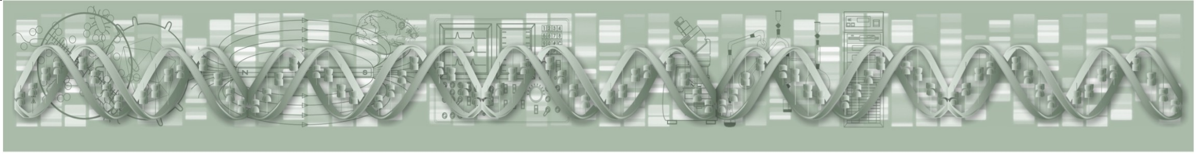


GENETIC DIVERSITY AND PHYLOGENETICS OF FOUR RELEASED COWPEA (*VIGNA UNGUICULATA* (L.) WALP) VARIETIES (FUAMPEA-1, FUAMPEA-2, FUAMPEA-3 AND FUAMPEA-4) USING SIMPLE SEQUENCE REPEATS MARKERS

Olalekan Joseph Olasan[1*], Celestine Uzoma Aguoru[1], Lucky Omoigui[2], Faith Oluma[1], Macsamuel Sesugh Ugbaa[2], Judith Ogechi Ezugwu[2], Godspower Ekeruo[2], Nater Iyorkaa[2], Teryima Iorlamen[2], Omoche Ojobo[1], Okekporo Efe Stephen[1], Ann Nnena Osuagwu[3]

[1]Plant Biotechnology Unit, Department of Botany, Federal University of Agriculture, Makurdi. Nigeria
[2]Department of Plant Breeding and Seed Science, Federal University of Agriculture, Makurdi. Nigeria
[3]Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike
[*] Corresponding author e-mail: olasan.olalekan@uam.edu.ng

Abstract

Cowpea improvement has continued to play key roles in the quest for national food security. This work elucidated the molecular genetic diversity and phylogenetics among four released cowpea varieties (FUAMPEA-1, FUAMPEA-2, FUAMPEA-3 AND FUAMPEA-4). DNA was extracted from 14 day old seedlings using the CTAB method. A total of nine (9) SSR (Simple Sequence Repeats) markers were employed in DNA amplifications. Binary matrix was generated from banding profiles of gel images and uploaded into the Minitab 17.0 software for analysis. Polymorphic Information Content (PIC) was computed for the primers and cowpea varieties. Cluster analysis was carried out using the Single Linkage method measured on Euclidean Distance. Four primers (CLM1190, CLM0342, RB38 and RB7) produced maximum of two bands each resulting in 15.4% polymorphism and 0.977 Polymorphism Information Content (PIC). Varietal Polymorphic Content (VPC) varied from 15.4% in FUAMPEA-2 as the lowest to 38.5% in FUAMPEA-1 as the highest. FUAMPEA-1 had the lowest genetic similarity index (19.82). Dendrogram further showed that FUAMPEA-2 and FUAMPEA-3 were more closely related than others with about 46.55 similarity index. Therefore, FUAMPEA-1 was the most divergent and genetically diverse variety followed by FUAMPEA-4. These are potential genetic resource material that can used to develop more cultivars when their inherent agronomic traits and genes coding for the traits are identified and tapped.

Keywords: Cowpea, Breeding, Phylogenetics, Molecular markers, Food security

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp), family Fabaceae, is an important leguminous food crop of the sub-Saharan African countries and it has become a staple food produced and consumed by almost every home in Nigeria. The grain is a source of cost effective plant based dietary protein (25%) and amino acids in addition to high fiber, vitamin and mineral contents needed to address malnutrition and poverty in developing countries (Aboki et al. 2013, Ahenkora et al. 2018). About 83.4% of the world's overall production comes from Africa while over 80% of African production comes from West Africa including Nigeria being the largest producer of

Received 28 December 2022; Accepted 6 March 2023; Published Online 16 March 2023; Published 3 April 2023

cowpea in Africa (Omoigui et al. 2016). The crop is well adapted to drought. It competes favourably well with other plants while playing an important role in nitrogen fixation, a process that adds nutrients to the soil (Uzogara and Ofuya 2012, Taiwo et al. 2014). Although there have been successes of cowpea production in the country, the crop is challenged by numerous biotic and abiotic stresses that limit its productivity or consumption (Omoigui et al. 2017). There is need for continuous improvement to ensure its sustainable production to feed the growing human population. The use of modern biotechnology cannot be over-emphasized in this regard (Singh et al. 2012).

To solve the numerous constraints to cowpea production, plant breeders have initiated many improvement programmes aimed at addressing specific challenges to meet the demand of farmers and consumers ((Singh et al. 2012). There are varieties that have been improved for resistance against parasitic weeds, fungal and bacterial diseases (Omoigui et al. 2016, 2017). Some varieties have been improved for nitrogen efficiency use and tolerance to pesticides and drought (Boukar and Fatokun 2019). The lack of genetic diversity has made further improvement in cowpea varieties an arduous task. New variation is critical for introducing interesting characteristics into breeding programs. Diversity in plant genetic resources is needed to provide opportunities for plant breeders to develop new improved cultivars with desirable traits. Varietal development is a crucial aspect of crop improvement that provides genetic resource to improve local landraces to achieve national food security (Boukar and Fatokun 2019).

Federal University of Agriculture Makurdi Nigeria (FUAM) in collaboration with IITA (International Institute of Tropical Agriculture) has been in the front burner of cowpea improvement programmes mainly to improve yield, maturity time and resistance to parasitic weeds, pests and diseases (Omoigui et al. 2015, 2016, 2017). Consequently, the National Variety Release Committee (NVRC) approved the release of two improved varieties (FUAMPEA-1 and FUAMPEA-2) in 2016 and additional two varieties (FUAMPEA-3 and FUAMPEA-4) in 2022. The overall aim is to boost cowpea production to achieve food security not only in Nigeria but also in Africa in addition to boosting farmers' income and improving their livelihood. Studies on the genetic diversity and phylogenetic relationship have not been conducted on these new varieties. This aspect is considered important in plant breeding and improvement programmes (Boukar and Fatokun 2019, Olasan et al. 2020). Simple Sequence Repeats (SSR) markers have been applied on a number of crops for cultivar identity, genetic diversity, determination of evolutionary relationship, marker assisted selection and construction of linkage maps (Dughdugh et al. 2017, Olasan et al. 2020, Tersoo et al. 2021, Aguoru et al. 2022). These repetitive DNA regions are reported to be polymorphic co-dominant markers with high resolutions (Olasan et al. 2020). The aim of this work was to assess the genetic diversity and phylogenetics among four newly released cowpea varieties using specific SSR markers.

Materials and Methods

Study area and plant sample collection

This study was carried out in the Molecular Biology Laboratory of the Federal University of Agriculture Makurdi (Now Joseph Sarwuan Tarka University Makurdi). Seeds of four new cowpea varieties developed by the Federal University of Agriculture Makurd (FUAM) were sourced from the Seed Store of the institution. They were: FUAMPEA-1, FUAMPEA-2, FUAMPEA-3 and FUAMPEA-4.

Molecular markers

A total of nine (9) cowpea specific SSR (Simple Sequence Repeats) primers were employed in DNA amplifications. They were part of the routine primers used in cowpea breeding work. The names and sequences of the primers are given in table 1.

Seed planting for molecular studies

Four seeds of each variety were planted in separate pots filled with soil inside the Screen House of the Department of Plant Breeding and Science of the same institution. Young leaves were collected from 14 day old seedlings.

DNA extraction

CTAB (Cetyltrimethylammonium bromide) method of DNA extraction method was used (Tersoo et al. 2021). Two leaves were placed in silica gel for three days to dry. The crispy dry leaves were squeezed into 2ml eppendorf tubes containing two steel balls and grounded vigorously using a vortex for one minute. Buffer (1M Tris-Hcl, 0.5M EDTA, 5M NaCl, 2-Mecarptoethanol) was added and incubated in a water bath for 30minutes at 60°C. Exactly 600uL chloroform: Isoamylalcohol (24:1) was added and spun for 10minutes at 4000rpm. The upper layer was transferred into new tubes and this step was repeated. 600uL of ice cold 2-propanol was added into the supernatant and inverted for few seconds. Tubes were kept in -20°C freezer to precipitate, after precipitation tubes were then centrifuge for 35minutes at 4000rpm to form pellets at the bottom of the tube and then supernatant was decanted. The pellets were washed with 400uL of 70% ethanol, centrifuge for 15minutes and decant ethanol. This process was repeated and the pellets were allowed to air dry for few hours by placing the opened tubes on paper towel. The dried pellets were suspended in 90uL of molecular grade water.

Polymerase chain reaction

SSR based PCR protocol was used (Omoigui et al. 2015). Each reaction contained 25uL of double distilled water and 1.5 µl of one specific primer pair mixed in a PCR tube already containing a customized BIONEER® Accupower PCR Premix (Top DNA polymerase (1U), dNTPs (250µM), Tris-HCl of 9.0 pH (10mM), KCl (30mM), $MgCl_2$ (1.5mM), stabilizer and tracking dye). The PCR tubes were loaded on the thermal cycler and programmed with the following temperature gradient profile as follows: 94°C denaturation temperature, 55°C annealing temperature, 72°C elongation temperature and 35cycle running for 3hours on a thermal cycler (Applied Biosystem in Life Technology version 2720).

Agarose gel electrophoresis

Exactly 0.8% agarose powder was poured into a beaker containing 1xTAE buffer and was swirled gently. The beaker was placed into the microwave for few minutes till the solution became crystal. The solution was allowed to cool and 5uL of EtBr was added and poured on an already gel plate with comb. Solution was placed into the electrophoresis tank (Galileo Bioscience tank connected to Consort EV243 electrophoresis power supply). The comb was removed gently 5uL of DNA sample was added into the PCR tube while 2uL of 6x loading dye was also added and spun for few seconds. Sample was gently loaded into the wells using pipette and finally 5uL of ladder was loaded. The gel ran at 120v for 45 minutes. DNA purity and quality was checked using UV spectrometer light (Omoigui et al. 2015).

DNA band visualization

The banding pattern of the samples resolved on agarose gel was viewed on a UV Bench top trans-illuminator and the gel image was captured using a camera for band scoring. Only distinct bands were scored as present (1) or absent (0) (Omoigui et al. 2015).

Data analysis

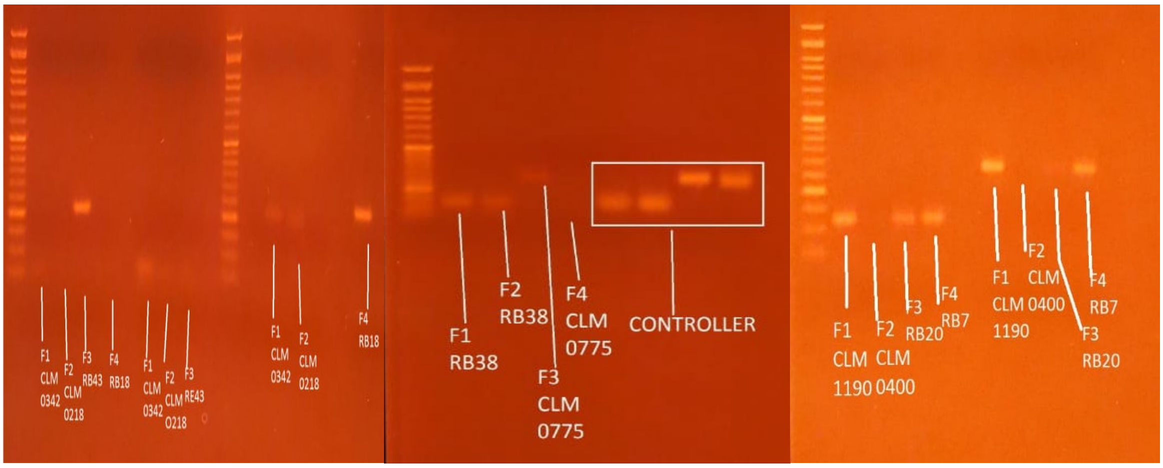
Binary matrix was generated from DNA banding profiles of gel images and uploaded into the Minitab 17.0 software for analysis. Polymorphic bands and % polymorphism were calculated for each SSR primer used. Polymorphism Information Content (PIC) was calculated using the formula adopted by Weir (1990) and Tersoo et al. (2021).

PIC = 1 - Σ *pi*2

Where *pi* is the frequency of the *i* th allele for each SSR marker *i* th summed across all alleles for the loci. Varietal Polymorphic Content (VPC) was calculated and expressed as percentages. Cluster analysis was carried out in two forms (i) the markers and (ii) the varieties. This was done using the Single Linkage method measured on Euclidean Distance resulting in the dendrogram (Olasan et al. 2018).

Results and discussion

Selected gel images produced from the study are shown in Plates 1a-c while banding patterns are pictorially given in figure 1. All the primers gave good amplification of DNA of the four varieties of cowpea studied. The resulting number of band depends on the SSR primer type and the cowpea variety. Three primers were clearly resolved in FUAMPEA-1 (CLM0342, CLM1190 and RB38) and FUAMPEA-3 (CLM0775, RB20 and RB43) while two primers were resolved in FUAMPEA-2 (CLM0218 and RB38) and FUAMPEA-4 (RB18 and RB7). Therefore, two to three primers have helped to differentiate the released varieties. This study is in agreement with previous reports on the high resolution of SSR markers in differentiating heterozygosity of traits as co-dominant markers (Jonah et al. 2011, Mason 2015, Tersoo et al. 2021). Different alleles may exist at a given SSR locus and this makes SSR markers more useful than other molecular markers including single nucleotide polymorphism (SNP) marker because repetitive regions linked to traits are highly unstable due to their high rate of mutation (Mason, 2015).



Plates 1. Selected Gel images of DNA from cowpea varieties as amplified by nine SRR primers

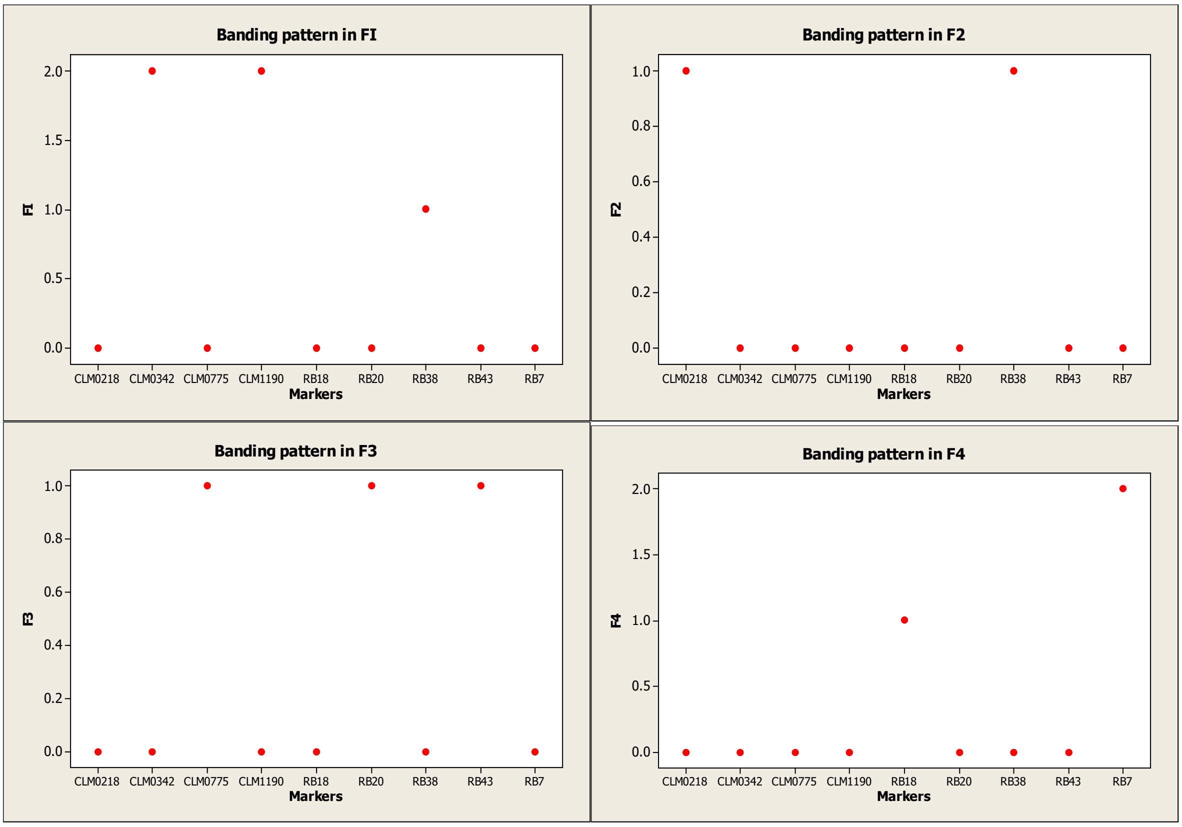


Figure 1. DNA Banding pattern of SSR molecular markers in the four varieties of cowpea (F1-F4 respectively)
Legend: FI= FUAMPEA-1; F2= FUAMPEA-2; F3= FUAMPEA-3; F4= FUAMPEA-4

Some levels of clustering were found among the primers based on their relatedness (Figure 2) as observed between CLM1190 and CLM0342 as well as CLM0775, RB43 and RB20. The divergent primers were RB38, CLM0218, RB7 and RB18. Four primers produced two bands each (CLM1190, CLM0342, RB38 and RB7) resulting in 15.4% polymorphism and 0.98 Polymorphic Information Content (PIC). Other primers produced one band each with 7.7% polymorphism and 0.941 PIC. All the primers had average of 1.4 polymorphic bands, 11.1% polymorphism and 0.957 PIC (Table 1). All the primers used in this study are highly informative as revealed by their high PIC values. According to Nelson *et al*. (2016), PIC values of 0.5, 0.4 and 0.2 are classified as highly informative, moderately informative and little informative respectively. The average PIC of 0.957 reported in this study was higher than values obtained in most breeding reports on SSR markers (Dhaliwal et al. 2014, Kumar et al. 2016, Dughdugh et al. 2017, Alfred et al. 2019, Tersoo et al. 2021). A marker is said to be polymorphic if it has at least two alleles and the allele frequency is up to 0.9 (Mason 2015). Therefore, four markers including CLM1190, CLM0342, RB38 and RB7 may be selected for cowpea improvement programmes because of their higher percentage polymorphism, PIC values and existence of heterozygosity for different traits. Moreover, other regions of the DNA identified by these primers may be coding for unique traits of breeding values. This outcome is consistent with the report given by Omoigui et al. (2015) who determined the suitability and use of two molecular markers to track race-specific resistance to *Striga gesnerioides* in cowpea.

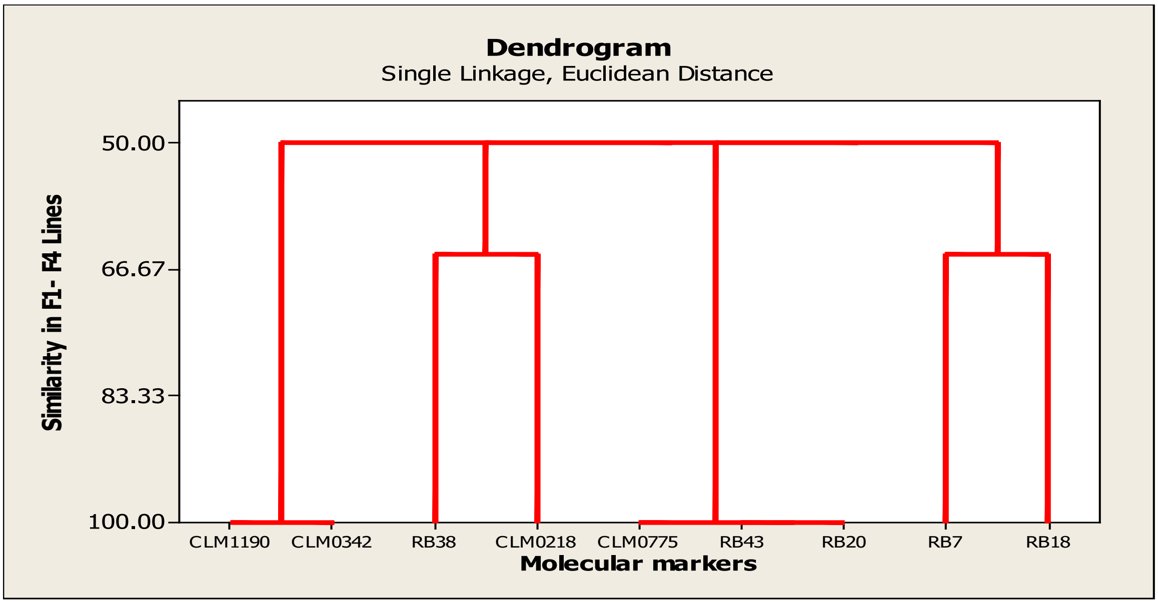


Figure 2. Relationship in banding pattern of SSR primers

Table 1. Primer name, Sequence and Polymorphism Information Content (PIC)

Primer name	Sequence	∑Polymorphic bands	% Polymorphism	PIC 1 - Σ *pi*2
CLM1190	ATTTGGCTGAATTGTTTCCA	2	15.4	0.9775
CLM0342	GGATTGGATATGTGTCTGGC	2	15.4	0.9775
RB38	GCGGCCGCTGCTCGTTCCCG	2	15.4	0.9775
CLM0218	TTTCCGATTTGCGATTTTTA	1	7.7	0.9410
CLM0775	GTGGCAGCACAAGTTAGTAG	1	7.7	0.9410
RB43	CCATGGTCGCCCCTGCTGCACCTTG	1	7.7	0.9410
RB20	CCATGGGGGCATCAACCTTGG	1	7.7	0.9410
RB7	GGGCGTTAATTAAGCCCACACA	2	15.4	0.9775
RB18	AAGCTTACTTGTACAGCTCGTCCATGCCG	1	7.7	0.9410
Total		13	100	8.615
Mean		1.4	11.1	0.957

Varietal Polymorphic Content (VPC) varied from 15.4% in FUAMPEA-2 as the lowest to 38.5% in FUAMPEA-1as the highest. FUAMPEA-3 and FUAMPEA-4 had VPC value of 23.1% each slightly below the mean VPC of 25.0% (Figure 3). Based on the phylogenetic relationship among the varieties, FUAMPEA-1 was the most divergent variety with 19.82 similarity index followed by FUAMPEA-4. Dendrogram further showed that FUAMPEA-2 and FUAMPEA-3 were more closely related than others with about 46.55 similarity index (Figure 4). This phylogenetic construction based on genetic diversity is useful in breeding programme to make an informed decision about a potential genetic material with high breeding value whose genes could be tapped. It has given us an understanding of the genetic changes that must have taken place in the course of the breeding work. This is consistent with findings in other crops (Aguoru et al. 2015, 2022)**.** As new varieties of cowpea are developed and released, plant breeders need to utilize them to improve other varieties to achieve national food security. This position is fully supported by plant breeders (Xu et al. 2010, Viswanatha et al. 2011, Atnafua and Endashaw 2014; Dughdugh et al. 2017).

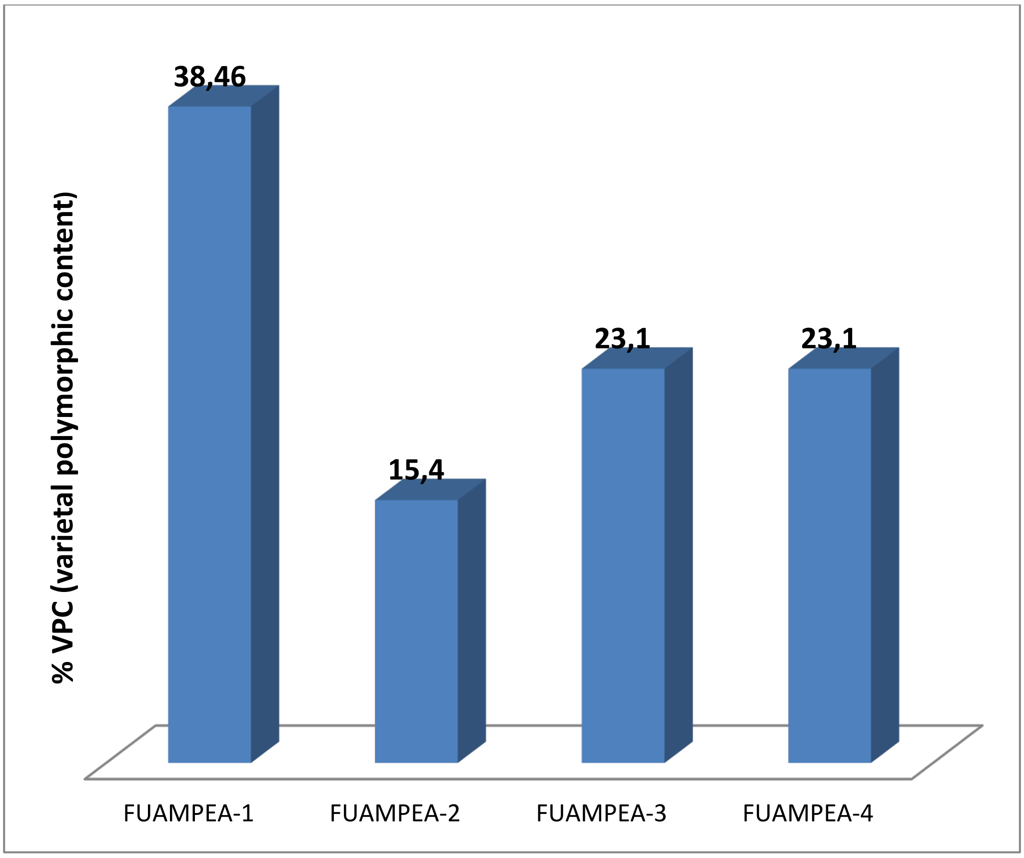


Figure 3. Varietal Polymorphic Content

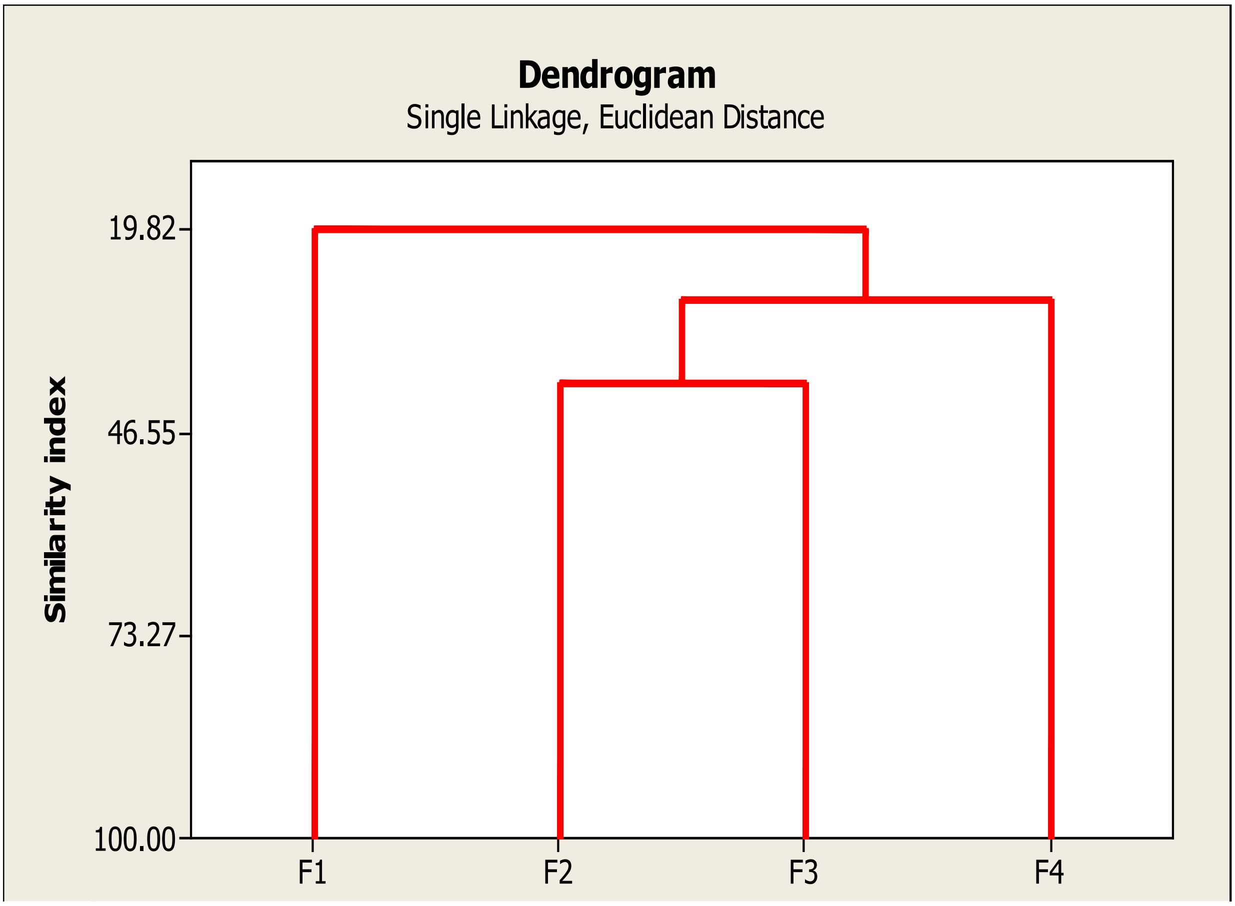


Figure 4. Phylogenetic relationship among the four cowpea varieties
Legend: FI= FUAMPEA-1; F2= FUAMPEA-2; F3= FUAMPEA-3; F4= FUAMPEA-4

Characteristically, FUAMPEA-1 (UAM09-1055-6) and FUAMPEA-2 (UAM09-1051-1) are high yielding, early maturing varieties that are resistant to *Striga* and *Alectra* (Omoigui et al. 2015, 2016, 2017) while FUAMPEA-3 and FUAMPEA-4 were developed of recent as an improvement over the previous varieties. They are brown, big seeded medium maturing types that carry high yield and resistant properties. The present investigation has showcased FUAMPEA-1as the most genetically divergent possibly in terms of other unidentified traits or variability at the gene locus of the improved traits. Other varieties are more related as they stem out of the parental source. Thus FUAMPEA-1 is a potential genetic resource material that can used to develop more cultivars when its inherent agronomic traits are fully known and the genes identified. FUAMPEA-4 is another divergent variety that may possibly carry useful genes on its genome that should be tapped.

Conclusions

The nine SSR primers employed are highly informative but four of the markers (CLM1190, CLM0342, RB38 and RB7) are selected for cowpea improvement programmes as they had higher PIC (0.98), % polymorphism (15.1%) than other primers. FUAMPEA-1 was the most divergent and genetically diverset variety followed by FUAMPEA-4. They are potential genetic resource material that can used to develop more cultivars when they are inherent agronomic traits are fully known while the genes identified and tapped. With a robust sustainable system of cowpea genetic improvement programme, national food security is assured.

Acknowledgement
The authors duly acknowledge all stakeholders including the Federal University of Agriculture Makurdi in collaboration with IITA. The contribution for the National Variety Release Committee (NVRC) is acknowledged.

References

Aboki E, Yuguda R. 2013. Determinant of profitability in cowpea production in Takum Local Government Area of Taraba State, Nigeria. Journal of Agriculture Science, 4: 33-37.

Aguoru CU, Olasan JO, Omoigui LO, Ekefan EJ. 2022. Marker assisted identification of suitable candidates for biosystematics and crop improvement among groundnut (*Arachis hypogaea* L.) breeding lines. Nigeria Journal of Plant Breeding, I(2): 43-54.

Aguoru CU, Omoigui LO, Olasan JO. 2015. Population genetic study of eggplants (*Solanum*) species in Nigeria, Tropical West Africa using molecular markers. International Journal of Plant Research, 5(1): 7-12.

Ahenkora K, Adu-Dapaah HK, Agyemang A. 2018. Selected nutritional components and sensory attributes of cowpea (*Vigna unguiculata* [L.] Walp.) leaves. Plant Foods and Human Nutrition, 52: 221–229.

Alfred UJ, Iheukwumere CC, Aguoru CU, Olasan OJ, Sesugh UM. 2019. Diversity analysis of sweet potato (*Ipomoea batatas* L. [Iam) accessions from North Central Nigeria using morphological and Simple Sequence Repeats Markers. Asian Journal of Biotechnology and Genetic Engineering, 2(2): 1-15.

Atnafua B, Endashaw B. 2014. Overview: Morphological and molecular markers role in crop improvement. International Journal of current Research in Life Sciences, 3(3): 035-042.

Boukar O, Fatokun C. 2019. Strategies in cowpea breeding. New approaches to plant breeding of orphan crops in Africa. Rome: FAO, 69-92.

Dhaliwal MS, Abhay Y, Jindal SK. 2014. Molecular characterization and diversity analysis in chilli pepper using simple sequence repeats (SSR) markers. African Journal of Biotechnology, 13(31): 3137-3143.

Dughdugh P, Adedzwa KD, Omoigui OL, Olasan JO, Ugbaa SM. 2017. Identification of genetic polymorphism among groundnut genotypes using simple sequence repeats (SSR) markers. Proceedings of the Genetic Society of Nigeria 41st Annual Conference, University of Agriculture Makurdi, pp 253-25.

Jonah P, Bello L, Lucky O, Midau A, Moruppa S. 2011. The importance of molecular marker in plant breeding programme. Global Journal of Science and Frontier Research, 11:ev-vers1.

Kumar D, Shukla N, Kumar V, Sahu DS, Chandel G. 2016. Assessment of genetic variation in tomato (*Solanum lycopersicum* L.) genotypes using SSR molecular markers. Ecology, Environment and Conservation, 22: 317-325.

Mason AS. 2015. SSR genotyping. In: Batley J. (ed). Plant genotyping. Springer, New York, NY, pp 77-89.

Nelson CA, Walter L, Martha O, Yacenia MC, Franco V. 2016. Use of microsatellites for evaluation of genetic diversity in cherry tomato. Plant Breeding, 76(2).

Olasan JO, Aguoru CU, Omoigui LO, Ekeruo GC, Ugbaa MS. 2020. Molecular genetic studies and QTL mapping for aflatoxin resistance in selected groundnut (*Arachis hypogaea* L.) breeding lines. Agricultural and Biological Sciences Journal, 6(4): 202-210.

Olasan JO, Aguoru CU, Omoigui LO, Ekefan EJ. 2018. Assessment of Genetic Diversity in Groundnut (*Arachis hypogaea* L.) Germplasm using Principal Component Analysis and Cluster Segmentation. Nigerian Journal of Genetics, 32: 173-181.

Omoigui LO, Alunyo GI, Kamara AY., Bello LL, Oluoch M, Timko MP, Boukar O. 2016. Identification of new sources of resistance to *Striga gesnerioides* in cowpea (*Vigna unguiculata*) accessions. Genetic Resources and Crop Evolution,

Omoigui LO, Ishyaku MF, Gwoda BS, Kamara AY, Timko MP. 2015. Suitability and use of two molecular markers to track race-specific resistance *Striga gesnerioides* in Cowpea (*Vigna unguiculata* (L.) Walp.). African Journal of Biotechnology, 4(27): 2179-2190.

Omoigui LO, Kamara AY, Moukoumbi YD, Ogunkanmi L, Timko MP. 2017. Breeding cowpea for resistance to *Striga gesnerioides* in the Nigerian dry savannas using marker-assisted selection. Plant Breeding, 77(5).

Singh BB, Ehlers JD, Sharma B, Freire FR. 2012. Recent progress in cowpea breeding. In: Fatokun CA, Tarawali SA, Singh BB, Kormawa PM, Tamo M, editors. Challenges and Opportunities for Enhancing Sustainable Cowpea Production. International Institute of Tropical Agriculture, Ibadan, Nigeria, 1: 22-40.

Taiwo LB, Taiwo MA, Shoyinka SA, Jegede SE, Okogun JA, Oyatokun OS, Adebayo GG. 2014. Interactive effects of virus and *Rhizobium* inocula on nodulation, growth and yield of cowpea. International Journal of Plant Physiology and Biochemistry, 6(3): 34-39.

Tersoo K, Iheukwumere CC, Omoigui LO, Olasan JO, Ugbaa MS. 2021. Characterization of some selected tomato (*Solanum lycorpersicum* L.) accessions using Simple Sequence Repeat Markers. Agricultural and Biological Sciences Journal, 7(2):46-50.

Uzogara SG, Ofuya ZM. 2012. Processing and utilization of cowpeas in developing-countries – A review. Journal of Food Processing and Preservation, 16:105-147.

Viswanatha KP, Pallavi MS, Khan PH. 2011. Sources of resistance to cowpea (Vigna unguiculata L.) bacterial leaf blight disease. Plant growth-promoting rhizobacteria (PGPR) for sustainable agriculture. Proceedings of the 2nd Asian PGPR Conference, Beijing, China, 45: 465-472.

Weir BS. 1990. Genetic data analysis-methods for discrete population genetics dData. Sunderland: Sinauer Associates Inc.

Xu P. 2010. “Development and polymorphism of *Vigna unguiculata* ssp. *unguiculata* microsatellite markers used for phylogenetic analysis in asparagus bean (*Vigna unguiculata* ssp. *sesquipedialis* (L.) Verdc.)”. Molecular Breeding, 25(4): 675-684.