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Characterization of mutant cowpea [*Vigna unguiculata* (L) Walp] lines using random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphism (AFLP) markers

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Phylogenetic relationship and polymorphism was detected in 10 cowpea lines comprising of leaf, flower and stem mutants, their putative parents and an exotic accession using 10 random amplified polymorphic DNAs (RAPDs) and three primer combinations of amplified fragment length polymorphism (AFLP) markers. These mutants were earlier obtained through the probable activities of transposable elements. The RAPD and AFLP markers revealed a genetic diversity of 47 and 31%, respectively, within the cowpea lines used. Genetic distance ranged from 0.05 to 0.30 based on AFLP markers, while it ranged between 0.13 and 0.44 for RAPD markers. Cluster analysis indicated that there are differences in RAPD markers between the various mutants and it grouped an exotic genotype separately. OPC-14 primer had the highest discriminatory capacity (11 polymorphic fragments). The AFLP analysis was able to group two of the flower mutants, leaf mutants and wild types separately. A combined analysis of the two markers gave a similar grouping as was obtained from the AFLP analysis. AFLP was more discriminatory in grouping the plant samples and the exotic line was distinguished based on both markers. Useful heterotic prediction can be done based on the genetic distance between the mutants and their parents. This will further broaden the genetic base of cowpea and enhance the use of these mutants which have some evolutionary significance. In addition, unique allele RAPD_OPC15-500bp can be harnessed in genetic identification of reduced petal mutant. This study further corroborates the discriminatory power of AFLP over RAPDs.

Key words: *Vigna unguiculata*, amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), mutants, transposable elements.

INTRODUCTION

Genetic diversity studies are usually done to assess the variation and the similarity within plant species. In breeding programs, this helps in determining the plant materials that can be crossed in order to obtain recombinants with the most favourable combination of desirable traits. Several mutants were obtained among

Table 1. List of the mutants and wild types and their sources.

Name	Description	Source
Ife brown	Non mutant cultivar	University of Ibadan , Ibadan Nigeria
Ife BPC	Non mutant cultivar derived from Ife Brown	IITA
Tvu1	Wild type	IITA
Tvu1509	Wild type	IITA
LM1	Unifoliate leaf form mutant	University of Ibadan , Ibadan Nigeria
LM2	Non petiolate and non-branching mutant	University of Ibadan , Ibadan Nigeria
RFM	Rose-like flower mutant	University of Ibadan , Ibadan Nigeria
RPM1	Reduced petal mutant	University of Ibadan , Ibadan Nigeria
RPM2	Reduced petal mutant	University of Ibadan , Ibadan Nigeria
Tvu 94051	Non mutant line/exotic line	University of California Davis, Davis CA USA

the progenies from crosses made by previous workers on cowpea. These mutants, with genetic evidence used in this study, have shown to be under the influence of transposable elements. Mutants are sources of variation, which may be useful in introducing unique and useful alleles to new populations. Genetic variation is required for crop improvement, hence the need to broaden the genetic base of any crop. This study was done in order to further enhance this in cowpea.

While assessing diversity and phylogenetic relationship with other mutants and their parents, each unique mutant was also characterized. Randomly amplified polymorphic DNA (RAPD) developed by Williams et al. (1990) has been successfully used in assessing diversity in many crops including cassava (Marmey et al., 1994), tropical pumpkin (Gwanama et al., 2000), somatic mutants of grapes (Maia et al., 2009) and more recently to assess diversity in Faba bean (Yahia et al., 2014) and cowpea (Anatala et al., 2014). It is an efficient marker for fingerprinting, evaluation of gene flow and studying traits such as pest resistance. Among PCR based assays, RAPD is more effective and easier than specific PCR based assays because they neither require sequence information nor any previous knowledge of the target genome, and moreover, they are relatively simple and rapid to perform (Mumtaz et al., 2009).

These cowpea lines were further characterized using amplified fragment length polymorphism (AFLP) developed by Vos et al. (1995). This PCR based marker, which has been widely used in genetic diversity studies, is reportedly more discriminatory in assessing diversity than the other markers like RAPDs and simple sequence repeats SSRs (Powell et al., 1996). Liu and Hou (2010) and Ojuederie et al. (2014) used AFLP in the assessment of genetic diversity of pigeon pea and African yam bean, respectively.

Baker et al. (1990), Garcia et al. (2004), Baraket et al. (2010) Ikechukwu et al. (2014) and Anatala et al. (2014) also used a combination of two or more molecular markers to assess more robust diversity or to compare the effectiveness of one marker relative to the other. The objectives of this study were to (i) compare the information provided by these markers in characterizing mutants and parents, (ii) compare the genetic distance information of these mutants and parents and (iii) show if there are unique alleles which can distinguish the mutant lines from their wild types (parents).

MATERIALS AND METHODS

Plant materials

Five mutants lines out of the 10 cowpea samples used for this study were obtained from the Department of Crop Protection and Environmental Biology, University of Ibadan. The mutants were obtained and selected from the progenies from crosses between several cowpea lines. They are namely: reduced petal mutant 1 (RPM-1), reduced petal mutant 2 (RPM-2), Rosa flower mutant (RFM), leaf mutants (LM-1) and LM-2. Their parents/wild types: Tvu 1509, Tvu 1, Ife brown, Ife BPC were obtained from the Gene bank of the International Institute of Tropical Agriculture and Tvu 94051 an 'exotic' cowpea cultivar (DNA) was obtained from University of California Davies. Ife Brown is the putative parent of Ife BPC (Table 1). The mutants are progenies of crosses between Ife brown and other lines.

DNA extraction

The seeds of the mutants and their parents were planted out in pots in the screen house, and young leaves were harvested from them at two weeks. DNA was extracted from these samples using the CTAB method (Dellarporta et al., 1983). The entire study was carried out at the Central Biotech Laboratory of International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria.

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Table 2. List of RAPD primers used for this study and their sequences.

Primer name	Sequence	No. of fragments	No. of polymorphic fragments
OPQ1	GGGACGATGG	10	7
OPQ15	GGGTAACGTG	10	6
OPP1	GTAGCACTCC	8	4
OPP13	GGAGTGCCTC	7	4
OPP15	GGAAGCCAAC	6	5
OPB06	TGCTCTGCCC	9	6
OPI20	AAAGTGCGGG	9	6
OPC10	TGTCTGGGTG	9	8
OPI04	CCGCCTAGTC	6	5
OPC14	TGCGTGCTTG	11	11
	Mean	81	62 (76%)

PCR amplification

For the RAPD study, OPERON primers OPQ1, OPQ15, OPP1, OPP13, OPP15, OPB06, OPI20, OPC10, OPI04 and OPC14 of 10mer oligonucleotides were used in this study (Table 2). Reactions were carried out in a total volume of 25 µl containing 1X buffer, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20, 2.5 mM each of dNTPs (Promega), 50 ng of genomic DNA and 1 unit of Taq polymerase (Bioline). PCR reactions were carried out in a thermal cycler (MJ Research). The samples were subjected to an initial denaturation at 94°C for 4 min, 45 cycles of 1 min at 94°C, one min at 37°C and 2 min at 72°C and then a final extension at 72°C for 5 min. Amplification products were analyzed on 1.5% agarose gels in 1X Tris Acetate EDTA (TAE) buffer at 100 V for 2 h and detected by staining with ethidium bromide.

AFLP marker analysis

PCR was carried out with genomic DNA obtained by using the same extraction protocol as used above. 400 ng DNA was digested with Mse and EcoR1 enzymes (5 u/µl) in a total volume of 25 µl. Ligations were carried out for 2 h using specific adapters. Pre-selective amplification was carried out on a 1:10 dilution of the ligated product in 10 µl following manufacturer's instructions. The mix was amplified using the following program: denaturation at 94°C for 2 min, 25 cycles of 2 min at 94°C, 1 min at 56°C and 1 min at 72°C and then a final extension at 72°C for 5 min. For selective amplification, 1:50 dilutions of pre amplification product was made and thereafter used as template for the reaction with the '+3' primers namely EAACMCAG, EAACMCAG and EAACMCTG.

The PCR was done using a modified touch down progam as follows: 94°C for 2 min, 12 cycles of 2 min at 94°C, one min at 65°C (-0.7°C per cycle) and 1 min at 72°C after which there were 23 cycles of 2 min at 94°C, 1 min at 56°C and 1 min at 72°C and then a final extension at 72°C for 5 min.

Gel electrophoresis

An aliquot of 5 µl of the product was mixed with equal volume of formamide dye, denatured for 5 min at 94°C and snap-chilled on ice. These products were separated on 6% PAGE gels. Electrophoresis was carried out in 1X TBE buffer at a constant power of 70 W for 2½ h at 50°C. Silver staining was carried out to visualize the bands on the gels.

Data analysis

Presence and absence of bands were scored using binary code of 1 or 0, respectively. Matrixes of distances were generated using SIMQUAL program of Numerical and Taxonomy system software (NTSYS) 2.0 (Rohlf, 1998). Clustering was done using the SAHN algorithm of the unweighted pair group method using average linkages (UPGMA) and a tree was obtained using the tree plot option of NTSYS, a rooted tree was also generated from the DARwin software version 4 (Perrier and Jacquemoud-Collet, 2006).

RESULTS

DNA fingerprints were obtained for all the mutants and their parents using both RAPDs and AFLP markers. Plate 1 shows the amplification obtained using RAPD primer OPP13 and Plate 2 shows the amplification obtained for the 10 samples using AFLP marker EAACMCAG. Based on the RAPD analysis, the total number of fragments obtained from the 10 primers on the 10 samples was 81 while the total number of polymorphic fragment was 62 (76%). The number of fragments for each primer varied from 6 to 11 fragments among the primers, with an average of 6.8, whereas OPC-14 primer showed the greatest capacity for discriminating polymorphism in the population studied (Table 2). From the DARwin analysis, a diversity of 31% was obtained from RAPD markers for these lines. Three mini groups were obtained from the use of the RAPD markers, while the exotic line Tvu 94051 and Reduced petal mutant 2 (RPM2) were grouped separately. Cluster I consisted of LM1 and RFM, cluster II consisted of Ife BPC, Ife brown and LM2, while cluster III consisted of Tvu1, Tvu1509 and RPM1. Line Tvu94051 from California was grouped alone in group II (Figure 1).

Conversely, a diversity of 47% was obtained from the AFLP analysis. Reduced petal mutant: RPM2, Tvu1 and Tvu 94051 clustered, separately. One cluster consisted of Ife BPC and Tvu1509 while another cluster consisted of RFM and RPM1, both flower mutants. In addition, the leaf

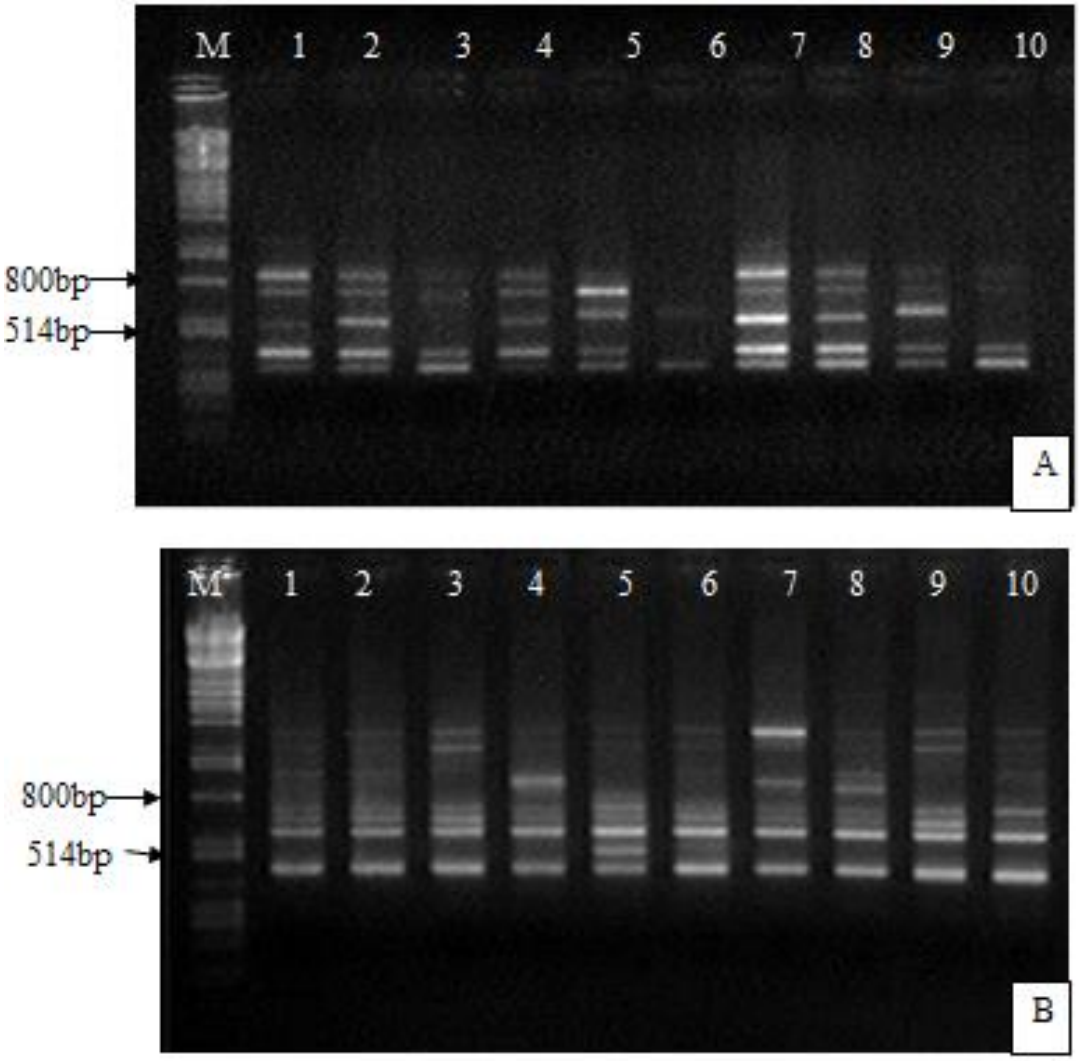

Plate 1. Amplification obtained from the mutant cowpea lines and parents using RAPD primers OPP13 (A) and OPQ15 (B). M is Lambda DNA pst1 digest. 1 = Ife Brown, 2 = Ife BPC, 3 = Tvu 1, 4 = LM1, 5 = RPM1, 6 = RPM2, 7 = RFM, 8 = LM2, 9 = Tvu1509, 10 = Tvu 94051 (arrow points at a band ca 520 bp distinguishing RPM1 from others).

mutants LM1 and LM2 clustered together with Ife brown (Figure 2). It was observed that the RAPD analysis was able to group the parental types together and the exotic line separately as compared to AFLP. For instance, the two cultivated parental lines, Ife Brown and Ife BPC clustered together while the wild types Tvu1 and Tvu 1509 were also found together in another cluster. However, in the case of AFLP, two of the flower mutants, were clustered together, while the two leaf mutants were in the same cluster along with Ife brown. Both RPM2 and Tvu 94051 (exotic line) were in a class of their own in both analyses. The AFLP analysis was more discriminatory as it gave a higher genetic distance among the lines and gave more separation based on the grouping of the mutants. It also gave a similar pattern to the combined analysis of the two markers.

The genetic distance ranged between 0.05 and 0.30, the lower value was obtained between Ife BPC and LM2, while the highest values were obtained between RFM and RPM2 and between LM2 and RPM2 based on RAPD analysis (Table 3). However, for the AFLP analysis, the genetic distance ranged between 0.13 and 0.44 which were obtained between LM2 and Ife brown and between RPM2 and Ife BPC, respectively (Table 4). A combined analysis of both AFLP and RAPD however gave a similar pattern to the AFLP in terms of grouping but a different genetic distance. The distance ranged from 0.13 to 0.36 with many pairs having the higher value. All the mutants and parents except RPM1 and exotic line had a genetic distance of 0.36 with RPM2. In addition, Tvu 94051 had the same distance of 0.36 with RFM, LM2 and Tvu 1509 (Table 5).

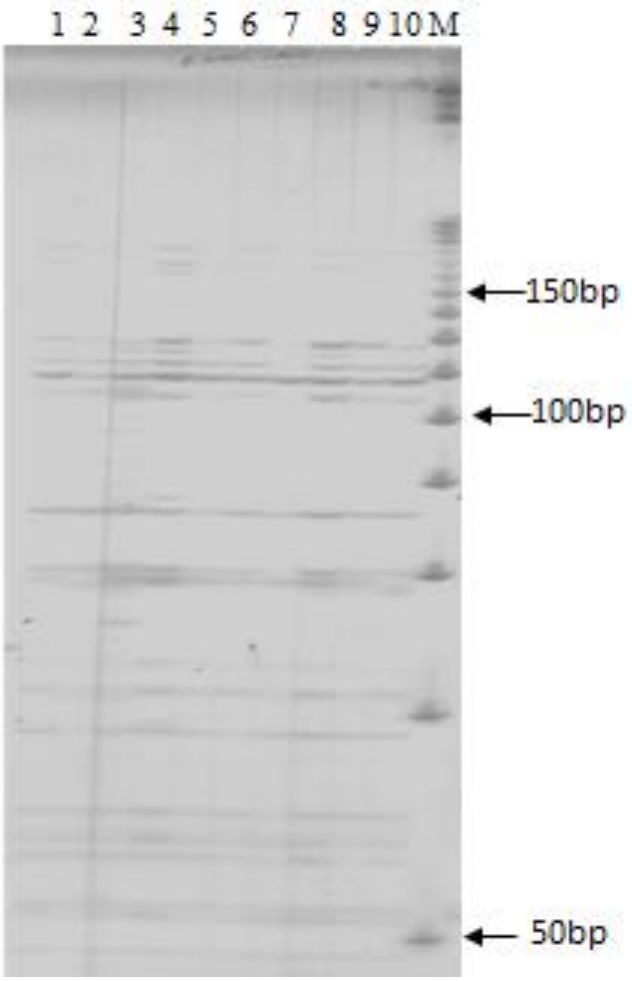

Plate 2. AFLP amplification of the mutant cowpea lines and their parents using primer EAACMCAG. Marker is 30 to 330 bp marker.1 = Ife Brown, 2 = Ife BPC, 3 = Tvu 1, 4 = LM1, 5 = RPM1, 6 = RPM2, 7 = RFM, 8 = LM2, 9 = Tvu1509, 10 = Tvu 94051.

The search for unique alleles revealed that there were no many bands distinguishing certain mutants from the other. A unique allele was however observed in the RAPD OPQ15_ 500 (about 500 bp) which distinguished the RPM1 from the others (Figure 1b).

DISCUSSION

The levels of differences obtained from the two molecular markers are relatively low; this is because they share a similar background but for the mutations on all the mutants which are derivatives of Ife Brown and Ife BPC. These are mutants that were obtained due to activities of transposable elements (TEs) and obtained from previous studies (Fawole, 1988a, 1997, 1998b, 2001, 2010). They have increased the genetic base of cowpea as additional sources of variation for future breeding work. The TEs have been characterized using molecular markers by Kolade et al. (2015).

The RAPD analysis gave a similar level of polymorphism, 62 out of 81 (76%) from 10 primers as compared to a previous study by Anatala et al. (2014) on cowpea genetic diversity which gave 67%, 81 out of 120 fragments. The clustering, which appeared to be based on the type of mutation or origin is an interesting observation. In this case, the fact that the leaf mutants, the flower mutants clustered close to each other and the wild species were found in the same cluster, while the exotic variety was found in separate cluster, indicated the usefulness of the markers for grouping based on the kind of mutation that exists in the sample and where the samples originated from. This has been observed by many authors, while using RAPD markers in population genetic analysis of biodiversity, relationships among species at different levels, to identify cultivars and to reveal phylogenetic relationships among them (Ba et al., 2004; Malviya and Yadav, 2010; Motagi et al., 2013; Anatala et al., 2014).

The more discriminatory power of the AFLP has been shown (Archak et al., 2003) for comparative analysis while using RAPD, ISSR and AFLP in Cashew (*Anachardium occidentalis*). This study also corroborates it but further showed that a combination of RAPD and AFLP is also slightly more discriminatory than when either is used singly. The reduced flower mutant, RPM2, might have clustered differently from the other flower mutants, because it is an unstable mutant. In addition, discrimination of the flower mutants from that of the wild types and the leaf mutants as revealed by AFLP analysis than RAPDs in the present studies agree with previous reports that compared two types of molecular markers (Barker et al., 1999; Garcia et al., 2004; Baraket et al., 2010).

Conclusion

This study reveals the phylogenetic relationship between these mutants and their parents. The exotic line was different from the rest with the two molecular markers, indicating the difference in their pedigree and geographical origin. AFLP analysis was found to be more discriminatory in characterizing the mutants than the RAPD analysis and gave a similar grouping pattern as obtained from the combined analysis. The result obtained here will be useful for breeders who are willing to explore the possibility of pyramiding genes that are responsible for rose like flower mutations, reduced petal mutation and others studied. The level of diversity as evident in these mutants can be harnessed in breeding for better varieties as the divergent genotypes are expected to result in high heterosis. The unique allele can be harnessed for genotype identity.

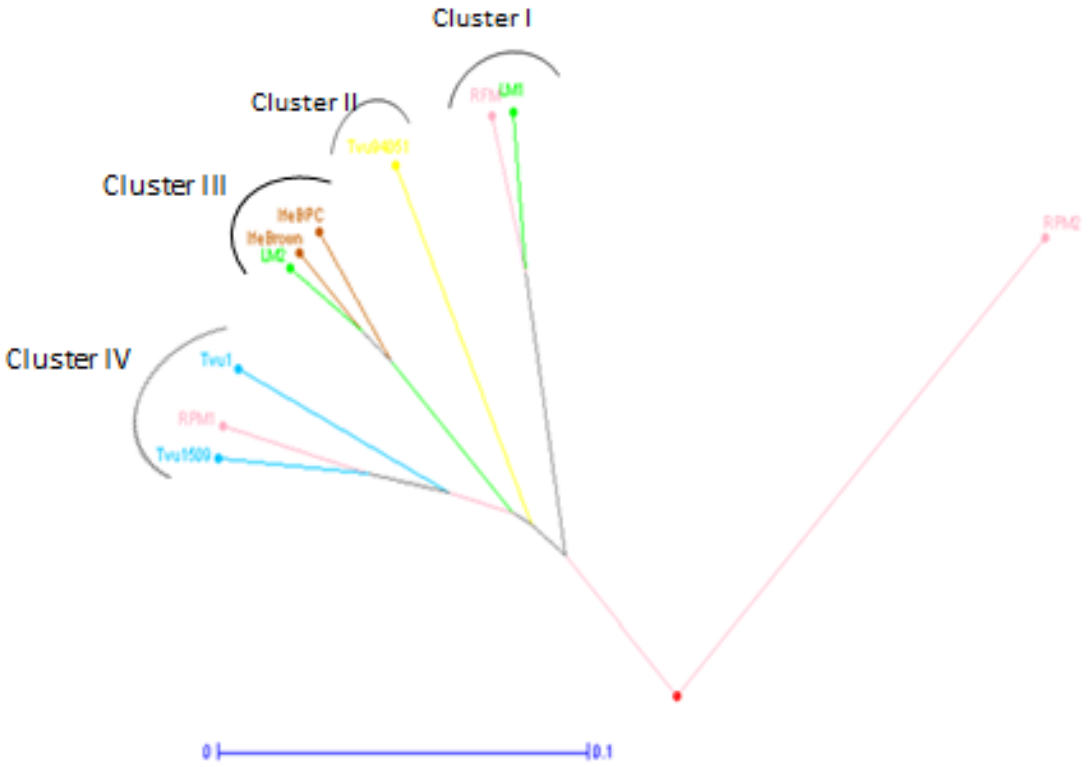

Figure 1. Rooted diagram (axial) obtained from the analysis of the mutant lines and parents using RAPD markers (DARwin) (pink represents flower mutants, blue represents wild type, green represents leaf mutants and yellow, exotic line).

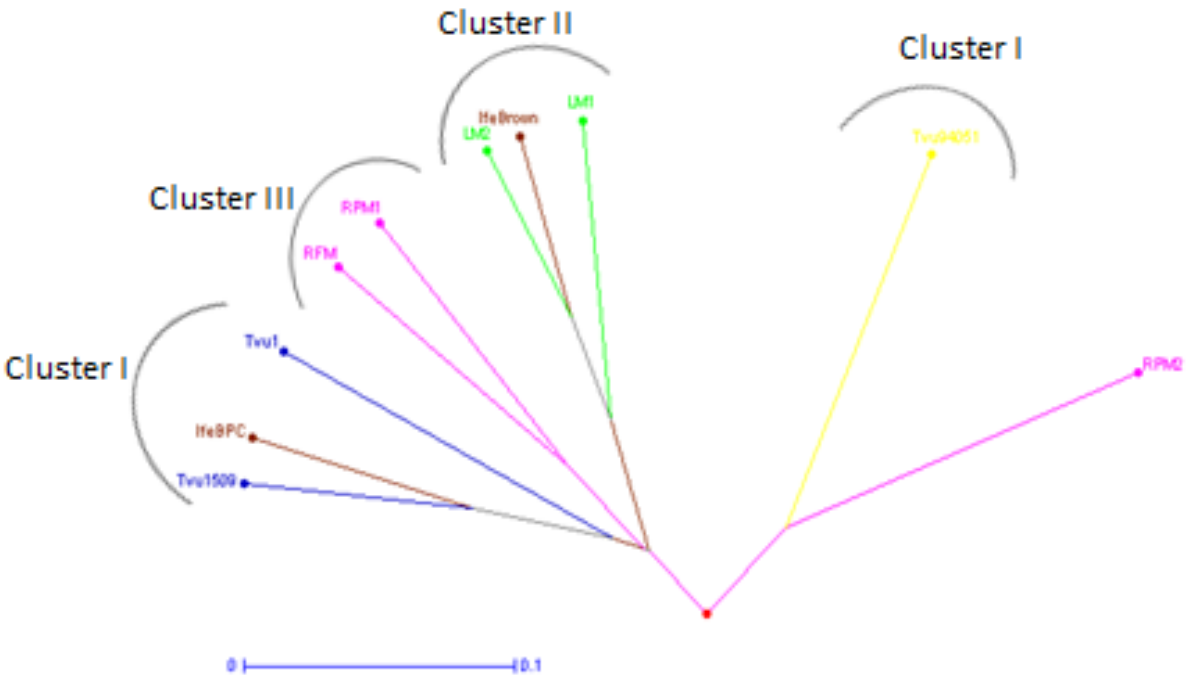

Figure 2. Rooted diagram (axial) obtained from the analysis of the mutant lines and parents using RAPD and AFLP markers (DARwin) (pink represents flower mutants, blue represents wild type, green represents leaf mutants and yellow, exotic line).

Table 3. Genetic distance matrix revealed by RAPD analysis.

Variables	Ife Brown	Ife BPC	Tvu1	LM1	RPM1	RPM2	RFM	LM2	TVu1509
Ife BPC	0.06								
Tvu1	0.13	0.17							
LM1	0.17	0.21	0.16						
RPM1	0.18	0.22	0.15	0.22					
RPM2	0.26	0.3	0.23	0.29	0.19				
RFM	0.17	0.22	0.17	0.07	0.23	0.3			
LM2	0.07	0.05	0.17	0.21	0.23	0.3	0.22		
Tvu1509	0.13	0.17	0.1	0.16	0.08	0.16	0.17	0.17	
Tvu94051	0.15	0.19	0.16	0.2	0.21	0.29	0.21	0.20	0.16

Table 4. Genetic distance matrix revealed by AFLP analysis.

Variables	Ife Brown	Ife BPC	Tvu1	LM1	RPM1	RPM2	RFM	LM2	Tvu1509
Ife BPC	0.33								
Tvu1	0.30	0.31							
LM1	0.19	0.34	0.31						
RPM1	0.26	0.31	0.28	0.28					
RPM2	0.33	0.44	0.41	0.35	0.37				
RFM	0.30	0.35	0.32	0.31	0.22	0.41			
LM2	0.13	0.36	0.33	0.22	0.29	0.36	0.33		
Tvu1509	0.26	0.17	0.24	0.28	0.25	0.37	0.28	0.29	
Tvu 94051	0.30	0.41	0.38	0.31	0.34	0.28	0.38	0.33	0.34

Table 5. Genetic distance matrix from combined RAPD and AFLP analyses.

Variables	Ife brown	Ife BPC	Tvu1	LM1	RPM1	RPM2	RFM	LM2	Tvu1509
Ife BPC	0.30								
Tvu1	0.30	0.27							
LM1	0.21	0.30	0.30						
RPM1	0.30	0.30	0.30	0.30					
RPM2	0.36	0.36	0.36	0.36	0.36				
RFM	0.30	0.30	0.30	0.30	0.22	0.36			
LM2	0.13	0.30	0.30	0.21	0.30	0.36	0.30		
Tvu1509	0.30	0.17	0.27	0.30	0.30	0.36	0.30	0.30	
Tvu 94051	0.36	0.36	0.36	0.36	0.36	0.28	0.36	0.36	0.36

Conflicts of Interests

The authors have not declared any conflict of interests.

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