SIMPLE SEQUENCE REPEATS (SSR) ANALYSIS OF GENETIC INTRASPECIFIC RELATIONSHIPS OF *MORINGA OLEIFERA* POPULATIONS FROM NIGERIA

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ABSTRACT: *Moringa oleifera Lam. (Moringaceae) is a multi-purpose economic plant whose commercial demand is on the increase in Africa. To meet this demand, it is significant to step up collections and diversity studies adaptable to higher productivity and utilization. The present study focuses on recently collected landraces/accessions of six populations of M. oleifera from different eco-geographical locations within Nigeria. A total of 70 accessions were evaluated for genetic intraspecific diversity using 20 SSR markers. Among the 20 SSR markers screened, 10 primer pairs (forward and reverse) were selected based on clear amplification products and reproducible scorable bands. Analysis of Molecular Variance (AMOVA), Principal Coordinates Analysis (PCoA) and cluster analysis (CA) were used to evaluate the genetic intraspecific diversity. A total number of 74 alleles with a range of 4 to 15 were detected among the 70 accessions. On the average, 7.4 alleles per locus were amplified in each accession. Allele frequency varied from 0.214 to 0.671 with a mean of 0.477; gene diversity from 0.487 to 0.885 with a mean of 0.669 while the average PIC value was 0.633. The observed and expected heterozygosity varied from 0.00 to 0.50 with a mean of 0.972 and from 0.00 to 0.250 with a mean of 0.567, respectively. AMOVA shows that 8 % of the genetic diversity was attributed to differences among the populations while 92 % of the variation (significant at p = 0.001) was due to differences within populations. Allelic patterns across the six populations aligned with the AMOVA result. The results of PCoA and CA identified high intraspecific similarities with few exceptions. Similarity coefficients (SC) of CA ranged from 0.53 to 1.00 and delineated the 70 accessions into seven groups. All accessions are distinguishable from each other at SC 1.00 except (soN066 and taN085) and (anN045 and anN047). The genetic relationships highlighted are significant for conservation, cultivation and genetic improvement of M. oleifera in view of the species socio-economic relevance to the people of Nigeria and Africa in general.*

Key word: *Moringa oleifera;* SSR; genetic intraspecific diversity; similarity coefficient (SC); Nigeria.

INTRODUCTION

Moringa oleifera Lam. is one of the most economic and cultivated species in the single genus *Moringa* of the (Moringaceae) family [1, 2]. It is a tree crop with enormous potential capable of contributing to improved food security and nutrition, medicine and health care, incomes and environment in Africa [3]. In recent time, various products including health care products such as Moringa organic powder, capsules, leaf tea, oil extracts among others have been prepared from the leaves, pods and seeds of *M. oleifera*. These confirms the significance of its medicinal, nutritional, food, phytochemicals and various economic values to the socio-economic lives of the people particularly in the Sub-Sahara Africa (SSA). Several authors have also reported the nutritional, food, medicinal, commercial and agricultural uses of the crop [3, 4, 5, 6]. Anwar *et al.* [7] reviewed detailed phytochemical composition, medicinal uses as well as pharmacological properties to include antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antihypertensive, cholesterol lowering, antioxidant, antibacterial among others.

In Nigeria, the increasing awareness on the economic values and usefulness of *M. oleifera* has led to the distribution or spread of landraces/ecotypes to different locations [3] even though there are little or no conservation management strategies for the present and future use. Recent database search by Leone *et al*. [5] also specified that there are no records of active germplasm banks worldwide on *M. oleifera* to represent 'core collections' of the taxa. The concept of 'core collections' represents the genetic diversity with a minimum duplication of accessions was introduced to effect a good and robust management of genetic resources for conservation and breeding purposes [8, 9]. In view of this, it is pertinent to step up genetic studies via germplasm collections and characterizations to possibly create core collections of *M. oleifera* in the near future. Also, to meet a stable and commercial demand for Moringa products and other economic values derivable from the crop, it is significant to intensify diversity studies to create variants adaptable to local needs. This is justifiable since genetic diversity is the key determinant of germplasm utilization in crop improvement [10]. Presently, the existing germplasms in Nigeria can be regarded as landraces/accessions with no elite varieties adapted to local conditions.

M. oleifera is a geitonogamous and xenogamous diploid species (2n = 28; n = 14 chromosomes) whose gene pool and genetic base are expected to be wide with higher productivity [11, 12, 13, 14]. Reports, however, are contrary to the above as gene pool/ genetic base is narrow/weak with unknown gene pool among cultivated and wild species [13, 15]. The genetic bases and relationships among the different ecotypes/landraces in Nigeria are still limited and very unclear though few genetic characterizations have been investigated based on phenotypic and molecular markers [15, 16, 17]. Hence, it is significant to further evaluate the genetic

relationships and provide useful information for the management and conservation of the genetic resources of the taxa towards breeding and improvement.

DNA based characterizations could provide additional information on the degree of diversity, genetic / eco-geographical relatedness of the collected landraces/accessions, avoid duplication of germplasms and maximize diversities. Globally, different DNA techniques including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSR), chloroplast gene rcbL among others have been used to assess the genetic diversities among the populations / some accessions of *M. oleifera*. Muluvi *et al*. [18] and Ulloa [19] used AFLPs to evaluate diversity among and within populations of *M. oleifera* from Kenya. High degree of genetic variations between cultivated and non-cultivated populations of *M. oleifera* from Tanzania was reported by Mgendi *et al*. [20] using RAPD. Da Silva *et al*. [21] also used RAPD markers to assess the genetic diversity of 16 Moringa accessions from Brazil. So far, very few studies are available where SSR markers have been used to assess genetic diversity of *M. oleifera* [22, 23, 24]. However, such studies did not cover West Africa nor include accessions from Nigeria. The studies of Shahzad *et al*. [22] combined SSR with a partial sequence of the chloroplast gene atpB to investigate genetic diversity and population structure of *M. oleifera* which covered a wide range of collections from Asia, Africa, North and South America and the Caribbean. Such studies have not been carried out on populations of *M. oleifera* in Nigeria. In the studies of Ganesan *et al*. [23] and Natarajan and Aslin-Joshi [24] both morphological and molecular markers were combined to assess genetic diversity among *M. oleifera* accessions restricted to Indian populations. SSR markers specific to *M. oleifera* were first developed by Wu *et al*. [25] which have been recommended as useful markers for detail genetic population studies and pollen-mediated gene flow within populations. SSR is used as a primer to amplify regions between microsatellites. The analyses of SSRs are highly polymorphic and reproducible with small quantities of template DNA, inherited co-dominantly, and particularly for the abundance distribution of repeat sequences throughout genomes [26, 27, 28]. SSRs over the years have found practical applications for evaluation of molecular diversity and germplasm classification of underutilized crops [28]. Assessment of genetic diversity is crucial for efficient *in situ* and *ex situ* conservation of the taxa to which SSRs have been proven to be most suitable because of ability to detect hyper variable allelic variations [29, 30, 25]. Therefore, the study was aimed at using SSR to analyze levels of genetic diversity within and among six populations of 70 accessions of *M. oleifera* collected from different locations in Nigeria. The study was also undertaken to provide information on genetic relationships based on differentiation of populations and clusters; and come up with strategies to adopt for conservation, management, breeding and genetic improvement of the species.

MATERIALS AND METHODS

Plant samples and Areas of Collection

A total of 70 accessions of *Moringa oleifera* were pooled from the survey and geographical distribution [3] and phenotypic intraspecific variability studies of *M. oleifera* in Nigeria [6]. Information about study sites, acquisition and passport data of plant materials/accessions are as described by Popoola and Obembe [3] and Popoola *et al*. [6]. The detail of accessions, codes and geographic source of the *Moringa oleifera* accessions used for this study are as listed in Table 1. Figure 1 shows the map of collection areas of the *M. oleifera* samples used for this study.

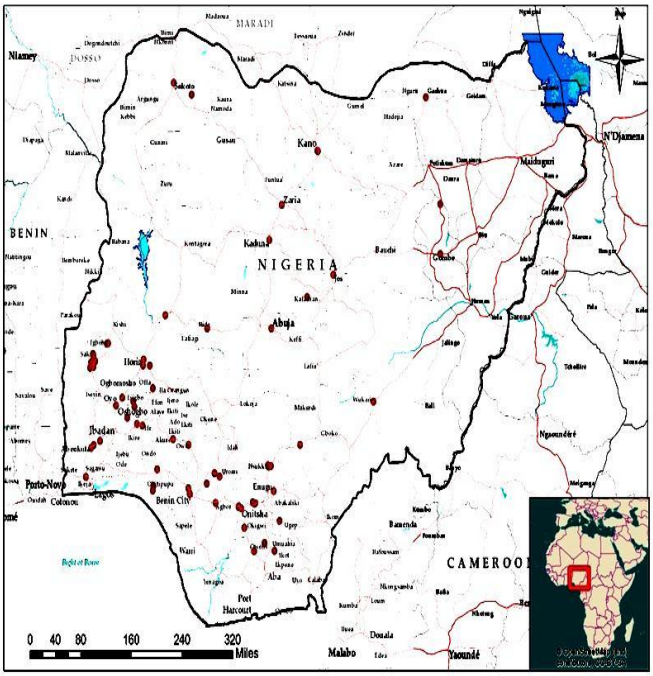

Figure 1. Map of Nigeria showing the areas of collection of *M. oleifera* samples used for this study. Scale in miles reflect sampling areas

Table 1. List Of Accessions, Codes, Area Of Collection And State Within Nigeria Where Samples Were Collected.

S/N	Accession Code	Area of Collection	L/G	State
1	abN057	Okpanku	Umuonoji	Abia
2	abN059	Umudike	Umudike	Abia
3	anN049	Enu Ifite	Awka	Anambra
4	anN051	Ihiala	Ihiala	Anambra
5	anN046	Unizik	Awka	Anambra
6	beN081	Otukpo	Otukpo	Benue
7	deN041	Asaba	Oshimili South	Delta
8	edN035	Ehanlen-Ewu	Esan Central	Edo
9	edN040	Ugbokwi	Ovia South	Edo
10	edN037	Benedicta Monastry	Esan Central	Edo
11	enN055	Ugwuomu	Enugu South	Enugu

12	enN053	Orba1	Nsukka	Enugu
13	goN068	Nafada	Nafada	Gombe
14	imN064	Ahiazu Mbaise	Mbaise	Imo
15	kaN033	Tudan Wada	Zaria	Kaduna
16	knN078	Kofar kudu	Kano Municipal	Kano
17	knN077	Army Barracks	Kano	Kano
18	kwN016	Igosun	Oyun	Kwara
19	kwN015	Ilorin	Ilorin West	Kwara
20	niN018	Bida	Bida	Niger
21	ogN025	Abeokuta	Abeokuta South	Ogun
22	ogN028	Covenant Univ	Ado-Odo	Ogun
23	ogN076	Kila	Odeda	Ogun
24	ogN026	Olodo	Odeda	Ogun
25	onN070	Owena road		Ondo
26	onN074	Ondo - Ore road		Ondo
27	onN072	OsusTech	Okitipupa	Ondo
28	osN019	Ipetumodu	Ife North Central	Osun
29	osN020	OAU	Ife North Central	Osun
30	osN024	Owode	Ede	Osun
31	oyN003	Aroje	Atisbo	Oyo
32	oyN001	Oke-oro / Saki	Saki West	Oyo
33	oyN005	Aba Oremeta / Irawo	Atisbo	Oyo
34	oyN004	Sango, Ago Are	Atisbo	Oyo
35	oyN009	Alariwo Village / Igboho	Oorelope	Oyo
36	oyN010	Okaka	Itesiwaju	Oyo
37	plN030	UniJos	Jos	Plateau
38	soN066	Sokoto	Sokoto	Sokoto
39	taN085	Wukari (Fed. Univ)		Taraba
40	yoN031	Bukarti / Karasuwa	Karasuwa	Yobe
41	kaN032	Kafanchan	Kafanchan	Kaduna
42	kaN034	Sabongari		Kaduna
43	niN017	Mokwa	Mokwa	Niger
44	soN067	Shagari	Shagari	Sokoto
45	goN069	Gombe		Gombe
46	kwN075	Unilorin	Ilorin East	Kwara
47	kwN014	Sobi	Ilorin West	Kwara
48	abN079	Wuse	Wuse	Abuja
49	oyN004	Agoare	Atisbo	Oyo
50	oyN007	Alakuko	Oorelope	Oyo
51	oyN012	Iseyin	Iseyin	Oyo
52	oyN029	Araromi	Oyo	Oyo
52	imN063	Obowo		Imo
53	imN065	Obowo		Imo
54	osN021	Iwo	Iwo	Osun
55	osN022	Ejigbo	Ejigbo	Osun
56	osN027	Owode		Osun
57	edN036	Agbede	Etsako West	Edo
58	edN038	Uniben	Benin City	Edo
59	edN039	Uselu	Egor	Edo
60	deN042	Parkinson	Oshimili South	Delta
61	deN044	Agbor		Delta
62	anN045	Onitsha	Onitsha	Anambra
63	anN047	Infite	Awka	Anambra
64	anN048	Aniocha	Aniocha	Anambra
65	enN052	UNN	Enugu South	Enugu
66	enN054	Orba2	Awka	Enugu
67	onN071	Idepe	Okitipupa	Ondo
68	onN073	Owo	Owo	Ondo
69	oyN080	Irawo-ile	Atisbo	Oyo
70	oyN011	Alagutan	Oorelope	Oyo
49	oyN004	Agoare	Atisbo	Oyo
50	oyN007	Alakuko	Oorelope	Oyo
51	oyN012	Iseyin	Iseyin	Oyo
52	oyN029	Araromi	Oyo	Oyo
52	imN063	Obowo		Imo
53	imN065	Obowo		Imo
54	osN021	Iwo	Iwo	Osun
55	osN022	Ejigbo	Ejigbo	Osun
56	osN027	Owode		Osun
57	edN036	Agbede	Etsako West	Edo
58	edN038	Uniben	Benin City	Edo
59	edN039	Uselu	Egor	Edo
60	deN042	Parkinson	Oshimili South	Delta
61	deN044	Agbor		Delta
62	anN045	Onitsha	Onitsha	Anambra
63	anN047	Infite	Awka	Anambra
64	anN048	Aniocha	Aniocha	Anambra
65	enN052	UNN	Enugu South	Enugu
66	enN054	Orba2	Awka	Enugu
67	onN071	Idepe	Okitipupa	Ondo
68	onN073	Owo	Owo	Ondo
69	oyN080	Irawo-ile	Atisbo	Oyo
70	oyN011	Alagutan	Oorelope	Oyo

SAMPLE PREPARATION

Young fresh leaf samples of the 70 accessions were harvested and lyophilized for three days and stored at - 20°C at Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria.

DNA extraction and Quantification

The native DNA was extracted using Cetyl Trimethyl Ammonium Bromide (CTAB) procedure described by FAO/IAEA [31]. The DNA was visually quantified using NanoDrop Spectrophotometer (Nanodrop Technologies, Inc. Wilmington, DE, U.S.A) at 230, 260 and 280 nm, on 1.5 % agarose gel. DNA samples were stored at - 20°C until use.

Source of SSR Primers

Twenty SSR polymorphic microsatellite markers specific to *M. oleifera* [25] were adopted and used for this study. The 40 oligonucleotides (20 bases F/R) with code number (IBOL0001) were synthesized and supplied by Inqaba biotechnical Industries (Pty) Ltd, South Africa.

PCR amplification reaction

The PCR reactions of 10 µl contained 3.0 µl of native DNA (100 ng / µl), 1.0 µl of 10 X PCR buffer, 0.4 µl of $MgCl_2$ (50 mM), 0.5 µl each of SSR primer mix (Forward and Reverse primers, 5 µM), 0.8 µl of DNTPs (2.5 mM), 0.8 µl of DMSO, 0.1 µl of taq polymerase (5 u/ul) and 2.9 µl of sterile double distilled water. The amplification reaction was performed using te Applied Biosystems thermal cycler (GeneAmp PCR system 9700, USA) with the following programs; initial denaturation at 94.0°C for 5 minutes, final denaturation at 94.0 °C for 15 seconds, annealing at 65.0°C for 20 seconds and extension at 72.0°C for 30 seconds (9 cycles). The reactions also followed another 35 cycles of 94.0°C for 15 seconds, 55.0°C for 20 seconds, 72.0°C for 30 seconds and a final extension at 72.0°C for 7 minutes. The PCR products were resolved on 1.5 % agarose gel and visualized using the automated trans-illuminator (ENDURO GDS) with digital camera compatible with Window/Vista. A 1000 bp ladder plus generuler (Thermo Scientific) was used to determine band sizes.

SSR PAGE Analysis

The amplified products were resolved on 6 % (w/v) polyacrylamide gel electrophoresis (PAGE) for 2.5 hours in 1 X Tris/borate/EDTA buffer with 7.5 M urea at 70 W according to the manufacturer's protocol. The gels were stained with silver nitrate [32, 33]. The size of DNA bands in base pairs was estimated using 1000-bp ladder (Thermo Scientific®). Gels output files were saved as TIFF format for scoring and analysis.

Statistical Analysis

Each SSR fragment was scored for their presence (1) /absence (0), size and polymorphisms. PowerMarker software program [34] was used to determine Nei [35] gene diversity, Shannon information index [36], number of alleles (Na), expected heterozygosity (*HE*) and observed heterozygosity (*Ho*). Allelic Polymorphic Information Content (PIC) and fixation index were estimated. Genetic similarity and genetic distance estimated by Nei's coefficient between pairs were analyzed using Popgene software version 3.5 [37]. The total numbers of alleles, number of alleles with a frequency of < 5 %, the number of private alleles, number of alleles found in more than 25 % and 50 % of the accessions from sub-groups according to source of samples, mean diversity and expected and unbiased expected heterozygosity were evaluated using GenAlEx 6.501 [38]. Analysis of Molecular Variance (AMOVA) using data matrix to partition the genetic variation into, within and among the populations' components was also determined using GenAlEx software [38]. Principal coordinate analysis (PCoA) and scores for the first and second components were plotted using Minitab software. Genetic similarity between different accessions were estimated based on Jaccard's similarity (J) coefficient using a SIMQUAL programme of NTSYSpc v. 2.20 [39]. Jaccard's similarity coefficients of different accessions were also used to construct UPGMA dendrograms for SSR markers using SAHN programme of NTSYSpc v. 2.20 [39].

RESULTS

SSR Primers Genetic Information/Polymorphisms

In this study, twenty polymorphic microsatellites [25] were used to analyze the intraspecific diversity among 70 accessions of *Moringa oleifera*. Ten SSR primers did not satisfactorily reflect/show clear polymorphisms among the accessions, and as such were not included in the analysis. The sequences and repeat motifs of the primers used are shown in Table 2 while Table 3 shows the summary of genetic estimates of the SSR primers used for this study. The primers generated polymorphic bands, which varied in size from 100 bp to 460 bp. A total of 74 alleles was detected in all the 70 accessions of *M. oleifera* with a mean value of 7.4 alleles per locus for each accession. Among these 74 alleles, 5 % were considered as rare (showed an allele frequency of < 5 %) (Table 3 and 4). The numbers of alleles per locus ranged from 4 in locus MO18 and locus MO61, to 15 in locus MO15 with an average of 7.4. Major allele frequencies observed varied and ranged from 0.214 for locus MO15 to 0.671 for locus MO13 and locus MO58, with a mean value of 0.477. Generally, the genetc diversity was high ranging from 0.487 for locus MO13 to 0.885 for locus MO15 with a mean value of 0.669. The polymorphic information content (PIC), which represents the allele diversity for a specific locus ranged from 0.430 for MO13 to 0.875 for MO15 with an average mean value of 0.633. The most informative markers were primers MO6, MO8, MO12, MO15 and MO46 with PIC values of 0.676, 0.782, 0.820, 0.875 and 0.701, respectively. For markers with higher PIC values, frequent alleles occurred in ≤ 45 % of the accessions. The observed heterozygosity per primer ranged from 0.00 in MO18, MO16 and MO48, to 0.500 in MO12 with an average of 0.972. The expected heterozygosity per primer ranged from 0.000 to 0.250 with an average of 0.567 while Shannon information index (*I*) ranged from 0.000 in locus MO13 to 0.347 in locus MO12 with a mean value of 0.048 (Table 3). The markers were more informative than the others with respect to allelic patterns across the populations in the assessment of the genetic diversity among the 70 accessions. For instance, locus MO6 and MO8 recognized 3 more private alleles each with frequencies of 0.083 and 0.250 for North-central and South-west accessions, while locus MO12 identified 5 private alleles with a frequency of 0.50 for South-south accessions. However, marker with locus MO15 detected a higher number of private alleles (10) and recorded the frequency of 0.834 for South-west accessions.

Allelic Pattern Across the Sub-groups/populations

Mean allelic patterns across the sub-groups/populations, according to the source of samples were also estimated to determine allele's variability among the populations. The numbers of different alleles (Na) were similar for North-central, Northeast, North-west, South-south and South-east subgroups with 0.80, 0.70, 0.90, 0.80 and 0.90, respectively, while South-west sub group/population was higher with 1.50 (Table 5). The analysis showed that only the South-west collections detected a higher number of different alleles (*Na*

= 1.50), effective alleles (*Ne* = 1.32) and private alleles (0.40). For Shannon Information Index (I), 0.02 was recorded for North-central; Northeast 0.00, North-west, South-east and South-south recorded 0.07 each while South-west recorded 0.27. There were no unique (private) alleles among the Northeast, North-west and Southeast subpopulations, North-central and South-south recorded 0.10 each while South-west recorded 0.40. Also, there were no locally common alleles (frequent ≥ 5 %) found in 25 % or fewer population across the six subgroup/populations. However, at 50 %, North-west and Southeast recorded 0.20 each, South-south 0.10 while South-west recorded 0.27. The expected heterozygosity (*He*) was low across board, with North-east (0.00), North-central (0.02), North-west (0.05), South-east (0.05), South-south (0.05) and South-west (0.07). The unbiased expected heterozygosity (*uHe*) was also low and ranged from 0.00 for northeast to 0.11 to southwest. This indicates that among the subgroups/populations, intraspecific diversity is low. The percentage of polymorphic loci across the populations shows the following; North-central 10.00 %, Northeast 0.00 %, North-west 10.00 %, South-south 10.00 %, Southeast 10.00 % and South west 40.00 %. With respect to accessions with number of one or more private alleles, one accession each was identified for North-central (KaN034) and South-south (edN039), while five accessions were recognized for the South-west population (onN070, onN074, onN072 and oyN005) by the markers.

Table 2: The Sequences And Repeat Motif Of The Primers Used For This Study.

Locus	Forward Sequence (5' – 3') Reverse (5' – 3')	Repeat Motif
MO6	**F**GCATAGCCACCTTTACTCCT **R**GACTTTTGAACTCCACCACC	$(AG)_T(AG)_6$
MO8	**F**GTAGATGGTGCAGCTACTCA **R**TGGGGTTCTTGTTCTTTATT	$(CT)_{13}$
MO12	**F**ACCGAAGATGATAAGGTGGG **R**CAAAAGGAAGAACGCAAGAG	$(CT)_{11}$
MO13	**F**TTTCGGGTTTTCTTTCACGG **R**AGCTCACTTTCCATCTCCAT	$(CT)_{15}$
MO15	**F**CCCCTCTATTTCCATTTTCC **R**GCTCCATAAACCCTCTTGCT	$(TC)_{10}CCT(TC)_6$
MO18	**F**TTTTCCTCCCTTATTGTGCC **R**CCGTTGCCCTTTGTGGTTCA	$(GA)_6A(AG)_{16}$
MO46	**F**ACCAAGGGTTTCAACTGCTG **R**CATTTTGCGACGGTCTCACG	$(AG)_5(GA)_6$
MO48	**F**AGAAGAACCCAACAGAGGAT **R**CTTTTCACTAACCACCACCC	$(TC)_8C(CT)_{15}A(AC)_7$
MO58	**F**TGGATTTCTTCTCCTGCTAT **R**CACAGTTCTTATTGTATTGG	$(CT)_6T(TC)_9$
MO61	**F**TGTGGGTCCTGCCTTTTCTC **R**CTTCTGTCTTTCTTCCTGCT	$(TC)_{11}$

Table 3: Summary Of Genetic Parameters Estimates Of The Ssr Markers Used For This Study

Locus	MAF	NA	GD	PIC	Ho	He	I
MO6	0.414	7	0.719	0.676	0.028	0.025	0.048
MO8	0.343	9	0.782	0.753	0.083	0.063	0.094
MO12	0.271	13	0.838	0.820	0.500	0.250	0.347
MO13	0.671	5	0.487	0.430	0.000	0.000	0.000
MO15	0.214	15	0.885	0.875	0.111	0.083	0.145
MO18	0.571	4	0.587	0.529	0.000	0.000	0.000
MO46	0.371	5	0.743	0.701	0.000	0.000	0.000
MO48	0.629	5	0.564	0.530	0.000	0.000	0.000
MO58	0.671	7	0.520	0.493	0.250	0.146	0.209
MO61	0.614	4	0.565	0.520	0.000	0.000	0.000
Total	**0.477**	**7.4**	**0.669**	**0.633**	**0.972**	**0.567**	**0.048**

MAF = Major Allele Frequency, NA = Number of alleles per locus, GD = Gene Diversity, PIC = Polymorphic Information Content, *Ho* = Observed heterozygosity, *He* = Expected heterozygosity, I = Shannon information index

Percentages of Molecular Variance among and within Populations (AMOVA)

The result of analysis of molecular variance (AMOVA) among and within populations of *M. oleifera* is shown in Table 4. Based on this, 8 % of the genetic diversity was attributed to differences among the populations while 92 % of the variation (significant at p = 0.001; after 999 permutations) were due to differences within populations. This indicates higher intraspecific diversity within the populations and less among the populations. Figure 3 shows the percentages of molecular variance among and within populations of *M. oleifera.*

Table 4. Amova Among And Within Populations Variations

Source	df	SS	MS	EV	TV %	p-value*
Among Pops	5	61.733	12.347	0.551	8%	<0.001
Within Pops	64	413.424	6.460	6.460	92%	<0.001
Total	69	475.157		7.010	100%	

Df = Degree of freedom, SS = Sum of square, MS = Mean Square, EV= Estimated variation, TV = Total Variation, *After 999 random permutations.

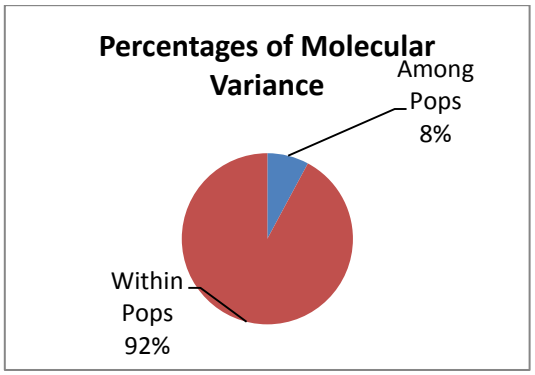

Figure 3. Percentages of molecular variance among and within populations

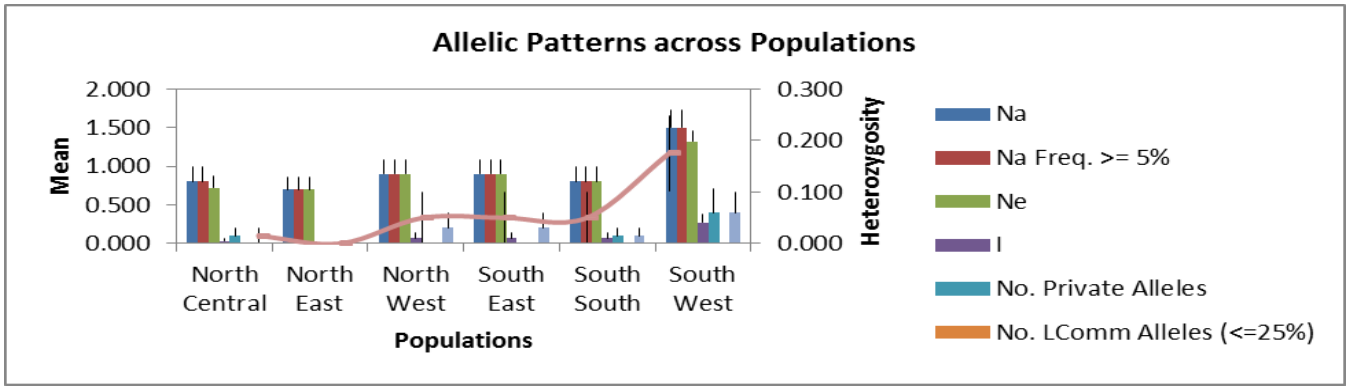

Figure 2. Allelic Patterns across the six populations. Na = number of different alleles, Na Freq. >= 5%, Ne = effective alleles, I = Shannon information index

Table 5: Alleles Pattern According To The Source Of Accessions Within Groups Of *Moringa Oleifera* Used For This Study

Population	NC	NE	NW	SE	SS	SW
No of accessions	12	4	4	15	9	26
Na	0.80	0.70	0.90	0.90	0.80	1.50
Na Freq. >= 5%	0.80	0.70	0.90	0.90	0.80	1.50
Ne	0.72	0.70	0.90	0.90	0.80	1.32
I	0.023	0.00	0.07	0.07	0.07	0.27
No. private alleles	0.10	0.00	0.00	0.00	0.10	0.40
No. frequent alleles (<=25%)	0.00	0.00	0.00	0.00	0.00	0.00
No. frequent alleles (<=50%)	0.00	0.00	0.20	0.20	0.10	0.40
He	0.02	0.00	0.05	0.05	0.05	0.18
uHe	0.02	0.00	0.10	0.07	0.10	0.25

NC – North-central, NE-North-east, NW-North-west, SE-South-east, SS-South-south, SW-South-west. *Na* = No. of Different Alleles, Na (Freq >= 5%)= No of different alleles with a frequency > = 5%, Ne = No. of effective alleles = 1 / (Sum pi^2), I = Shannon's Information Index = -1* Sum (pi * Ln (pi), No. of private alleles = No. of alleles unique to a single population, No. LComm Alleles (<=25%) = No. of Locally Common Alleles (Freq. >= 5%) Found in 25% or Fewer Populations, No. LComm Alleles (<=50%) = No. of Locally Common Alleles (Freq. >= 5%) Found in 50% or Fewer Populations, He = Expected Heterozygosity = 1 - Sum pi^2, uHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He

Nei Genetic Identity (I) and Distance (D) among and within the populations of *Moringa oleifera*

The genetic similarity coefficients among the populations of *M. oleifera* was evaluated based on Nei [35] unbiased genetic identity and distance. The genetic identity varied from 0.632 (lowest) between Northeast and South-south accessions to 0.884 (highest) between North-west and South-west accessions with an average value of 0.736. The genetic distance ranged from 0.123 between North-west and South-west accessions to 0.458 between Northeast and South-south accessions with an average of 0.311. Nei genetic distance = -1* Ln(Nei Identity I) and Nei unbiased genetic distance = -1 * Ln(Nei unbiased Identity) Nei [35] and where I = 0.00 shows no common alleles while I = 1.00 shows equal gene frequencies. These suggest that North-east and South-south accessions shared most of the alleles while North-west and South-west accessions displayed higher diversity. The higher genetic distance (0.458) observed between any accessions was between the Northeast and South-south accessions while the least distance (0.123) was that between the North-west and South-west accessions (Appendix 1 and 2).

Principal Coordinate Analysis (PCoA) of the 70 accessions of *M. oleifera*

The SSR data matrix was used to generate the principal coordinate analysis the 70 accessions of *M. oleifera*. The first five PC accounted for 64.10 % of the total variation out of which PC1 (24.30%) and PC2 (13.60%) extracted 37.90 % molecular similarity. All accessions were labeled with different colors according to different population to indicate region of collection (Figure 4). The scatter plot of the PCoA clustered the 70 accessions into three major cluster groups. Cluster group A comprised a total of 48 accessions (68.57 %) from all the subgroups/populations while cluster group B and C of 15 and 7 accessions, respectively are mainly of southeast, south-south and southwest accessions.

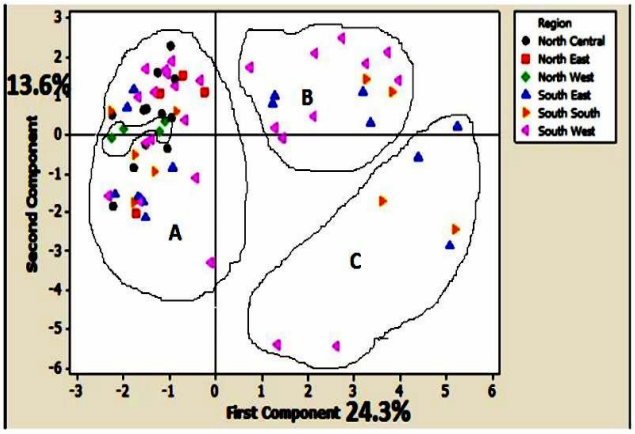

Figure 4. Scatter plot of 70 *Moringa oleifera* accessions based on first and second components of principal coordinate analysis using SSR data.

Cluster Analysis

A dendrogram was generated from the combined data of the ten SSR polymorphic markers using NTsys-pc software program. The similarity coefficient (SC) of the dendrogram ranged from 0.53 to 1.00 and delineated the 70 accessions into four (4) major groups at SC = 0.72. The clusters/groups are A, B, C and D as depicted in Figure 5. All accessions can be distinguished from each other at the level of similarity coefficient of 1.0 except (soN066 and taN085) and (anN045 and anN047). Cluster group A was further segregated into four subclusters A1, A2, A3 and A4. The subcluster A1 consisted of 8 accessions (abN057, anN049, abN059, anN051, anN046, edN035, beN081 and onN072) while A2 contained 30 accessions segregated as follows (deN041, edN040, kaN032, soN067, knN077 and ogN025), (edN037, niN017, goN069, kaN034, oyN001, abN079, enN053, yoN031, niN018, ogN028, kwN075 and kwN014), (onN070 and oyN005), (ogN076, plN030 and oyN010) and (enN055, goN068, **soN066 and taN085*, imN064, kaN033, ogN026). The subcluster A3 has 2 accessions (oyN004, oyN007) and A4 consisted of 7 accessions (knN078, edN039, osN019, osN022, osN020, kwN016 and kwN015). Cluster group B contained 2 accessions (onN074, oyN029), cluster group C contained 17 accessions (osN024, oyN009, oyN003, osN027, imN063, osN021, edN038, deN044, deN042, oyN004, edN036, **anN045 and anN047*, anN048 and oyN080, imN065 and oyN012) while cluster group D has 4 accessions (enN052, enN054, onN071 and onN073).

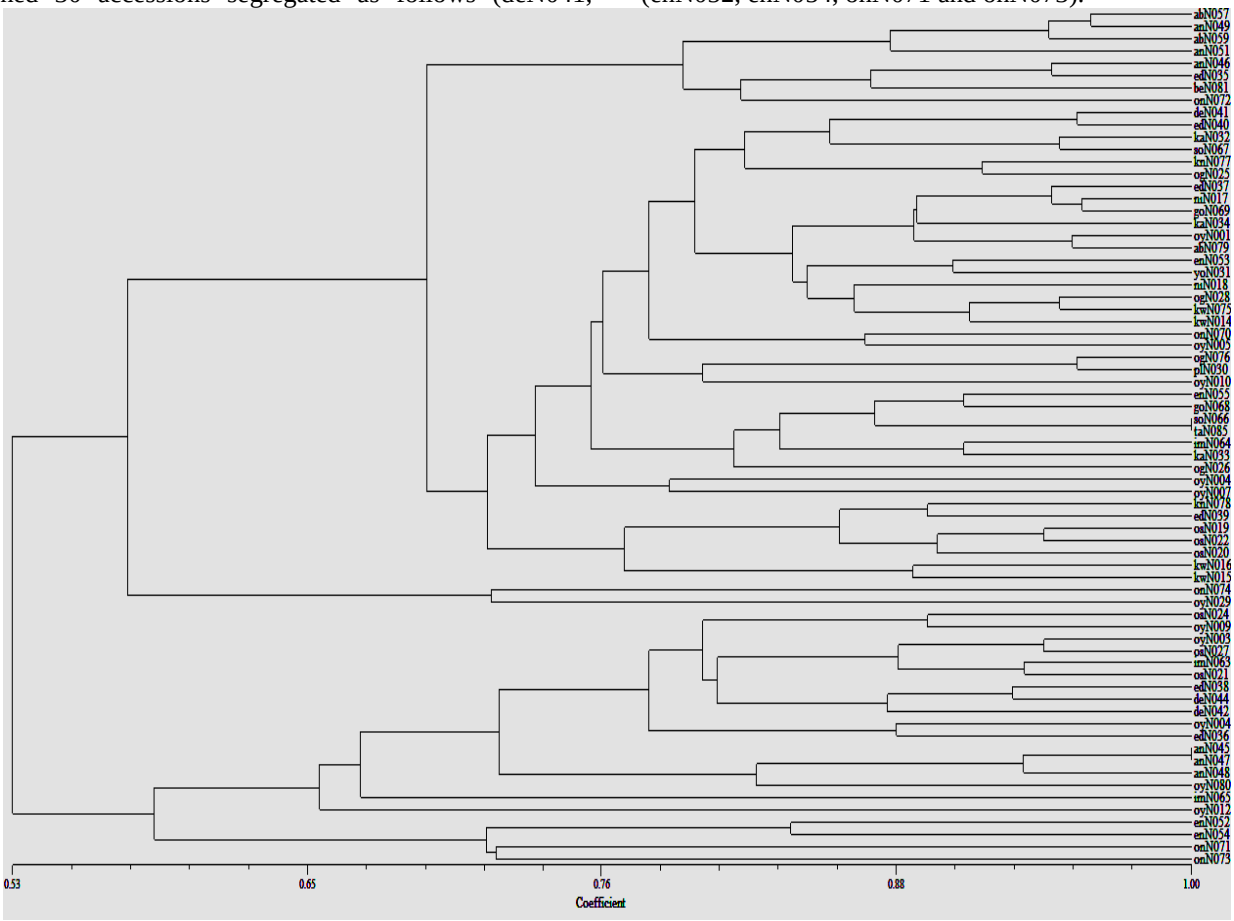

Figure 5: **Dendrogram generated from SSR markers used for the 70 accessions of *Moringa oleifera***

DISCUSSION

In this study, twenty pairs of polymorphic microsatellite primers developed specifically for *Moringa oleifera* [25] were adopted and optimized for the investigation of intraspecific diversity studies of *M. oleifera* in Nigeria. Ten primers were, however, selected based on clear amplification and reproducible scorable bands.

SSR markers and Genetic diversity among the accessions/populations

The genetic informativeness of the ten SSR markers used in this study are not in doubt as the markers effectively delineated the accessions into groups; some along collections regions/areas while others disparately based on genetic similarities. The study confirms the polymorphic nature of the SSR markers used in the assessment of the genetic intraspecific diversity among the 70 accessions of *M. oleifera* in Nigeria. The mean observed number of alleles (*Na*) per locus (7.4) detected was higher than 3.32 reported by Wu *et al*. [25] but closer to 8.3 alleles/per SSR primer reported by Shahzad *et al*. [22]. The range of numbers of alleles per primer (4 to 15) recorded in this study is contrary to range of 2 to 6 reported by Wu *et al*. [25] but comparable to that of Shahzad *et al*. [22] which reported a range of 6 to 13. However, two markers MO18 and MO46 with numbers of alleles per locus (4 and 5, respectively) tallied with the reported values for the two markers by Wu *et al*. [25]. Varying range of number of alleles per locus has been reported by several authors. Wangari *et al*. [40] reported a range of (2 to 15) for *Morus species*. In the study of Doumbia *et al*. [33] six primers detected higher numbers of alleles (a range of 7 to 27) per locus in the assessment of *Vigna unguiculata* L. Walp. germplasms from Ghana and Mali using SSR markers.

The mean of polymorphism information content (PIC) obtained in this study is within the range of the previous studies. Shahzad *et al*. [22] obtained a comparable 59 % PIC value on *Moringa oleifera* accessions from Asia, Africa, North and South America and the Caribbean. Natarajan and Aslin-Joshi [24] reported 0.52 PIC value from Indian populations while Ganesan *et al.* [23] obtained extremely low PIC value of 0.15 from 12 Indian *M. oleifera* populations. The relatively high level of polymorphisms recorded for *M. oleifera* in this study could be attributed to continuous spread and domestication of the species in different regions of Nigeria. Mean values of other genetic estimates such as gene diversity (0.669), observed and expected heterozygosity (*Ho* = 0.97, *He* = 0.57) recorded in this study also compares favorably and in consonance with values reported from previous studies [22, 23, 24, 25]. All these indicate the efficiency and effectiveness of SSR markers in the genetic intraspecific diversity of underutilized species like *M. oleifera* [25, 28]. In addition, high values for all the genetic diversity measures indicated allelic richness among the studied accessions of *Moringa oleifera* which can be tapped and used in breeding programs to get the desired variants for commercial cultivation. Though, the heterozygosity among the accessions can be improved; the study indicated that such heterozygosity can be possibly leveraged upon in the quest for diversity for genetic improvement purpose. However, insignificant amount of rare (private) alleles identified among the regions indicated that the populations are genetically similar and that these alleles contributed very minimally to the overall genetic diversity of the population.

Allelic Pattern / distribution of populations of *M. oleifera* accessions studied

The results of mean allelic pattern structured/partitioned the 70 accessions into subgroups/populations according to source/region of collections (North-central, North-west, North-east, South-east, South-south and South-west) with little or no diversity. Five out of the six subgroups/populations exhibited similarity in allelic measurements such as number of different alleles (*Na*), effective alleles (*Ne*) and private alleles (unique) while only South-west subgroup displayed/detected higher values *Na* = 1.50; *Ne* = 1.32 and unique alleles 0.40 (Table 2). This also reflected in the percentage polymorphic loci (North-central 10.00 %, North-east 0.00%, North-west 10.00 %, South-south 10.00 %, South-east 10.00 % and South-west 40.00 %) indicating low allelic diversity among the subgroups. The fixation index of alleles in this study was also low with a mean value (-0.64 ± 0.05), which invariably support inbreeding and hence reduced heterozygosity among the accessions of each subgroup/population. The plot of allelic pattern across population revealed a gradual increase in allelic richness from North-central down North-east to South-west (Fig. 2), though there was no clear difference between the observed pattern among the North-west, South-east and South-south populations compared to South-west population, which displayed higher allelic richness. This suggests a strong connectivity among the subgroups due to similarity or commonality of alleles and exchange of a number of alleles at a particular locus. For instance, locus MO15 detected no alleles (0) for (North-central, North-west, North-east, South-east, and South-south) and three (3) alleles for South-west while locus MO8 detected one allele each for the five subgroups with similar allelic patterns and two (2) alleles for South-west subgroup/population (Fig.2). These, therefore, suggested that these accessions except the South-west accessions are becoming more genetically homogenous, which may be attributed to indirect gene flow among nearby populations possibly aided by agents of pollination, including wind, insects and birds. According to Balloux and Lugon-Moulin [41], genetic structuring reflects the number of alleles exchanged between populations influenced by gene flow and homogenizes allele frequencies between populations. This assertion might be responsible for the pattern described in this study and possibly corroborate the narrow gene pool reported by NRC [13]. With respect to the allelic patterns again (Fig. 2), we observed a slight rise of connection of subgroups/populations from the North-central to a steady rise from North-east and to a stable connection to South-east and South-south via North-east route and finally climaxed among South-west population. This allelic connectivity finding is interesting and comparable to sources of introduction and domestication of *M. oleifera* from Northern Nigeria to other regions/areas via exchange of planting materials and trade routes. The sources of introduction and domestication may have positively

influenced the distribution pattern across the geographical areas within Nigeria [3]. Generally, the finding from this study provides insight into the genetic relationships and possible source of introduction of *M. oleifera*, which is traceable from North-central/North-east to other regions of Nigeria. It is also remarkable that the allelic result analyzed is in perfect harmony with earlier study of Popoola and Obembe [3] claiming that *M. oleifera* must have been originally introduced to Northern Nigeria where the species has become practically endemic and spread to other regions of Nigeria. However, given the limited sample size of population collected from North-east and North-west regions (4 accessions each) as a result of insecurity (Boko Haram insurgency), additional accessions from the regions and others should be collected and included in future studies to confirm the present findings.

Genetic relationships among the accessions of *M. oleifera*

The SSR primers showed a relatively high level of diversity within populations (92 %) and very low genetic differentiation among population (8 %) (Significant at p = 0.001). Generally, outcrossing and woody species have been reported to possess higher levels of genetic diversity within populations compared to less genetic differentiation among populations [40, 42, 43]. Previous studies have also consistently identified high genetic diversity within populations than between populations of *M. oleifera* [18, 23, 44]. The SSR markers used in this study provided higher genetic diversity within populations and lower genetic differentiation among populations than similar study that utilized AFLP in genetic diversity of *M. oleifera* [18, 45]. The report of Muluvi *et al*. [18] identified significant differences between region and population and concluded that there was high genetic variation within populations of *M. oleifera* in Kenya. The present study also compares favorably with the phenotypic intraspecific variability of a subset (40 accessions) of *M. oleifera* in Nigeria [6], which if combined can successfully guide in robust selection procedure for breeding trial of the species in Nigeria. In addition, the results of this study are closely correlated to the biological behavior and breeding mechanisms of the species. *Moringa oleifera* is both geitonogamous (pollination between two flowers of the same plant resulting into genetically similar flowers) and xenogamous (outcrossing resulting into genetically different flowers/plants). These modes of pollination coupled with different methods of propagation (seedling and stem vegetative), easy fruit dehiscence and seed dispersal means may have collectively and effectively enhanced gene flow thereby increasing within population diversity and reduced genetic differentiation among populations. These observations have been reported for several outcrossing and cleistogamous species including *M. oleifera* [6, 11, 43, 46].

The pairwise population matrix of Nei genetic identity and distance suggests very close relationships which indicates that although all the subgroups/populations are geographically distinct but are genetically close/similar due to lower genetic distance values, which corresponds with the allelic pattern analysis. A minimum genetic distance of 0.123 was recorded between the North-west and South-west accessions while a maximum of 0.458 was recorded between the North-east and South-south accessions. These suggest that North-east and South-south accessions shared most of the alleles while North-west and South-west accessions displayed high intraspecific diversity.

The results of the cluster (CA) and PCoA analyses further provided additional insights into the relationships of all the 70 accessions from the six eco-geographical regions. The study revealed high degree of similarity among the accessions particularly at > 75 % genetic similarity coefficient (Fig. 5). Some of the main cluster groups obtained from the UPGMA tree and PCoA were correlated with the geographic regions/areas of collection of the accessions. For instance, cluster group A in Fig. 4 and Fig. 5 consists of accessions from all the sub regions/populations and corresponds to their geographical regions of collection. This is in agreement with the studies of Matus and Hayes [47] on Barley (*Hordeum vulgare* L.), which coincided with geographic origin. Several other researchers have also reported correlation between germplasms collections and geographical distribution [43, 48, 49]. In addition, the grouping of 68.57 % of the accessions in cluster A of PCoA (Fig. 4) and the intermixing of colors across the coordinate further support the allelic connectivity earlier observed among the subpopulations. The accessions from the North-west were the most distinct and centrally placed within the scatter group A which suggest the North-west as the primary point of spread of *M. oleifera* to other regions (Fig. 4). Also the closeness of North-central accessions to the North-west presupposes that *M. oleifera* accessions may have been introduced to Southern Nigeria from North-west via North-central and then to all south regions of Nigeria.

The clustering system of CA indicated that cluster groups shared a large number of alleles hence resulting into overlap/random distribution of accessions across the cluster groups except sub cluster A3. The widespread distribution of accessions particularly in cluster group A and C showed the ability of the *Moringa oleifera* accessions to adapt to varying/diverse climatic conditions across Nigeria. *M. oleifera* is highly adaptable to varying ecological conditions both in its native and introduced ranges in temperate, semi-arid and arid regions [3, 50, 51]. Similarly, accessions collected from the same source were grouped in the same cluster group indicating close affinity between accessions while accessions with different genetic background were clustered in single cluster (Figure 5). Clustering of over 50 % (47) accessions in cluster group A (Fig. 5) buttressed the view that geographical distribution and genetic divergence do not follow the same pattern as many accessions from differing locations were clustered together [52]. Similar observations have been reported in many tree species including *M. oleifera* [22, 44, 53]. The cluster analysis with low genetic distances indicated that the accessions are closely related and could have a recent common ancestor. For instance, in cluster group A sub cluster A1 with 8 accessions, beN081 collected from Benue (North-central) Nigeria, where *M. oleifera* is apparently endemic may probably be the ancestor accession/source for other accessions. Likewise, for sub cluster A2 with greater number of collections and hence wide ancestral relationship may likely be traced to either plN030 from Plateau State or

KaN034 from Kaduna State and alternatively from yoN031 (Yobe State) through exchange of planting materials and trade routes. The closeness of accessions abN057 and anN049 in sub cluster A1and the similarities of accessions (soN066 and taN085) in sub cluster A2 and accessions (anN045 and anN047) in cluster C also reinforced the possibility of similar ancestral relationship. These two pairs of accessions (soN066 and taN085) and (anN045 and anN047) appeared to be 100 % identical; however, accessions anN045 and anN047 collected from the same locations (Anambra state) might be regarded as duplicates while other accessions were distinguishable at similarity coefficient of 1.0. Two accessions (soN066 and taN085), were geographically divergence (Sokoto and Taraba) but genetically identical indicating possibility of same ancestral relationship. Similar reports have suggested same ancestors for genetically closely connected accessions/species in other plants including *M. oleifera* [45, 52, 54].

The spread of planting materials in form of cuttings, seeds and seedling via exchange may have also enhanced rates of gene flow between adjacent populations and thereby contributing to the distribution of accessions in cluster groups and intraspecific relationship among the accessions. The clustering of accessions in cluster group B and D, however, was quite different without any connection with accessions from any of endemic areas of *M. oleifera* in Nigeria. Cluster group B contained only two accessions (onN074 from Ondo and oyN029 from Oyo) and cluster group D had four accessions (enN052 and enN054 from Edo State; onN071 and onN073 from Ondo Sate) indicating that the accessions have potential inherent diversity that can be exploited for genetic improvement and breeding purposes. Therefore crossing between accessions of cluster A and cluster B or D might possibly create more variability for increased yield and utilization of the species.

Implication for conservation and utilization

The use of SSR markers have apparently enriched our understanding of the level of genetic relationship existing among the accessions of *M. oleifera*, which can be exploited for future Moringa breeding program. The collections are of great significance as major landraces in Nigeria with valuable agronomic traits [6], which can be cultivated as Moringa plantation for utilization as leafy vegetables, food and medicine, oil from seeds and also for other product development. Since there was no clear genetic differentiation among the populations and duplications among the accessions other than 100 % resemblance of two pairs of accessions (soN066 and taN085) and (anN045 and anN047) observed; all other accessions were independent at similarity level of 1.00, these accessions could, therefore, be given high priority *in situ* conservation. Likewise, there was no suspicion of loss of genetic intraspecific variation among the accessions studied; combining the *in situ* and *ex situ* conservation as management strategies will practically enhance utilization for breeding program and other socio-economic uses of *M. oleifera* in Nigeria and elsewhere. The allelic patterns plot, which highlighted the possibility of genetically homogenous accessions could be mitigated by systematic increase in collection of accessions, which can also enhance the creation of core collection of *M. oleifera* for conservation and utilization to meet the increasing demand for Moringa products.

CONCLUSION

The present study significantly contributes basic information towards the implementation of appropriate conservation and utilization plans as well as potential breeding trial programs for *Moringa oleifera* genetic resources in Nigeria. Simple sequences repeats (SSRs) markers are indeed very efficient in the genetic intraspecific diversity study of *M. oleifera* in Nigeria. Relatively high genetic diversity within population and low among populations are useful as accessions/planting materials for continuous use for cultivation, breeding and utilization purposes. As a result of weak gene pool arising from exchange of same planting materials and increased gene flow among the accessions, there is need to further broaden the genetic diversity of the species via germplasm collections particularly from the endemic Northern regions for systematic characterizations. On the whole, this study is a timely contribution considering the multi-purpose economic importance of the species, its wide distribution, adaptation and ease of integration into commercial agricultural production.

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Table (Appendix 1): Pairwise Population Matrix of Nei Unbiased Genetic Distance

	North Central	North East	North West	South East	South South	South West
North Central	0.000					
North East	0.360	0.000				
North West	0.308	0.304	0.000			
South East	0.326	0.322	0.269	0.000		
South South	0.450	0.458^{H}	0.365	0.424	0.000	
South West	0.251	0.247	0.123^{L}	0.141	0.312	0.000

Appendix 2: Pairwise Population Matrix of Nei Unbiased Genetic Identity

	North Central	North East	North West	South East	South South	South West
North Central	1.000					
North East	0.698	1.000				
North West	0.735	0.738	1.000			
South East	0.722	0.725	0.764	1.000		
South South	0.638	**0.632^{L}**	0.694	0.655	1.000	
South West	0.778	0.781	**0.884^{H}**	0.868	0.732	1.000

L = Lowest, H = Highest