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Research article

Genetic diversity of *Ocimum* species (Scent leaf) landraces from South Nigeria using inter-simple sequence repeat (ISSR) markers

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Abstract: *Ocimum* species, commonly known as scent leaf or basil are aromatic or scented shrubs that belong to the Lamiaceae family. This study investigated the genetic diversity of twenty-five scent leaf genotypes using ten Inter-Simple Sequence Repeat (ISSR) markers. Seeds of 25 *Ocimum* genotypes were obtained from five different Local Government Areas in six southern states of Nigeria. The ten ISSR primers generated a total of 679 bands with 629 bands been polymorphic (92.64%). Primer UBC813 gave the highest number of polymorphic bands (143). The unweighted pair-group method with arithmetic average cluster analysis grouped the 25 genotypes of the scent leaves into six major clusters with a dissimilarity distance from 0.015 to 0.288. Genotypes Udu-DT and Ikp-EDO were the most closely related at a dissimilarity coefficient of 0.015. The polymorphic information content values ranged from 0.381 (UBC834) to 0.913 (UBC813) with a mean of 0.677. UBC813 was the most polymorphic and informative ISSR marker for diversity studies on scent leaves. An extremely high genetic diversity was observed across the genomic loci of the genotypes which showed the effectiveness of ISSR markers in determining the extent of genetic variability in *Ocimum* species. Among the genotypes, Uyo-AKS had the highest values of genetic parameters including the effective number of alleles (1.4511), Nei's gene diversity (0.3108) and Shannon's information index (0.4898). The findings of this study gave valid procedures for the management of *Ocimum* genetic resources which will enhance the utilization, genetic conservation, and improvement of the species in Nigeria.

Keywords: Genetic improvement - Medicinal plant - Molecular characterization - *Ocimum* species - Polymorphism.

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INTRODUCTION

The Lamiaceae family is regarded as one of the most essential herbal families which comprises an extensive variety of plants with biological and medical applications (Uritu *et al.* 2018) accounting for approximately 150 identified species in the tropics and warm areas especially in Africa (Matasyoh 2012). *Ocimum* species, commonly known as scent leaf or basil are aromatic or scented shrubs which is a prominent member of the Lamiaceae. They are found in humid countries of Asia, Central and South America, with a vast majority of species in Africa (Kenya, South-Africa, Burundi, Ghana, Cameroon, Liberia, Nigeria, etc. (Nweze & Eze 2009). Scent leaf plant is among the well-known herbs grown globally and due to its popularity, it is often referred to as the "king of the herbs." Scent leaf is known by different names, but the well-known name basil was copied from

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the Greek words *basileus* meaning "king" or *basilikon* which means "royal." Therefore, *Ocimum* species are regarded as the 'king of herbs' (Makri & Kintzios 2008, Moghaddam *et al.* 2011). The general name *Ocimum* is derived from the Greek word 'Okimon' which means 'aromatic herb' (Matasyoh 2012).

In southern Nigeria, the plant is highly utilised and commonly known by its local names in the South-Southern states as "Ntong" (Cross-River/Akwa-Ibom), "Tchayo" (Edo), "Kunudiri" (Rivers), "Forokana" (Bayelsa), and "Ufuo-yibo" (Delta) (Ndukwu & Ben-Nwadibia 2005). Despite its medicinal and industrial importance, there is the paucity of information on the use of molecular markers for the characterization of *Ocimum* species in Nigeria.

The *Ocimum* genus has over two basic chromosome numbers and diverse haploid chromosome numbers (Carovic *et al.* 2006, Kumar *et al.* 2016). The basic chromosome number in *Ocimum* species is x = 12 (Carović-Stanko *et al.* 2010, Kumar *et al.* 2016), although *Ocimum basilicum* L. and *Ocimum americanum* L., has been stated to be tetraploid (2n = 4x = 48) and hexaploid (2n = 6x = 72), respectively (Kumar *et al*. 2016). *Ocimum tenuiflorum* L. a perennial shrub, on the other hand, has a basic chromosome number of x = 8 (Darrah 1980, Gupta & Chadha 1995, Kumar *et al*. 2016, Edet 2018).

Plants from the genus *Ocimum* are well known for their therapeutic, culinary, and nutritive benefits. It has been reported by several researchers that they possess insect repellent, antimicrobial, antiulcer, anti-inflammatory, hepatoprotective, chemoprotective, free radical scavenging, antioxidant, stimulating, anticataract, antihypertensive, antifertility, antiathritis anti-dermatophytic and anthelminthic properties (Mahajan *et al.* 2013, Uritu *et al.* 2018). Thus, some members of the genus such as *Ocimum tenuiflorum*, *Ocimum gratissimum* L. and *Ocimum campechianum* Mill. have been widely used in different medicines and are currently considered as potential sources for novel drugs (Uritu *et al.* 2018), to possibly prevent and treat malaria, common cold, fever and in the treatment of some skin diseases, urinary tract infections and aches. *Ocimum americanum* leaves have been used either alone or jointly with norfloxacin against strains of *Staphylococcus aureus* (Oyedemi *et al.* 2017). *Ocimum americanum* had a powerful antioxidative ability due to its high phenolic compound which depleted 1 diphenyl-2-picryl-hydrazyl (IC50:146.5 µg ml^{-1}), lipid oxidation (152 µg ml^{-1}), protein glycation inhibition; (47.6 µg ml^{-1}) and Ferric reducing antioxidant power (122.75 µmol Fe (II) g^{-1}) (Oyedemi *et al.* 2017).

Members of the genus *Ocimum* contain aromatic compounds and essential oils comprising of biologically active constituents and secondary metabolites. Some of the essential oils are eugenol, methyl eugenol, linalyl acetate, geraniol, citral, camphor, thymol, linalool, methyl chavicol, methyl cinnamate, and safroleetc (Patel *et al.* 2015). The Lamiaceae family also contains high amounts of secondary metabolites dominated by flavonoids and terpenoids, and then monoterpenoids, sesquiterpenoids, diterpenoids, and iridoid glycosides. Alkaloids and flavonoids have therapeutic properties. The essential oils found in leaves, seeds, flowers, and roots of the species are used as medicines (Matasyoh 2012).

Genetic diversity which involves any disparity in the nucleotides, genes, chromosome, or complete genomes of organisms, can be studied with molecular, chemical, morphological, and cytological approaches to identify the traits of domestication, propagation, and breeding methods as well as conservation of plant genetic materials. Genetic variability can be influenced by the geographical, seasonal, and edaphic factors of the environment (Lal *et al.* 2012). Plant populations can be differentiated by their display of distinctive or unique genetic traits. Plant populations can be differentiated by their display of distinctive or unique genetic traits. However, larger populations have a wider diversity of traits when they are likened to small populations. Members of small populations are inherently, physically, and physiologically more alike than the members of bigger populations and are consequently unlikely to adapt to diverse environments. Genetic diversity is, therefore, the main component for conservation efforts connected with population management (Andayani *et al.* 2001). Phylogenetic reconstruction may also aid in the discovery of greater plant diversity and assist biologists in choosing areas or species to prioritize in their conservation efforts (Patel *et al.* 2015). The genus *Ocimum* is very variable and has a wide array of genetic diversity and the genetic variability can be noticed at inter-specific and intra-specific levels (Stebbins 1957, Lal *et al.* 2012). Members of the genus show considerable morphological variations besides growth characteristics, reproductive behaviour, and chemical constituents among their species (Carović-Stanko *et al.* 2010, Chowdhury *et al.* 2017). The genus is very extensive and complicated, this complexity is thought to be due to genetic diversity influenced by cross-pollination and several environmental factors (Chowdhury *et al.* 2017).

Molecular markers are important tools in evaluating biodiversity because they are not affected by the age of the plant, physiology, and environmental conditions. These genetic markers assist in locating the positions of desirable traits; therefore, the study of polymorphism is best done at the level of arrangement of nucleotide

bases in DNA, which is the primary source of biological information (Mukharib *et al.* 2010).

Inter-simple sequence repeats are regions in the genome flanked by microsatellite (short sequence repeats) sequences. Amplification of these regions using polymerase chain reaction (PCR) uses a single primer but produces multiple amplicons that could serve as a dominant multi-locus marker system for the study of genetic variation (Ng & Tan 2015). The ISSR's markers are valuable in phylogeny, gene tagging, genome mapping and evolutionary biology investigations (Reddy *et al.* 2002). It is one of the most widely used molecular markers for genetic diversity studies as they are highly reproducible and polymorphic. Inter-simple sequence repeat (ISSR), Random Amplified Polymorphic DNA (RAPD) as well as Amplified fragment polymorphism (AFLP) markers have been used for genetic diversity studies in *Ocimum* Species (Carovic *et al.* 2006, Harisaranraj 2008, Moghaddam *et al.* 2011, Patel *et al.* 2015). The ISSR markers utilize microsatellite sequences that are highly variable and ubiquitously spread across the genome (Al Salameen *et al.* 2018) and achieve a higher reproducibility and less expensive and time-consuming when compared to using RAPD and AFLP markers. This makes ISSRs ideal genetic markers for several studies, mostly on genetic variation/diversity of crop species (Wang *et al.* 2012, Shafiei-Astani *et al.* 2015). ISSR markers has been employed to assess the genetic diversity in various medicinal and aromatic plants, such as *Ocimum* (Aghaei *et al.* 2012, Chen *et al.* 2013, Patel *et al.* 2015, Alves *et al.* 2019), *Cordia curassavica* (Jacq.) Roem. & Schult. (Brito *et al.* 2016, Alves *et al.* 2019), and *Croton tetradenius* Baill. (Almeida-Pereira *et al.* 2017, Alves *et al*. 2019).

There is no information on the assessment of the genetic diversity of *Ocimum* species in Nigeria using molecular techniques. A problem is equally envisaged with regards to the various species available, although there are existing reports on the morphological characterization of scent leaves in Nigeria (Edet 2018). There is also an increase in the need to exploit plants for medicinal purposes especially in developing countries due to the resistance of many microorganisms to existing antibiotics and the reduced effectiveness of some antibiotics. The South-southern region of Nigeria is one of the most productive ecosystems that constitute a unique geographical area which is noted for extremely high rainfall, high diversity of species, predominantly rain forest vegetation which contains the third largest mangrove in the world (George-Ukpong 2012). *Ocimum* species is well pronounced in this geographical area as it is well used as a remedy for several ailments like headaches, nose bleeding, and indigestion as well as for some other culinary uses such as the preparation of major delicacies like pepper-soup, white soup and for porridges. But despite all these uses, no study has been established to ascertain the genetic basis of *Ocimum* in anticipation of identifying similar landraces that could be related in their medicinal properties. It is equally important to study the genetic diversity of *Ocimum* species from this location, for its utilization, genetic conservation, and genetic improvement in Nigeria. Although genetic characterization of some *Ocimum* species have been reported by some researchers outside Nigeria, characterization of the species in Nigeria have been carried out using morphological, phytochemical, and ethnobotanical approaches (Ojo *et al.* 2012, Olugbade *et al.* 2017). This study, therefore, investigated the genetic diversity of *Ocimum* species from five states in South-South Nigeria using Inter-Simple Sequence Repeats markers with the view of enhancing the utilisation, conservation, and improvement of the plant. Thus, we established the phylogenetic relationship among the collected *Ocimum* genotypes and identified the most polymorphic ISSR marker for future phylogenetic studies on *Ocimum* species.

MATERIALS AND METHODS

Plant materials

The six states in the South-South region of Nigeria; Akwa-Ibom, Bayelsa, Cross-River, Delta, Edo, and Rivers were selected for the collection of plant samples (Fig. 1). The average temperature, rainfall, longitude, and latitude of the different sites were recorded (Table 1) Seeds were collected from healthy plants in five (5) different locations in each state and the sampling was carried out from October to December 2017 using the random sampling method.

Planting and seedling maintenance

The seeds obtained were planted in pots containing about 7 kg of fertile soil. The pots were labelled, and the planted seeds watered once a day. Weeds were controlled manually, and the seedlings were thinned down to one stand per pot after four (4) weeks. Figure 2 shows an *Ocimum* seedling prior to DNA extraction.

Genomic DNA extraction

Genomic DNA extraction was done using a modified Dellaporta *et al.* (1983) method. Fresh young leaves (200 mg) of each sample were ground thoroughly with 700 µl of pre-heated extraction buffer (100 mM Tris-HCl pH 8 + 50 mM EDTA + 500 mM NaCl + 1.25% SDS, freshly prepared 0.07% β-mercaptoethanol and 1% PVP

to form a homogenous mixture) using a mortar and pestle and transferred to an Eppendorf tube. The eppendorf tubes were incubated in a water bath at 65ºC for 25 min with occasional mixing and then allowed to cool for 5 min before adding 400 µl of ice-cold potassium acetate with gentle inversion. Chloroform-Isoamyl-alcohol (350 µl) in the ratio (24:1) was added to the mixture and centrifuged at 12,000 rpm for 15 min for purification. The supernatant was transferred into new tubes without disturbing the interface and the DNA precipitated with 200 µl of ice-cold isopropanol mixed by inverting the tubes 2–5 times. The precipitated DNA was kept in a -20ºC freezer for 1h. After precipitation, the mixture was centrifuged at 4,000g for 20 min and then the supernatant was discarded. The DNA pellet obtained was washed twice with 300 µl of 70% ethanol. The DNA was pelleted down by centrifugation at 12000 rpm for 10 min and then air-dried. The air-dried DNA pellets were later re-suspended in 50 µl sterile distilled water and stored at -20ºC.

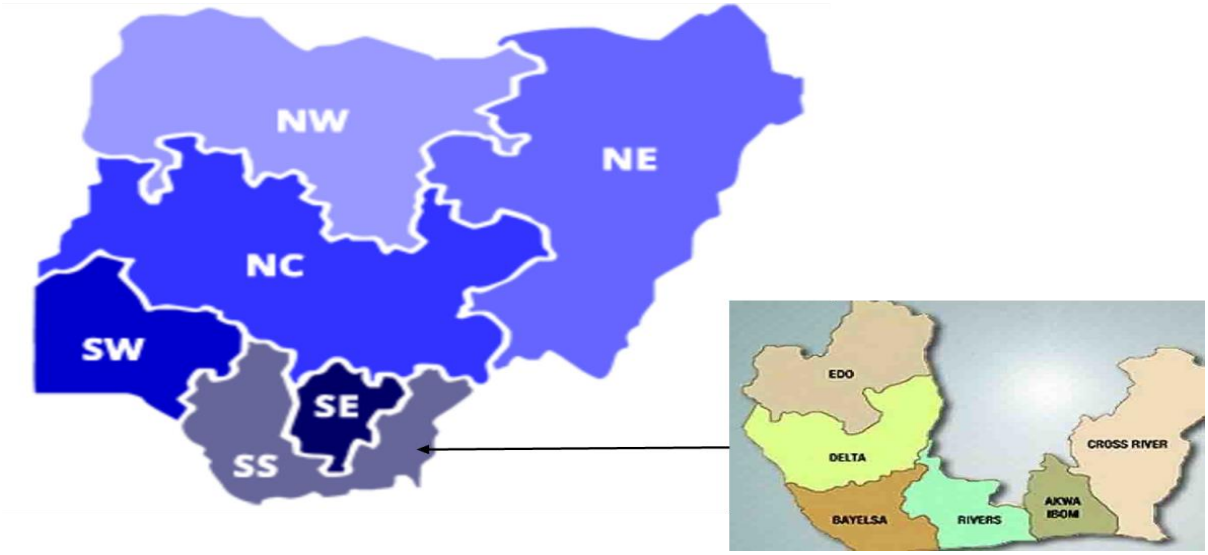

Figure 1. Map of Nigeria showing the six (6) geographical zones and the collection sites of *Ocimum* populations (South-South Nigeria). [Source: www.uspf.org.ng http://www.wikipedia.com/Nigeria/southsouth]

Table 1. Geographical data of collection sites.

Collection state	Natural vegetation	Latitude	Longitude	Average temp. (ºC)	Average rainfall (mm)
AkwaIbom	Mangrove, Rainforest	4˚ 32' 05.33" N	7˚ 25' 08.25" E	26.4	2509
Bayelsa	Mangrove, Fresh water swamp	4˚ 53' 07.99" N	5˚ 53' 26.17" E	26.7	2899
Cross-River	Mangrove, Rainforest	6˚ 12' 08.31" N	8˚ 39' 01.37" E	26.1	2750
Delta	Fresh water swamp	5˚ 32' 02.67" N	5˚ 53' 49.53" E	26.9	2133
Edo	Rainforest	6˚ 33' 53.10" N	5˚ 53' 37.86" E	26.1	2125
Rivers	Fresh water swamp, Mangrove	4˚ 51' 42.16" N	6˚ 55' 10.91" E	26.5	2874

Source: Sowunmi [32] ; Climate-Data (2018)

Figure 2. An *Ocimum* seedling prior to DNA extraction.

DNA quantification and qualification

The DNA samples were run on a 1% agarose gel containing 10 µL ethidium bromide solution. The DNA concentration and quality were determined using a Nano-drop spectrophotometer (N.D.1000) at an absorbance ratio of 260/280 nm. DNA (2 µL) was loaded into the well of the Nano-drop spectrophotometer and the concentrations measured at A260/A280. Optimally pure DNA should be a ratio between 1.6–1.8 ng μl^{-1} while values between 1.4 and 2.0 ng μl^{-1} are accepted.

Inter simple sequence repeats (ISSR) analysis

A total of ten ISSR primers were used for PCR amplification of the DNA samples. These ISSR primers of UBC (University of British Columbia) series were selected from the report of Matasyoh (2012) and Patel *et al*. (2015) and the primers with the highest polymorphism were used for this study. The list of ISSR primers used in the present study is given in table 2.

Table 2. List of the 10 ISSR markers used in the study.

Primer Name	5'Sequence3	Annealing temp. (ºC)
UBC443	ACACACACACACACACACT	42
UBC807	AGAGAGAGAGAGAGAGT	40
UBC808	AGAGAGAGAGAGAGAGC4	42
UBC813	CTCTCTCTCTCTCTCTT	40
UBC816	CACACACACACACACAT	40
UBC825	ACACACACACACACACT	42
UBC834	AGAGAGAGAGAGAGAG(CT)T	42
UBC840	GAGAGAGAGAGAGAGAYT	42
UBC841	GAGAGAGAGAGAGAGAYC	40
UBC855	ACACACACACACACACY	42

Note: Y = A single letter abbreviation for mixed base positions for C and T (pyrimidine).

Pilot experiments were carried out with small sets of scent leaf DNAs and the ISSR primers were screened for reproducible bands. The annealing temperatures used in previous studies and stated by the manufacturer yielded no bands. The annealing temperatures for the primers were therefore optimized using different temperatures (40ºC, 42ºC, 45ºC, 50ºC, 53ºC, 54ºC, 55ºC and 60ºC). The final reactions were carried out with the optimized temperatures that gave optimum polymorphism. All the PCR reactions were carried out in 0.2 ml capacity thin-walled PCR tubes (Inqaba Biotec) at a total volume of 15 µl containing 3 µl of 20 ng of genomic DNA, 3 µl of PCR Master mix which contained 2 units of Hot FIRE Pol Taq DNA polymerase, Proofreading enzyme, 5X Blend Master Mix Buffer, (which was brought down to 1X concentration containing 1X Blend Master mix buffer) (Solis Biodyne), 2.0 mM $MgCl_2$and 200 µM of each deoxynucleoside triphosphates (dNTPs) (Solis Biodyne), 0.5 µl of the primer (Inqaba Biotec) and 8.5 µl double-distilled H_2O. The reaction mixture was gently mixed by tapping against the tube followed by a short spinning (~3,000 rpm for 30 secs) and the tubes subsequently placed in the thermocycler (Bio-Rad, California) for amplification with the following programme: Initial denaturation temperature at 95ºC for 5 min, followed by 38 cycles of 95ºC for 15 secs, annealing temperature at 42ºC for 40 secs, an extension at 72ºC for 1 min followed by a final extension at 72ºC for 10 min.

Electrophoresis of PCR products

The amplification products were resolved on 2.0% agarose gel electrophoresis using 1x TBE buffer (45 mM Tris-acetate, 5 mM Boric acid, and 1mM EDTA, pH 8.0) at 100 V for 1.5 h. A 100 bp ladder (New England Biology) was included as a molecular size marker. Gels were visualized by staining with ethidium bromide solution (0.5 µg ml^{-1}) and banding patterns were photographed over UV light using UV trans-illuminator (Model-2, Upland, CA, USA).

Data analysis

The number of polymorphic and monomorphic bands were determined. Amplified DNA bands were scored as '1' for presence and '0' for absence at a position and the binary scores were translated into a character matrix as described by Ojuederie *et al*. (2014). The gene diversity and polymorphic information content were calculated using Power marker software. Phylogenetic relations were determined by cluster analysis with pairwise distance matrices, compiled by numerical taxonomic and multivariate analysis system (NTSYS-pc) software version 2.02 (Rohlf 2000), using the Jaccard's similarity coefficient (Jaccard 1908, Ojuederie *et al.* 2014). A dendrogram was constructed by unweighted pair-group method of arithmetic averages (UPGMA) and the Factorial Coordinate Analysis (FCoA) used for multivariate analysis with the DARwin software package Version 5.0.0.158 (Perrier & Jacquemoud-Collet 2006, Ojuederie *et al.* 2014).

RESULTS

Qualitative and quantitative analysis of genomic DNA

The quality of the DNA of the extracted scent leaf samples as determined by the Nano-drop spectrophotometer were in the range of 1.60 to 2.38 (Table 3). The genotype from Okrika, Rivers State had the highest DNA concentration of 2174.5 ng μl^{-1} while the genotype from Eket, Akwa-Ibom State had the least (256.9 ng μl^{-1}).

Table 3. Qualitative and quantitative analysis of genomic DNA.

S.N.	Location	Nanodrop reading / 1 µl of DNA	Purity
1	Uruan-AKS	791.7	2.07
2	Itu-AKS	1062.7	1.77
3	Etinan-AKS	318.3	2.08
4	Uyo-AKS	469.2	2.02
5	Eket-AKS	256.9	1.74
6	Yenagoa-BY	2008.2	1.61
7	Southern Ijaw-BY	374.9	1.6
8	Kolokomo/Opokuma-BY	334.6	1.96
9	Ogbai-BY	1540.5	2.00
10	Calabar Municipality-CRS	597.3	1.75
11	Etung-CRS	716.6	1.97
12	Calabar South-CRS	785.9	1.94
13	Akpabuyo-CRS	549.6	1.94
14	Odukpani-CRS	567.4	1.83
15	Uvwie-DT	753	2.38
16	Ethiope East-DT	550.2	2.02
17	Udu-DT	1093.2	2.02
18	Ikpoba Okha-EDO	1178.5	1.88
19	Egor-EDO	500.9	1.89
20	Oredo-EDO	907.6	1.71
21	Ovia North-East-EDO	1587.8	2.02
22	Orhionmwa-EDO	1386.6	1.63
23	Abuloma-RV	414.3	1.74
24	Bonny-RV	524.6	2.03
25	Okrika-RV	2174.5	1.82

Note: AKS = Akwa-Ibom State, BY = Bayelsa State, CRS = Cross River State, DT = Delta State, EDO = Edo State, RV = Rivers State.

Polymorphism revealed by inter simple sequence repeats (ISSR) markers

The 10 ISSR primers used to assess the diversity in *Ocimum* genotypes, amplified successfully and produced polymorphic and monomorphic bands which were selected for DNA profiling. The total number of generated fragments and associated polymorphisms are shown in table 4. A total of 679 amplicons were obtained from the ten primers out of which 629 were found to be polymorphic (92.64%). The number of amplified fragments ranged from 26 (UBC 816) to 143 (UBC813) and the primers identified a minimum of 14 and a maximum of 25 genotypes.

Table 4. Polymorphism revealed by ISSR primers in scent leaf genotypes.

ISSR Primers	Number of amplified bands	Polymorphic bands	Percent Polymorphism	Number of genotypes identified	Allele frequency	PIC
UBC 443	80.0	80.0	100.0	25	0.20	0.872
UBC 807	58.0	33.0	56.9	25	0.72	0.419
UBC 808	39.0	14.0	35.9	25	0.48	0.527
UBC 813	143.0	143.0	100.0	24	0.16	0.913
UBC 816	29.0	29.0	100.0	14	0.44	0.724
UBC 825	26.0	26.0	100.0	16	0.36	0.727
UBC 834	50.0	50.0	100.0	24	0.76	0.381
UBC 840	64.0	64.0	100.0	24	0.48	0.676
UBC 841	100.0	100.0	100.0	19	0.24	0.836
UBC 855	90.0	90.0	100.0	24	0.44	0.693
Average	67.9	62.9	89.28	22	0.43	0.677

Note: PIC = Polymorphic information content.

Eight of the primers revealed 100% polymorphism (UBC443, UBC813, UBC816, UBC825, UBC834, UBC840, UBC841, and UBC855) while primers UBC807 and UBC808 revealed 56.9% and 35.9%

polymorphism, respectively. The average number of polymorphic bands per primer revealed was 62.9. Polymorphic information content (PIC) ranged from 0.381 (UBC834) to 0. 913 (UBC813) with an average value of 0.688 across all ten primers. Allele frequency ranged from 0.16 (UBC813) to 0.76 (UBC834). The band sizes for the primers ranged from 200 kb to 1517 kb. The DNA banding patterns of the 25 scent leaf genotypes using primers UBC813, UBC840, UBC855 and UBC807 are presented in figure 3.

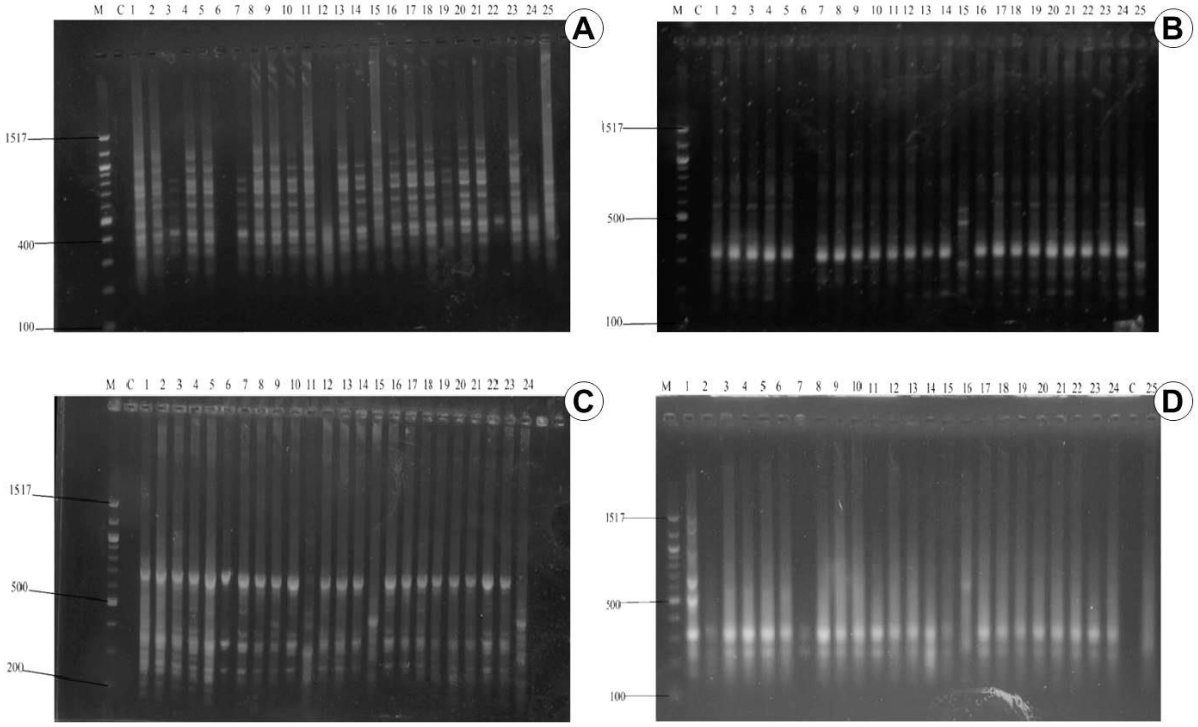

Figure 3. ISSR banding profiles of twenty-five *Ocimum* genotypes using primer: **A**, UBC813; **B**, UBC840; **C**, UBC855; **D**, UBC807. [Lane M- 100 base pair marker, Lane C- control, Lanes 1-25 represents profiles of DNA from 25 *Ocimum* genotypes, respectively]

Genetic diversity indices of 25 Ocimum landraces revealed by ISSR markers

Within the 25 genotypes of *Ocimum* species assessed with ISSR primers, the effective number of alleles (Ne), Nei's gene diversity, and Shannon's information index (I), varied from 1.3757–1.4511, 0.1464–0.3108 and 0.2775–0.4898, respectively (Table 5). Among the genotypes, the Uyo-AKS had the highest values of genetic parameters including the Ne (1.4511), H (0.3108) and I (0.4898), while the lowest genetic index was detected in the genotypes Okr-RV from Rivers State with Ne (1.1714), H (0.1464) and I (0.2775). The genetic diversity parameters identified in these genotypes constitutions were ranked as Uyo-AKS, > Soi-BY, > Eke-AKS, > Ogb-BY, > (Uru-AKS, Itu-AKS, Eti-AKS) > (Koo-BY, Cam-CRS) > Akp-CRS, > Ikp-Edo, > (Eth-DT, Ore-Edo) > Udu-DT, > Etu-CRS, > Abu-RV, > Odu-CRS, > Ego-Edo, > Yen-BY, > Cas-CRS, > Ovn-Edo, > (Orh-Edo, Uvw-DT) > Bon-RV, > Okr-RV, from high to low based on polymorphic loci of the selected ISSR primers. The overall mean values of Ne, H and I and their respective standard deviations across the diverse genomes were 1.3125 ± 0.0801, 0.2353 ± 0.0477 and 0.3961 ± 0.0618 (Table 5).

Table 5. Genetic diversity indices obtained from 25 genotypes of *Ocimum* species using inter-simple sequence repeat markers.

***Ocimum* genotypes**	**Sample size**	**Observed number of alleles**	**Effective number of alleles**	**Nei's gene diversity**	**Shannon's Information index**
Uru-AKS	25	2	1.3757	0.2731	0.4449
Itu-AKS	25	2	1.3757	0.2731	0.4449
Eti-AKS	25	2	1.3757	0.2731	0.4449
Uyo-AKS	25	2	1.4511	0.3108	0.4898
Eke-AKS	25	2	1.4079	0.2897	0.4649
Yen-BY	25	2	1.2405	0.1939	0.3440
Soi-BY	25	2	1.4186	0.2951	0.4713
Koo-BY	25	2	1.3651	0.2674	0.4380
Ogb-BY	25	2	1.3864	0.2787	0.4517
Cam-CRS	25	2	1.3651	0.2674	0.4380
Etu-CRS	25	2	1.3124	0.2380	0.4015

Cas-CRS	25	2	1.2305	0.1873	0.3351
Akp-CRS	25	2	1.3544	0.2617	0.4310
Odu-CRS	25	2	1.2916	0.2258	0.3858
Uvw-DT	25	2	1.2007	0.1672	0.3073
Eth-DT	25	2	1.3333	0.2500	0.4165
Udu-DT	25	2	1.3228	0.2440	0.4090
Ikp-EDO	25	2	1.3439	0.2559	0.4238
Ego-EDO	25	2	1.2608	0.2069	0.3612
Ore-EDO	25	2	1.3333	0.2500	0.4165
Ovn-EDO	25	2	1.2106	0.1739	0.3167
Orh-EDO	25	2	1.2007	0.1672	0.3073
Abu-RV	25	2	1.3020	0.2319	0.3937
Bon-RV	25	2	1.1811	0.1534	0.2877
Okr-Rv	25	2	1.1714	0.1464	0.2775
Mean	25	2	1.3125	0.2353	0.3961
Std. Dev			0.0801	0.0477	0.0618

Note: Uruan-AKS (Uru-AKS), Itu-AKS, Etinan-AKS (Eti-AKS), Uyo-AKS, Eket-AKS (Eke-AKS), Yenagoa-BY (Yen-BY), Southern Ijaw-BY (Soi-BY), Kolokomo/Opokuma-BY (Koo-BY), Ogbai-BY (Ogb-BY), Calabar Municipality-CRS (Cam-CRS), Etung-CRS (Etu-CRS) Calabar South-CRS (Cas-CRS), Akpabuyo-CRS (Akp-CRS, Odukpani-CRS (Odu-CRS), Uvwie-DT (Uvw-DT, Ethiope East-DT (Ete-DT), Udu-DT (Udu-DT), Ikpoba Okha-EDO (Ikp-EDO), Egor-EDO (Ego-EDO), Oredo-EDO (Ore-EDO), Ovia North-East-EDO (Ovn-EDO), Orhionmwa-EDO (Orh-EDO), Abuloma-RV (Abu-RV), Bonny-RV(Bon-RV), Okrika-RV (Okr-RV).

Brown'Multilocus structure analysis of 25 Ocimum genotypes revealed by ISSR markers

The multi-locus structure indices obtained with ISSR varied across the genotypes of *Ocimum* species, as suggested by the proportion of disequilibrium variance (VD) in the total variance from ISSR (VD = 4.669172) in all the genotypes (Table 6). Also, for the single locus effect, results showed that Wahlund's effect (WH) was low in all the populations based on the values from the marker system (ISSR, WH = 0.570413). This implies that the observed multi-locus structure is attributable to selection rather than population admixture or founder effect. The total variance obtained was 62.62%.

Table 6. Brown's multilocus analysis of ISSR diversities and linkage disequilibria of the populations of 25 genotypes of *Ocimum* species.

Single locus effect:	
Mean gene diversity (MH):	3.502310
Variance of diversity (VH):	0.370588
Wahlund's effect (WH):	0.570413
Two-locus effect:	
Mean disequilibrium (MD):	44.680361
Wahlund's effect (WC):	9.123826
Interaction between MD and WC(AI):	4.380945
Variance of disequilibrium (VD):	4.669172
Covariance of interaction (CI):	-7.552346
Total variance (σ2t):	62.628443
Average variance(σ2a):	49.680443

Note: σ^2t = MH+VH+WH+MD+WC+AI; σ^2a = MH+MD+AI+VD+CI; ISSR = Inter-simple sequence repeat.

Unweighted pair group method of arithmetic (UPGMA) cluster analysis of Ocimum genotypes

The dendrogram constructed using UPGMA placed the 25 *Ocimum* genotypes into 6 distinct clusters (Fig. 4). Cluster 1 had six genotypes with Udu from Delta State and Ikpoba-Okha from Edo State been the most closely related (dissimilarity index of 0.015). Also present in this cluster were genotypes Eth-DT, Ore-EDO, Abu-RV and Etu-CRS which cut across four States. Etu-CRS was genetically isolated from other genotypes in the group. Cluster II had only three genotypes namely: Uru-AKS, Ego-EDO and Odu-CRS.

Cluster III had the highest number of genotype (8) comprising of Ogb-BY, Koo-BY, Itu-AKS, Cam-CRS, Eke-AKS, Uyo-AKS, Eti-AKS and Soi-BY which was genetically isolated from other genotypes in the cluster. The IV cluster had only one genotype Yen-BY from Bayelsa State. The 5th Cluster contained just genotype Okr-RV from Okrika in Rivers State while the 6th cluster had only four genotypes namely Ovu-EDO, Orh-EDO, Bon-RV and Cas-CRS cutting across Edo, Rivers and Cross Rivers States.

Factorial coordinate analysis of twenty-five Ocimum genotypes

Factorial coordinate analysis (FCoA) generated placed the 25 *Ocimum* genotypes into five groups (Fig. 5). Four genotypes clustered together in group one, three from Akwa Ibom State: (Etinan, Uyo, Eket) and one from

Bayelsa State (Southern-Ijaw). Group II had the highest number (seven) of genotypes; Ovia-Edo, Orhionmwa-Edo, Calabar-South (CRS), Okrika-RV, Bonny-RV, Uvwie-DT, and Egor-Edo. The genotype from Yenagoa, Bayelsa was placed in group III and was genetically isolated from every other genotype as established by the dendrogram.

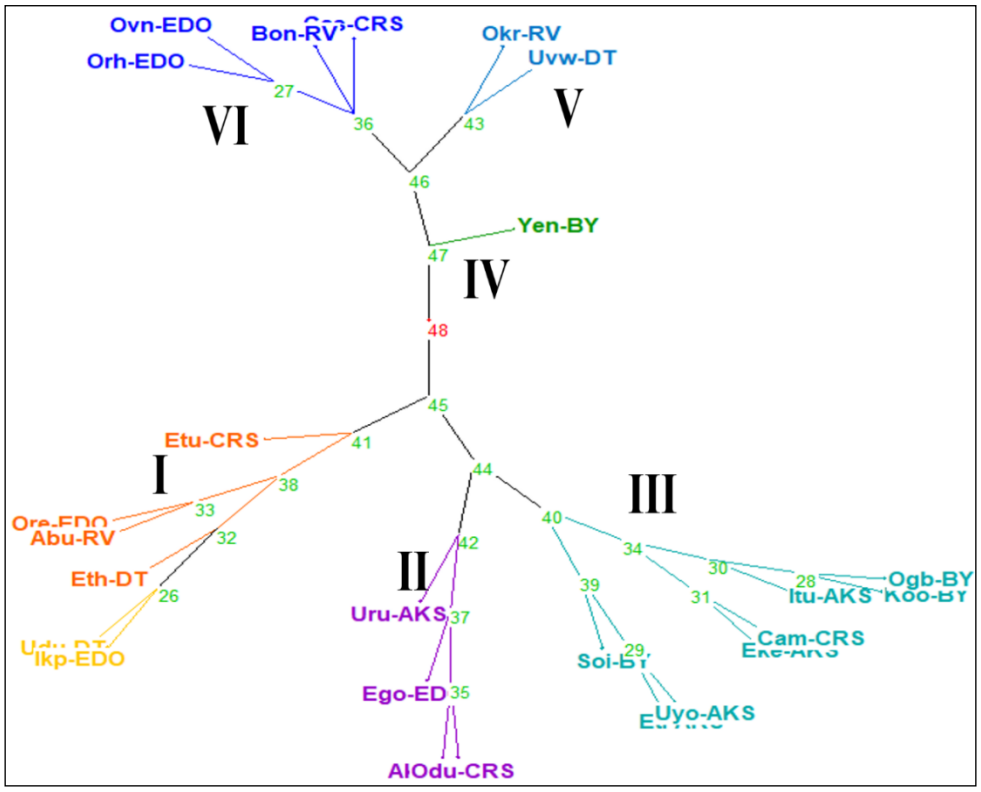

Figure 4. Dendrogram showing the relationship between 25 *Ocimum* genotypes as revealed by UPGMA cluster analysis.

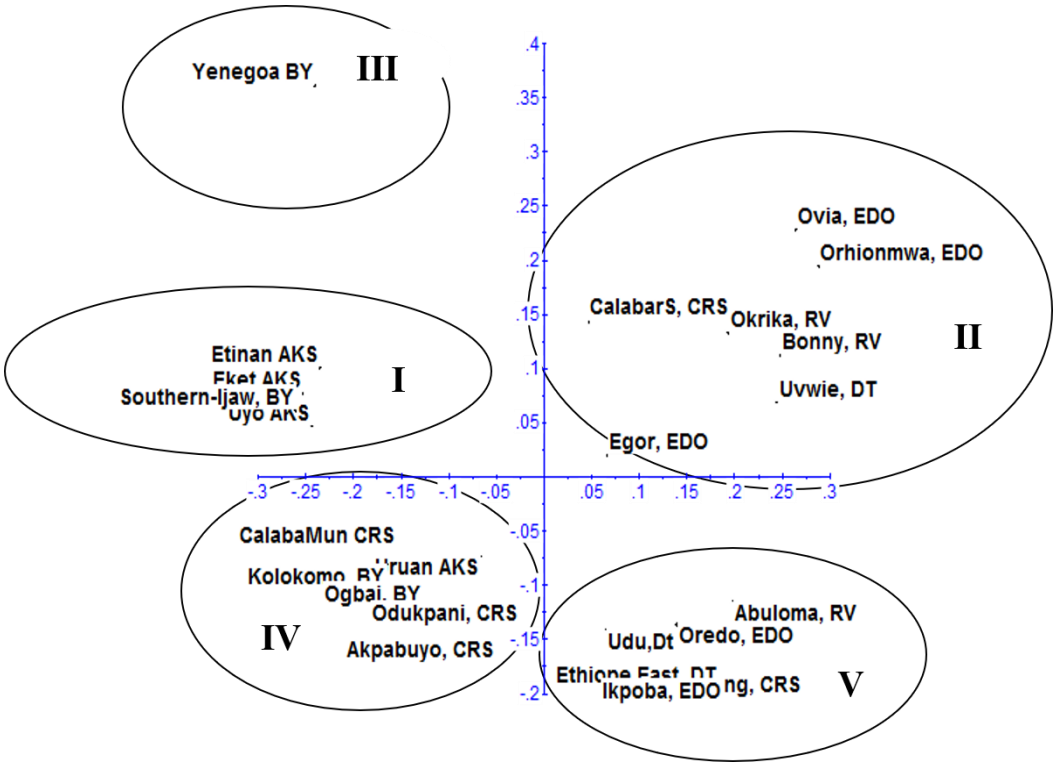

Figure 5. Factorial coordinate analysis as generated by DARwin software version 5.0.0.158 using dissimilarity coefficient matrix.

Group IV had three genotypes from Cross-River State: Calabar Municipality, Odukpani, and Akpabuyo, as well as genotypes from Uruan-AKS and Kolokomo-BY and Ogbai-BY. Group V had six genotypes clustered together: Abuloma-RV, Oredo-Edo, Udu-DT, Ethiope East-DT, Etung-CRS, and Ikpoba-EDO.

Genetic dissimilarity indices of 25 Ocimum genotypes revealed by ISSR markers

The genetic dissimilarity index ranged from 0.015 to 0.288 with the least dissimilarity obtained between genotypes Udu-DT and Ikp-EDO (0.015) (Table 7). Close genetic similarity was also observed between

Table 7. Dissimilarity coefficients of allelic data from 25 *Ocimum* genotypes obtained from ten ISSR primers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
2	0.106																							
3	0.152	0.091																						
4	0.159	0.098	0.053																					
5	0.144	**0.083**	0.098	0.076																				
6	0.265	0.205	0.174	0.197	0.182																			
7	0.182	0.106	0.091	0.114	0.098	0.189																		
8	0.129	0.053	0.144	0.136	0.091	0.197	0.129																	
9	0.144	0.053	0.114	0.136	0.106	0.212	0.098	**0.045**																
10	0.114	0.068	0.114	0.106	0.061	0.227	**0.083**	0.091	0.106															
11	0.152	0.136	0.197	0.205	0.159	0.295	0.182	0.129	0.129	0.129														
12	0.182	0.152	0.167	0.205	0.205	0.144	0.197	0.159	0.189	0.159	0.182													
13	0.106	0.106	0.152	0.159	0.144	0.250	0.152	0.083	0.098	0.098	0.121	0.152												
14	0.136	0.106	0.106	0.129	0.159	0.250	0.167	0.129	0.129	0.114	0.152	0.167	0.091											
15	0.174	0.159	0.189	0.212	0.197	0.197	0.250	0.182	0.182	0.167	0.159	0.159	0.174	0.144										
16	0.167	0.136	0.182	0.174	0.174	0.265	0.197	0.114	0.114	0.144	0.136	0.182	0.091	0.106	0.159									
17	0.144	0.114	0.205	0.182	0.182	0.242	0.205	0.106	0.121	0.136	0.114	0.159	0.098	0.144	0.121	**0.068**								
18	0.129	0.098	0.189	0.167	0.167	0.258	0.189	0.091	0.106	0.121	0.098	0.144	0.083	0.129	0.136	**0.053**	**0.015**							
19	0.144	0.129	0.144	0.167	0.152	0.197	0.205	0.136	0.152	0.121	0.159	0.144	0.098	0.098	0.106	0.144	0.121	0.136						
20	0.152	0.136	0.212	0.174	0.174	0.280	0.212	0.098	0.144	0.144	0.091	0.152	0.106	0.167	0.159	0.121	**0.083**	**0.068**	0.144					
21	0.197	0.182	0.182	0.220	0.220	0.174	0.212	0.189	0.220	0.174	0.182	0.076	0.197	0.182	0.159	0.197	0.174	0.159	0.144	0.152				
22	0.220	0.205	0.205	0.242	0.258	0.167	0.220	0.212	0.227	0.212	0.189	0.098	0.189	0.174	0.152	0.189	0.152	0.167	0.152	0.174	0.038			
23	0.159	0.144	0.189	0.167	0.212	**0.288**	0.220	0.136	0.152	0.167	0.114	0.174	0.144	0.129	0.167	0.144	0.106	0.091	0.152	**0.068**	0.129	0.152		
24	0.189	0.189	0.174	0.212	0.212	0.197	0.220	0.212	0.197	0.197	0.144	0.098	0.189	0.159	0.121	0.174	0.167	0.152	0.167	0.174	0.083	0.091	0.167	
25	0.212	0.197	0.197	0.250	0.220	0.265	0.242	0.220	0.235	0.189	0.197	0.212	0.212	0.182	0.129	0.182	0.174	0.189	0.159	0.197	0.167	0.174	0.205	0.159

Note: 1-Uruan-AKS, 2-Itu-AKS, 3-Etinan-AKS, 4-Uyo-AKS, 5-Eket-AKS, 6-Yenagoa-BY, 7-Southern Ijaw-BY, 8-Kolokomo/Opokuma-BY, 9-Ogbai-BY, 10-Calabar Municipality-CRS, 11-Etung-CRS, 12-Calabar South-CRS, 13-Akpabuyo-CRS, 14-Odukpani-CRS, 15-Uvwie-DT, 16-Ethiope East-DT, 17-Udu-DT, 18-Ikpoba Okha-EDO, 19-Egor-EDO, 20-Oredo-EDO, 21-Ovia North-East-EDO, 22-Orhionmwa-EDO, 23-Abuloma-RV, 24-Bonny-RV, 25-Okrika-RV.

genotypes Koo-BY and Ogb-BY (0.043), between Eth-DT and Ikp-EDO (0.053), between Eth-DT and Udu-DT, Ikp-EDO, and Ore-EDO (0.068) as well as between Itu-AKS and Eku-AKS, Soi-BY and Cam-CRS, and Udu-DT and Oredo-EDO (0.083). The most distant genotypes were Abu-RV and Yen-BY with a dissimilarity coefficient of 0.288 (Table 7).

DISCUSSION

The present study investigated the genetic diversity and relationship among twenty-five *Ocimum* sp. genotypes collected from six southern states in Nigeria, using ten ISSR markers. It was also carried out to identify the most polymorphic ISSR markers for future phylogenetic studies on the species. A suitable genetic marker for diversity studies possesses high variability and its ability to create multi-locus information from the genome under study (Anne 2006, Al Salameen *et al.* 2018). ISSR markers have been efficiently utilized to unravel genetic polymorphism in some other crops like rice, millet, cowpea (Ghalmi *et al.* 2010), sesame (Woldesenbet *et al.* 2015), amaranth (Singh *et al.* 2013), as well as on *Ocimum* species (Aghaei *et al.* 2012, Patel *et al.* 2015).

A total of 679 amplicons were recorded from the 10 ISSR primers for the twenty-five scent leaf genotypes with the amplified bands per marker ranging from 26 to 143. This range was lower than the range of 94 to 155 polymorphic bands reported by Patel *et al*. (2015). Eight primers recorded 100% polymorphism while two (UBC807 and UBC808) recorded 56.9% and 35.9% respectively, which is quite low when compared to the report of Patel *et al*. (2015) who recorded 92.30% polymorphism from UBC 808 among *Ocimum* landraces. Lal *et al*. (2012) investigated the genetic diversity in six species of *Ocimum* using ISSR markers and found 100 % polymorphism.

To determine the level of polymorphism in markers, polymorphic information content can be used (Shete *et al.* 2000) and markers with polymorphic information content (PIC) above 0.5 are considered highly informative (Molosiwa 2012). The average PIC score of all primers was 0.677, with the PIC values ranging from 0.381 to 0.913. Eight of the primers had PIC values above 0.5, indicating a high resolving power of the ISSR markers. The PIC value obtained in this study was higher than the values reported by (Chen *et al.* 2013) who obtained a mean PIC of 0.19 and values ranging from 0.10 to 0.29.

This study confirmed the efficiency of the ISSR molecular markers for genetic diversity of *Ocimum* species as those reported in previous studies in the genus *Ocimum* (Aghaei *et al.* 2012, Chen *et al.* 2013, Patel *et al.* 2015, Alves *et al.* 2019). Five species of the *Ocimum* genus namely, *Ocimum basilicum*, *Ocimum americanum*, *Ocimum tenuiflorum*, *Ocimum gratissimum*, and *Basilicum polystachyon* (L.) Moench were investigated by Patel *et al*. (2015) using 12 ISSR markers. They obtained a high level of polymorphism (98.17%) from 238 fragments with an average PIC value of 0.92. In our study, 89.28% polymorphism was obtained using 10 ISSR primers but primer UBC813 which had the highest PIC (0.91) had 100% polymorphism. This polymorphism from UBC813 was much higher than 88.9% polymorphism reported by Alves *et al*. (2019).

The clustering of the maximum of 8 genotypes in cluster III showed low genetic variability among the genotypes which may be because of autogamous propagation of *Ocimum* sp. The factorial coordinate analysis grouped the 25 scent leaf genotypes into five groups. Although the grouping of the factorial coordinate analysis and the dendrogram were similar, some differences were observed. The genotypes from Okrika, Rivers state, and Uvwie, Delta state which were genetically isolated from other genotypes in the dendrogram were closely related to some other genotypes in the factorial coordinate analysis. The clustering pattern in the dendrogram and factorial coordinate analysis displayed considerable genetic variations among the 25 *Ocimum* genotypes and showed that the grouping is not based on geographical location. This agrees with the finding of Aghaei *et al.* (2012). The grouping of the genotypes could be based on the phytochemical constituents, exhibited traits, chromosome number and their responses to various physiological conditions.

CONCLUSION

The efficacy and suitability of ISSR markers as a molecular tool for diversity studies and assessment of phylogenetic relationships among populations of *Ocimum* species was revealed. High levels of genetic variation among the 25 *Ocimum* genotypes were obtained, indicating the robustness of ISSR markers for diversity studies of the species. Scent leaves are beneficial herbs and the findings of this study offer a better understanding of the plant populations in the South-South states of Nigeria. The high percentage of polymorphism obtained (89.28%) from the genotypes used, shows the heterogeneity of the genotypes. Primer UBC813 which yielded 100% polymorphism and the highest PIC of 0.913 was the most informative ISSR for diversity assessment of *Ocimum* genotypes. The genetic similarity among the genotypes is important for its utilization, genetic conservation, and

crop improvement. The UBC813 primer is therefore recommended for diversity studies on scent leaf. Physiological, morphological and karyotype characterization would be carried out on these genotypes of scent leaf to establish the correlation among the attributes and traits with the molecular data.

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