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Full Length Research Paper

Genetic variability of cultivated cowpea in Benin assessed by random amplified polymorphic DNA

A. Zannou[1,2,3]*, D. K. Kossou[1], A. Ahanchédé[1], J. Zoundjihékpon[4], E. Agbicodo[5], P.C. Struik[3] and A. Sanni[5]

[1]Faculté des Sciences Agronomiques, Université d'Abomey-Calavi, 01 BP 526 Cotonou, Benin.
[2]Technology and Agrarian Development Group, Wageningen University, Wageningen, The Netherlands.
[3]Centre for Crop Systems Analysis, Wageningen University, Wageningen, The Netherlands.
[4]Laboratoire d'Ecologie Appliquée, Faculté des Sciences Agronomiques, Université d'Abomey-Calavi, 01 BP 526, Cotonou, Benin.
[5]Laboratoire de Biochimie et de Biologie Moléculaire, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, 01 BP 526, Cotonou, Benin.

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Characterization of genetic diversity among cultivated cowpea [*Vigna unguiculata* (L.) Walp.] varieties is important to optimize the use of available genetic resources by farmers, local communities, researchers and breeders. Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic diversity in 70 cowpea accessions collected throughout Benin. Nine random primers were screened on 24 accessions to assess their ability to reveal polymorphisms in cowpea and four of them were selected for use in characterizing the total sample. A total of 32 amplified bands were generated by the four primers. The number of loci detected varied from 5 to 11. RAPD profiles were analysed and amplified polymorphic DNA fragments were used to construct a dendrogram, clustering the accessions into nine groups at a similarity index of 71% based on the Unweighted Pair-Group Method using Arithmetic Averages. The genetic diversity among the cowpea cultivars investigated was large and the RAPD proved to be a useful technique to characterise it. Based on the molecular variance, the fixation index suggests a large differentiation of cowpea cultivars in Benin.

Key words: Cowpea germplasm, farmers, local communities, genetic diversity, RAPD, *Vigna unguiculata.*

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp, Leguminosae (2n = 2x = 22), is an essential food crop in less-developed countries of the tropics and subtropics, especially in sub-Saharan Africa, Asia, and Central and South America (Singh et al., 1997). The wild forms are endemic to Africa (Pasquet, 1999; Coulibaly et al., 2002). In Benin, the crop is grown for its young leaves and grains and these are used in the meals of both the rural and urban populations (Kossou et al., 2001; Zannou et al., 2004). Recent studies conducted at the International Institute of Tropical Agriculture on more than 14,000 accessions of cowpea collected throughout the world revealed that those from West African countries such as Nigeria, Niger, Burkina Faso and Ghana displayed very high levels of genetic diversity (Ng, 1995). However, in Benin the molecular diversity of the cultivated cowpea has not been studied at the DNA level yet. When we analysed – together with farmers – the agronomic and physiological traits of cowpea and the constraints this crop was facing, it became obvious that it was necessary to really understand the genetic traits of the crop (Zannou et al., 2004).

Recent taxonomic studies of *Vigna* (Ng and Maréchal, 1985; Pasquet, 1993a, b, 1997, 1999) divided cowpea into ten perennial subspecies and one annual subspecies (ssp. *unguiculata*). These studies split the ssp. *unguiculata* into var. *unguiculata* and var. *spontanea* (Schweinf.) Pasquet (annual wild cowpea) (Pasquet, 1999). The annual cultivar group (var. *unguiculata*) is composed of the cultivated cowpea varieties on which this study focused.

*Corresponding author. E-mail: afiozannou@yahoo.com. Tel.: +229.97.44.92.55.

Abbreviations: **AMOVA**, analysis of molecular variance; **C**, central; **CTAB**, cetyl trimethyl ammonium bromide; **NE**, North East; **NW**, North West; **SE**, South East; **UPGMA**, unweighted pair-group method using arithmetic averages.

Cultivated cowpea germplasm diversity studies based on isozyme diversity and proteins have shown very low genetic diversity (D'Urzo et al., 1990; Pedalino et al., 1990; Panella et al., 1993; Vaillancourt et al., 1993), and cultivar group *sesquidalis* could not be distinguished from the cultivar group *unguiculata* (Vaillancourt et al., 1993). Molecular markers based on differences in DNA sequences between individuals generally detect more polymorphisms than morphological and protein-based markers and constitute a new generation of genetic markers (Botstein et al., 1980; Tanksley et al., 1989). DNA markers survey both functional as well as neutral genetic variation. Alternative molecular markers showing higher levels of polymorphism among closely related genotypes include RAPDs (Williams et al., 1993; Mignouna et al., 1998; Ba et al., 2004; Diouf and Hilu, 2005), microsatellites (Sonnante et al., 1994; Akkaya et al., 1995; Diouf and Hilu, 2005), and AFLPs (Vos et al., 1995; Coulibaly et al., 2002; Gillaspie et al., 2005). RAPD markers have been shown to be useful in assessing intraspecific or interspecific genetic variability in many crops species (Haley et al., 1994; Mignouna et al., 1998; Ba et al., 2004; Diouf and Hilu, 2005).

This study assessed the genetic diversity of the cowpea cultivated by farmers in Benin, based on RAPD techniques. The main objective of the present study was to characterise the genetic diversity of cultivated cowpea germplasm in Benin for the best use of the genetic potential of the crop and for a better use and management of cultivated cowpea varieties.

MATERIAL AND METHODS

Plant material

The plant material used in this study only includes cowpea varieties grown by farmers; they all belong to *V. unguiculata* ssp. *unguiculata* var. *unguiculata* (Table 1). A total of 70 cowpea accessions were collected from farmers of the transitional Guinea Sudan of Benin, some from the South East and others from some main market places of these areas in Benin. Five seeds of each accession were grown in pots and leaf samples were collected at seven days age from all the plants for DNA isolation and analysis.

DNA isolation

Fresh leaves from young plants were collected and frozen in liquid nitrogen. Leaves were ground with a mortar and pestle. DNA was isolated according to the cetyl trimethyl ammonium bromide (CTAB) protocol described by Rogers and Bendich (1985), with slight modifications as described below. Up to 200 mg of ground leaf tissue was transferred to 2 ml eppendorf tubes, mixed with 500 µl of 2 × CTAB extraction buffer and incubated in a 65°C water bath with frequent agitation for 90 min. The tubes were removed from the water bath and allowed to cool until room temperature before 500 µl of phenol was added and mixed thoroughly. The mixture was centrifuged at 12,000 rpm for 10 min and the upper supernatant phase collected in a new tube. A second extraction was performed with 500 µl of a mixture of 24% of phenol/chloroform and 1% of isoamyl alcohol (v/v). After centrifugation, the supernatant was treated with RNase and the last extraction was performed with chloroform isoamyl alcohol. The upper phase was transferred into a new tube and DNA was precipitated with equal volumes of 2–propanol and sodium-acetate. The DNA pellet was washed with 70% ethanol and was dried for 5 min in a heating block of 60°C. The resulting DNA pellet was dissolved in 100 µl of distilled and sterilized water (SIGMA). DNA integrity was tested, using 1.5% agarose gel electrophoresis, and its concentration was determined with a UV spectrophotometer. DNA was then diluted to 25 ng/µl for PCR amplification.

PCR amplification

Preliminary PCR amplification trials were conducted on four accessions, arbitrarily selected in order to standardize the DNA amplification conditions. These accessions were Vu5 (Azangban), Vu30 (Tanguieta), Vu33 (Tchabè Funfun), and Vu41 (Soui Kerri). Different concentrations of $MgCl_2$, DNA, dNTPs, and Taq DNA polymerase were tested to obtain the most reproducible and reliable DNA amplification profiles. Optimal conditions which revealed clear and reproducible amplification fragments were used in the study as earlier described.

PCR reactions were performed in 25 µl volume in a mixture containing 3 mM $MgCl_2$, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0), 0.1 mM of each dNTPs, 0.1 µM of random decamer primer, 50 ng of DNA and 1 unit of Taq DNA polymerase. The PCR amplification process was conducted in T3 Thermocycler Biometra. For each amplification process, an initial heat denaturation of DNA at 94°C for 3 min was followed by 45 cycles consisting of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C. A final incubation for 7 min at 72°C was performed and the amplification products analysed on 2% agarose gels in Tris-borate buffer gels were stained in ethidium bromide, visualized under UV and photographed using a digital camera. A 100 bp ladder (Sigma) was used as molecular size standard.

Selection of the most informative primers

Mignouna et al. (1998) used 120 RAPD markers to investigate the genetic diversity of 95 cowpea accessions from diverse geographical origin across Africa, America and Asia and nine markers were the most informative. These nine primers were pre-selected for this study (Table 2). PCR amplification was performed on 24 cowpea accessions using each of the nine primers individually in order to select the primers that showed the highest number of polymorphic amplification fragments. As a result, four primers were selected for the whole study. The random primers used for DNA amplification were 10 base sequences obtained from Invitrogen Life Technologies as listed in Table 2.

Data analysis

After electrophoresis separation, amplified DNA fragments detected in each accession were scored for presence (1) or absence (0) of a particular DNA fragment of a similar length. Faint fragments were omitted and only reproducible fragments were considered for the analysis. A data matrix was prepared based on different analyses.

To estimate genetic diversity, a pairwise similarity matrix was generated using the Nei – Li similarity index (Nei and Li, 1979):

$$S=2N_{AB}/(N_A+N_B)$$

where N_{AB} is the number of RAPD fragments shared by two genotypes or cultivars (A and B); N_A and N_B are the total number of RAPD fragments analysed in each genotype (Levi et al., 2001).

A dendrogram was then constructed based on the similarity matrix data using the UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) cluster analysis of NTSYSpc-2.02j (Numeral Taxonomy and Statistical Analysis; Rohlf, 1998).

The genetic structure of the cultivars was investigated by an Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992). The total molecular variance (σ_T^2) was partitioned into variance components due to differences among clusters (σ_{SC}^2) and within clusters (σ_{ST}^2). To analyse the genetic structure, the fixation index is a measure that is more and more used (Weir and Cockerham, 1984; Excoffier et al.,

Table 1. List of cowpea germplasm accessions and their regions of collection used for RAPD analysis.

Codes	Cultivar names	Villages	Region	Codes	Cultivar names	Villages	Region
1	Adjaïkoun ancien	Bohicon	C	43	Sowétin	Gbékandji	SE
2	Yawari petit grain	Dani	C	44	Aïglo	Glazoué	C
3	Djohozin (Adjohozin)	Gbékandji	SE	45	Kacripia	Alfakpara	NW
4	Moussa	Dani	C	46	Atchawékoun (Bohicon)	Bohicon	C
5	Kpohoundjo	Dani	C	47	Malanville petit grain	Dani	C
6	Sèwékoun	Glazoué	C	48	Niger	Save	C
8	Tawa petit grain	Dani	C	49	Zerma soui	Marégourou	NE
9	Adjaïkoun	Bohicon	C	50	Kpodjiguèguè		SE
10	Wankoun	Ouèdèmè	C	51	Sokan	Gbékandji	SE
11	Tontouin	Gbékandji	SE	52	Yèringo		NW
13	Kpodji wéwé	Bohicon	C	53	Glessissoafoado	Dani	C
14	Djèté	Dani	C	54	Soui Kpika	Sonoumon	NE
15	Atchawe ou Tola (Bohicon)	Bohicon	C	55	Togo grain	Ouèdèmè	C
16	Kpeïkoun (Bohicon)	Bohicon	C	56	Tanguieta	Dani	C
17	Kakè	Bohicon	C	57	Boto wéwé	Dani	C
19	Soui Zerma	Marégourou	NE	58	Katché Django	Alédjo-Kpataba	NW
20	Tchabè Funfun	Diho	C	60	Kaki	Yagbo	C
22	Azobahundé (Kpodjiguèguè)	Dannou	SE	61	Olodjou Maria	Pira	C
23	Ewa Egbessi	Egbessi	C	63	Boto vovo	Dani	C
24	Olikpokpo-doudou	Dani	C	64	Yanti Kpika	Donga	NW
25	Assitchénongbinhami	DamèWogon	SE	65	Katché Koukpédon	Alédjo-Kpataba	NW
26	Mahounan	Yagbo	C	67	Soui Kerri	Sonnoumon	NE
27	Téhivigboto	Dannou	SE	68	Mosso	Ouassa	NW
28	Wan akpavi	DamèWogon	SE	69	Kplobè rouge	Dani	C
29	Atama	Save	C	70	Djètoko	Glazoué	C
30	Malanville gros grain	Save	C	71	Kwx	Dani	C
32	Boto	Ouèdèmè	C	72	Egni-awo	Glazoué	C
33	Yawari gros grain	Dani	C	73	Kplobè wéwé	Dani	C
34	Sèhèkoun original	Ouèdèmè	C	74	Ewa Nigeria	Diho	C
35	Sindjinnansin	Dannou	SE	75	Tchawa koubanguè / Grand Tchawa	Alédjo-Kpataba	NW
36	Tonton	Dani	C	76	Toura	Ouassa	NW
37	Tchadilè djofè	Diho	C	77	Nanwi	Dannou	SE
38	Tawa gros grain	Dani	C	78	Tola	Glazoué	C
39	Azangban	Dani	C	79	Ewa Zaffé	Glazoué	C
40	Atchawe Dangbo	Dannou	SE				
41	Matamaéko	Ouoghi	C				

C: Central, NE: North East, NW: North West, SE: South East.

1992; Weir, 1996; Schneider et al., 2000; Rousset, 2001; Dugoujon et al., 2004; Kiambi et al., 2005). This index, also called Wright's (1969) fixation index, was calculated for polymorphic loci and notated F_{ST}. F_{ST} is considered as the standardized variance of allele frequencies among subdivisions (Excoffier, 2001). It reveals the proportion of the total variance of allele frequencies among clusters that could be explained by the group structure.

The hierarchical AMOVA was performed based on a pairwise squared Euclidean distance matrix using Arlequin ver 3.01 software (Excoffier et al., 2006).

RESULTS

RAPD primers' selectivity on cowpea genetic resources

Figure 1 shows PCR amplification fragments on the 24 accessions as detected by the primer OPB-05. This primer revealed monomorphic bands in the size of about 470 bp on 23 accessions, and only on one accession (lane 22) an additional band of 600 bp was shown. A similar amplification pattern was also detected by primer OPB-10 which revealed monomorphic amplification bands in 23 accessions and only in one accession (lane 24) two additional bands were detected (Figure not shown). So, among the 24 accessions screened, primers OPB-05 and OPB-10 were able to distinguish only one accession from the others. Similarly, three other primers OPA-01, OPB-13, OPC-06 were also unable to distinguish among accessions. Hence, these primers were not considered for the study.

Table 2. List and sequence of the 10-base nucleotide primers used for the RAPD analysis.

Selected primers		Not selected primers	
Primer code	**Nucleotide sequence**	**Primer code**	**Nucleotide sequence**
OPA-04	5'-AATCGGGCTG-3'	OPA-01	5'-CAGGCCCTTC-3'
OPB-01	5'-GTTTCGCTCC-3'	OPB-05	5'-TGCGCCCTTC-3'
OPC-05	5'-GATGACCGCC-3'	OPB-10	5'-CTGCTGGGAC-3'
OPD-18	5'-GTGTGCCCCA-3'	OPB-13	5'-TTCCCCCGCT-3'
		OPC-06	5'-GAACGGACTC-3'

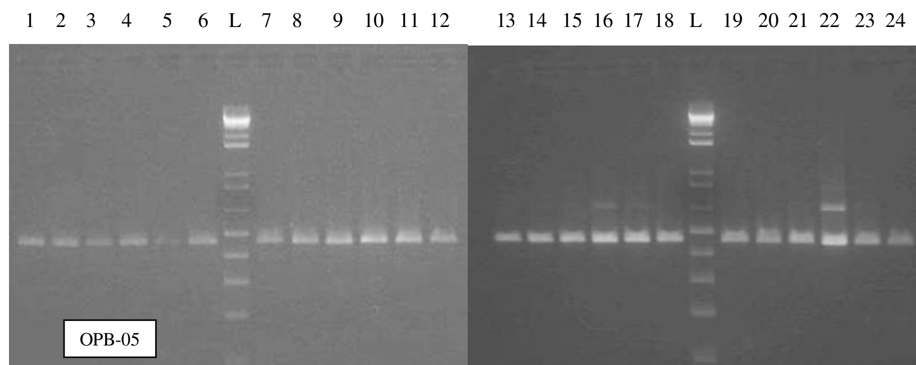

Figure 1. RAPD profile of 24 randomly selected cowpea accessions using the primer OPB-05. Lanes 1 = Adjaïkoun (ancien), 2 = Yawari petit grain, 3 = Djohozin, 4 = Moussa, 5 = Kpohoundjo, 6 = Sèwékoun, 7 = Tawa gros grain, 8 = Adjaïkoun, 9 = Tontouin, 10 = Kplobè rouge, 11 = Djètè, 12 = Atchawé ou Tola (Bohicon), 13 = Kpeïkoun (Bohicon), 14 = Kakè, 15 = Soui Zerma, 16 = Tchabè Funfun, 17 = Ewa Egbessi, 18 = Assitchénongbinhami, 19 = Boto, 20 = Yawari gros grain, 21 = Sèhèkoun (original), 22 = Tonton, 23 = Katché Funfun, 24 = Niger, and L = ladder (100 bp).

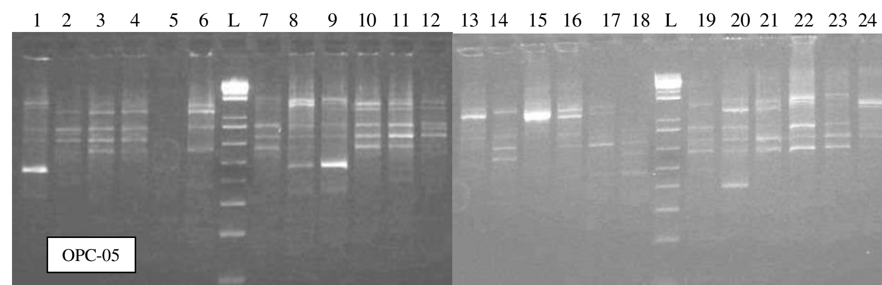

Figure 2. RAPD profile of 24 randomly selected cowpea accessions using the primer OPC-05. Lanes 1 = Adjaïkoun (ancien), 2 = Yawari petit grain, 3 = Djohozin, 4 = Moussa, 5 = Kpohoundjo, 6 = Sèwékoun, 7 = Tawa gros grain, 8 = Adjaïkoun, 9 = Tontouin, 10 = Kplobè rouge, 11 = Djètè, 12 = Atchawé ou Tola (Bohicon), 13 = Kpeïkoun (Bohicon), 14 = Kakè, 15 = Soui Zerma, 16 = Tchabè Funfun, 17 = Ewa Egbessi, 18 = Assitchénongbinhami, 19 = Boto, 20 = Yawari gros grain, 21 = Sèhèkoun (original), 22 = Tonton, 23 = Katché Funfun, 24 = Niger, and L = ladder (100 bp).

Taking into account their ability to reveal polymorphic bands, four primers (OPW-04, OPC-05, OPD-18, OPB-01) were selected. Figure 2 shows DNA polymorphism detected in the 24 accessions screened using primer OPC-05. While the primer OPB-05 was unable to distinguish these accessions (Figure 1), the primer OPC-05 detected polymorphic bands showing important variation among these accessions. However, PCR amplification profiles were similar in some of the accessions as for example shown by lane 3 (Djohozin) and lane 4 (Moussa), and by lane 10 (Kplobè rouge) and lane 11 (Djètè) (Figure 2). Similarly, DNA polymorphisms were also detected by the three other primers used in the study. These four primers were therefore used to characterise genetic diversity of the 70 cultivated germplasm accessions investigated (Table 1).

Cluster analysis and genetic estimates

The number of RAPD marker loci detected was 5 for the

Table 3. Analysis of molecular variance.

Source of variation	D.F.	Sum of Squared Deviations	Variance components	Percentage of variation	F-statistics
Among groups	8	113.115	1.452	26.27	F_{ST} = 0.2627***
Within groups	61	248.571	4.075	73.73	
Total	69	361.686	5.527		

***Average F-statistics over all loci: F_{ST} = 0.2627, highly significant ($p < 0.0001$).

primer OPB-01, 8 for the primers OPC-05 and OPD-18, and 11 loci for primer OPA-04. The size of the amplified bands ranged from 0.3 to 2 kb. A total of 32 amplified DNA bands were generated by all primers. None of the primers considered was individually able to distinguish all accessions. Considering together all the fragments generated by the four primers selected for the present study, investigated accessions could be distinguished because of some unique bands.

From the presence or absence of DNA fragments, the estimates of distances among accessions were based on Nei and Li's similarity index and used to construct the dendrogram (Figure 3). At an agglomerative coefficient of 0.71 (similarity level) on the dendrogram, the cowpea accessions were clustered into nine groups. One group, cluster 2, contained the largest number, consisting of 27 accessions from different geographical origins: 15 from the centre, 5 from South East, 4 from North West and 3 from North East (Table 4). The groups 1, 5, 6, 7 and 8 were mainly or predominantly consisting of cultivars from the centre.

Genetic diversity

The frequency of the 32 amplified DNA fragments reveal-ed by the four primers is shown in Figure 4. From the analysis of molecular variance (AMOVA), the percentage of genetic variation among cultivars explained 73.73% of the total variation whereas the among groups differentiation explained 26.27% of the variation (Table 3). The fixation index (F_{ST} = 0.26l) is relatively high indicating that there was a large differentiation of cowpea cultivated varieties.

DISCUSSION

RAPD analysis was performed to evaluate genetic diversity in 70 cowpea accessions collected throughout Benin. All accessions analysed belonged to the cultivar group *unguiculata*. Significant genetic diversity was detected in the cowpea germplasm investigated herein confirming the results of Mignouna et al. (1998) who identified extensive genetic variability particularly in the cultivar group *unguiculata* compared to the groups *sesquipedalis* and *textilis*. In comparison to our results, the genetic diversity detected by Mignouna et al. (1998) was higher probably because of the higher number of accessions (95 compared to 70) and the geographically worldwide origins of their collections. The high genetic diversity detected in the cowpea accessions analysed, probably indicated that accessions were originally generated by different ancestors of cowpea in the past.

In this study within the informative markers, the primers OPA-04 and OPD-18 showed 11 and 8 polymorphic bands compared to 10 and 8 polymorphic bands respectively in Mignouna et al. (1998). Conversely, the primer OPB-10 which detected 10 polymorphic amplified bands was unable to distinguish accessions we investigated by showing monomorphic pattern. This monomorphic pattern was the same for the five primers which were not selected in this study.

Wright (1978) cited by Hartl (1987) and Kiambi et al. (2005) suggested that an F_{ST} range of 0 – 0.05 indicates little differentiation, 0.05 – 0.15 moderate differentiation, 0.15 – 0.25 large differentiation, and above 0.25 indicates a very large differentiation. In this study, basing on the AMOVA analysis, the fixation index is 0.26 suggesting a large differentiation of cultivars in Benin.

At agglomerative coefficient of 0.71, the dendrogram shows nine clustering groups which contain large and small numbers of accessions. The classification of acces-sions into different groups is independent of collection zones, agro-ecozones and market places. Accessions of morphologically different characters including shape of seeds, seed coat colour, etc., are very close according to the dendrogram constructed based on the presence or absence of amplified DNA fragments of a particular size. The discrepancy between molecular genetic diversity and morphological diversity has been well documented (Doebley, 1989). This result shows that during the process of domestication, modifications in a few genes can lead to marked phenotypic differences. Also as self-pollinated crop, cowpea accessions maintained some parts of their genetic components during the process of domestication. This can explain the monomorphism pattern shown by some of the primers like those in Figure 1.

Additionally, this study shows the presence of important genetic variability among the Benin cowpea germplasm which can be used to broaden the genetic bases of the crop for better use of its genetic potential. For germplasm management, it is important, in addition to morphological characterisation, to reveal the extent of genetic diversity present in a collection, using other means such as molecular markers.

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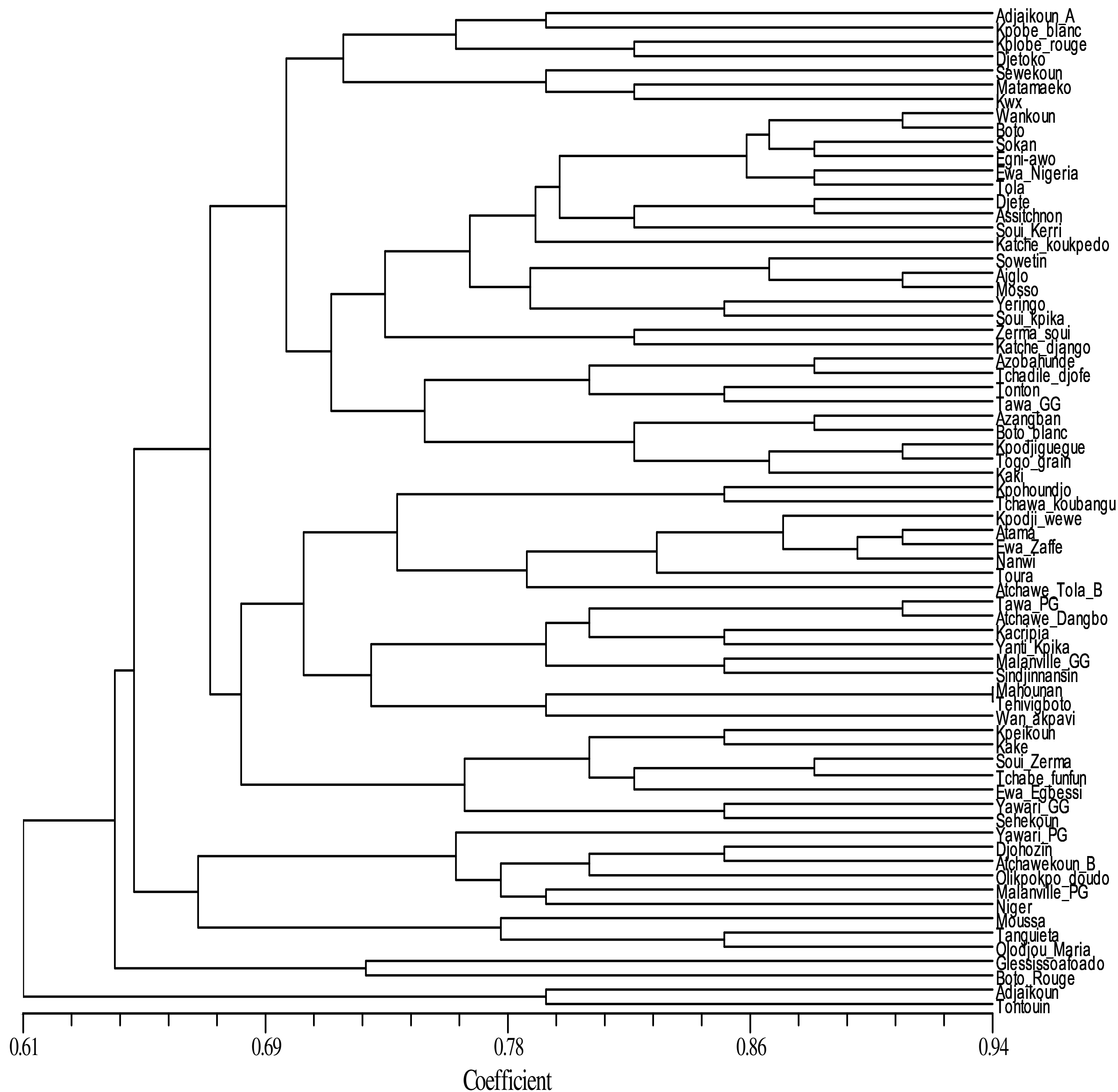

Figure 3. Dendrogram of the cowpea accessions based on coefficient of similarity matrix.

Table 4. Cultivar groups revealed by RAPD with respect to their origins.

Cluster	Cultivar name	Origin		Cluster	Cultivar name	Origin
1	Adjaïkoun ancien	C		3	Kpohoundjo	C
	Sèwékoun	C			Kpodji wéwé	C
	Matamaéko	C			Atchawe ou Tola (Bohicon)	C
	Kplobè rouge	C			Atama	C
	Djètoko	C			Ewa Zaffé	C

Table 4. Contd.

	Kwx	C		Tchawa koubanguè	NW
	Kplobè wéwé	C		Toura	NW
2	Wankoun	C		Nanwi	SE
	Djèté	C	4	Malanville gros grain	C
	Mahounan	C		Atchawe_Dangbo	C
	Boto	C		Malanville petit grain	C
	Tonton	C		Boto vovo	C
	Tchadilè djofè	C		Kacripia	NW
	Tawa gros grain	C		Yanti Kpika	NW
	Azangban	C		Téhivigboto	SE
	Aïglo	C		Wan akpavi	SE
	Togo grain	C		Sindjinnansin	SE
	Boto wéwé	C	5	Kpeïkoun (Bohicon)	C
	Kaki	C		Kakè	C
	Egni-awo	C		Tchabè Funfun	C
	Ewa Nigeria	C		Ewa Egbessi	C
	Tola	C		Yawari gros grain	C
	Zerma soui	NE		Sèhèkoun original	C
	Soui Kpika	NE		Soui Zerma	NE
	Soui Kerri	NE	6	Yawari petit grain	C
	Yèringo	NW		Tawa petit grain	C
	Katché Django	NW		Atchawékoun (Bohicon)	C
	Katché Koukpédon	NW		Niger	C
	Mosso	NW		Djohozin (Adjohozin)	SE
	Azobahundé	SE	7	Moussa	C
	Assitchénongbinhami	SE		Olikpokpo-doudou	C
	Sowétin	SE		Tanguieta	C
	Kpodjiguèguè	SE		Olodjou Maria	C
	Sokan	SE	8	Glessissoafoado	C
			9	Adjaïkoun	C
				Tontouin	SE

C: Central, NE: North East, NW: North West, SE: South East.

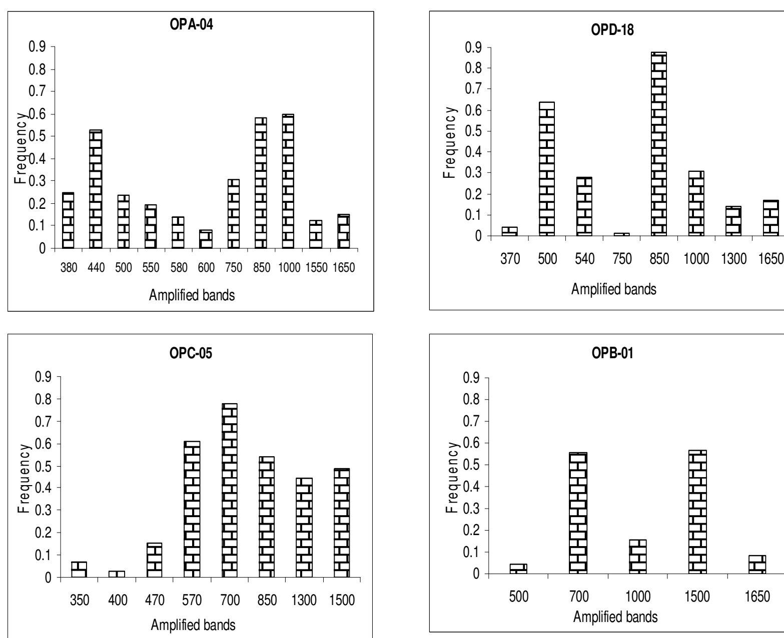

Figure 4. Amplified DNA fragments frequency of the cowpea accessions as revealed by the four primers.

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