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Research Article

Assessment of diversity among cowpea (*Vigna unguiculata* (L.) Walp) genotypes based on RAPD analysis

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Abstract
Random Amplified polymorphic DNA (RAPD) is used to determine genetic diversity of cowpea genotypes collected from different regions of India. A high diversity within population and high genetic differentiation among them were analyzed. Total 194 bands were generated among them 152 bands were found polymorphic with an average 7.6 bands per primer. The average percentage of polymorphism across 20 primers was 78.83 %. A high level of average genetic diversity was observed. A dendrogram produced by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on Jaccard's similarity coefficient revealed two groups. The overall range of genetic similarities ranged from 0.533 to 0.790 in 20 genotypes of cowpea which indicates there was high variability among the genotypes. Based on genetic distance in RAPD analysis the genotypes *viz*., C-152, PGCP-11 and PGCP-6 appeared as most divergent and could be used in breeding programme of cowpea. Our results indicate that RAPD approach analysis seemed to be best suited for assessing with high accuracy the genetic relationships among distinct cowpea genotypes.

Key words
Cowpea, Geneticdiversity, RAPD

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.), a member of the family Fabaceae, is a crop grown throughout the tropics and the substropics covering Africa, Asia, South America, parts of Southern Europe and the United States (Singh *et al*. 1997). In India, cowpea cultivated area is 3.9 million ha with 2.2 million tonne seed production and 567 kg/ha productivity (Anonymous, 2011). In Gujarat, it is cultivated in an area about 0.26 lakh ha with 0.16 lakh tonne seed production and 615 kg/ha productivity (Anonymous, 2008). Cowpea seeds possess high nutritive value. The plants are well adapted to grow under high temperature and drought and tolerate low soil fertility due to their high rate of nitrogen fixation and ability to form effective symbiotic mycorrhizae. Therefore, cowpea can play an important role in agricultural development (Ghalmi *et al.,* 2009). Cowpea is called as vegetable meat due to more than 25% protein in grain as well as in young leaves (dry weight basis) with better biological value. It is a major source of minerals and vitamins in daily diets of human and is equally important as nutritious fodder for livestock (Singh *et al*., 2007). During the last few years, the characterization and evaluation of genetic diversity and relationships within and between species and genotypes were performed generally by molecular techniques that substituted the classic ones such as morphological and physiological characters (Katsiotis *et al*. 2009). DNA-based molecular markers have the advantage of being free from environmental modulations. Random amplified polymorphic DNA (RAPD) markers have proved to be a very useful tool to provide a convenient and rapid assessment of the genetic differences between genotypes (Williams *et al*. 1990). Moreover, RAPDs use arbitrary primers that provide a large number of multilocus markers and can be applied to analyze almost any organism, even those for which no previous genetic or molecular information is available. RAPDs have advantages such as rapidity, requiring little genomic DNA as template and they are able to detect variation in coding and noncoding regions of the genome (Gajera *et al*. 2010). However, most RAPD loci are assumed to possess only two alleles and segregate as dominant markers, leading to an underestimation of the genetic diversity. To date, few studies have been performed in cow pea using RAPDs (Akundabweni 1995; Mene´ndez *et al.* 1997; Mignouna *et al.* 1998; Tosti and Negri 2002). The present research had the following objectives: Assessment of diversity among cowpea (*Vigna unguiculata* (L.) Walp) genotypes based on RAPD analysis.

Material and method

*Sample collection:*The plant materials were collected from various parts of India (Table 1) during August and March 2012. Field experiments were conducted at the Centre of Excellence for Research on Pulses, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar. Sardarkrushinagar is situated at 24.12 °N latitude and 72.12 °E longitude with an elevation of 154.52 meters above the mean sea level. A total of 20 genotypes of *Vigna unguiculata* (L.) Walp. were used in this study. The details of 20 genotypes and their sources were tabulated (Table 1). All the collected genotypes were sawn in *summer*, 2012 in a randomized block design with three replications under irrigated condition. Each entry was accommodated in a three rows of 4.0 m length with a spacing of 45 x 10 cm.

*Genetic diversity assessment of Vigna unguiculata (L.) Walp. using RAPD-PCR marker analysis:*DNA was isolated using a modified Cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof *et al*. 1984). For each accession, about 5 g of bulked leaf tissue collected from five plants each was ground to a fine powder using liquid nitrogen and then suspended in 20 mL of extraction buffer (20 mM EDTA (pH 8.0), 100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 2% CTAB, and 1% β-mercaptoethanol). The suspension was mixed well, incubated at 60°C for 45 min, followed by chloroform–isoamyl alcohol (24:1) extraction and precipitation with 2/3 of the volume of isopropanol at −20°C for 1 h. The DNA was pelleted down by centrifugation at 12,000 rpm for 10 min and suspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA (pH 8.0)). The DNA was purified from RNA and proteins by standard procedures (Sambrook *et al*. 1989). and DNA concentration was estimated by agarose gel electrophoresis and staining with ethidium bromide.

*DNA quantification:*The isolated genomic DNA from the plants were quantified spectrophotometrically by measuring absorbance at 260 nm and calculated their concentration using the formula given below and expressed in microgram per microliter (µg/µL). DNA was diluted to make a working solution of 15 ng/µL for RAPD marker analysis. DNA concentration of solution A (µg/mL) = OD 260 of diluted sample × dilution × 50 µg/µL.

*Primer screening:*Fourty decamer primers from Operon, Advanced Biotecchnologies Inc., Almeda, USA were initially screened using one individual clone to determinate the suitability of each primer for the study. After preliminary testing on a few samples, twenty primers were selected for further analysis based on their ability to detect distinct clearly resolved and polymorphic amplified products within the population. To ensure reproducibility the primers generating no, weak, or complex patterns were discarded.

*PCR amplification:*PCR amplification was performed in a total volume of 25 µL containing 10× Taq buffer witn $MgCl_2$ (2.5 µL) (Genei, Bangalore), dNTPs (2.5 µL) (10 Mm each) (Genei, Bangalore), Taq DNA polymerase (0.5 µL) (3 U/µL) (Genei, Bangalore), Primer (1 µL) (40 µM/µL) (Sigma Aldrich, Bangalore), template DNA (0.5 µL) (40 ng/µL) and sterile nanopure water (18 µL) in Eppendorf Master Cycler (AG 22331 Harmburg, Germany) using the following conditions: (a) initial denaturation at 94°C for 5 min; (b) 45 cycles each consisting of denaturation step at 94°C for 1 min, annealing step at 35°C for 1 min, amplification at 72°C for 2 min step; and (c) final extension at 72°C for 5 min followed by arresting the reaction at 4°C for infinite period. Control reactions without template DNA (negative control) were also run in the experiments. All the experiments were repeated thrice to ensure reproducibility.

*Agarose gel electrophoresis:*A 1.5% solution of agarose was prepared and the slurry was heated in a microwave oven until the agarose is completely melted into a clear solution. When melted the solution was cooled into 60°C and added with 10 µL (10 mg/mL) of ethidium bromide. The gel casting tray was set up on an equal plane so that the agarose will be distributed throughout the mold and the comb was kept in position. The solution was poured into the tray slowly without the formation of any air bubbles. The mold was allowed to be undisturbed for about 30–45 min until the agarose solidifies completely. After that the tray was placed on the buffer tank and 1× TBE buffer was poured into the tank until it covered the gel to a depth of about 2 mm. The DNA samples mixed with gel loading dye (6×) were loaded carefully into the wells. DNA marker (1 kb ladder; High range) was loaded at first or last well of agarose gel. Electric supply of 120 V was given consistently for a period of 1 h. Electrophoresis was stopped when samples stained with bromophenol blue crossed more than 3/2 of the length of the gel. The DNA profile was photographed using UV PRO (Plentinum) gel documentation system.

Data scoring and statistical analysis of RAPD data:
RAPD bands were scored using binary matrix '1' for presence and '0' for absence. Data was analyzed using NT-SYS-pc version 2.1 (Rohlf *et al*. 2002).

Pair-wise genetic similarities between accessions were estimated using the Jaccard's similarity coefficient (Jaccard *et al*. 1908). A dendrogram was constructed based on similarity coefficient values by adopting the sequential hierarchical agglomerative non-overlapping (SHAN) clustering technique of unweighted pair group method of arithmetic mean (UPGMA) which is a variant of the average linkage clustering algorithm (Sneath and Sokal 1973).

Result and discussion

After screening 40 primers 20 primers produced polymorphic and repeatable products (Table 2). The banding profile and polymorphism generated are shown in Fig. 1. Total 194 bands were generated among them 152 bands were found polymorphic with an average 7.6 bands per primer. The average percentage of polymorphism across 20 primers was 78.83 %. The number of DNA fragments varied from 4 to 14 with an average 9.7 per primer. The PIC value ranged from 0.683 to 0.923 (Table 2) with an average of 0.844. The percentage of polymorphism of cowpea genotypes ranged from 58.33 (OPA-04) to 100% (OPA-06 and OPB-02) with an average 78.83%. The maximum fragments (14) were generated by primer OPB-01, OPB-04 and OPB-06, whereas primer OPA-06 amplified minimum (4) fragments which were found polymorphic (Table 2).

*Cluster analysis:*Based on electrophoretic banding pattern of RAPD primers, pair wise genetic similarity among 20 genotypes (Table. 2) was estimated and dendogram was generated using Unweighted Pair Group method with "UPGMA" sub programme of "NTSYS"-pc "(Fig. 2). Cluster analysis revealed that genotypes of cowpea under study fell into two groups, major group A and minor group B. Major group A divided into two sub groups, first subgroup containing 16 genotypes *viz.* GC-3, GC-4, GC-601, GC-706, GC-505, GC-516, PGCP-5, PGCP-13, PGCP-14, Pant Lobia-1, Pant Lobia-2, RC-101, KM-5, C-152, IT-38956-1 and TVX-944. Second sub group had only 1 genotype PGCP-12. Group B divided into two sub group, first sub group containing only one GC-5 genotype. Second sub group having two genotypes PGCP-6 and PGCP-11. These genotypes were diverged from other genotypes and placed at end of the cluster

*Similarity matrix:*The genetic distance was computed considering all the genotypes from the pooled data and the dendrogram were constructed the distance similarity matrix is based on Jaccard's pair-wise similarity coefficients (Table 3). The overall range of genetic similarities ranged from 0.533 to 0.790 in 20 genotypes of cowpea which indicates there was high variability among the genotypes. The average genetic similarity among these 20 genotypes was 0.660 (Table 3). The highest similarity index value was found between C-152 and IT-38956-1 (0.790) followed by PGCP-14 and Pant Lobia-1 (0.777), whereas lowest similarity (0.533) was found between PGCP-11 and C-152 followed by PGCP-6 and C-152 (0.536) (Table 3).

The results of this study indicated that RAPD are sufficiently informative and powerful to assess genetic variability in *V. unguiculata (L.) Walp*. Based on genetic distance in RAPD analysis the genotypes *viz*., C-152, PGCP-11 and PGCP-6 appeared as most divergent and could be used in breeding programme of cowpea. The RAPD analysis revealed substantial polymorphism in cowpea. The technique may be used to obtain reasonably precise information on genetic relationship among the cowpea genotypes. Such information may be useful for selecting the diverse parents and monitoring the genetic diversity periodically in the breeders working collection of cowpea.

Overall, it is concluded that the genetic diversity obtained in this study might be useful in future cowpea improvement programmes and selection in cowpea may be based on the phenotypic traits as well as on the molecular markers. Hence, studies on morphological and molecular markers are quite useful in analyzing the genetic diversity in cowpea.

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Table 1. Genotypes and collection details of cowpea from the study area

Sr. No.	Genotype	Location	District	Country	latitude	longitude
1.	GC-3	Gujarat	Banaskantha	India	24.31°N	72.31° E
2.	GC-4	Gujarat	Banaskantha	India	24.31°N	72.31° E
3.	GC-5	Gujarat	Banaskantha	India	24.31°N	72.31° E
4.	GC-601	Gujarat	Banaskantha	India	24.31°N	72.31° E
5.	GC-706	Gujarat	Banaskantha	India	24.31°N	72.31° E
6.	GC-505	Gujarat	Banaskantha	India	24.31°N	72.31° E
7.	GC-516	Gujarat	Banaskantha	India	24.31°N	72.31° E
8.	PGCP-5	Uttarakhand	Udham Singh Nagar	India	29.02°N	79.48° E
9.	PGCP-6	Uttarakhand	Udham Singh Nagar	India	29.02°N	79.48° E
10.	PGCP-11	Uttarakhand	Udham Singh Nagar	India	29.02°N	79.48° E
11.	PGCP-12	Uttarakhand	Udham Singh Nagar	India	29.02°N	79.48° E
12.	PGCP-13	Uttarakhand	Udham Singh Nagar	India	29.02°N	79.48° E
13.	PGCP-14	Uttarakhand	Udham Singh Nagar	India	29.02°N	79.48° E
14.	Pant Lobia-1	Uttarakhand	Udham Singh Nagar	India	29.02°N	79.48° E
15.	Pant Lobia-2	Uttarakhand	Udham Singh Nagar	India	29.02°N	79.48° E
16.	RC-101	Rajasthan	Bikaner	India	28.09°N	73.35° E
17.	KM-5	Karnataka	Bangalore	India	13.03°N	77.58° E
18.	C-152	Karnataka	Bangalore	India	13.03°N	77.58° E
19.	TVX-944	Karnataka	Bangalore	India	13.03°N	77.58° E
20.	IT-38956-1	Karnataka	Bangalore	India	13.03°N	77.58° E

Table 2. Analysis of RAPD banding pattern for cowpea genotypes

Sr. No	Primers	Molecular Weight Range (bp)	Total No. of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands	Percent Polymorphism	PIC Value
1.	OPA-01	400-3960	10.0	8.0	2	80.00	0.873
2.	OPA-02	205-2233	10.0	9.0	1	90.00	0.827
3.	OPA-03	680-1675	8.0	5.0	3	62.50	0.833
4.	OPA-04	102-1600	12.0	7.0	5	58.33	0.899
5.	OPA-05	423-1827	10.0	6.0	4	60.00	0.875
6.	OPA-06	801-1566	4.0	4.0	0	100.00	0.684
7.	OPA-07	211-3454	12.0	10.0	2	83.33	0.874
8.	OPA-08	265-4023	8.0	7.0	1	87.50	0.833
9.	OPA-09	410-1785	7.0	5.0	2	71.42	0.683
10.	OPA-10	450-2081	6.0	5.0	1	83.33	0.772
11.	OPB-01	446-3279	14.0	12.0	2	85.71	0.910
12.	OPB-02	308-3433	12.0	12.0	0	100.00	0.906
13.	OPB-03	443-3783	10.0	8.0	2	80.00	0.880
14.	OPB-04	376-1837	14.0	13.0	1	92.85	0.923
15.	OPB-05	428-2229	10.0	6.0	4	60.00	0.874
16.	OPB-06	383-2005	14.0	9.0	5	64.28	0.918
17.	OPB-07	373-1538	12.0	9.0	3	75.00	0.859
18.	OPB-08	364-1515	5.0	4.0	1	80.00	0.756
19.	OPB-09	281-4566	8.0	7.0	1	87.50	0.863
20.	OPB-10	270-1670	8.0	6.0	2	75.00	0.842
		Total	194	152	42	1576.75	16.88
		Average	9.7	7.6	2.1	78.83	0.844

PIC- Polymorphic Information Content

Table 3. Jaccard's similarity coefficient for different cowpea genotypes based on RAPD data analysis

Genotype	GC-3	GC-4	GC-5	GC-601	GC-706	GC-505	GC-516	PGCP-5	PGCP-6	PGCP-11	PGCP-12	PGCP-13	PGCP-14	Pant Lobia-1	Pant Lobia-2	RC-101	KM-5	C-152	TVX-944	IT-38956-1
GC-3	1.000																			
GC-4	0.723	1.000																		
GC-5	0.612	0.613	1.000																	
GC-601	0.667	0.611	0.596	1.000																
GC-706	0.678	0.681	0.653	0.732	1.000															
GC-505	0.671	0.627	0.589	0.728	0.738	1.000														
GC-516	0.690	0.657	0.619	0.639	0.707	0.752	1.000													
PGCP-5	0.691	0.671	0.551	0.699	0.665	0.658	0.709	1.000												
PGCP-6	0.587	0.556	0.637	0.592	0.626	0.586	0.604	0.639	1.000											
PGCP-11	0.605	0.584	0.623	0.633	0.678	0.572	0.644	0.658	0.685	1.000										
PGCP-12	0.647	0.579	0.542	0.573	0.632	0.600	0.620	0.612	0.561	0.590	1.000									
PGCP-13	0.698	0.606	0.579	0.622	0.635	0.593	0.657	0.649	0.608	0.686	0.637	1.000								
PGCP-14	0.643	0.563	0.570	0.662	0.616	0.619	0.662	0.597	0.589	0.631	0.593	0.762	1.000							
Pant Lobia-1	0.624	0.556	0.586	0.630	0.655	0.659	0.691	0.580	0.582	0.613	0.621	0.649	0.777	1.000						
Pant Lobia-2	0.662	0.607	0.636	0.646	0.692	0.650	0.669	0.606	0.568	0.639	0.580	0.618	0.633	0.687	1.000					
RC-101	0.671	0.616	0.623	0.667	0.724	0.683	0.667	0.658	0.587	0.649	0.624	0.674	0.655	0.709	0.772	1.000				
KM-5	0.685	0.688	0.603	0.591	0.669	0.650	0.681	0.649	0.578	0.628	0.626	0.607	0.599	0.650	0.712	0.759	1.000			
C-152	0.641	0.619	0.559	0.590	0.626	0.617	0.636	0.617	0.536	0.533	0.580	0.573	0.610	0.677	0.667	0.701	0.716	1.000		
TVX-944	0.636	0.590	0.574	0.573	0.556	0.566	0.620	0.601	0.582	0.613	0.621	0.590	0.593	0.646	0.687	0.672	0.699	0.703	1.000	
IT-38956-1	0.664	0.596	0.548	0.684	0.593	0.652	0.614	0.651	0.546	0.564	0.615	0.630	0.647	0.664	0.655	0.713	0.679	0.790	0.730	1.000

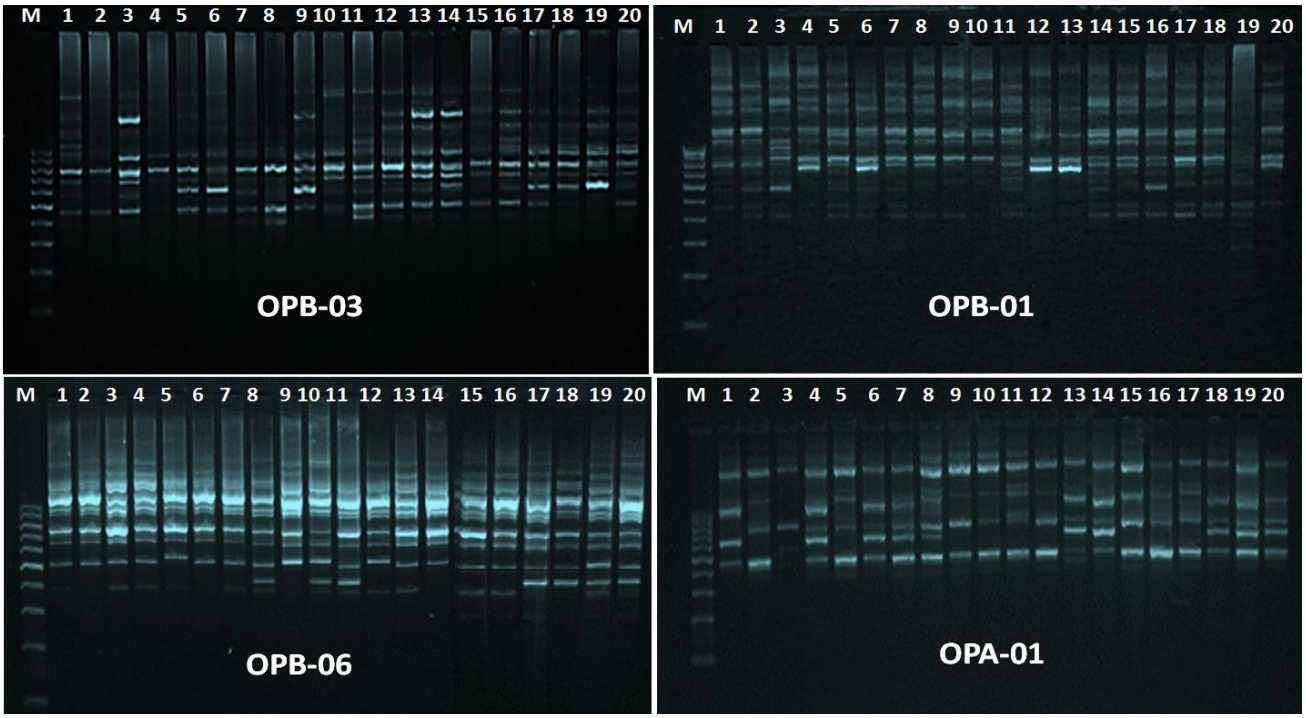

Fig.1. RAPD-PCR amplification of genomic DNA of cowpea M: marker; Lanes 1–20

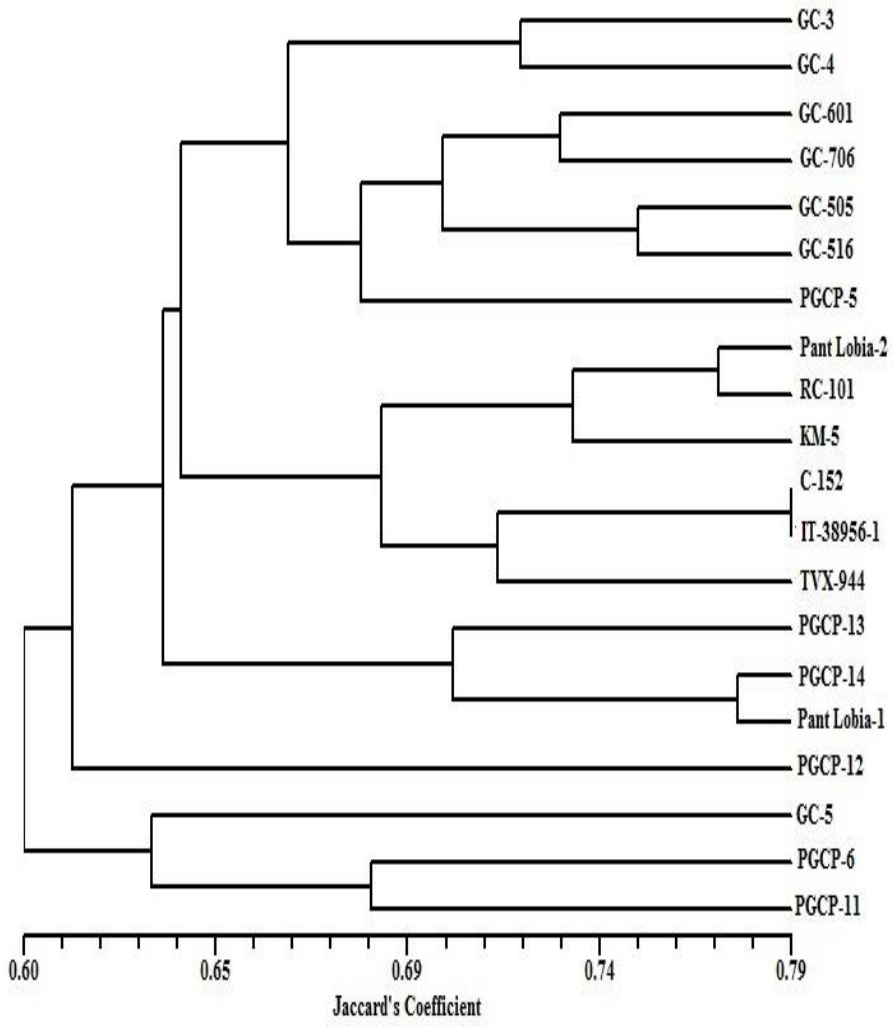

Fig.2. Dendrogram showing clustering of 20 cowpea genotypes constructed using UPGMA based on Jaccard's similarity coefficient obtained from RAPD analysis