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Research

Molecular Characterization of Twenty-nine Okra Accessions Using the Random Amplified Polymorphic DNA (RAPD) Molecular Marker

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Abstract

Twenty –nine okra accessions collected from different agro-ecological regions in Nigeria were raised in plastic pots. Genomic DNA from the young apical leaves of the plants were extracted and analyzed by the random amplified polymorphic DNA (RAPD) technique to access genetic variability in twenty-nine okra accessions. Eighty-four amplified products and 53 RAPD bands were scored with an average of 61.4% of them revealing polymorphism across accessions. Primer OPX 17 yielded the highest polymorphic band (8), with 67% polymorphism, while OPY 02 yielded 6 polymorphic bands with the highest percentage polymorphism (75%). The least number of polymorphic bands (3) as well as least percentage polymorphism (50%) was in OPX 18. The amplification products of Okra DNA assayed with OPM 18 and OPM 16 respectively revealed outstanding band patterns among the accessions signifying genetic differences amongst them. The dendogram revealed five distinct clusters which joined to form one major cluster at 55% level of similarity. The similarity indices of the RAPD dendogram ranged between 50% and 100% averagely high enough to suggest useful variability for genetic conservation and plant breeding. The two most divergent accessions revealed by the molecular dendogram are OK 20 and Enugu-1 suggesting that the origins or sources of these accessions were quite diverse.

Keywords: RAPD; Polymorphism; Okra; Dendrogram; Primer

Introduction

Okra, *Abelmoschus esculentus* (syn. *Hibiscus*) [L.] Moench is a Dicotyledonae, belonging to the order Malvales and family Malvaceae (Schippers, 2000). It is an important vegetable crop widely grown in the tropical and subtropical regions of the world (Tindall, 1983). In addition it has a wide range of uses which includes curing ulcer, suppressing the pains and effects of haemorrhoid and its mucilage serves as plasma replacement or blood volume expander (Siemonsma & Kouame, 2004). World production of okra (*A. esculentus and A. callei*) as fresh fruit-vegetable is estimated at 6 million t/year (FAOSTAT, 2004). In Africa, Nigeria is the largest producer of okra (0.70 million tonnes/year) followed by Ghana (0.10 million tonnes). The primary objectives of okra germplasm characterization have typically been to identify high yielding genotypes with resistance to YVMV, fruit borer, jassid and higher vitamins C content in the world species that can be utilized for the improvement of *A. esculentus* (Bisht & Bhat, 2006). However, characterization based on morphological traits are not easily reproducible especially, since morphological traits are influenced by variations in environment (Staub *et al*., 1996).

Apart from the use of morphological traits in the characterization of plant species other markers are equally available and are often more reliable than morphological traits. This includes the use of isozyme makers (IBPGR, 1991) and molecular markers (Archak *et al.*, 2002; Singh *et al.*, 2007). Genomic investigation using morphological and isozyme markers have some limitations, which include problems of phenotypic penetrance or heritability, and low map resolution (Vogel *et al*., 1996). Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among species (Chakravarthi & Naravaneni, 2006). Genetic diversity in *A. esculentus* and the four related species (*A. ficulneus, A. manihot*, *A. moschatus* and *A. tuberculatus*) was studied using isozyme electrophoresis and RAPD techniques (Bhat *et al*., 1995). This study covered allelic variations at 13 isozyme loci and 189 amplification products obtained by random amplification of genomic DNA using 22 random primers of 10 nucleotide lengths. They reported moderate genetic diversity within *A. ficulneus,* A. *moschatus* and *A. esculentus*. *A. moschatus* genome was observed to be quite distinct from that of the other four species. Gene duplication was common in all the species studied. Similarly, Aladele (2007) reported a significant genetic diversity between *A. esculentus* and *A. callei* using 125 RAPD bands and 13 primers.

RAPD techniques involve PCR amplification of genomic DNA using a single arbitrary

primer (usually 10 nucleotides), separating amplified products by electrophoresis, and visualizing them using ethidium bromide staining (Baird *et al*., 1996). Two correctly-oriented primer sites must exist within a limited distance (typically under 300 bp) in the genomic DNA for amplification to be successful. Amplification will not occur if primer sites are much further apart in the genome (Whitkus *et al*., 1994). Most variation among individuals for RAPDs probably arises from pair substitutions or insertion/deletions that modify (or eliminate) the primer site, or insertions in the genomic sequence that separate the primer sites to a distance that will not permit amplification (Williams *et al*., 1990). Among other advantages of RAPD over morphological, isozyme and other molecular markers include cost effectiveness and utilization of little quantity (a few hundred nanograms) of genomic DNA for analysis. Furthermore, RAPDs reveal high levels of polymorphism even within and among species that show little Restriction Fragment Length Polymorphism (RFLP) or isozyme polymorphism (Paran *et al*., 1991; Van Heusden & Bachmann, 1992, Vogei *et al*., 1996). However, the reproducibility of amplified products is usually low (Rajput *et al*., 2006). The objective of this work is to access genetic variability in twenty-nine okra accessions using RAPD molecular marker.

Materials and Methods

Raising of seedlings for DNA extraction

Three seeds of each accession were planted in plastic pots in a screen house at an average temperature range of 24 ^{o}C and 30 ^{o}C. Seeds germinated between five and seven days after planting. Two weeks after germination in the screen house, the second set of young leaves were harvested from four randomly selected seedlings of each accession for DNA extraction. The harvested leaf samples were put in well labelled polythene bags inserted into an ice pack and quickly taken to the laboratory. This is to preserve the leaf samples and the quality of DNA to be extracted.

DNA-extraction

Extraction of genomic DNA was done according to the procedure reported by Thottappilly *et al*. (1999), with modifications. The method is as follows:

1. 0.5 g of leaf per sample per accession harvested were grounded into fine powder in liquid nitrogen using 1.5 ml eppendorf tube. These tubes were properly labeled and the samples treated or stored at -80 ^{o}C until use.
2. The samples in eppendorf tubes were then suspended in 600 µl of 2xCTAB buffer (50mM Tris-HCl [pH 8.0], 0.7mM NaCl,

10mM EDTA, 2% hexadecyltrimethylammonium bromide, and 0.1% 2-mercaptoethanol) and incubated for 65 ^{o}C for 20 mins. At 65 ^{o}C in the extraction buffer, DNA was extracted from the plant cell.

3. Isolated DNA was purified by two extractions with phenol: chloroform: isoamyl alcohol (24:25:1) and precipitated with -20 ^{o}C absolute ethanol (Chloroform removes RNA, starch, peptide and unwanted material extracted along with the DNA. Isopropanol was used to precipitate the DNA)
4. The supernatant was carefully decanted off making sure that the pellet was not dislodged from the bottom of the tube after centrifuging at 9000 rpm for 8 min, using SS-34 Coex-adapted rotor
5. The pellets were washed in 2 ml of 70% cold ethanol for 20 min.
6. The DNA pellet was air dried to remove the remaining ethanol. The pellet was resuspended in 200 µl of sterile distilled water.
7. Resuspended DNA was transferred into 1.5 ml eppendorf tubes, properly labeled and stored at -20^{O}C until ready for use.

Checking the quality and estimating the concentration of DNA

Quantification and quality of isolated DNA was measured by the use of DU-65UV spectrophotometer (Beckman instruments inc., Fullerto CA, USA). 5 µl of each DNA sample was mixed with 95 µl distilled water, which gave a dilution factor of 100. Readings were taken at 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of DNA in the samples.

$$\text{The concentration in ng/µl} = \frac{\text{OD260} \times 100 \text{ (dilution factor)} \times 50 \text{ ng/µl}}{1000}$$

50 is a constant for double stranded molecules (Sambrook *et al*., 1989; Hoisington *et al*., 1994).

The quality and purity of DNA was determined by calculating the ratio of readings at 260 nm and 280 nm. Values between 1.8 and 2.0 are an indication of high quality DNA. Ratios below 1.8 is an indication of presence of protein contamination while ratios above 2.0 is an indication of chloroform and phenol contamination

PCR amplification of DNA

Five hundred primers in the OPM, OPX and OPY series were screened for reproducibility and to eliminate sporadic amplification products. This was done by preparing two concentrations of DNA (24 ng and 96 ng per reaction) from four randomly selected okra samples and used to screen these

primers for their ability to amplify the okra sample's DNA. After the initial primers screening analysis, potential primers that revealed high levels of DNA polymorphism were selected. Each of the selected primers was then used to amplify the DNA of the remaining sample accessions of okra.

PCR amplification of the DNA was performed in 25µl reaction mixtures consisting of 24ng DNA template, 1X reaction buffer (Promega), 100µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM Operon random primer, 2.5 µM $MgCl_2$ and 1unit of Taq polymerase (Boehringer, Germany). A single primer was used per reaction. The reaction mixture was overlaid with 50 µl of sterile mineral oil to prevent evaporation. The tubes were properly labelled, capped and amplified using 96-well thermal cycler (Perkin Elmer programmable thermal controller model 9600). The cycling programme used for amplification consisted of

1. 1 cycle of 94 ^{o}C for 3 min
2. 45 cycles of 94 ^{o}C for 20 s. for denaturing, 37 ^{o}C for 20 s. for annealing of primer and 72 ^{o}C for 40 s. for extension and
3. A final extension at 72 ^{o}C for 7 min.

Gel electrophoresis of PCR products

The amplification products were resolved by electrophoresis in 1.4% agarose gel using TAE buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 2 h. A 1 kb maker (Life Technologies, Gaithersburg, MD, USA) was included as DNA size maker. 10 µl of each of the PCR amplification products were mixed with a loading buffer and then loaded on the agarose gel for electrophoresis. When the maker dye was ¾ of the way to the end of the gel, the power was tuned -off. The gel was then taken and put in a tray containing ethidium bromide solution (0.5µg/ml) for staining and visualizing. The gel was allowed to stain for 5 min. and then rinsed in a tray containing water for about 10 min. The stained banding patterns were then visualized under UV-light in the transiluminator. Photograph was taken under UV-light using a red filter. The photographed stained banding pattern consists of different DNA fragments or RAPD bands that revealed different fingerprints for each okra DNA analyzed.

Scoring of RAPD bands

The position of the RAPD bands was scored as presence (1) or absence (0) to produce a binary data matrix. A fragment is considered polymorphic if present in at least one accession but absent in the others. Using the NTSYS-pc, the transformed binary character marix data were first transferred into the software data collection module from which a pair wise distance matrices was calculated (Rohfl, 1995), using the Jaccard coefficient of similarity (Jaccard, 1908). Using the output data and the graphical model of the software, a

phylogenetic (dendrogram) tree was generated by the Unweighted Pair-Group Method of Arithmetic average cluster analysis (UPGMA) (Sneath and Sokal, 1973; Swafford and Olsen, 1990).

Results

Molecular characterization of twenty-nine accessions of okra using RAPD analysis

Table 1 shows the DNA yield per unit volume of DNA sample from each of the twenty-nine okra accessions studied. Clemson spineless gave the highest DNA yield per volume with 68.10 ng/µl, followed by Dajofolowo-1 with 56.20 ng/µl. The least yield in DNA was recorded in NH99/28 with 1.89 ng/µl. The average DNA yield in all the accessions was 13.97 ng/µl. Also the ratio of OD 260 nm to OD 280 nm determined by spectrophotometry ranged between 1.8 and 2.00 for 78.05% of the samples.

Table 2 presents the RAPD primers that showed polymorphism. Out of the five hundred decamer random primers (Operon Technologies, OPM, OPX, OPY series) ten revealed high levels of DNA polymorphism. The ten primers generated a total of 53 RAPD bands most of which were polymorphic across accessions because they were able to differentiate at least two of the Okra accessions. Each primer revealed a unique band pattern for all the twenty-nine accessions of okra tested at the molecular level. This also brought out clearly genetic variations amongst the accessions studied using RAPD. Primer OPX 17 revealed the highest level of amplification (12) followed jointly by OPM 18 and OPY 04 with (10). The lowest amplification was recorded in OPX 18 with (6). The ten primers selected produced between 3 and 8 polymorphic bands, with an average of 5.3 bands. Primers produced fragments that ranged between 200 bp and 3 kb. OPX 17 yielded the highest polymorphic band (8), with a corresponding 67% polymorphism. OPM 18 yielded 7 polymorphic bands with a corresponding 70% polymorphism, while OPY 02 yielded 6 polymorphic bands with the highest percentage polymorphism 75%. OPX 18 yielded the least number of polymorphic bands (3) as well as the least percentage polymorphism of 50%.

Plates 1 and 2 show the amplification products of Okra DNA assayed with OPM 18 and OPM 16, respectively. The arrows on each of the plates points to unique genotypes with outstanding banding patterns. These outstanding patterns also signify genetic differences amongst the accessions. Plate 1 (primer OPM 18) revealed that all the accessions differ since none of them had the same banding pattern with each other. This is an indication of polymorphism in the accessions studied. Furthermore, it also

revealed a particular accession LD88/1-8-5-2 (7), which is deficient in bands situated between 2400 and 2800 bp present in all other accession.

Four accessions Enugu-1 (16), V-35 (19), CCN2005/2 (24) and CCN2005/1 (29) have a common band pattern in the region of 2100bp. All the accessions have the common band pattern in the regions of 1400, 2100 and 3000 bp except LD88/1-8-5-2 (7). Within the region of 760bp all accessions share similarity in band pattern except, Ola kg 1-6-05 (2), Ola V1 (3), OLA K2005 (4), LD88/1-8-5-2(7),

Table 1: Determination of DNA concentration in 29 accessions of okra

S/N	Accession	Volume of DNA (µl)	Concentration (ng/µl)
1	Lady's finger	5.0	5.03
2	Ola kg1-6-05	5.0	3.50
3	Ola V1	5.0	13.05
4	Ola k2005	5.0	2.98
5	Ila Gidi	5.0	4.19
6	LD88/1-8-11-1	5.0	10.26
7	LD88/1-8-5-2	5.0	3.02
8	Short Mouth Ibarapa	5.0	8.12
9	Clemson spineless	5.0	68.10
10	V45-2	5.0	2.88
11	NH99/DA	5.0	10.01
12	LD88/1-8-16-2	5.0	20.16
13	0LA99/13	5.0	4.96
14	OSADEP Purple Tall	5.0	13.10
15	47-4-5	5.0	12.68
16	Enugu-1	5.0	4.60
17	47-4	5.0	11.97
19	V2-OYO	5.0	9.16
19	V-35	5.0	8.02
20	OLA 3 Local	5.0	3.90
21	OK 20	5.0	45.10
22	NH99/28	5.0	1.89
23	Dajofolowo-1	5.0	56.20
24	CCN2005/2	5.0	5.16
25	NH88/1-8-16-2	5.0	16.22
26	NH88/82	5.0	3.16
27	NH99/9	5.0	18.16
28	Jokoso-2	5.0	21.66
29	CCN2005/1	5.0	20.11
	Average		13.97

Volume: amount of DNA expressed in µl; *Concentration:* Actual amount of DNA per unit volume

Table 2: List of RAPD primers that showed polymorphism

Primer Code	Sequence	No of Fragments amplified	No of Polymorphic bands	Percentage Polymorphism
OPM 16	5'-GTAACCAGCC-3'	8	5	62.5
OPM 18	5'-CACCATCCGT-3'	10	7	70
OPX 14	5'-ACAGGTGCTG-3'	7	4	57
OPX 17	5'-GACACGGACC-3'	12	8	67
OPX 18	5'-GACTAGGTGG-3'	6	3	50
OPY02	5'-CATCGCCGCA-3'	8	6	75
OPY 03	5'-ACAGCCTGCT-3'	9	5	56
OPY 04	5'-GGCTGCAATG-3'	10	6	57
OPY 17	5'-GACGTGGTGA-3'	8	5	62.5
OPY 18	5'-GACTAGGTGG-3'	7	4	57
	Total No of bands	84	53	–
	Mean No of bands	8.4	5.3	–
	Mean (%) polymorphic band	–	–	61.4

LD88/1-8-16-2 (12), OSADEP Purple Tall (14), Enugu-1(18) and V2-OYO (18). Plate 2 primer OPM 16, also shows polymorphism amongst the accessions, however, LD88/1-8-5-2 (7), Clemson spineless (9), 47-4-5 (15), and NH88/1-8-18-2 (25) were clearly different from all other accessions due to absence of some bands between the 1410bp and 3000bp. NH88/82 (26) and Jokoso-2 (28) has an identical polymorphic band situated at 1750bp region that makes them different from all other accessions. Meanwhile, there was a strong relationship between OLA K2005 (4) and Ila Gidi (5) as shown by their identical banding pattern. Similarly, V_2-OYO (18) and 0LA 3 Local (20), appear also identical in banding pattern. Ola Kg 1-6-05 (2), OLAV (3), LD88/1-8-11-1 (6), LD88/1-8-5-2 (7), NH99/DA (11), LD88/1-8-16-2 (12), OSADEP Purple Tall (14), V2-OYO (18), V-35 (19), OLA 3 Local (20), NH99/28 (22), Dajofolowo-1 (23) and CCN2005/1 (29) were different from others in the region of 200 bp.

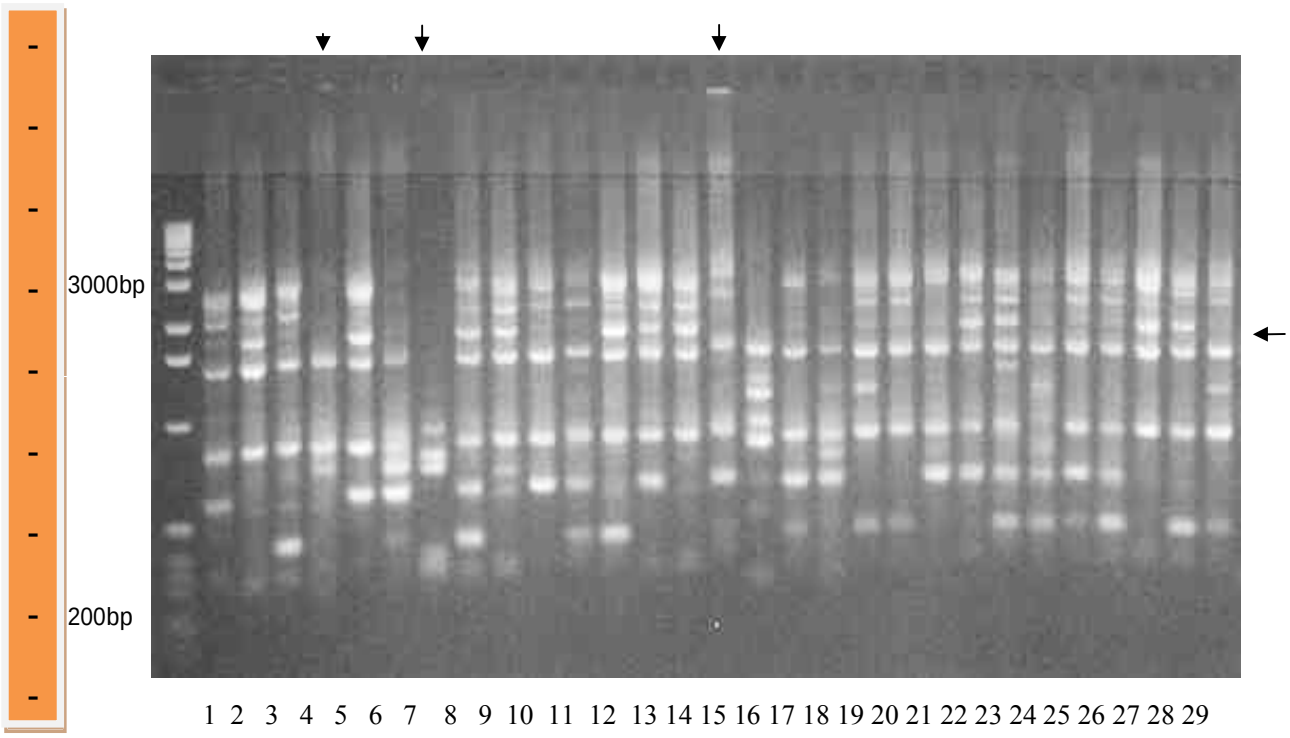

Plate 1: RAPD profiles obtained with primer OPM 18 revealing polymorphism

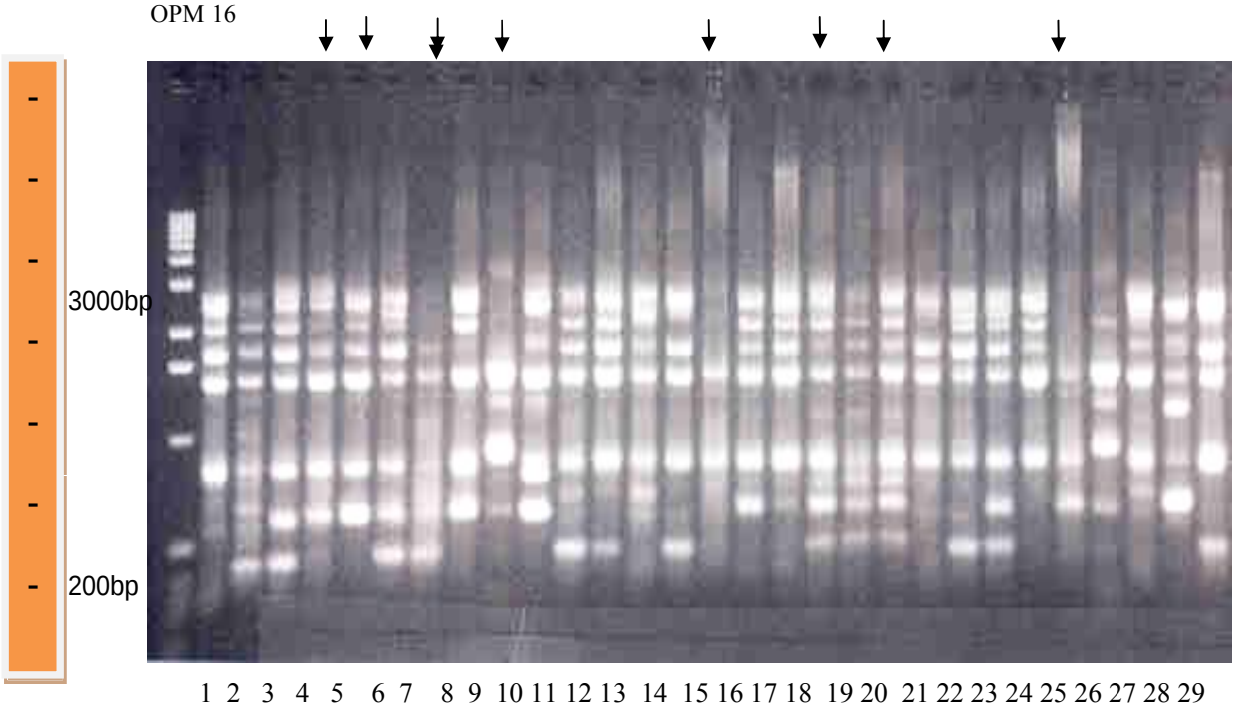

Plate 2: RAPD profiles obtained with primer OPM 16 revealing polymorphism

The dendrogram generated from the data on similarity matrix from the base pairs using the unweighted pair-group method of arithmetic average (UPGMA) and the Neighbour (NJ) method is presented in Fig. 1. The molecular dendrogram revealed five distinct clusters which have joined to form one major cluster at 0.55 level of similarity. Cluster I is composed of six accessions joining to form one cluster at 0.66 level of similarity. These accessions include V2-OYO, OSADEP Purple Tall, Jokoso-2, Ola 3 local, NH88/1-8-16-2, and Ila Gidi. Cluster II, is composed of two accessions, 47-4 and OLA99/13, joining at 0.68 level of similarity. Cluster III is composed of twelve accessions, NH88/82, NH99/9,47-4-5, CCN2005/1, LD88/1-8-5-2, LD88/1-8-11, OLA K2005, LD88/1-8-16-2, OLA V1, NH99/28, NH99/DA and OLA KG 1-6-05 joined at 0.61 level of similarity. Cluster IV is composed of two accessions, which includes, OK 20, Enugu-1, which joined at 61% level of similarity to form one cluster. Finally, cluster V has seven accessions which include CCN2005/2, V-35, and Dajofolowo-1, Clemson spineless, V45-2, Short Mouth Ibarapa, and Lady's Finger, joined at 0.63 level of similarity to form a cluster. At 0.85 level of similarity all the accessions were distinct while at 0.83 level of similarity, Jokoso-2 and Ola 3 local were the most similar amongst the accessions. The two most distant accessions from all others were 0K 20 and Enugu-1, which joined to a neighbour at 0.63 level of similarity

Discussion

The result of the RAPD analysis revealed that the use of 24 ng concentration of DNA produced a better result in terms of DNA reproducibility. This observation will reduce the limitations of DNA reproducibility in PCR due to small DNA concentration. It could help in minimizing cost of running this analysis since DNA concentrations as minimal as 24 ng will produce reasonable results. It further suggests that RAPD analysis requires little quantity of DNA to produce meaningful result. Primers in the OPX, OPY and OPM series revealed the highest amplification probably due to high homology between the primers and the okra DNA fragments. Furthermore, the variation in the quantity of DNA per unit volume of DNA may be due the ratio of euchromatin and hetero-chromatin present in the DNA according to Verma & Agarwal (2005).

The variation of the amplified banding pattern as revealed by the RAPD results is an indication of polymorphism and suggests that there was variation amongst the accessions tested in this research. However, each of the primers used in this study revealed different banding patterns with similar accessions, an indication of different sequencing in the base pair of the primers used. The amplification product of the okra accession DNA reveals specific genetic variations among the accessions. Since RAPD primers were able to

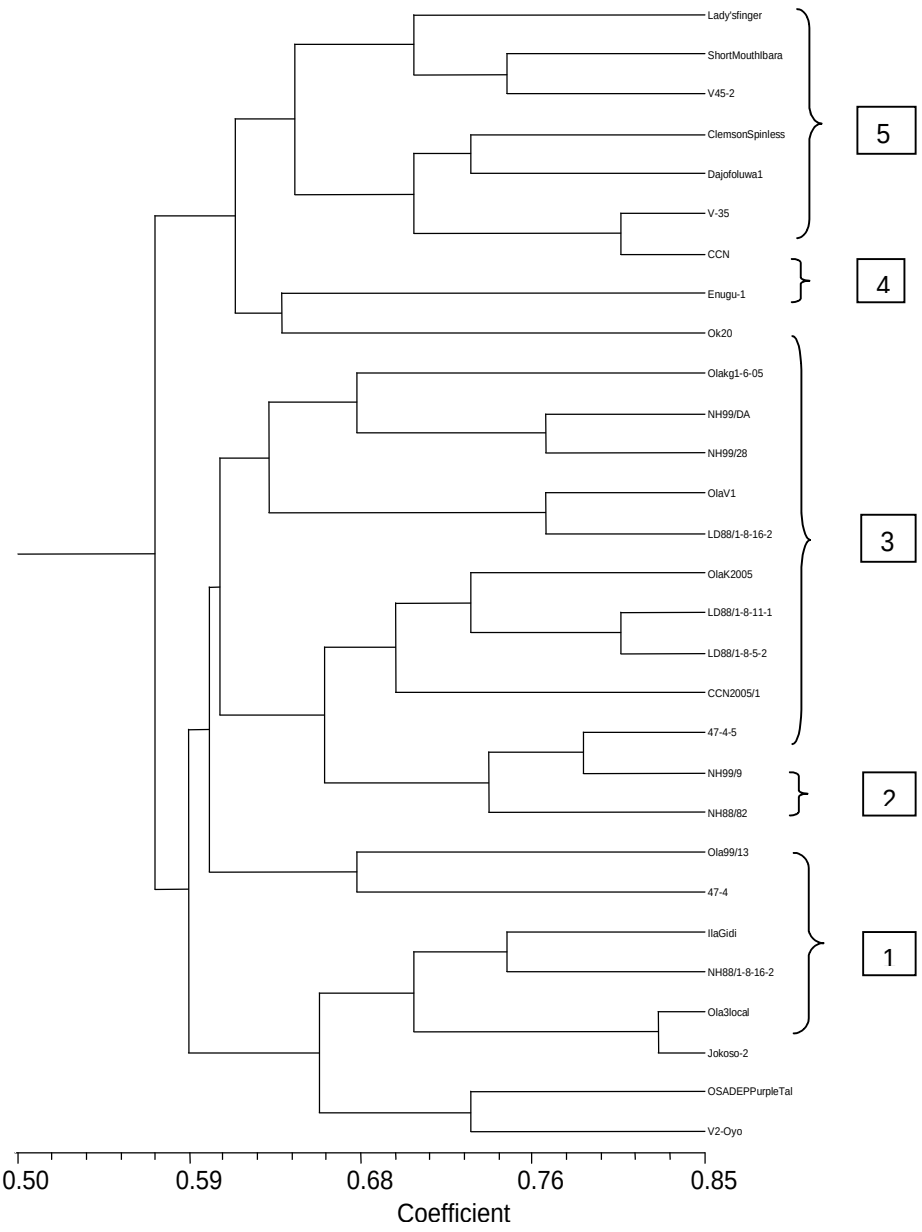

Figure 1: Molecular dendrogram showing level of similarity amongst 29 okra accessions

amplify more than one band per accession, residual heterogenicity within the accessions is apparent. This agrees with the report of Ogunbayo *et al*. (2005).

The similarity indices of the RAPD dendrogram ranged between 50% and 100% and were averagely high enough to suggest useful variability for genetic conservation and plant breeding (Torkpo *et al*., 2006). None of

the accessions was exactly identical to each other, a fact that suggests genetic variation among the accessions. The two most divergent accessions revealed by the molecular dendrogram are OK 20 and Enugu-1 suggesting that the origins or sources of these accessions were quite diverse. OK 20 was an entry from NIHORT, while Enugu-1 was an entry from NACGRAB. These accessions may likely have originated from diverse localities and assembled in these research institutes. The molecular dendrogram reveals that the accessions with the highest level of relatedness were Ola 3 local and Jokoso 2, with over 80% level of relatedness. This suggest that these two must have similar ancestral decent and may have similar genes and therefore have closely related origins. It further implies that crosses between such accessions may not be recommended, since the result of such a cross may not produce much difference. Information on genetic relatedness among genetic resources of crop plants is useful not only for breeding purposes but also for the conservation of germplasm (Torkpo *et al*., 2006). The current work elucidates the usefulness of molecular markers in establishing distinct relationships between accessions where phenotypic expression using morphological methods alone may not be able to determine differences between accessions, especially since phenotypes are influenced by the environment.

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