primer specific annealing temperature 65°C to 50°C for 30 sec, 72°C for 1 min. A final extension at72 °C for 5 min was carried out for primer extension and hold at 4°C for 10 min. The amplified products were electrophoresed in 2.5% agarose and stained with Ethidium Bromide (1µg/ml) in 1 X TAE Buffer. Electrophoretic patterns were scored and checked with a100-bp DNA ladder (Bangalore GeNei) which was used to estimate allele sizes. Then gel was observed on Gel Documentation System (Syngene) and the best annealing temperature for each primer were selected. Once, the best annealing temperature was determined, the PCR reaction mixture prepared as mentioned above and was analyzed for amplification at their respective annealing temperature.

The PCR products were then electrophoreses on 2.5% agarose gel stained with Ethidium Bromide in 1X TAE buffer at 180V. Themolecular fragments were estimated using low range ruler (Bangalore GeNei), and 100 bp molecular marker. Gel Documentation System (Syngene) was used to take gel photographs.

Results and discussion

RAPD markers: The amplification profiles of total genomic DNA from the24 accessions of *M. oleifera* genotypes with 25 random primers produced a total of 137 fragments ranging in size from 0.4–3.3 kb with 87.63% polymorphic loci (Table- 2, e. g. Figure-2).

Among the 24 genotypes the mean Nei's gene diversity was 0.25± 0.1535 while mean Shannon's Information index was 0.39±0.21. The range of Similarity Coefficients varied from 0.11–0.82 as per the similarity matrix obtained using Nei and Li's coefficient[25].

The same were used to generate a dendrogram. Maximum genetic distance (0.9883) was observed between JRK /JLS (both Jharkhand) and WHP (West Bengal) while minimum genetic distance (0.0017) was between some genotypes form Jharkhand (JRB and JSC; JSC and JGD; JEB and JEG; JWJ and JWN) and Orissa (OSH and ODH; OSH and ORB; ORB and OSH).

Three major clusters were revealed in the cluster analysis based on similarity indices. Out of these three clusters, one cluster contained only one genotype i.e. BGA (B-3 South Bihar Alluvial Plains) where as one cluster contained four genotypes and another cluster contained rest of nineteen genotypes (Figure-3). Interestingly the four genotypes falling in one cluster belong to four different agro-climatic zones viz. WHP (WB-3 Old Alluvium Zone), BDM (B-1North West Alluvial Plains), JRD (JH-2 Central and Western Plateau Zone) and OCB (OR-4 East and South-eastern Coastal Plain Zone) in four different states. The cluster analysis further revealed proximity among genotypes from same geographical areas/states. For example JFB, JGD, JSC, JGB and JLS which are the accession of Jharkhand, were present in one sub-cluster.

SSR markers: High quality of DNA bands were obtained and the extent of genetic diversity across the 24 *M. oleifera* genotypes was assessed using 10 microsatellite markers dispersed across the genome (e. g. Figure-4). All except primer MSSR01 generated polymorphic patterns. The primer MSSR01 showed no proper separation which indicates it requires further PAGE separation.

The remaining 9 markers distinguished total 87 alleles with a mean of 9.66 allele per locus. The number of alleles per locus had a range of 5 to 15 alleles. The marker showed 100 % polymorphism. The highest number of allele (15) was detected in the locus 7. In contrast the locus 2 revealed the lowest number of alleles (5).

Genetic distance and genetic identity between the different genotypes were estimated through Nei's analysis of genetic distance and identity. Highest genetic identity was recorded between genotypes JRK and JRD of Jharkhand i.e. 0.8250 and the maximum genetic distance was found between the genotype OCB belonging to Orissa and WHP of West Bengal i.e. 3.4657. The dendrogram based on Nei's analysis divided the genotypes into two groups (Figure-5). One genotype i.e. Genotype WHP belonging to West Bengal stood separate in the dendrogram while rest of the 23 genotypes were grouped in one group. Further the major group was subdivided and the genotype WSK of West Bengal again stood alone. Two sub-groups were found in the larger group.

One of the sub-groups consist 16 members belonging to Jharkhand, Odisha and Bihar. It has been separated from a common ancestor because historically Bihar, Jharkhand, Odisha shared same state. According to the dendrogram, the genotype JRK and LGB of Jharkhand were highly similar to each other with respect to genotype JSC of Jharkhand and genotype BPG of Bihar which seems to be recently separated. Similarly genotype (JRD and JGD), (JEG and JWJ), (JRB and JLS), were extremely related at genetic level to each other and all belonging to the same region i.e. Jharkhand with respect to genotype OSH, ODH and JGH belonging to Jharkhand, Odisha, Jharkhand respectively and genotype BGA of Bihar, JWN and ORB of Jharkhand and Odisha had recently separated from them.

The other group was divided into two sub-groups, in which genotype OCB of Odisha and WMR belonging to West Bengal are closely related with each other but separated from the above division, where genotype JLK and genotype JEB are genetically close to each other with respect to genotype JEK and all three belonged to Jharkhand and genotype BDM belonging to Bihar obtained from nearby area or Jharkhand wasn't divided from Bihar.

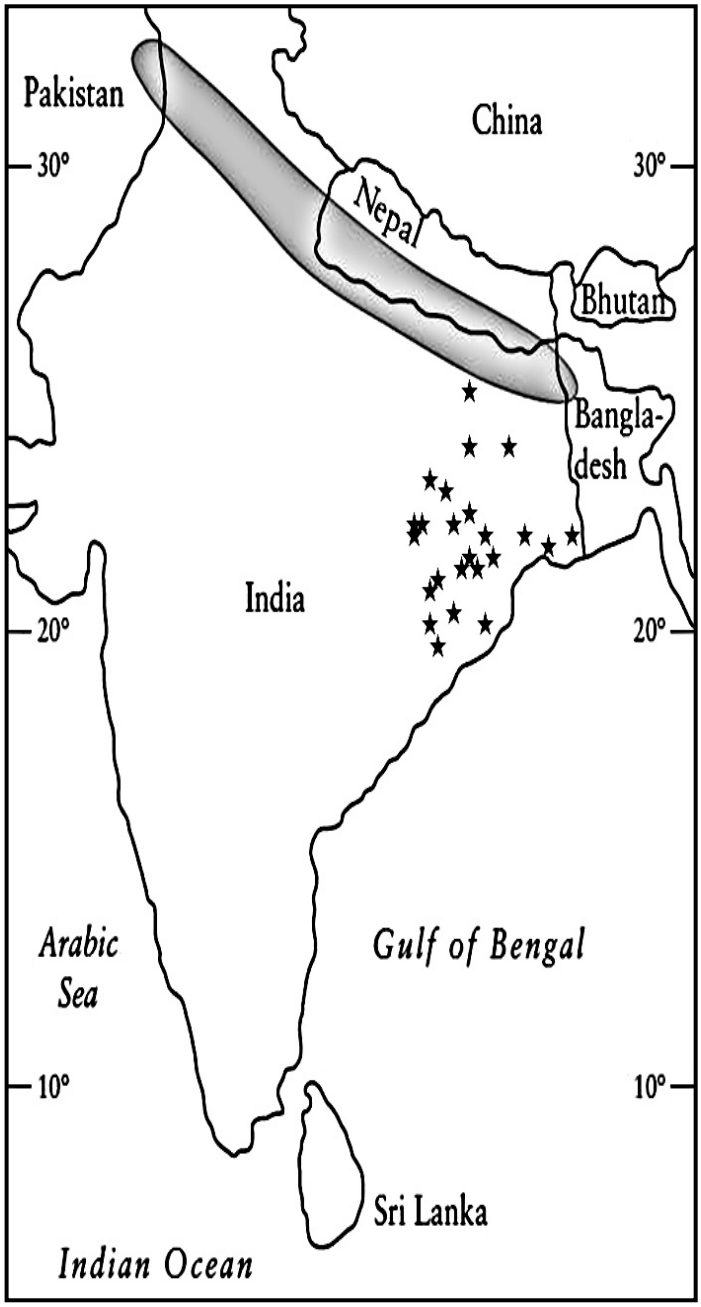

Figure-1: Centre of origin of *Moringa oleifera* Lam. and locations of genotype collections.

Table-1: Details of accessions from different areas of eastern India.

Sl. No.	Accession Code	Location (District)	State	Latitude	Longitude	Altitude (m)	Agro-climatic Zone
1	JRK	Kharsidag (Ranchi)	Jharkhand	23°15’6″N	85°22’42.5″E	370	JH-2 Central and Western Plateau Zone
2	JRD	Dahutoli (Ranchi)	Jharkhand	23°19’48″N	85°12’7.5″E	718	JH-2 Central and Western Plateau Zone
3	JRB	Bundu (Ranchi)	Jharkhand	23°9’44.5″N	85°35’45″E	610	JH-2 Central and Western Plateau Zone
4	JLK	Kudu (Lohardaga)	Jharkhand	23°26’23″N	84°41’12″E	380	JH-2 Central and Western Plateau Zone
5	JLS	Senha (Lohardaga)	Jharkhand	23°23’6″N	84°39’7″E	640	JH-2 Central and Western Plateau Zone
6	JGB	Bishnupur (Gumla)	Jharkhand	23°22’33.5″N	84°22’28″E	790	JH-2 Central and Western Plateau Zone
7	JGD	Dibdih pataratoli (Gumla)	Jharkhand	23°02’44″N	84°33’17″E	642	JH-2 Central and Western Plateau Zone
8	JGH	Hundratoli (Gumla)	Jharkhand	23°03’45″N	84°32’23″E	640	JH-2 Central and Western Plateau Zone
9	JSC	Chandil (Saraikela)	Jharkhand	22°57’12″N	86°03’33″E	630	JH-3 South Eastern Plateau Zone
10	JEB	Bahragora (E. Singhbhum)	Jharkhand	22°16'40.5"N	86°43'29.4"E	75	JH-3 South Eastern Plateau Zone
11	JEG	Ghatshila (E. Singhbhum)	Jharkhand	22°35'14.9"N	86°28'27.3"E	109	JH-3 South Eastern Plateau Zone
12	JEK	Kashidiha (E. Singhbhum)	Jharkhand	22°25’57″N	86°25’47″E	126	JH-3 South Eastern Plateau Zone
13	JWJ	Jagannathpur (W. Singhbhum)	Jharkhand	22°13’09″N	85°38’32″E	429	JH-3 South Eastern Plateau Zone
14	JWN	Nawamundi (W. Singhbhum)	Jharkhand	22°9’46″N	85°30’35″E	590	JH-3 South Eastern Plateau Zone
15	OSH	Hira kund (Sambhalpur)	Odisha	21°32’14″N	83°53’41″E	159	OR-9 West-central Table Land Zone
16	ODH	Hindol (Dhenkanal)	Odisha	20°36’47″N	85°12’46″E	192	OR-10 Mid-central Table Land Zone
17	ORB	Bisra (Sundergarh)	Odisha	22°15’81″ N	84°59’40″E	221	OR-1 North-western Plateau Zone
18	OCB	Bahugram (Cuttack)	Odisha	20°28’54″ N	86°00’43″E	23	OR-4 East and South-eastern Coastal Plain Zone
19	BDM	Mirzapur (Darbhanga)	Bihar	26°8’59″N	85°53’34″E	56	B-1North West Alluvial Plains
20	BGA	Atri (Gaya)	Bihar	24°56’38″N	85°18’23″E	108	B-3 South Bihar Alluvial Plains
21	BPG	Gai ghat (Patna)	Bihar	25°36’39″N	85°12’6″E	63	B-3South Bihar Alluvial Plains
22	WSK	Kultali (South 24 Parganas)	West Bengal	22°06’46″N	88°34’31″E	5	WB-6 Coastal & Saline Zone
23	WHP	Pandua (Hugli)	West Bengal	23°05’00″N	88°16’59″E	14	WB-3 Old Alluvium Zone
24	WPR	Rangamati (Purulia)	West Bengal	23°19’58″N	86°11’34″E	270	WB-5 Lateritic & Red Soil Zone

Table-2: The details of the characteristics of RAPD primers and their banding patterns.

Sl. No.	Primer	Sequence	Total number of amplified products	Number of polymorphic products	Number of monomorphic products	Polymorphism (%)	Tm (°C)	Annealing Temperature (°C)
1	RPI#1	AAAGCTGCGG	4	4	1	80	32	35
2	RPI#2	AACGCGTCGG	7	7	0	100	34	35
3	RPI#3	AAGCGACCTG	12	12	1	92.3	32	40
4	RPI#4	AATCGCGCTG	11	11	2	84.6	32	45
5	RPI#5	AATCGGGCTG	0	0	5	0	32	40
6	RPI#6	ACACACGCTG	9	9	0	100	32	35
7	RPI#7	ACATCGCCCA	5	5	0	100	32	45
8	RPI#8	ACCACCCACC	1	1	3	25	34	50
9	RPI#9	ACCGCCTATG	0	0	0	0	32	35
10	RPI#10	AGCATGAGCG	6	6	0	100	32	40
11	RPI#11	ACGGAAGTGG	6	6	0	100	32	30
12	RPI#12	ACGGCAACCT	8	8	1	88.88	32	40
13	RPI#13	ACGGCAAGGA	0	0	0	0	32	40
14	RPI#14	ACTTCGCCAC	3	3	0	100	32	40
15	RPI#15	ACCTGAAGCC	5	4	1	80	32	40
16	RPI#16	AGGCGGCAAG	11	9	2	81.8	34	40
17	RPI#17	AGGCGGGAAC	4	4	0	100	34	40
18	RPI#18	AGGCTGTGTC	0	0	0	0	32	40
19	RPI#19	AGGTGACCGT	3	3	0	100	32	-
20	RPI#20	AGTCCGCCTC	5	2	3	40	34	40
21	RPI#21	CACGAACCTC	8	7	1	88.88	32	45
22	RPI#22	CATAGAGCGC	7	7	0	-	32	-
23	RPI#23	CCAGCAGCTA	0	0	0	-	32	-
24	RPI#24	CCAGCCGAAC	13	12	1	66.66	34	45
25	RPI#25	GAGCGCCTTC	5	5	0	90	34	40
Total			146	125	21	-	-	-
Average			5.84	5.00	0.84	87.63	32.63	40

Table-3: Characteristics of 10 polymorphic microsatellite loci in *M. oleifera.*

S. No.	SSR Primer Sequence	Length of the Primer	Tm (°C)	Repeat Motif	Product Size (Bsp)
MSSR1	F:AGAGGATAAACCCTGCAAGACA	22	63.7	$(TC)_8$, $(CT)_{15}$ $(AC)_7$	125
	R:TTGTTGCTCTCTCGAAAATCTG	22	63.3		
MSSR2.	F:TACAGAACGATGAAACCAATCG	22	63.6	$(AG)_8$	108
	R:CTCTTTCCCTCCATTCAACCT	21	63.2		
MSSR3.	F:AATACGCCAAGTAAGCAAAACC	22	62.8	$(AG)_{13}$	339
	R:CTTCACGCATAAAACCCGAT	20	63.6		
MSSR4.	F:CTTCTCCTGCTATATGCTTGCC	22	63.8	$(CT)_6$, $(TC)_9$	171
	R:TGAATTTGAAGCGAAAGGTCTC	22	64.2		
MSSR5.	F:TTTCTCTCTCTTTTGTCTCGCC	22	63.7	$(CT)_{11}$	284
	R:TCGTCTTTCTTCCTGCTTGGT	22	64.0		
MSSR6.	F: CGGCAAAGAAACTCATCTCTC	21	59.066	$(TC)_{10}$	153
	R: AGGGTAGTTGAATCCATTTTCG	22	59.372		
MSSR7.	F: GGAGCACCCAGCTTTGTATATC	22	59.993	$(AG)_{16}$	149
	R: CCTTTGTGGTTCATGCTTCA	20	59.691		
MSSR8.	F: AAATGCAACTTCCCTCCTTATG	22	59.502	$(CT)_{15}$	210
	R: CACTTTCCATCTCCATGAAACA	22	59.971		
MSSR9.	F: CTGCTACAGTGGAATGCGATAA	22	60.285	$(CT)_{11}$	166
	R: CAAAAGGAAGAACGCAAGAGG	21	61.246		
MSSR10.	F: ACACCTCAGTATCCCTCTCTGC	22	59.768	$(CT)_{20}$	289
	R: TGTTCTCGTTTCTTGCTATGGA	22	59.883		

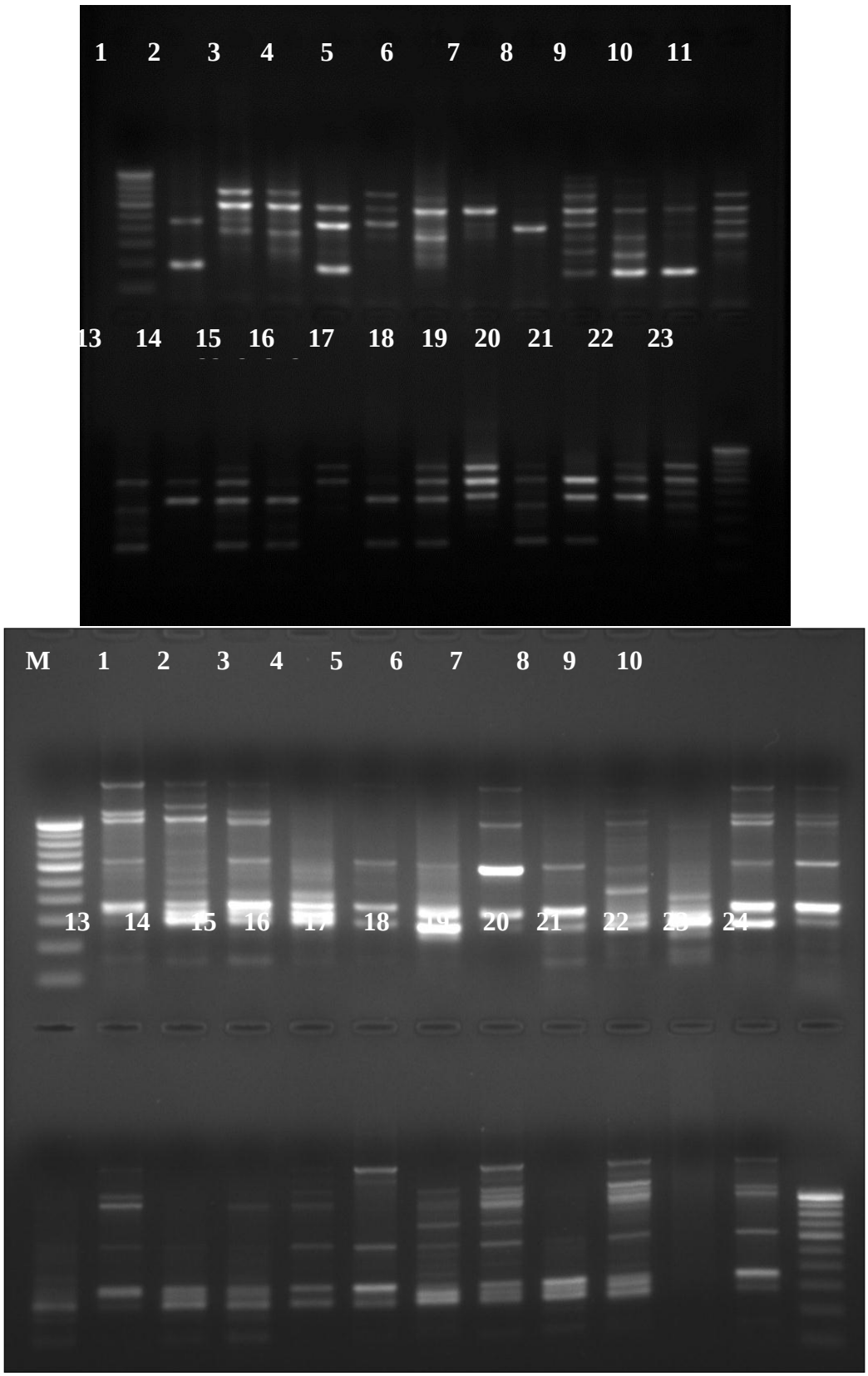

Figure-2: RAPD patterns of 24 accession of *Moringa oleifera* generated by primer RPI 22 (above) and RPI 25 (below): M – 100 bp molecular weight ladder.

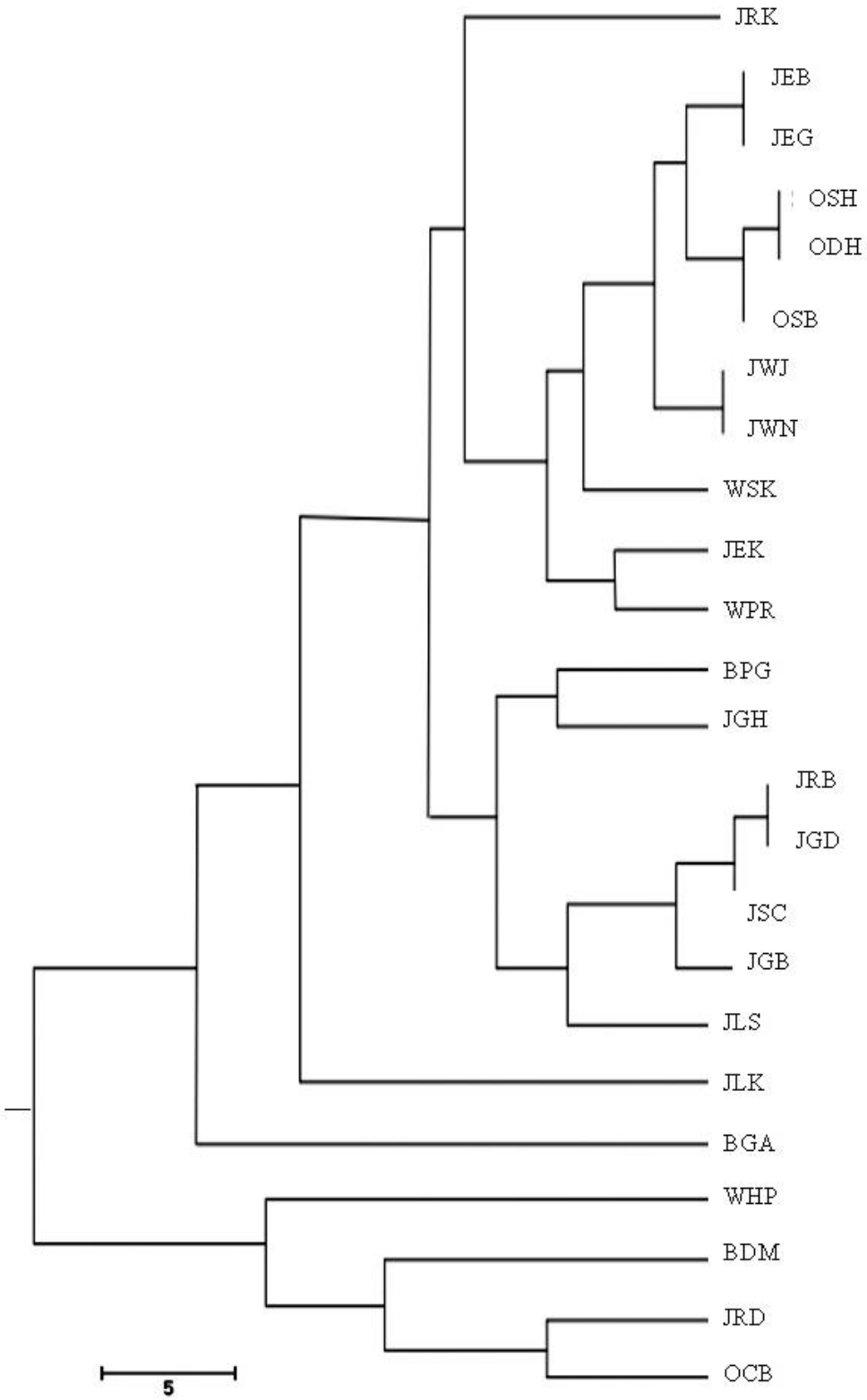

Figure-3: Cluster generated from the Jaccard similarity coefficient and UPGMA clustering using RAPD markers between 24 *Moringa oleifera* Lam. genotypes.

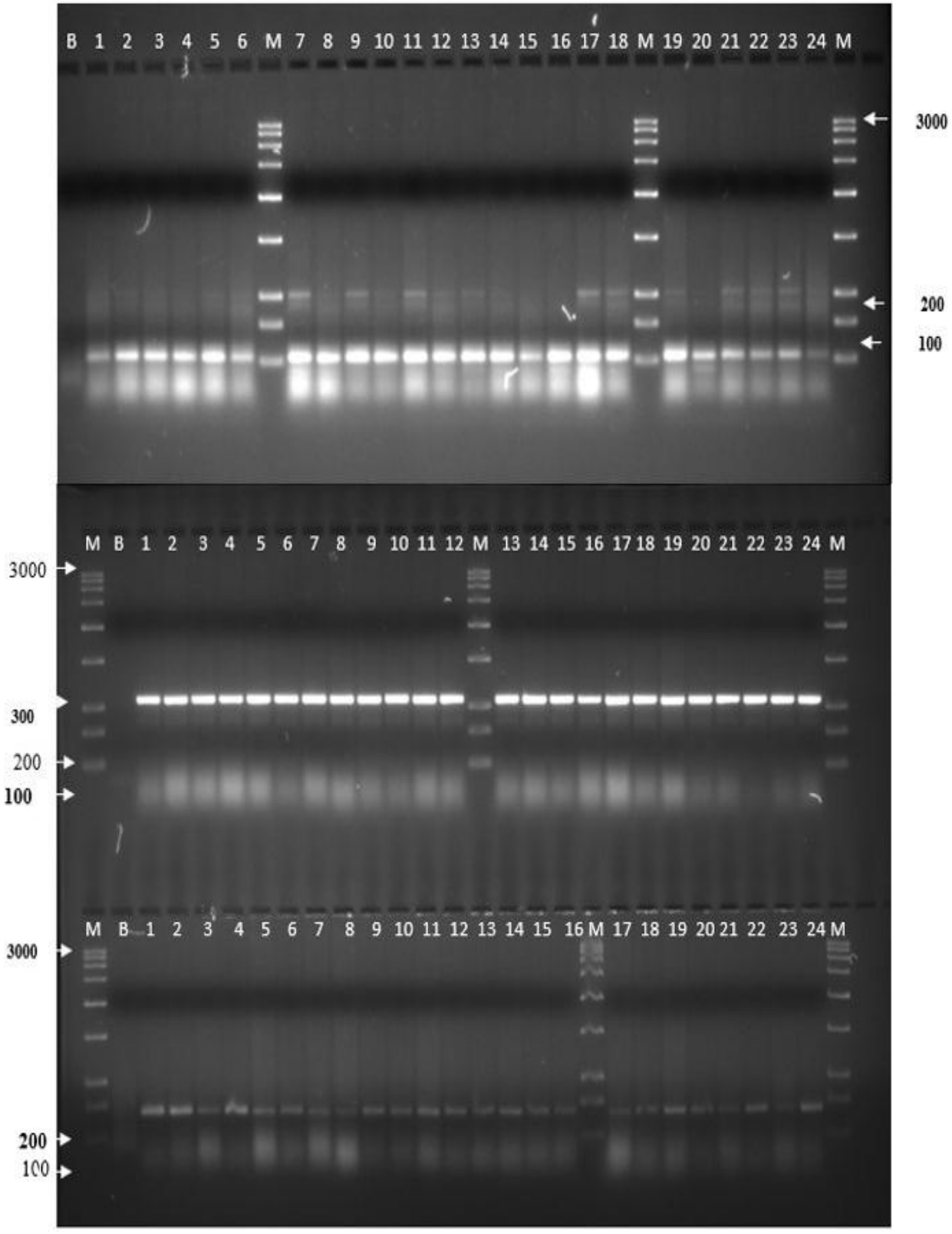

Figure-4: Agarose gel (2.5%) electrophoresis of amplicons (Primer MSSR# 2, 3 and 4).

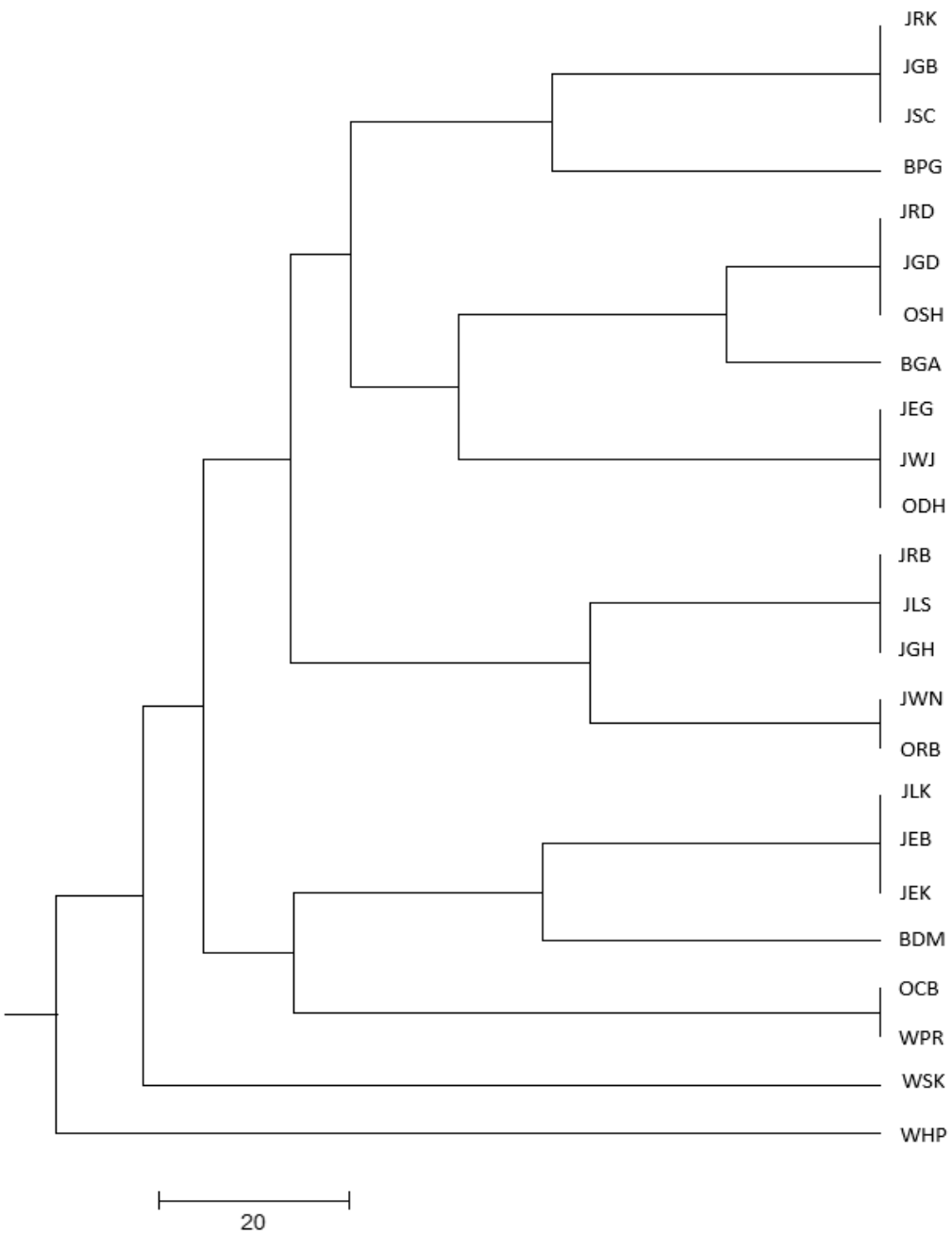

Figure-5: Dendrogram generated on the basis of Nei's genetic distance and genetic similarity analysis among the *M. oleifera* accessions through SSR markers

Discussion: In response to a change in ecology or population genetic structure sexual reproductive traits can evolve relatively quickly in flowering plants[26,27]. Further the wild individuals/ populations evolve differently at genetic level from domesticated cultivated ones[28-32] as genetic changes slow down under cultivation due to reduced competition and natural selection since environmental conditions are kept at optimum and pests are artificially controlled[33]. Further in case of extensively clonally propagated crops the selective pressures acting on sex are altered. Established clones and sexually produced volunteers are the two compartments of populations of most clonal crops which experience different selective environments. Compared to seed-propagated crops, in clonally multiplied species interactions between traits become much more complex with selection often acting in conflicting direction. Preferred clonal propagation by cultivators can significantly impact species' responses to disturbance which include composition and diversity of the species and the overall vegetation structure[34,35].

M. oleifera can serve as model species to analyze patterns of genetic diversity in such complex scenario for three reasons: i. the species once naturally occurring in the forests has now been reduced to semi-domesticated one depending on patronage of farmers for its survival; ii. clonal propagation has been traditionally practiced and preferred over sexual reproduction for cultivation all over the natural range; iii. the tree is never allowed to attain its original dimensions due to persistent lopping every year to collect pods and leaves. This becomes more complex when the species also has a mix mating system. In the present study the genotypes were identified in 11 different agro-climatic zones in four Eastern Indian states in close proximity to Centre of origin of the species.

Study of variability among the germplasm collections allows identification of duplicates, estimation of genetic linkages and identification of suitable breeding genotypes. DNA based molecular markers such as RAPD and SSR can be very useful in assessment of genetic variability among such genotypes. Successful use of RAPD technique has been previously made for identification of genetic diversity between and within the *Moringa* species[36-41]. The level of polymorphism (87.63%) recorded in the present study however surpasses these previous reports.

The SSR markers also demonstrated to be extremely informative in assessment of genetic diversity among *M. oleifera* populations from geographically different locations. Hundred percent polymorphismas well as Nei's mean estimates of diversity-H (equal to or higher than 0.55) recorded in the present study is sufficiently high. In other tree species similar H values have been reported *e. g. Populus tremuloides* (0.58 to 0.69), *Fitzroya cupressoides* (0.42 to 0.56) and *Swietenia macrophylla* (0.41 to 0.27)[42-44]. The findings indicate towards sufficient genetic diversity among the genotypes which should be incorporated in improvement and breeding programmes for the species. SSR makers have previously been used effectively for assessing genetic diversity and population structure in *M. oleifera*[45-48].

In contrast to general apprehension, studies have underlined that predominantly clonally propagated plant species, in general do not account for reduced genetic diversity as measured by allelic diversity or heterozygosity[49,50]. Variable diversity (low, moderate or high) levels have been reported in previous studies in the species were probably due to use of differential germplasm collections mostly from small geographical area or limited number of accessions from germplasm banks.

Three major clusters were revealed in the cluster analysis based on similarity indices employing RAPD markers. Out of these three clusters, one cluster contained only one genotype i.e. BGA (B-3 South Bihar Alluvial Plains) where as one cluster contained four genotypes and another cluster contained rest of nineteen genotypes. Interestingly the four genotypes falling in one cluster belong to four different agro-climatic zones viz. WHP (WB-3 Old Alluvium Zone), BDM (B-1North West Alluvial Plains), JRD (JH-2 Central and Western Plateau Zone) and OCB (OR-4 East and South-eastern Coastal Plain Zone) in four different states. However, these genotypes belong to urban centers where probably frequent movement and exchange of propagules may have occurred considering highly commercial nature of this multiple utility species. The cluster analysis further revealed proximity among genotypes from same geographical areas/states. For example JFB, JGD, JSC, JGB and JLS which are the accessions of Jharkhand, are present in one sub-cluster. It is established that in most plant populations genetic structure is influenced by historical relationships. The populations having recent common ancestry are have more genetic similarity compared to those having more distant common ancestry[51].

At population level environmental variation and genetic drift affects the impact and course of natural selection that distributes the total genetic variation among the populations of a species[52]. However, many times separation of genotypes based on geographic origin has also been recorded[53]. Being a slow evolutionary process total genetic separation between the two groups takes extremely long time span. Thus, presence of most accessions from Jharkhand in a single sub-cluster emphasized their close genetic similarity to be considered as a single population.

Contrastingly, when genotypes from same state are positioned in different clusters, lacking geographical association, it signifies high level of genetic variation within the population. It may be due to use of varied seeds/clonal propagules by the farmers; mutations and/or breeding systems. Expectedly most variations have to be maintained within populations than between populations considering predominantly out-crossing nature of *M. oleifera.* It was reflected in fact that highest genetic identity was present between genotypes JRK and JRD of Jharkhand i.e. 0.8250 and the maximum genetic distance was found between the genotype OCB belonging to Orissa and WHP of West Bengal i.e. 3.4657, as recorded through SSR markers.

In cluster analysis through SSR markers the genotypes falling in the major cluster belonged to 10 different agro-climatic sub zones from four different states indicating frequent movement and exchange of germplasm as recorded in clustering patterns through RAPD markers. However, further observation at sub cluster level revealed proximity among genotypes from same geographical areas/states. For example JRK, JGB and JSC which are the accession of Jharkhand, are present in one sub-cluster but separated from Bihar accession, BPG. However, a careful observation of UPGMA analysis suggests presence of sufficient genetic variation among genotypes from different areas of eastern India.

Lack of clustering according to geographical areas in the present investigation indicates that the accessions collected are inherently not significantly different at genetic level due to evolution in similar agro climatic conditions, rapid spread of the

species through preferred clonal means, frequent seed exchange and/or significantly high gene flow between contiguous populations. This is in agreement with the prior reports involving SSR markers in India[47, 54] but oppose to certain studies where clustering has been found based on their geographic origin[21]. The pattern of clustering shown in our study may be linked to the wide spread of propagules and high rate of gene flow[41]. Reasonably outcrossing rates might vary widely within and between populations spatially as well as within a single population, temporally[55]. Contrary to it high genetic variation within population owing to the variability of the seed sources, mutation or breeding system may have resulted in clustering of individuals from the same population in different sub clusters. This conforms to the fact that *M. oleifera* is principally an out-crossed species[56].

Conclusion

The outcome of the present study reveal that both RAPD and microsatellite markers appeared as highly informative for genetic diversity assessment of among the populations of *M. oleifera* from geographically different areas in eastern India. The study also exhibits that there exists sufficient genetic variation among genotypes for selection of materials for improvement from different areas of eastern India, incidentally the native range of the species, despite the fact that *M. oleifera* has acquired status of a principally clonal propagated species in the region.

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