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Screening of SSR Molecular Markers for Polymorphism in Aphid Resistant and Susceptible Cowpea Varieties

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Abstract:
The aim of this study was to screen SSR markers for polymorphism in aphid resistant and susceptible varieties. Twenty-Two (22) Simple Sequence Repeat (SSR) markers were screened on aphid resistant (TVNu-1158 and TVu-2876) and susceptible (Aloka Local and Keffi local). varieties. These molecular markers were used to identify polymorphism between the resistant and susceptible cowpea varieties using the PCR technique. Polymorphic indices of primers were generated on the PICcalc DEMoMa application v2012. Primers showed different banding and clustering patterns. Results showed that 17/22 (77.3%) of the primers produced a total of 79 visible bands. Six (6) primers (27.3%) had PIC >0.50 and were considered polymorphic. They were: EX-78, EX-79, C42-B, RB-45, JL 31/32 and CP-253/254. The first five primers had PIC of 0.99 each as the highest value recorded. The maximum Marker Index (MI) recorded was 2.96 while the Effective Multiplex Ratio (EMR) was highest in CP-253/254. Polymorphism was higher in the aphid susceptible varieties than the resistant varieties in the following order: Keffi Local (27%), Aloka Local (26%), TVu-2879 (24%) and TVNu-1158 (23%). The highly polymorphic primers as stated in this report are effective candidates for developing varieties for aphid resistance in cowpea breeding.

Keywords: Aphid resistance, Cowpea, SSR marker, DNA polymorphism, Breeding

INTRODUCTION

Cowpea [*Vigna unguiculata* [L.] Walp] is one of the most important food legumes of vital importance to the livelihoods of millions of people in West and Central Africa (Boukar *et al.*, 2018). Cowpea belongs to the Fabaceae family and is grown agriculturally for food, animal feed and generation of cash (Mhango *et al.*, 2013). It is an annual herbaceous legume largely grown in the West and Central African countries. Nigeria produces about 3.5 million tons of cowpea from a cultivated land area of 3.5 million ha, making it the world largest producer (FAOSTAT, 2017). Nigeria is the 2nd highest consumer of cowpea in the whole world. The crop is one of the most important food legumes of vital importance to the livelihoods of millions of people because it is a multipurpose crop that is used as food, fodder and as a source of income. The seed provides a cheap source of protein to the subsistence farmers and also provides a range of essential micronutrients and vitamins (Boukar *et al.*, 2018). However, cowpea is affected by pests and diseases at different stages of development leading to a drastic reduction in yield (Adegbite and Amusa, 2010; Boukar *et al.*, 2018) and deterioration of seeds during storage (Keneni *et al.*, 2011).

Cowpea aphid (*Aphis craccivora* Koch), is a specie of insect pests that affect cowpea production, in that it causes significant yield losses (Ikwelle and Okello, 2021).

Conventional breeding seems to offer a possible solution to several biotic stresses of cowpea. However, it may require a decade or more to develop and release a new cowpea cultivar because it involves screening and identifying appropriate resistant germplasm sources and then introgressing the resistance trait (Omoigui *et al.*, 2019). Molecular breeding tools, including marker-assisted selection, have the potential to accelerate and improve the effectiveness of breeding for disease resistance in many crops. DNA polymorphism refers to the presence of genetic variation within a population or species. It is a condition where there are multiple forms or variants of a particular DNA sequence or gene at a specific location in the genome (Jiang *et al.*, 2021). In spite, of greater efforts in discovering the aphid resistance genotypes, resistance to aphids (*A. craccivora*.) of most of the identified cowpea cultivars has recently broken down, due to the occurrence of resistance-breaking down biotypes, in various plant-aphid systems. Lack of polymorphic DNA markers needed in the identification of new sources of resistance to *A. craccivora* has been a major challenge in the breeding system. The aim of this study was to screen SSR markers for polymorphism in aphid resistant and susceptible varieties

MATERIALS AND METHODS

Genetic Resources

Seeds of aphid resistant (TVNu-1158 and TVu-2876) and susceptible (Aloka Local and Keffi local) varieties were obtained from the Molecular Biology Laboratory, Department of Plant Breeding and Seed Science, Joseph Sarwuan Tarka University.

Screening of Molecular Markers

Twenty-Two (22) Simple Sequence Repeat (SSR) markers designed for resistance to biotic stresses were screened on aphid resistant and susceptible varieties.

Planting of Cowpea Seeds in the Screen House and Collection of Leaf Samples

The four varieties of cowpea were planted in pots containing top soil. Three seed from each variety was planted in each pot containing top soil and was tinned to two seed at ten (10) days after planting to maintain two plants per pot. Leaf samples from young cowpea plants were collected for each variety at fourteen days after planting. The leaf samples were collected and stored in polythene zip-lock bags containing silica gel to dry for three days.

DNA Extraction (CTAB Method)

The CTAB (Cetyltrimethylammonium bromide) method of DNA extraction as described by Omoigui *et al.* (2012) was modified for cowpea leaf to obtain quality DNA for PCR reaction. The resulting pellets were washed with 600uL of 70% ethanol and suspended in 80uL of RNase water.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was performed using 15µl total reaction volume. The components of the reaction included the following mixture: PCR premix beads (containing PCR Buffer, $MgCl_2$, DNTP's, and Taq Polymerase), distilled water, 1µl of each primer and 1µl of DNA (50ng). Simple Sequence Repeats (SSR) based PCR protocol (Omoigui *et al.*, 2012) was used in carrying out PCR amplification where 25 µl of molecular biology grade water was added into 0.2 ml eppendorf tubes containing the PCR beads. The mixture was then divided into two for two PCR reaction, 1 µl primer (marker) and 1 µl DNA sample to serve as template was added into each 0.2

ml eppendorf tube. A total of 35 cycles were programmed on the thermocycler, each cycle consisting of denaturation stage (94°C for 4.0 minutes), annealing stage (55°C for 1 minute), extension stage (72°C for 5 minutes) and final hold at 6°C.

Agarose Gel Electrophoresis and DNA Visualization

The Procedure used by (Omoigui *et al*., 2018) was adopted. Exactly 3.5% agarose powder was measured on the weigh balance and poured into a beaker containing 350ML 1xTAE buffer. The solution was allowed to cool and 30uL of Etbr was added and swirled gently then solution was poured on an already gel plate with comb. 1uL of DNA sample was added into the PCR tube then 1uL of 6x loading dye was also added. Samples were gently loaded into the wells using pipette and finally 5uL of ladder was loaded then electrophoresis tank was closed and gel start running 120v for 45minutes. DNA purity and quality was checked using Uv spectrometer light. 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 according for band scoring and only distinct bands were scored.

Data Analysis

Binary matrix was generated from DNA banding profiles of gel images and analyzed on Minitab 17.0 software for clustering pattern. Polymorphic indices of primers were generated on the PICcalc DEMoMa application v2012 (Hansi, 2022). The information uploaded on the software included: number of alleles detected from each marker, number of examined genotypes, number of different bands in each primer, number of polymorphic bands in each primer, total number of bands in the gene pool and frequencies of alleles. The output generated the following indices of polymorphism: H- value (Heterozygosity of primers), PIC (Polymorphic Information Content), EMR (Effective Multiplex Ratio), M1 (Marker Index) and RP (Resolution Power).

RESULTS AND DISCUSSION

Banding Patterns of SSR Primers in Aphid Resistant and Susceptible Cowpea Varieties

Plates 1 and 2 show the agarose gel images of 22 screened Simple Sequence Repeats (SSR) Marker employed in the DNA amplification for polymorphism between Aphid resistant cowpea varieties (TVu-2876 and TVNu-1158) and susceptible cowpea varieties (Aloka Local and Keffi local). The primers showed varying degrees of genetic polymorphism depending on the DNA of the cowpea varieties amplified and the SSR primer used. Similar results on genetic polymorphism were reported in different cowpea varieties selected for Striga resistance using SCAR markers. (Omoigui *et al.,* 2012). All primers used in the present study produced visible bands except in Ex-15, MS118 and 1989-1F. DNAs of aphid resistant varieties (TVu-2876 and TVNu-1158) were well resolved in primers Ex77, Ex78, RB45, 59f/r and 48f/r primers. In susceptible varieties (Aloka Local and/or Keffi local), sharp bands were observed in RB45, Ex24, Y24, 570-1F and 570-2F primers. The following primers produced sharp bands in at least one resistant and susceptible varieties: C42B, Ex20a, Ex39, CP253/254, CF2-5, MS143, JL31/32. The following primers were noted for producing sharp bands in the DNA of four varieties of cowpea amplified: C42B, CP253/254 and JL31/32. Resistance to aphid could be explained from the genetic point of view and it shows that the presence of genes controlling aphid resistance as previously reported by Braimah *et al.* (2022).

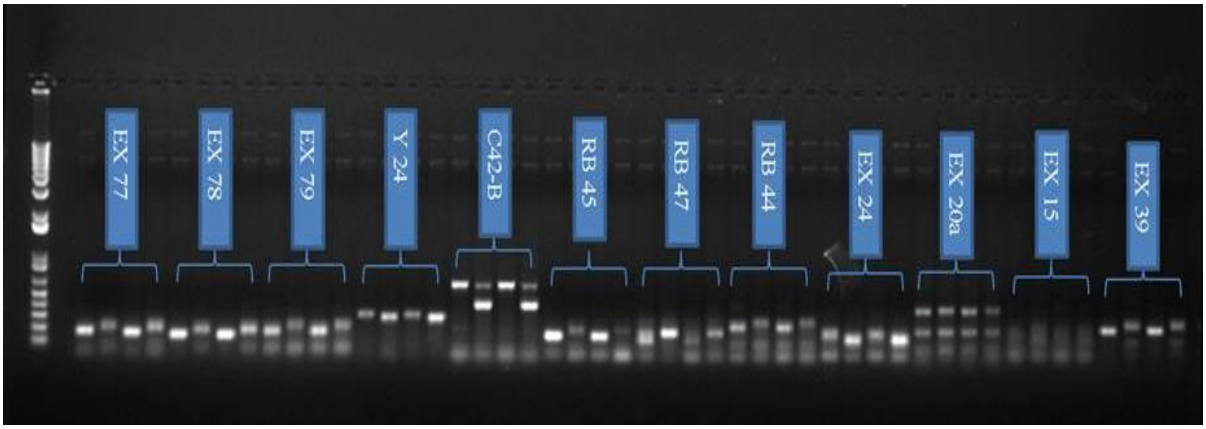

Plate 1: Agarose gel image showing screening of Twelve SSR markers (SET 1) for polymorphism between Aphid resistant cowpea varieties (TVu-2876 and TVNu-1158) and susceptible cowpea varieties (Aloka Local and Keffi local). Each group of four represents screening with a single marker as labelled. Lane 1 and 3 in each group is DNA from resistant parents TVu-2876 and TVNu-1158 respectively, while Lane 2 and 4 is DNA from susceptible parents Aloka Local and Keffi local respectively.

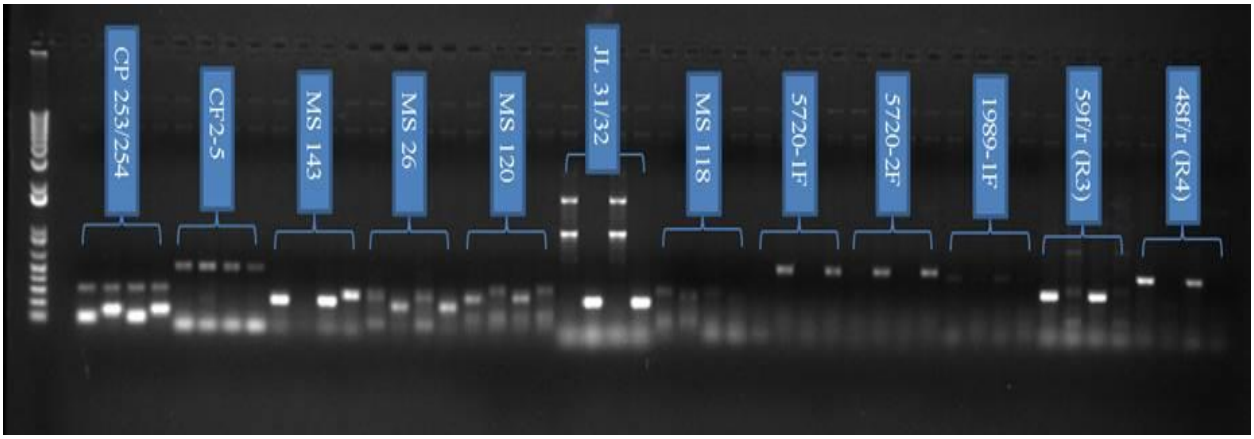

Plate 2: Agarose gel image showing screening of Twelve SSR markers (SET 2) for polymorphism between Aphid resistant cowpea varieties (TVu-2876 and TVNu-1158) and susceptible cowpea varieties (Aloka Local and Keffi local). Each group of four represents screening with a single marker as labelled. Lane 1 and 3 in each group is DNA from resistant parents TVu-2876 and TVNu-1158 respectively, while Lane 2 and 4 is DNA from susceptible parents Aloka Local and Keffi local respectively.

Table 1 presents the banding pattern of 22 SSR primers employed to identify polymorphism between aphid resistant and susceptible varieties of cowpea. Presence of bands were indicated by 1 or 2 to represent single or double bands respectively while absence of band was indicated as zero (0). Double bands were recorded in the four varieties as amplified by primers: CP253/254, EX77, X20a and CF2-5. Double bands were characteristic of only resistant varieties in primer JL 31/32. Double bands were characteristic of only susceptible varieties in primers C42B, RB45, EX-78 and EX-79. Band analysis in TVu-2876 variety (resistant type) showed 14 bands representing 63.6% of the total primers employed in the DNA amplification. There were 5 double bands (22.7%) and 9 single bands (40.9%). In Aloka Local (susceptible variety), there were 13 (59.1%) bands grouped into 8 double bands (36.4%) and 5 single bands (22.7%). In TVNu-1158 variety (resistant type), there were 13 (59.1%) bands grouped into 5 double bands (22.7%) and 8 single bands (36.4%). In Keffi Local (susceptible variety), there were 13 (59.1%) bands grouped into 8 double

bands (36.4%) and 5 single bands (22.7%). The above findings are indications of the co-dominant nature of SSR markers as revealing heterozygosity of alleles at specific loci. Researchers have demonstrated the presence of multiple repeats of sequences along the DNA known to detect polymorphism and monitor inheritance of resistance to specific diseases in plants (Duangsong *et al.*, 2018).

Table 1: Banding Pattern of SSR Primers to Identify Polymorphism Between Aphid Resistant and Susceptible Varieties of Cowpea

Primer	Sequence (F/R Primer)	TVu-2876	Aloka Local	TVNu-1158	Keffi Local
CP-253/254	GAAAGGGAAGGATTATGGGATA GGCAAATAGCGGGGTAGAGT	2	2	2	2
Y-24	GGTTTCCTAGTTGGGAAGGAA ATTATGCCATGGAGGGTTCA	0	1	0	1
EX-39	CAAGAGTCATTCGGCTCCTT GCTGCACCGTTTTCCGAAAT	1	0	1	0
EX-77	GATCCAACATTTCCTGTGTCTC GGAGCACCCGACAAGCCCCT	2	2	2	2
EX-78	ACTTCGCACACAGATCCAAC AATTGCCGGCTTTCCCATTG	1	2	1	2
EX-79	TTCAGACAGCATAGCTCCCA GGCCGTATCAAGGATGAACA	1	2	1	2
MS-120	TTTCTAGGCAGTGAAGATAATCA AAACAAAATACCAACTACCA	0	0	0	0
MS-143	ATGTTTCAGATCGGTTTAGA GAGCTGAAAAAATCGGTGTC	1	0	1	1
C42-B	GTAGGGAGTTGGCCACGATA CAACCGATGTAAAAAGTGGACA	1	2	1	2
RB-45	GAAGGCCCTTTAGGATCACC CATCGTTCAGCTGATGTTCG	1	2	1	2
RB-47	CAAAGGGTCATCAGGATTGG TTTAAGCAGCCAAGCAGTTGT	1	1	0	0
RB-44	CTACGCTGGTTATTCTAGGGGA GATAGAAGAAGAATGAGTAAGTAA	0	0	0	0
X-24	CGCTCCTCGCTGGCAAAAG CCTTCCCTACAGTGATATTTCCC	0	1	0	1
X20a	GCGCTGGATGGTCAGAGACA CAAGAGAAAAATGGTATAGA	2	2	2	2
X15	GGACTTGTTATAAACTATAG CCCGTAGAAGAGTACAAGAA	0	0	0	0
CF2-5	GTTTGTGCATAGATATCCCC CCCCCTCATCCTATAAACTCC	2	2	2	2
JL 31/32	GATGAGTGTGTGAACAAAGGAG CCCACATTCCATCATCCC	2	1	2	1
MS118	GACTTACTCCACTTAAACAAC GTTCCTGGTCCATCCTAATTTTCCG	0	0	0	0
5720-1F	TGCGGTTGAGATTTTGACGT CGTGAAGTTGAATGTGAAT	0	1	0	1
1989-1F	GTGGGCAGTGTCACCTTTTT GTGGAGCAACTGATTGCAGA	0	0	0	0

59f	GCAGAATCCTTGTGAACCTG TTTCGCAATATGCCCTTTTC	1	0	1	0
48f	GCAGAATCATTGTCAACCAG CGTCGCAATATGCCCATTTC	1	0	1	0

Determination of Relationships in Markers and Varieties

Primers were clustered on the basis of the DNA amplification results as shown in the dendrogram (figure 1). Genetic distance ranged from 1.11 to 2.12 with similarity coefficient of 76.7 to 55.7 respectively. There were two main clusters. The first cluster (cluster 1) comprised primers that amplified DNAs of the four varieties of cowpea (2 resistant and 2 susceptible). Four primers which produced double bands in all varieties (CP253/254, Ex77, X20a and CF2-5) formed a sub-cluster while JL31/32 primer which produced double bands in resistant varieties and single bands in susceptible varieties was a divergent lone entity among them. There were four primers that produced single bands in resistant and double bands in susceptible varieties (Ex78, Ex79, C42B and RB45) forming a sub-cluster. The second cluster (cluster 2) comprised primers that did not amplify DNAs of all the four varieties. Primers with single band DNA amplification only in the susceptible varieties but absent in the resistant types (Y24, X24 and 5720-1F) formed a sub-cluster. Primers with single band DNA amplification only in the resistant varieties but absent in the susceptible types (Ex39, 59f and 48f) formed a sub-cluster. MS143 was a divergent primer as it amplified DNA of only one resistant variety (TVu-2876) but two susceptible varieties. Primer RB47 was the most divergent in this group as it amplified DNA of the two resistant varieties but failed to amplified that of susceptible variety. Primers that failed to produce any amplification (MS120, RB44, X15, MS118 and 1989-1F) also formed a sub-cluster. Figure 2 shows the dendrogram of the four varieties of cowpea. They formed two groups on the basis of their responses to the 22 primers employed in the amplification of the DNA of aphid resistant and susceptible varieties. The resistant types (TVNu-1158 and TVu-2876) formed a group of higher similarity level (98.2) than the susceptible type (Aloka Local and Keffi Local) grouped together (79.4). Coefficients of similarity between the two groups were 0.04 and 0.41 respectively. It orders to apply these markers; it is important to understand the genetic inheritance pattern and heritability of aphid resistance in cowpea although it was reported to be controlled by two duplicate genes and makers were previously found linked to aphid resistance in cowpea. Also, QTLs controlling different aspects of aphid resistance in cowpea and other crops have been identified using SNP markers although monogenic aphid resistance has been established in the crop (Omoigui *et al.,* 2018). The selection of divergent SSR markers as presented in the dendrogram may be explored to further substantiate the nature of genes controlling aphid resistance,

Assessment of Polymorphic Indices

Table 2 presents indices of polymorphism of SSR primers among those that amplified DNA bands of cowpea varieties. It also gives information on the total number of bands and relative polymorphic bands (RPB) of the SSR primers. The 22 SSR primers produced 79 bands. Maximum of 8 bands were recorded in four primers (CP-253/254, Ex77, X20a and CFS-5) each with RPB of 10.1%. Heterozygosity of primers (H) ranged from 0.18 to 0.99. Results showed that 17/22 (77.3%) of the markers produced visible bands. Out of these, 6 primers (27.3%) had PIC >0.50 and were considered polymorphic. They were: EX-78, EX-79, C42-B, RB-45, JL 31/32 and CP-253/254. The first five primers had PIC of 0.99 each as the highest value recorded. The maximum Marker Index (MI) was 2.96. Effective Multiplex Ratio (EMR) of primers was highest in CP-253/254. Resolution Power (RP) was between 32.0 and 35.5. The markers screened in this work had high power of

resolution (>30) but 6 polymorphic out of 17 primers that produced bands. Primers are considered polymorphic when the 0.50 bench mark is surpassed (Ogunkanmi *et al.*, 2014; Olasupo *et al.*, 2018). The PICs of primers reported in many studies were lower than the 0.99 maximum value reported in this work. For instance, maximum PIC of 0.51 was reported by Olasupo *et al.* (2018) while 0.71 was reported by Ogunkanmi *et al.* (2014). The above finding is consistent with other reports where markers were screened and selected for their effectiveness in breeding work based on their polymorphic indices (Boukar *et al.*, 2016; Qin *et al.*, 2017; Omoigui *et al.*, 2019). Polymorphism was higher in the aphid susceptible varieties than the resistant varieties in the following order: Keffi Local (27%), Aloka Local (26%), TVu-2879 (24%) and TVNu-1158 (23%) as shown in figure 3. It suggests that the local landraces could be repository of genetic resources harboring diverse genes of interest. This view was earlier suggested in the work of Adejumo *et al.* (2021) who analysed the cytogenetic of some wild and cultivated species of cowpea. The outcome of this study is in conformity with some findings on the usefulness of SSR marker in varietal identity and selection as well genetic diversity of improved cowpea varieties (Dhakal *et al.*, 2019; Olasan *et al.*, 2023). Meanwhile Braimah *et al.* (2022) earlier elucidated the genetic diversity and relationships among cowpea genotypes for resistance to cowpea aphid using SSR markers. The outcome of their work agreed supports the present report. Marker-assisted selection (MAS) is embraced to allow timely development of new crop varieties. The principle is based on markers linked to quantitative trait loci (QTL) which are regions within a genome containing genes associated with a particular quantitative trait (Collard *et al.*, 2005). It helps to deal with complex and low-heritability traits. The complexity in the inheritance patterns of resistance in cowpea and the challenges associated with the measurements of the trait in the field or greenhouse make aphid studies a perfect target for MAS (Frejus *et al.*, 2020). Omoigui *et al* (2017) successfully developed and applied a marker-assisted selection strategy that employs a single backcross programme to introgress *Striga* resistance into farmer preferred varieties of cowpea for the Nigeria savannas. They introduced the *Striga* resistance gene from the donor parent IT97K-499-35 into an elite farmer preferred cowpea cultivar 'Borno Brown'. The selected 47 BC1F2 populations confirmed the recombinants with desirable progeny having *Striga* resistance gene(s). Therefore, the selected markers with high PIC and MI values reported in this study could be applied in the improvement of the aphid susceptible varieties using the resistant lines. This is because the development and deployment of cowpea varieties with resistance to pest infestation is the most cost effective and economically friendly approach to combat insect pests (Duangsong *et al.*, 2018).

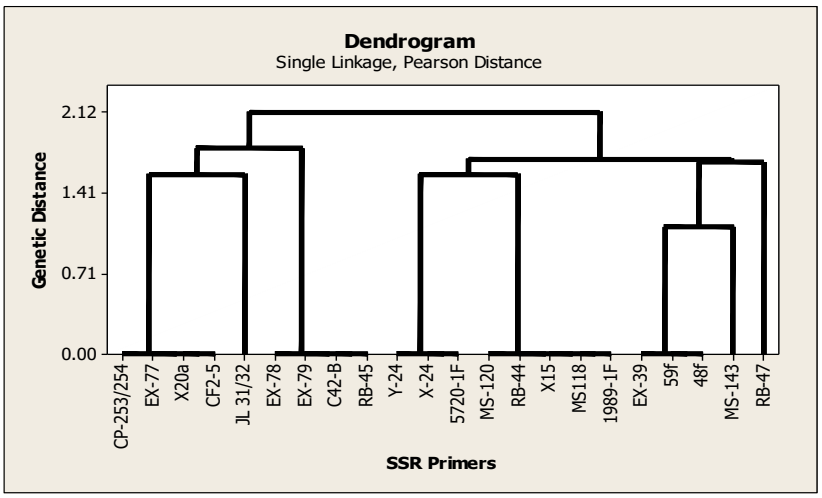

Figure 1: Dendrogram of SSR Primers

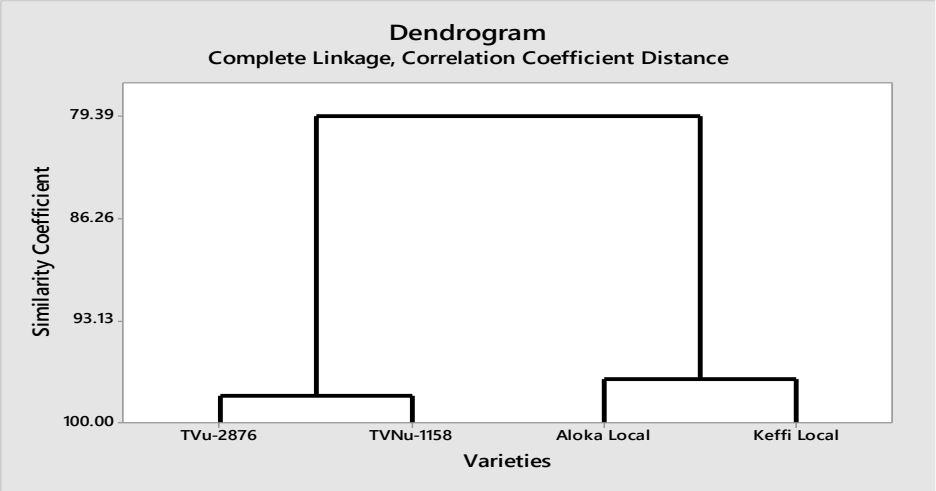

Figure 2: Dendrogram of Cowpea Varieties

Table 2: Polymorphic Indices of SSR Primers

Primer	Total number of bands	H value	PIC	EMR	MI	RP
CP-253/254	8	0.75	0.70	4.00	2.81	31.5
Y-24	2	0.18	0.16	0.50	0.08	35.5
EX-39	2	0.18	0.16	0.50	0.08	35.5
EX-77	8	0.54	0.47	1.33	0.62	32.0
EX-78	6	0.99	0.99	3.00	2.96	33.5
EX-79	6	0.99	0.99	3.00	2.96	33.5
MS-143	3	0.50	0.38	0.50	0.19	34.0
C42-B	6	0.99	0.99	3.00	2.96	33.5
RB-45	6	0.99	0.99	3.00	2.96	33.5
RB-47	2	0.18	0.16	0.50	0.08	35.5
X-24	2	0.18	0.16	0.5	0.08	35.5
X20a	8	0.54	0.47	1.33	0.62	32.0
CF2-5	8	0.34	0.31	1.33	0.42	33.5
JL 31/32	6	0.99	0.99	3.00	2.96	33.5
5720-1F	2	0.18	0.16	0.50	0.08	35.5
59f	2	0.18	0.16	0.50	0.08	35.5
48f	2	0.18	0.16	0.50	0.08	35.5
Total	79					

H= Heterozygosity of primers; PIC= Polymorphic Information Content of primers; EMR= Effective Multiplex Ratio of primers; M1= Marker Index; RP= Resolution Power

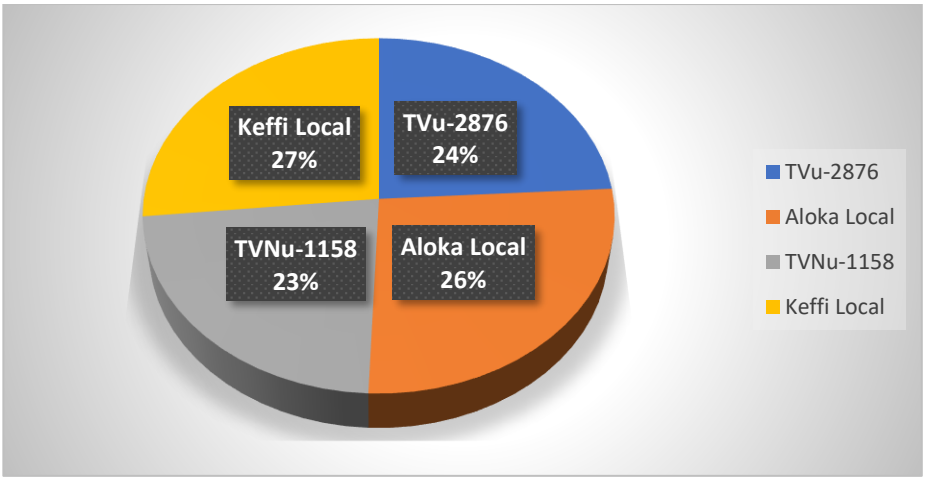

Figure 3: Percentage Polymorphism among Four Cowpea Varieties

CONCLUSION

The markers screened in this work had high power of resolution (>30). Results showed that 17/22 (77.3%) of the markers produced visible bands. Out of these, 6 primers (27.3%) had PIC >0.50 and were considered polymorphic. They were: EX-78, EX-79, C42-B, RB-45, JL 31/32 and CP-253/254. The first five primers had PIC of 0.99 each as the highest value recorded. The highly polymorphic primers as stated in this report are effective candidates for developing cowpea varieties for aphid resistance. There is need to screen more cowpea varieties and markers for resistance and polymorphism respectively to facilitate breeding programme.

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