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Assessment of Genetic Diversity in some *Moringa oleifera* Lam. Landraces from Western Nigeria using RAPD Markers

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ABSTRACT

Genetic diversity in 10 *Moringa oleifera* Lam. landraces from Western Nigeria was assessed using 10 arbitrary random amplified polymorphic DNA (RAPD) markers. A total of 595 fragments were obtained, out of which 485 (81.5%) were polymorphic. Primer OPT-5 exhibited the least percentage of polymorphism (26.8%) compared to other primers used. Primers OPB-3, OPB-6, OPH-2, OPH-5, OPH-6, OPT-3, and OPT-4 gave 100% polymorphism. The number of amplified fragments per primer ranged from 44 (OPT-2) to 123 (OPT-5) with a polymorphic information content (PIC) ranging from 0.8301 to 0.9280. Unweighted Pair Group with Mean Average (UPGMA) clustering placed the genotypes into two major groups. 'MO2' and 'MO4' showed very close relatedness from the dendrogram obtained while 'MO10' was genetically isolated from the other genotypes. 87% dissimilarity was revealed by the dendrogram. The high degree of polymorphism obtained suggests that RAPD is very useful for genetic diversity studies in *M. oleifera*.

Keywords: dendrogram, genetic resources, genotypic characterization, *Moringaceae*, polymorphic information content, principal coordinate analysis
Abbreviations: PCoA, principal coordinate analysis; **PCR**, polymerase chain reaction; **PIC**, polymorphic information content; **RAPD**, randomly amplified polymorphic DNA; **UPGMA** unweighted pair group with mean average

INTRODUCTION

Moringa is the sole genus in the flowering plant family *Moringaceae* (Quattrocchi 2000). The most widely cultivated species is *Moringa oleifera*, a multipurpose tree cultivated throughout the tropics. *M. oleifera* also known as Drum stick tree, is a perennial plant native to tropical Africa and is also widely distributed in India, Sudan, Ceylon and Madagascar (Eilert 1978; Abdellatef and Khalafalla 2010). *Moringa* leaves are highly sought after as vegetables or taken in powdered form with meals or tea. It has been cultivated in tropical regions all over the world for the following characteristics: high protein, vitamins, mineral and carbohydrate content of entire plants; high value of nutrition for both humans and livestock; high oil content (42%) of the seed which is edible, and with medicinal uses (Awodele *et al*. 2012). The dried powdered seeds have been used, both in crude form and following extraction of their active principle through a hot percolation in petroleum ether as an effective and low-cost coagulant for removing turbidity and reducing bacterial and viral contamination from drinking water in rural communities in the Sudan, Malawi, India, Myanmar, and Indonesia (Nyein *et al*. 1997; Mandloi *et al.* 2004). Few diversity studies have been reported over the years in Asia and parts of Africa. Genotypic characterization using molecular markers to assess genetic diversity has become a paradigm to devise effective sampling strategies for pre-breeding programmes. Developing DNA markers of all the genetic resources of the medicinally and industrially important plants is a necessity for generating a molecular database to catalogue as well as to utilize the information in a systematic way (Khanuja *et al.* 2000). Molecular markers detect polymorphisms to improve the efficiency and precision of conventional plant breeding hence, possesses enormous potential to explore genetic diversity (Sarikamis *et al.* 2010). Genetic markers are used as a source of genetic information for analysis of genetic diversity in crops and indirect selection of characters for which phenotypic markers are affected by environmental conditions, stage of plant development and lack of precise evaluation methods (Kumar 1999). Muluvi *et al.* (1999) analyzed seven populations in Kenya, using amplified fragment length polymorphism (AFLP) markers; the results indicated high levels of differentiation in the populations. Random amplified polymorphic DNA (RAPD) is one of the molecular tools deployed especially in the characterization of genetic resources for rapid quantification of diversity (Silveira *et al*. 2009). It was first developed by Williams *et al*. (1990). RAPD markers are inexpensive, rapid and utilize arbitrary primers with no need of initial sequence information. Though a dominant marker, it is still useful for genetic diversity studies in orphan or underutilized plants like *Moringa*. RAPD markers were used to estimate the diversity and separation of cultivars in *Morinda* species (Singh *et al.* 2011), in *Moringa* from coastal regions of Tanzania (Mgendi *et al.* 2010) and germplasm bank in Brazil (Cruz da Silva *et al*. 2012). Genetic diversity study on *M. oleifera* in Nigeria has only been reported by Abubakar *et al.* (2011) in 75 accessions collected from the Sudan and Guinea savannah zones of northern Nigeria analyzed using RAPD markers. High degree of polymorphism (74%) was obtained indicating the effectiveness of RAPD markers for genetic diversity studies especially in orphan or underutilized crops and trees. Limited information is available on the genetic diversity of *M. oleifera* in Nigeria especially as regards genetic conservation and utilization in the South-Western regions of the country. Morphological and molecular characterizations are required for effective improvement for better utilization. The objectives of this research therefore, were to assess the genetic diversity within some cultivated

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Fig. 1 Phenotypic variability in leaf size, color and stem pigmentation in *Moringa oleifera* landraces studied.

M. oleifera landraces from Oyo town and Ile Igbon town in Ibadan Western Nigeria to see if there is any genetic relatedness as well as to evaluate ten arbitrary RAPD primers for genetic diversity assessment. The information obtained from this study will be used to develop effective strategies for the conservation and utilization of *Moringa* genetic resources in Nigeria.

MATERIALS AND METHODS

Source of materials

This study was conducted in the Bioscience Center of the International Institute of Tropical Agriculture IITA Ibadan and the Biotechnology laboratory of Bells University of Technology, Ota. Young leaves from 10 landraces of *M. oleifera* of the same age (16 months) from the apex of the trees were collected from a farmer's *Moringa* field at Ibadan containing genotypes from Oyo town and Ile Igbon town in Ibadan, Oyo State, Nigeria (**Table 1**).

Table 1 Area of collection of *Moringa oleifera* Lam. landraces in Western Nigeria.

Moringa oleifera landraces	Area of collection	City
MO1	Ile Igbon	Ibadan
MO2	Ile Igbon	Ibadan
MO3	Ile Igbon	Ibadan
MO4	Ile Igbon	Ibadan
MO5	Ile Igbon	Ibadan
MO6	Ile Igbon	Ibadan
MO7	Oyo	Oyo town
MO8	Oyo	Oyo town
MO9	Oyo	Oyo town
MO10	Oyo	Oyo town

DNA extraction

A modified Dellaporta (1983) DNA extraction protocol devoid of chloroform-isoamyl-alcohol steps was used. Young leaf samples were collected from the field with an ice box and 100-200 mg of each fresh young leaves was weighed prior to DNA extraction. Leaf samples were thoroughly ground in liquid nitrogen, mixed with 700 µl of preheated extraction buffer (50 mM EDTA, 500 mM NaCl, 100 mM Tris-HCl pH 8.0, SDS (1.25%), freshly prepared 0.07% β-mercaptoethanol and 1% (w/v) PVP [MW 40000] incubated at 65°C for 30 min using water bath with occasional mixing, allowed to cool for approximately 1 min before adding 400 µl of ice-cold potassium acetate to each tube with gentle inversion. After centrifugation at 12000 revolutions per minute

Table 2 Polymorphism obtained from 10 RAPD primers in *M. oleifera* Lam.

Primers	Sequences	Amplified bands	Polymorphic bands	% Polymorphism	PIC
OPB-3	CATCCCCCTG	50	50	100	0.8872
OPB-6	TGCTCTGCCC	50	50	100	0.8600
OPH-2	TCGGACGTGA	63	63	100	0.9050
OPH-5	AGTCGTCCCC	56	56	100	0.9292
OPH-6	ACGCATCGCA	48	48	100	0.9253
OPH-8	GAAACACCCC	57	47	82.5	0.8950
OPT-2	GGAGAGACTC	44	34	77.3	0.8301
OPT-3	TCCACTCCTG	51	51	100	0.9243
OPT-4	CACAGAGGGA	53	53	100	0.9230
OPT-5	GGGTTTGGCA	123	33	26.8	0.9280
Polymorphism (average)				81.5	
Total		595	485		

PIC: polymorphic information content

(rpm) for 15 min, the supernatant was transferred to fresh tubes without disturbing the interface. 200 µl of ice-cold isopropanol was added to the supernatant and mixed by inverting the tubes 2-5 times to precipitate the DNA and subsequently kept at -80°C for 1 h. The DNA was pelleted down by centrifugation at 12000 rpm for 10 min and the dried DNA pellets obtained were re-suspended in 200 µl of GIBCO water (Invitrogen, Carlsbad, CA, USA) and 2 µl of 10 mg/ml RNase (Qiagen Valencia, CA, USA) was added to each of the samples and kept at 4°C for 24 h to get rid of RNA. In the spectrophometric readings using NanoDrop (ND-1000), 1.5 µl of the extracted DNA samples was used to obtain 1.8-2.0 ratio at 260/280 absorbance level and concentration through which working dilutions were prepared for polymerase chain reaction (PCR). On 1.5% agarose gel for electrophoresis, 2 µl of the stock DNA samples were loaded and visualized under a UV light Transilluminator (Model-2, Upland, CA, USA) to check the quality of the extracted DNA molecules.

RAPD PCR amplification

Ten arbitrary RAPD decamer primers obtained from Operon Technology (Almeda, CA, USA) were used for PCR amplification (**Table 2**). PCR amplification was performed in 25 µl total volume containing 2.0 µl genomic DNA (100 ng/ul), 2.5 µl of 10X PCR buffer, 1.2 µl $MgCl_2$ (50 mM), 2.0 µl of dNTPs (2.5 mM), 0.2 µl BIOTAQ™ DNA polymerase (Bioline, Massachusetts, USA), 1.0 µl of each primer (10 ρmol), 1.0 µl of dimethyl sulfoxide (Duchefa, Haarlem, The Netherlands) and 15.1 µl of GIBCO water (Invitrogen, Carlsbad, CA, USA). PCR amplifications were performed using Applied Biosystems thermocycler at an initial denaturation temperature of 94°C for 3 min (1 cycle), denaturation at 94°C for 20 s, annealing at 38°C for 35 s and extension at 72°C for 1 min, back to 94°C for 20 s for 44 cycles followed by final extension at 72°C for 5 min. Amplified fragments were separated electrophoretically on 1.5% (w/v) agarose (Sigma Aldrich, USA) gels with 1X TBE (Tris-Boric acid-EDTA) buffer and stained with ethidium bromide (0.5 mg/ml). The molecular fragments were estimated using 100-bp step DNA marker (Biolabs, New England). Each sample was run twice for PCR amplification and only reproducible, relatively intense bands were scored.

Analysis of RAPD profiles

Data matrix of RAPD profiles for fragments of similar molecular weight from each individual were scored as present (1) or absent (0). The data obtained from scoring the RAPD bands were subjected to genetic similarity matrix using Jaccard's similarity coefficient (Jaccard 1908). Phylogenetic relations were determined by cluster analysis using UGPMA (unweighted pair-group method with arithmetic averages) with the NTSYS-pc software version 2.02 (Rohlf 1998). Multivariate grouping was done using principal coordinate analysis (PCoA) with DARwin software version 5.0.0.157 while polymorphic information content (PIC) was calculated using the method of Botstein *et al*. (1980).

RESULTS AND DISCUSSION

This research was conducted to assess the degree of genetic diversity amongst 10 landraces of *M. oleifera* Lam. from Western Nigeria to determine if there were genotypes that could be used for genetic improvement of the crop or if they were related. The degree of success in the genetic improvement of any crop species to a large extent depends on the amount of genetic diversity existing among advanced and primitive cultivars as well as their wild relatives. Such variation would make up a valuable source of parents for hybridization and subsequent development of improved cultivars. Cultivars with a great amount of diversity, if collected and appropriately evaluated, would provide a great likelihood of promising genetic rearrangement for breeders and selecting and disseminating most appropriate plants (Bainiwal and Jatasra 1980; CIRAD 1999). Analysis of 10 *M. oleifera* genotypes with 10 arbitrary RAPD decamer primers in this study identified a total of 595 fragments of which 485 were polymorphic (81.5%) (i.e., two or more

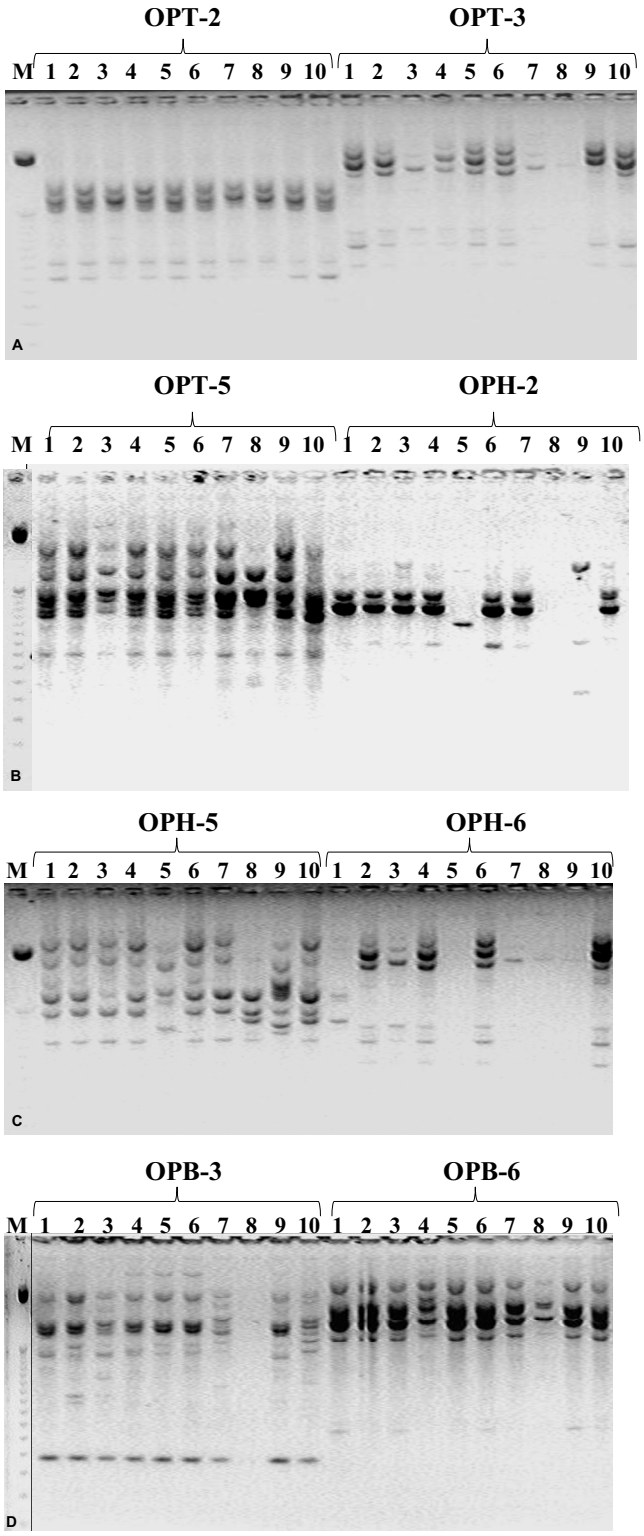

Fig. 2 RAPD banding profiles of ten *Moringa oleifera* landraces using 8 primers. (**A**) OPT-2 and OPT-3, (**B**) OPT-5 and OPH-2, (**C**) OPH-5 and OPH-6, (**D**) OPB-3 and OPB-6. Lanes: M, 100 bp markers; Lanes 1-10 represent respective primer sets.

discontinuous fragments or variants regularly and simultaneously in the same population between two or more genotypes). Representative profiles of RAPD products (amplified with primers OPT-2, OPT-3 OPT-5, OPH-2, OPH-5, OPH-6, OPB-3 and OPB-6) from all ten *Moringa* genotypes is shown (**Fig. 2A-D**). All the selected primers amplified fragments across the 10 genotypes studied, with the number of amplified fragments per primer ranging from 44 (OPT-2) to 123 (OPT-5). Polymorphic fragments were generated by each of the primers. Polymorphic information content (PIC) values ranged from 0.8301 to 0.9280 (**Fig. 3**). The percen-

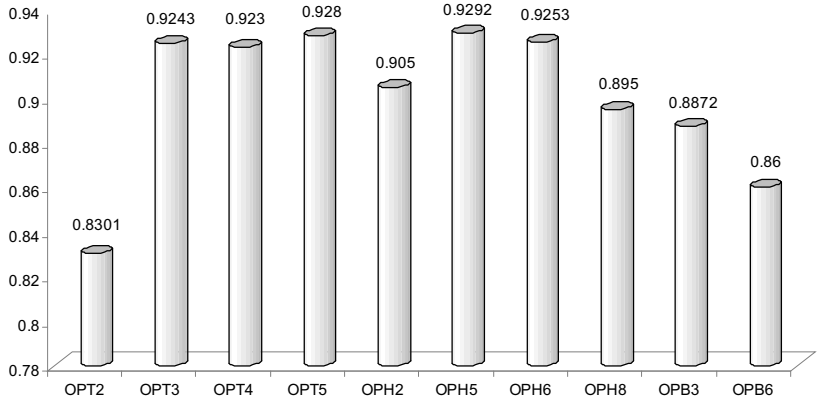

Fig. 3 PIC for 10 RAPD primers.

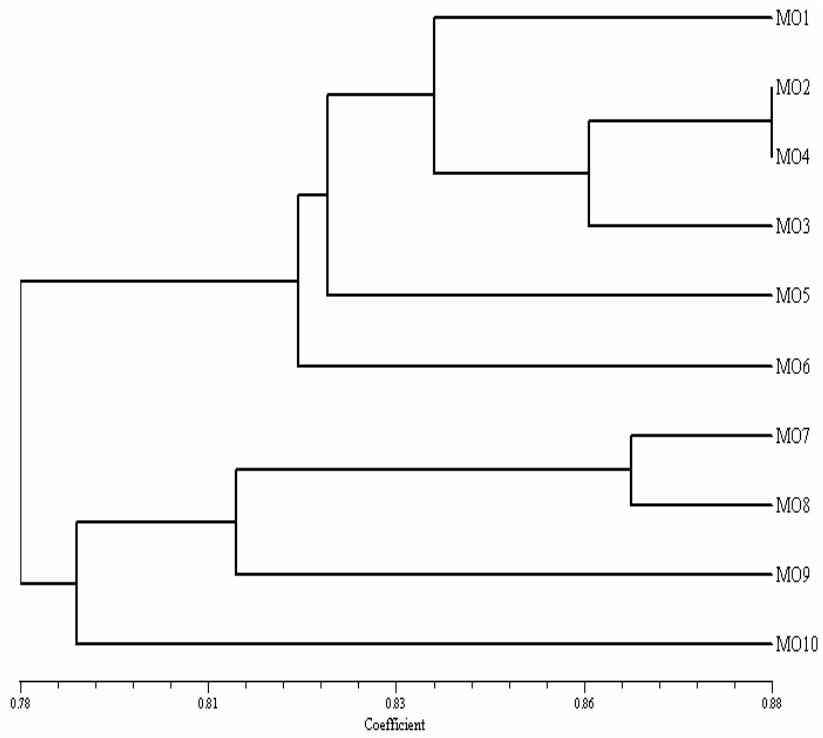

Fig. 4 Dendrogram showing genetic diversity in 10 *Moringa oleifera* cultivars.

tage of polymorphic amplicons varied from 26.8 to 100%. OPT-5 RAPD primer gave the least level of polymorphism (26.8%) while OPB-3, OPB-6, OPH-2, OPH-5, OPH-6, OPT-3, and OPT-4 gave the highest polymorphism (100%). The polymorphisms revealed by the 10 decamer primers indicate that they are good and reliable for genetic diversity assessment in *M. oleifera* and there is a high degree of diversity in the landraces studied. The polymorphism obtained in this study (81.5%) is very high compared to that obtained in some other studies. In a study to determine the genetic diversity in 16 accessions from the germplasm bank of Brazil, 17 RAPD primers generated a total of 95 fragments of which 59 (62%) were polymorphic (Cruz da Silva *et al.* 2012). In another study by Mgendi *et al.* (2010) on diversity studies in cultivated and non-cultivated *M. oleifera* in Tanzania, 89.6% polymorphism was obtained from 86 fragments using 12 RAPD primers while Abubakar *et al.* (2011) obtained 74% polymorphism using 24 RAPD primers from genetic diversity studies on *Moringa* in northern Nigeria (**Table 3**). AFLP markers, for diversity studies in *Moringa* in Kenya and India populations were only reported by Muluvi *et al*. (1999) in which 157 (66.5%) fragments out of 236 amplification products were polymorphic. All of these reports indicate the genetic potential of *M. oleifera*. Polymorphism in a given population is often due to existence of genetic variants represented by the number of alleles at a locus and their frequency of distribution in a population. According to UPGMA clustering taking into account the presence (1) or absence (0) of the bands, two distinct groups were identified at 0.78. The first group (G1) was formed by six genotypes; 'MO1'. 'MO2', 'MO3', 'MO4', 'MO5', and 'MO6' while the second group (G2) by four genotypes; 'MO7', 'MO8', 'MO9', and 'MO10'. RAPD markers can measure the polymorphisms between the genomes of two organisms of the same species. Two closely related organisms would be expected to yield more similar banding patterns than two organisms that are distant in evolutionary terms (Miesfeld 1999). When the bands are converted into binary matrix and grouped with UPGMA, the closely related genotypes are placed together. In G1 there were 4 subgroups of which 'MO2' & 'MO4' showed close relatedness (**Fig. 4**) and 'MO6' was genetically isolated from the others. In G2, 'MO10' stood totally alone, indicating that this genotype is the most genetically distant from others. From the results of morphological characterization, it had the largest leaf size and could be useful for genetic improvement of the crop with other genotypes (**Fig. 1**). Genotypes 'MO10' and 'MO9' were also genetically isolated. The dendrogram revealed about 87% genetic variability in the 10 landraces studied. This percentage is higher than that obtained by Abubakar *et al*. (2011) using a different set of RAPD primers. The RAPD primers used for

Table 3 Comparison of genetic diversity studies in *Moringa oleifera* Lam.

Number of genotypes studied	Country	RAPD primers used	% Polymorphism	Researchers
96	Tanzania	KFP-1, KFP-3, KFP-4, KFP-5, KFP-6, KFP-7, KFP-8, KFP-9, KFP10, KFP 11, KFP-13, KFP-21	89.6%	Mgendi *et al.* 2010
75	Northern Nigeria	OPA-7, OPA-8, OPA-10, OPA-13, OPA-17, OPA-18, OPA-19, OPA-20, OPB-17, OPB-20, OPF-20, OPH-19, OPM-6, OPM-8, OPO-2, OPO-3, OPO-13, OPQ-2, OPBB-7, OPBC-2, OPBC-10, OPBD-18, OPBD-19, POU-17	74%	Abubakar *et al.* 2011
16	Brazil	A3, A4, A8, A12, A15, A16, A18, IDT02, IDT3, IDT15, S01, S18, W02, W13, W19, B02, B18	62%	Cruz da Silva *et al*. 2012
10	Western Nigeria	OPB-3, OPB-6, OPH-2, OPH-5, OPH-6, OPH-8, OPT-2,OPT-3, OPT-4, OPT-5	81.5%	Present study Ojuederie *et al.*

RAPD: random amplified polymorphic DNA

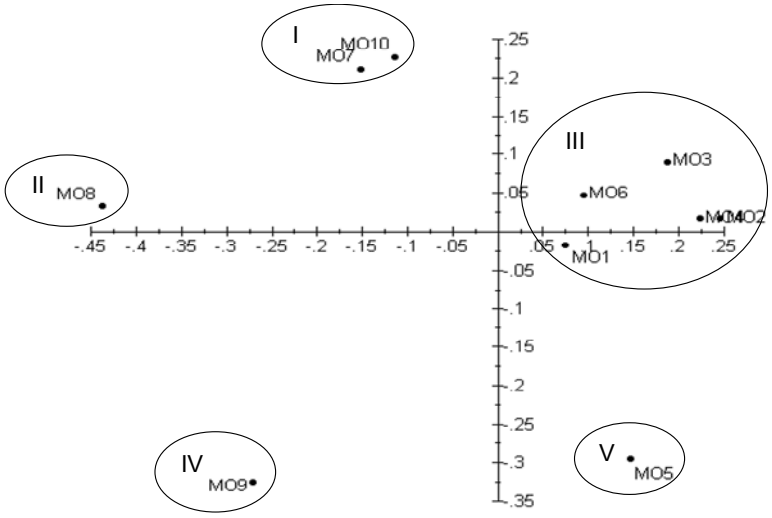

Fig. 5 Principal Coordinate Analysis (PCoA) obtained from Jaccard's similarity coefficient using 10 RAPD markers between 10 landraces from moringa (*Moringa oleifera* Lam.).

this study have the ability to detect more polymorphisms compared to those used by other researchers on diversity study in *M. oleifera*. Genetic diversity ranged from 0.78 to 0.87 (**Fig. 4**). Principal coordinate analysis (PCoA) grouped the *Moringa* landraces into five groups. 'MO7' and 'MO10' (G1), 'MO8' (G2), 'MO1', 'MO2', 'MO3', 'MO4' and 'MO6' (G3), 'MO9' (G4) and lastly, 'MO5' (G5). 'MO2' and 'MO4' are very much related compared to other Ibadan genotypes (**Fig. 4**). The genotypes 'MO5' from Ibadan, 'MO8' and 'MO9' from Oyo were genetically isolated and the information generated can be used to design strategies for improvement and conservation of germplasm in future breeding programs of the species. Unlike the results in the dendogram where 'MO10' was isolated, principal coordinate analysis linked it to 'MO7' (**Fig. 5**). Principal coordinate analysis also placed the landraces from the two locations on separate axes. 'MO8' and 'MO9' from Oyo were genetically isolated while 'MO5' was genetically isolated amongst the six landraces from Ibadan. It however showed clearly that 'MO2' and 'MO4' are very much related compared to other genotypes from Ibadan. This was also similar with the result from the dendrogram (**Fig. 5**).

CONCLUSION

Molecular markers are indispensable tools for measuring the diversity of plant species. The RAPD molecular markers used in this study revealed 81.5% polymorphism, indicating a high degree of diversity in the *Moringa* landraces studied. For breeding purposes, the genotypes in the two clusters presented by the dendrogram could be used especially 'M010' which was genetically isolated from other genotypes with a useful attribute of possessing broad leaves which is important for industries that utilize the *Moringa* leaves. The RAPD primers used in this study especially OPB-3, OPB-6, OPH-2, OPH-5, OPH-6, OPT-3, and OPT-4 are efficient in generating polymorphisms in *M. oleifera* and could be used for diversity studies in uncharacterized genotypes of *Moringa* and for selection of parents for breeding and integration into marker-assisted selection (MAS).

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