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Phylogenetic Relationship Between Some Cultivars of Cucurbita Species in Southwest Nigeria Using RAPD Analysis

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Abstract
Cucurbits are economic crops in Nigeria, which are not only taken to overcome nutritional deficiency but are also good sources of income for farmers. The taxonomy of the members of the family *Cucurbitaceae* had been a challenge to botanists in Nigeria as a result of many overlaps in the group, hence confusion the proper identification of the members. Randomized Amplified Polymorphic DNA (RAPD) markers were employed to study the genetic diversity among species of *Curcubita* species collected from three Southwestern states in Nigeria. Six out of the thirteen studied primers amplified a total of 74 polymorphic bands and 83 Allele numbers. The polymorphic bands exhibited a very high percentage of polymorphism. The result of the cluster analysis showed that the least distance was observed between samples collected from ONWL II, IFCL III, ISEL III, and IFCL I. The result also showed that samples from the three study locations (ONWL II, IFCL III, and ISEL III) exhibit the strongest level of homogeneity. The high level of heterozygosity of Curcubita species can provide valuable information to improve the breeding of *Curcubita*. It can also be useful in studying germplasm material or gene flow for further evaluation.
Keywords: *Curcurbitae,* RAPD, Heterozygosity, Polymorphism, Accession, Allele
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1. Introduction

Cucurbits are economic crops in Nigeria, which are not only taken to overcome nutritional deficiency but are also good sources of income for farmers. *Cucurbita* species commonly known as pumpkin and locally called "Elegede" in Southwest Nigeria. *Cucurbita* is grown for its young leaves and vines in the Oyo, Osun, Ogun, and Ekiti states of Southwest; it is consumed as a green vegetable among rural dwellers. Cucurbit is a common homestead garden crop in Southwest Nigeria and is most cultivated by women. It is a cheap source of protein, vitamins, fibers, and antioxidants in their diet. Generally, in Nigeria, it is used as food, it is also used locally to treat tapeworm and had been used in other regions of the world to treat the early stages of prostate disorder (FAO 1995; Matus 1993). The seed has been use in traditional medicine as an anti-helminths agent and supportive treatment for functional disorders of the bladder and difficulties in urination.

Among several methods, molecular biology provides many techniques that could be applied in resolving diversity issues and classifying species into their ideal genetic groups. Different types of DNA makers such as RFLPs (Garcia-Mas *et al* 2000), SSRs (Barzregar *et al* 2013, Rreghami *et al* 2014), and RAPD (Oshingboye *et al* 2013) have been used to determine genetic diversity in different species of *Cucurbtaceae*. Random Amplified Polymorphic DNA (RAPD) has been used in DNA fingerprinting gene mapping isolation of polygenetic relationships of many organisms and taxonomy within many families (Chandra, 2004), and Radwan *et al* (2014) also found RAPD useful as a molecular technique for cultivar identification. Wilson *et al.*, (1992) also explored Chloroplast DNA diversity analysis and placed cultivated species in different groups.

There is disagreement about the taxonomy of the genus as the number of accepted species varies from 13 to 30. Several authors have described this plant by the shape and colours of their flowers, petals, calyx, number of stamens, and thickness of their pedicels among many other taxonomic features (Whitaker and Bemis, 1975; Wilson *et al.*, 1992). Despite the nutritional and agronomic potentials of *Cucurbita,* reports in comparison to other countries imply that there is a dearth of knowledge of the genetic diversity and phylogeny of the Nigerian landraces. Most importantly, landraces are sources of genetic diversity for the improvement of cultivated species as they could be applied in the breeding program. There is a need to explore our landraces to estimate the amount of genetic diversity within and among accessions and cultivars of *Cucurbita*, gene flow among accession and to examine the phylogenetic relationship between cultivars.

The objective of this study was to identify genetic diversity and phylogenetic relationship among and within some cultivated species of *Cucurbita* from different growing areas in Southwest, Nigeria using RADP molecular marker.

2. Materials and Methods

2.1 Collection of plants

Thirteen *Cucurbit* fruits were collected between December 2021 and January 2022 for the study. They were sourced from different Southwestern states of Nigeria where the traditional communities usually grow *Cucurbita*. The location where the fruits were collected was used to code for the accession names. The Collected fruits were deposited at the Department of Biology, Faculty of Science; Adeyemi College of Education, Ondo. Morphological traits of the selected fruit accessions were characterized using the ECPGR descriptors for *Cucurbita* spp. (2008). Two to three accessions were sampled per cultivar according to availability (Table 1). The geographical sites of collection were identified in Figure 1. The seeds of each accession were carefully processed and grown out on a plot of land in the Institution Staff quarters for DNA extraction. The seeds were grown between February and June 2018. During the experiment, the mean upper and lower temperatures of the screen house were 25.1 and 18.2^{0}C and the mean relative humidity was 62 mmHg. To maximize geographical and taxonomic diversity, at least two different individuals for each accession were amplified.

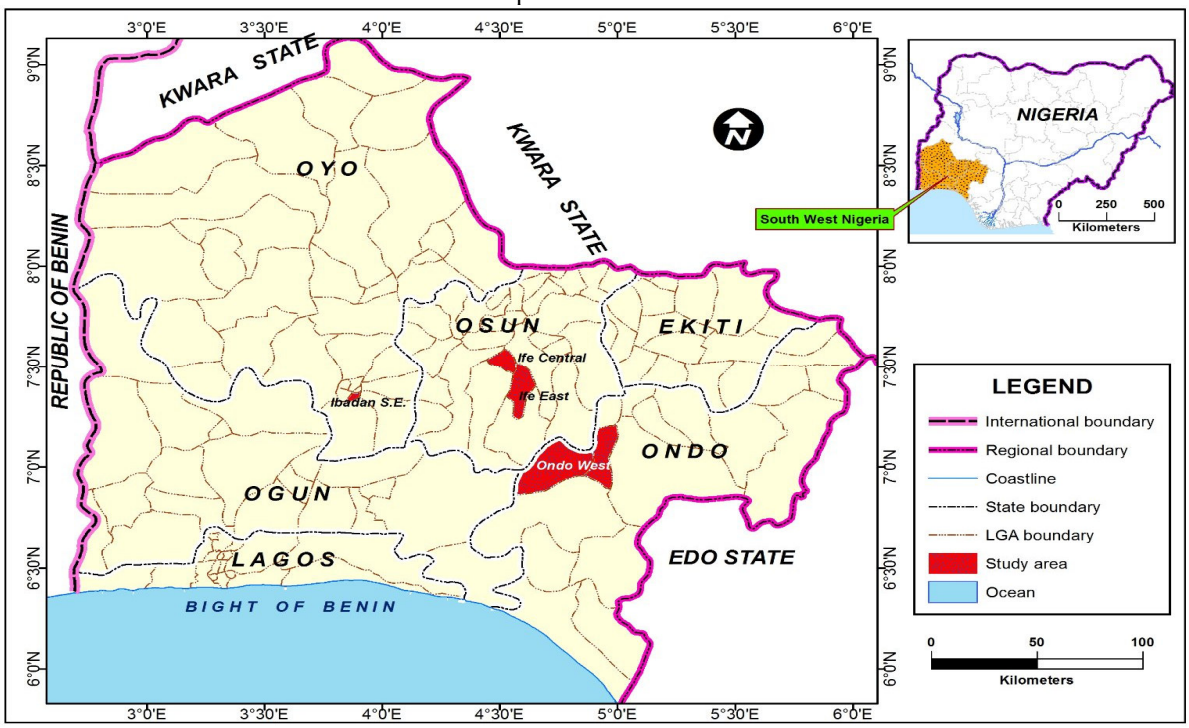

Figure 1: Map of study areas in South-west, Nigeria.

Table 1: Location and Sources of Fruits of 13 *Cucurbita* Accessions

S/N	State of collection	Place of collection	Location	Accession Name
1	Osun State	Oja-Oba Market	Ife-central Local Govt	IFCL I
2	Osun State	Oja-Oba Market	Ife-central Local Govt	IFCL II
3	Osun State	Oja-Oba Market	Ife-central Local Govt	IFCL III
4	Osun State	Oja-Oba Market	Ife-east Local Govt	IFEL II
5	Ondo state	Moferere market	Ondo-west Local Govt	ONWL I
6	Ondo state	Moferere market	Ondo-west Local Govt	ONWL II
7	Ondo state	Moferere market	Ondo-west Local Govt	ONWL III
8	Ondo state	Moferere market	Ondo-west Local Govt	ONWL IV
9	Ondo state	Omiluri Market	Ondo-East Local Govt	ONEL I
10	Oyo State	Bode Market	Ibadan South-East Local Govt	ISEL I
11	Oyo State	Bode Market	Ibadan South-East Local Govt	ISEL II
12	Oyo State	Bode Market	Ibadan South-East Local Govt	ISEL III

2.2 DNA Extraction

Less than 100mg of the plant tissue was placed in a mortar and homogenized with liquid nitrogen. The homogenate was transferred into a DNAse-free 1.7ml microcentrifuge tube. Thereafter, 500ml of lysis buffer and 1µl of RNAse was added to the homogenate. Pellets were formed. The pellets were incubated in an incubator for 10 minutes at 65ºC. During incubation, the homogenate was mixed about 2 – 3 times by inverting the tube. 100µl of binding buffer was added, mixed thoroughly, and incubated for 5 minutes to form the lysate. The lysate was centrifuged for 2 minutes at 14,000 rpm. The supernatant was pipetted into a DNAse-free Eppendorf tube. An equal volume of 70% ethanol was added to the lysate collected above and vortexed to mix. Ethanol was added to 650µl of the lysate and centrifuged for 1 minute at 10,000 rpm. The Resin formed was washed in a wash solution A and centrifuged for 1 minute. The resin was dried by centrifuging for 2 minutes at 4, 000 rpm.

2.3 DNA Elution

A column was placed in 1.7ml of elution tube. Thereafter, 100µl of elution buffer B was added to the column and incubated at room temperature for 1 minute. This was centrifuged at 1,000 rpm for 1 minute to get clean DNA. The pure genomic DNA was stored at 4ºC.

2.4 Screening of RAPD - PCR

The plant genotypes were studied for the polymorphism using thirteen decamer primers including OPA, OPB, OPD, OPE, and OPF primer kits from Operon Technologies and SIGMA-D series from Sigma Technologies. PCR amplifications were carried out according to the method described by Williams *et al.,* (1990) using the Multi-master mix protocol.

The thermoprofile for the PCR reaction was as follows: Denaturation of DNA at 94ºC for 4 minutes followed by 45 cycles of PCR amplification for 30 seconds of denaturation at 94ºC. This was followed by 1 minute of annealing at 36ºC and 2 minutes of Primer extension at 68ºC. The primer extension was completed by incubating for 4 minutes at 72ºC. The amplified products were electrophoretically resolved on 1% agarose gel with 1X TBE (40mM Tris-borate, 1mM EDTA) at 100 volts for 55 minutes and photographed on a UV transilluminator. The sizes of the products were determined by comparison with a 1000bp DNA ladder size standard. The gels were scored as 1 or 0 based on the presence or absence of a band.

2.5 Data Analysis

Data were scored for the presence or absence of amplification fragments from each RAPD primer and entered into a binary data matrix. The data matrix was used to calculate the genetic similarity within and among species based on Euclidean's similarity coefficients, and a dendrogram displaying relationships among the 12 genotypes was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using the PAST-pc statistical package version 5.0.

3.0 RESULTS

3.1 Genetic diversity of *Cucurbita sp.* using RAPD markers

Thirteen decamer primers were evaluated for DNA polymorphism of *Curcubita species* (Table 3). Among these primers that were amplified, OPA -2, OPA-3, OPD-07, OPD-13, OPD-02, and SIGMA-D-01 cross amplified as single, intense bands of approximately 500bp in all the samples of *C. pepo*. The amplicon was polymorphic to all the specimens.

Table 3: Sequences of thirteen RAPD Primers

Primer	Sequence (5' – 3')
OPA-2	TGCCGAGCTG
OPA-3	AGTCAGCCAC
OPA-13	CAGCACCCAC
OPB-08	GTCCACACGG
OPD-02	GGACCCAACC
OPD-07	TTGGCACGGG
OPD-08	GTGTGCCCCA
OPD-13	GGGGTGACGA
OPE-07	AGATGCAGCC
OPF-14	TGCTGCAGGT
SIGMA-D-01	AAACGCCGCC
SIGMA-D-14	TCTCGCTCCA
SIGMA-D-P	TGGACCGGTG

From the thirteen primers that were used, only six of these primers cross-amplified. These primers are represented in Table 4. A total of seventy-four (74) polymorphic bands and 83 allele numbers were observed

between the studied species. Among these primers that cross-amplified was OPA - 3, OPA-13, OPD-02, OPD-13, OPD-17, and SIGMA-D-01 series (Table 4). The bands were renamed CUB 1, CUB 2, CUB 3, CUB 4, CUB 5, and CUB 6 (Plates 1 - 6) respectively. The number of RAPD bands varied between 11 – 18 and the size of the bands ranged between 200 – 800bp

Table 4: The sequences of amplified RAPD primers

Primer	Sequence (5' – 3')	Allele Number(AN)	Size range of bands (bp)	Polymorphic band (PB)	% Polymorphism
OPA-13	CAGCACCCAC	11	350 - 600	9	82
OPA-3	AGTCAGCCAC	12	200 - 800	11	92
OPD-07	TTGGCACGGG	13	250 - 700	12	92
OPD-13	GGGGTGACGA	15	200 - 500	13	87
OPD-02	GGACCCAACC	14	300 - 500	14	100
SIGMA-D-01	AAACGCCGCC	18	200 - 400	15	83
	TOTAL	83		74	

% Polymorphism = PB/AN X100

**PB means Polymorphic Band, **AN means Allele Number*

The RAPD profiles of the amplified genomic DNA of *Cucurbita spp.* are presented in Plates 1 – 6.

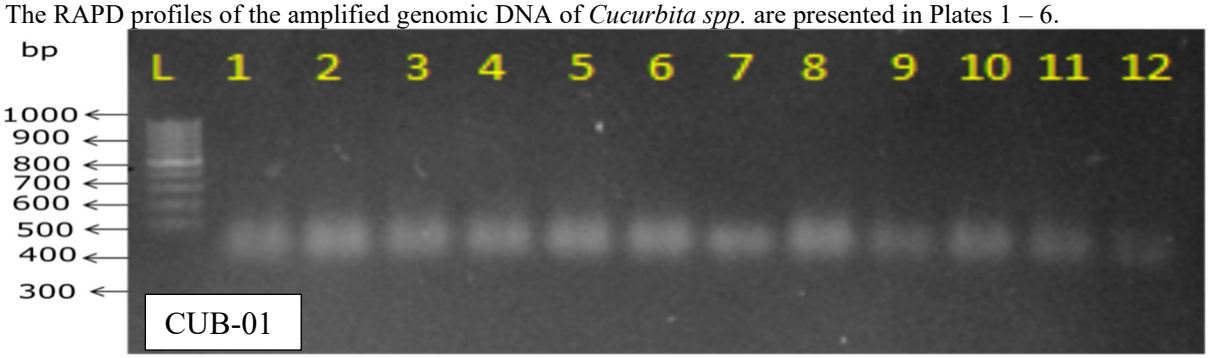

Figure 1: RAPD profile of genomic DNA of *Cucurbita spp.* from OPA-13 primer (renamed as CUB-01)

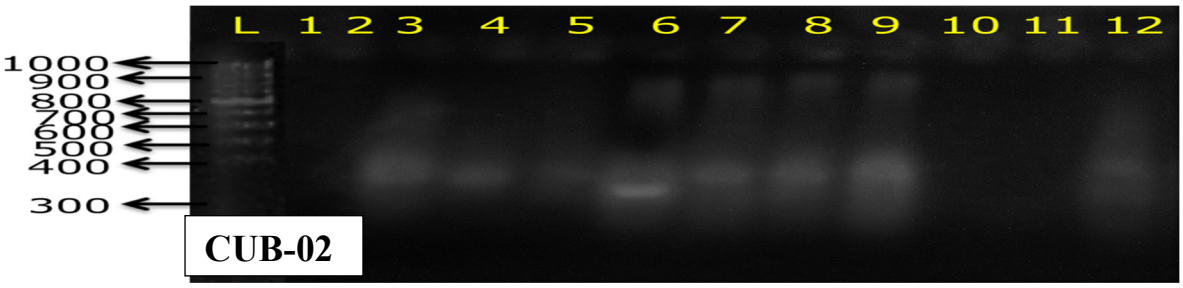

Figure 2: RAPD profile of genomic DNA of *Cucurbita spp.* from OPA-3 primer (renamed as CUB-02)

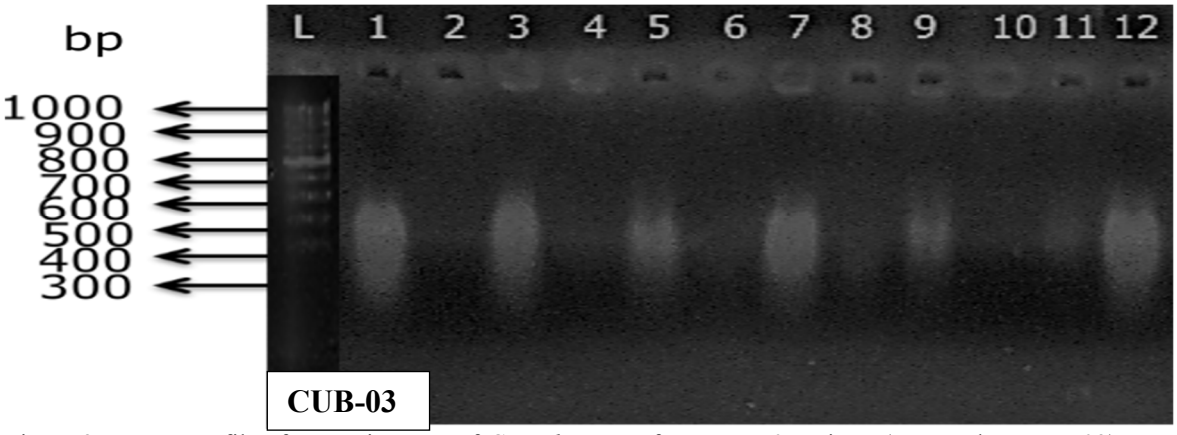

Figure 3: RAPD profile of genomic DNA of *Cucurbita spp.* from OPD-07 primer (renamed as CUB-03)

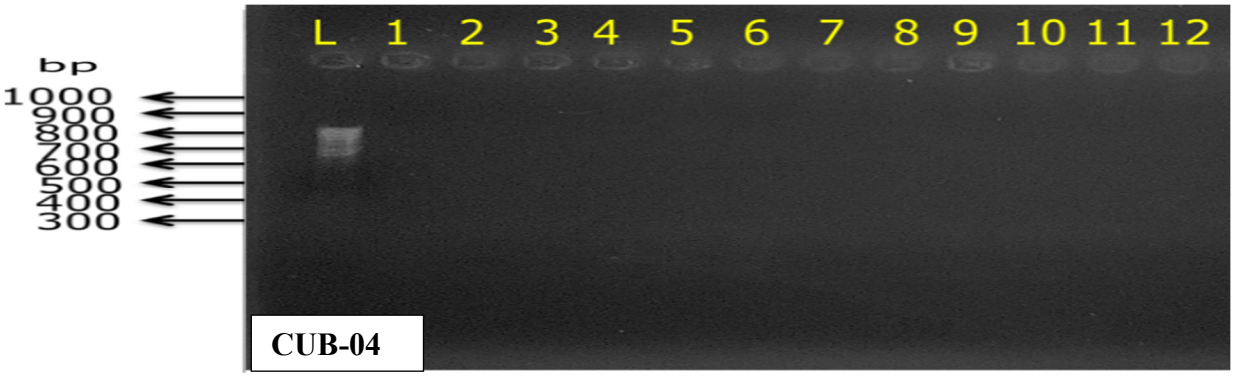

Figure 4: RAPD profile of genomic DNA of *Cucurbita spp.* from OPD-13 primer (renamed as CUB-04)

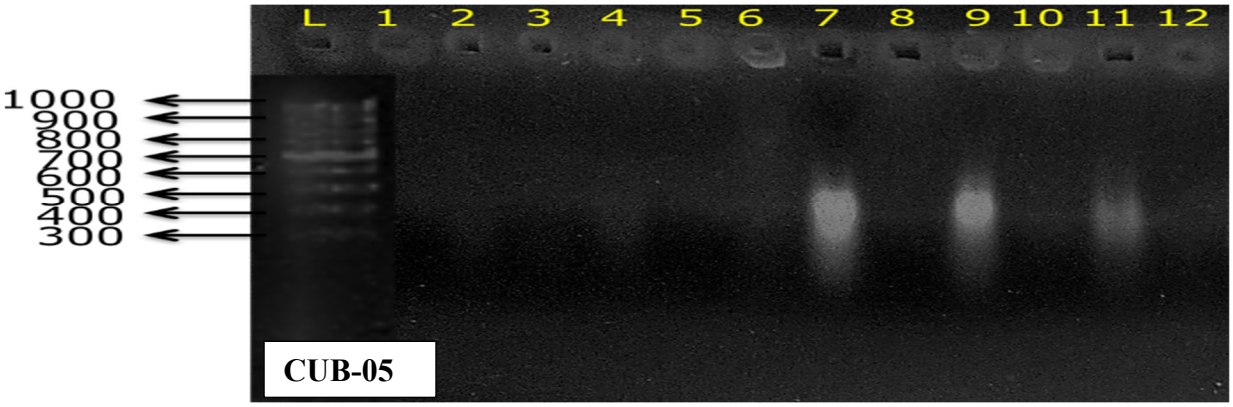

Figure 5: RAPD profile of genomic DNA of *Cucurbita spp.* from OPD-02 primer (renamed as CUB-05)

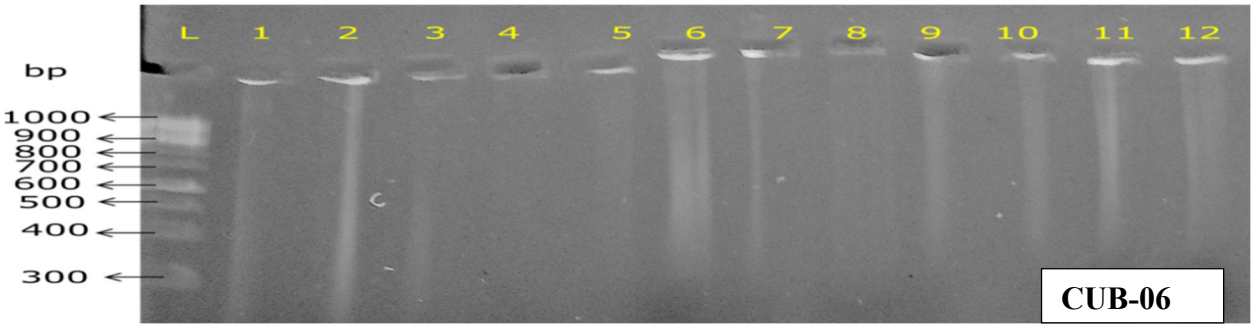

Figure 6: RAPD profile of genomic DNA of *Cucurbita spp.* from SIGMA-D-01 primer (renamed as CUB-06)

Based on the presence or absence of 74 RAPD bands, cluster analysis of the genetic similarity estimates was performed using PAST version 5.0 to generate a UPGMA dendrogram shown in Figure 7. The RAPD profiles produced a dendrogram that fully resolved the taxa of Cucumerinae into 2 clades.

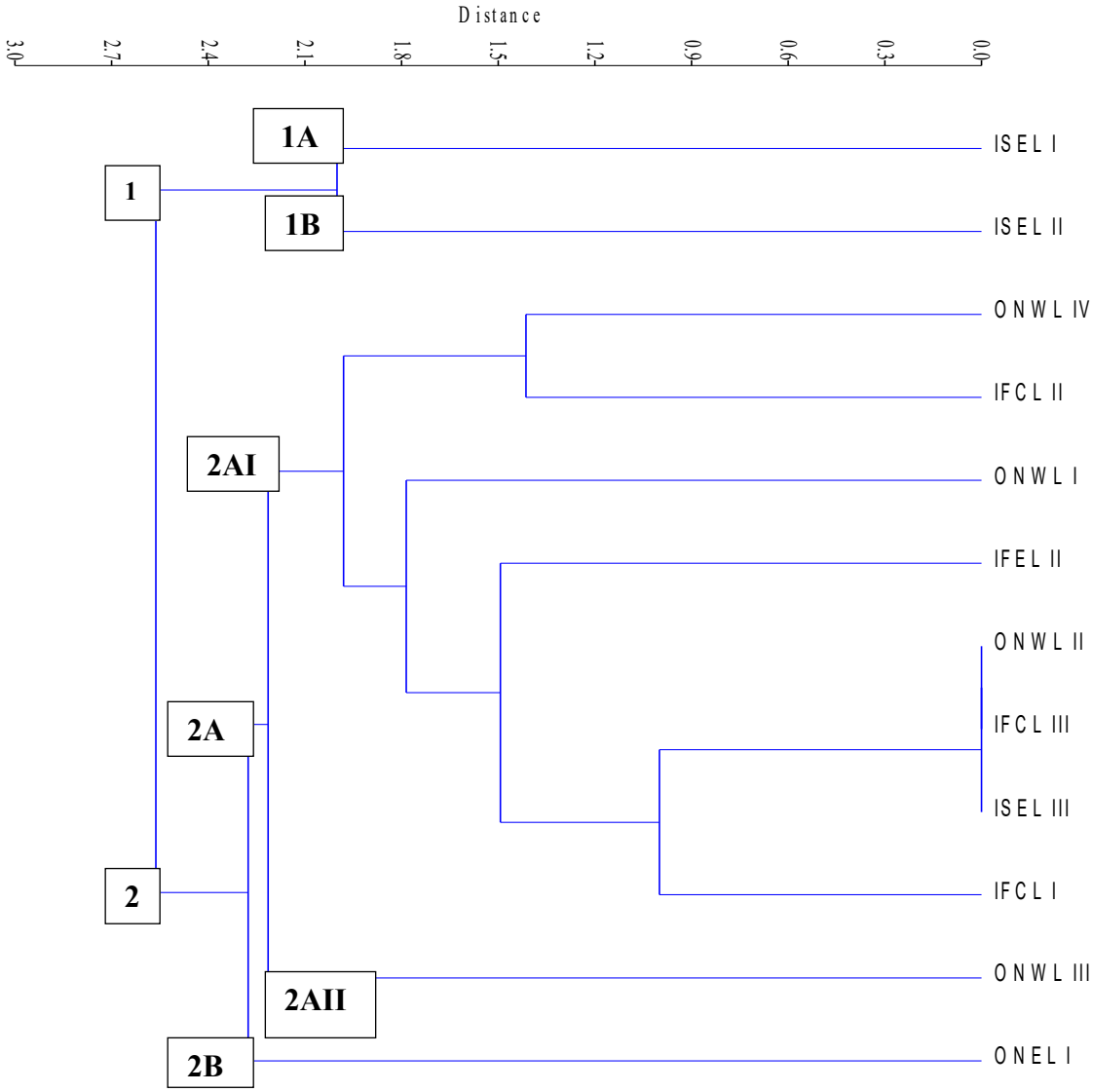

Figure 3: Dendrogram, showing the relationship among the population of *Cucurbitaceae species* from three Southwestern states of Nigeria constructed by using UPGMA based on RAPD banding profiles.

The dendrogram shows the genetic relationship of *Curcubita species* in South-west, Nigeria. The dendrogram has two clades, 1 and 2. These clades show that the studied species of *Curcubita* have a common ancestral relationship. They differ at a genetic distance of 2.7. Clade 1 is divided into two gropes namely 1A and 1B. The two gropes in clade 1 differ at a distance of 2.0. The two samples in clade one are fruits from Ibadan Southeast Local Government (ISEL I) and Ibadan Southeast Local Government (ISEL II). The second clade (clade 2) is divided into two clusters viz: 2A and 2B. These clusters deviate at a similarity distance of 2.3 Cluster 2A is divided into two clusters, 2AI and 2AII while 2B has the specie of *Curcubita* collected from Ondo East Local Government (ONEL I). Cluster 2AI has most of the samples on it. On the same cluster, samples from Ondo-west Local Government (ONWL IV) and Ife-central Local Government (IFCL II) at a similarity distance of 1.4. Samples from ONWL II, IFCL III, and ISEL III have the highest similarity value among the studied species of *Curcubitaceae.* Hence, they exhibit the strongest level of homogeneity. According to the dendrogram linkage joining rule, the sample from Ife-Central Local Government I (IFCL I) is more distantly related and separated from the other members of the group at a similarity value of 1.0.

4. Discussion

Molecular markers such as Amplified Fragment Length Polymorphism (ALFP), Simple sequence repeat (SSR), Inter simple sequence repeat (ISSR) have been used to analyze the genetic diversity of *Curcubita spp* such as *C. pepo* (Katizir *et al.,* 2000; Paris *et al.,* 2003; Chiba *et al*., 2003, Ferriol *et al.,* 2014) but much work have not been done about the genetic diversity within *Curcubita*family species (Radwan *et al.,* 2014).

RAPD analysis can reveal a high degree of polymorphism (Prajak and Saensouk, 2012). This fact is confirmed

by the result obtained from this study. The percentage polymorphism ranged between 82 and 100%. Due to the high levels of polymorphism, it can be predicted that there is a strong genetic relationship among the studied species of *Curcubita* collected from the different study locations. RAPD also has high levels of reproducibility. The different reproducible bands by each primer depend on the sequence of the primer and the extent of variation in specific genotypes (Chan and Sun, 1997; Shukla *et al.,* 2006; Shiran, 2007). The result obtained from the study showed that some fragments were incomparably amplified and such bands include samples from Ondo-east Local Government I (ONEL I), Ondo-West Local Government III (ONWL III), Ondo-West Local Government I (ONWL I) and Ife-East Local Government II (IFEL II). This result is similar to the result of Al-Anbari *et al.,* (2015) on the genetic diversity of some taxa of the *Curcubitaceae* family. These fragments from ONEL I, ONWL III, ONWL I, and IFEL II have an important interest in the optimal management of germplasm collections. They indicate a unique banding pattern over the other samples; hence, they have specific genotypes. They can also provide the identification of taxa and duplicates and also verify possible contamination of the seeds during cultivation and/or conservation activities.

The RAPD-PCR analysis and index matrix based on all DNA fragments that are isolated by the six primers showed that the strongest homogeneity existed between samples of *C.pepo* collected from ONWL II, IFCL III, and ISEL III (all from the three different states and study locations). The result is similar to the findings of Al-Anbari *et al.,* (2015). The result is also similar to the findings of Saengprajak and Saensouk, (2012) on cultivar species of *Cucumerinae* using RAPD and SCAR markers.

5. Conclusion

Awareness of the phylogenetic relationship relationships in the genus *curcubita* is important because the germplasm and natural composition can provide valuable information to improve pumpkin breeding. RAPD is a reliable method of analysis of *Curcubita spp.* because it provides an effective procedure to understand evaluating germplasm material or gene flow for further evaluation. This study also shows that a high degree of correlation exists among the samples of the studied species in the three geographic locations.

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