Cluster Analysis and Association Between Simple Sequence Repeat Makers With Qualitative Trait in Some Nigerian *Achishuru* Cowpea Land Races

Amos Cyrus[1], Mohammed F. Ishiyaku[2], Yusuf Mansir[1], US Abdullahi[1]

[1]Department of Plant Science Faculty of Agriculture, Ahmadu Bello University, Zaria. Nigeria
[2]Institute of Agricultural Research, Ahmadu Bello University, Zaria, Nigeria.
mramoscyrus@yahoo.com
+234-7031114820

Abstract: Most studies on cowpea in Nigeria are restricted to the mainstream cowpea germplasm with little attention to *achishuru* type despite its age-long importance in the survival of over one million people of the mid central Nigeria. A total of twenty *achishuru* cowpea accessions where collected for genetic diversity studies through grouping the accessions into similar agronomic characteristic using the cluster analysis, since little is known on the genetic architecture of the crop, Morphological data was taken in a completely randomized block design. Accessions were characterized based on ten quantitative and thirteen qualitative traits. The Cluster analysis shows that cluster I consist of ten accessions with similar earliness to maturity, cluster II consist of six accessions with similar days to grain filling, cluster III and IV consist of two accessions each. For the qualitative traits, cluster I consist of seven accessions whose members are tolerant to cowpea *bruchid*. Six polymorphic simple sequence repeat, were amplified using the six primers (VM31, VM35, VM36, VM68, VM39 and VM70) using the PCR technique. The level of association between the simple sequence repeat primers and some qualitative traits were analyzed using a non parametric statistics. No SSR marker was suspected to be associated with any of the qualitative trait except for twinning tendency and VM39 ($P<0.05$).

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Keyword: simple sequence repeat; *achishuru*; association; PCR; landrace;

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp] is one of the ancient human food sources and has probably been used as a crop since Neolithic times Suliman, (2000). Major diversity of cowpea is found in Asia and Africa but the precise origin of cowpea has been a matter of speculation and discussion for many years (FAO, 2008). Food and Agriculture Organization estimated that 5.4 million metric tones of cowpea grain were produced worldwide in the year 2014 and 91 % of that production were from Africa (FAOSTAT, 2014). West Africa is the key cowpea producing zone with countries like Nigeria, Niger, Senegal, Ghana, Mali, and Burkina Faso taking the lead (FAOSTAT 2014). Most studies on cowpea in Nigeria, is generally restricted to the mainstream cowpea germplasm with little attention on the *achishuru* with its high food security value occupying a small but special production niche, and its age-long importance in the survival of over one million people of the mid-central Nigeria. The land races are sown and harvested just when all other food reserves have been depleted (June-August) and so the local name *achishuru* meaning "feed with ease". As important as this *achishuru* type of cowpea, not much is known about the genetic architecture of these groups. In order to access their genetic potential, there is therefore the need to access the level of diversity within this group. Intra-species genetic diversity is one of the resources enhanced in crop breeding which provide vital resources for developing new cowpea varieties with economic attribute.

Plant height: Taken at fifty percent flowering from the stem (soil level) up to the meristematic regions at 50% flowering.

Petiole length: Measured at fifty percent flowering from the attached site of the leaf on the stem to where the lamina starts.

Number of pods per plants: The number of dry pod at harvest per plant for each pot were counted.

Pod length: The length of the pod found per plant was taken at maturity from the three replicates.

Days to maturity. Days taken from the day when the dicotyledon has emerged to when they reached physiological maturity.

Days to 50% flowering: Days were taken from the day when the dicotyledon has emerged to when the plant has opened flower.

Days to grain filling: Days were taken after each accession has flowered to when the pods have reached physiological maturity.

100seed weight: One hundred seeds from each harvested accession were counted manually to a hundred seed, and latter weights on a scale in grams.

Grain yield: The total weight of the grains from each block at harvest was taken for each accession and weight was recorded in grams.

Qualitative Traits Characterization of the Twenty *Achishuru* Cowpea Accessions.

Variables that were categorical (define into classes) and discrete were characterized and scored according to IBPGR (1993) descriptor for Cowpea.

Genomic DNA extraction.

Plant genomic DNA was used in detecting the variable SSR region of the twenty *achishuru* accessions, extraction was done the column was transferred to a sterile 1.5ml microfuge. To elude the genomic DNA, 100ul of elution buffer was added to the center of the column membrane, and incubated for five minutes at room temperature and later centrifuge for one minute at 8000xg (10000rpm), the second elution step was done using another 100ul of elution buffer using a different elution appendorf tubes. The purified DNA was stored at -20^{oc}.

DNA quality Confirmation of the twenty *achishuru* accessions.

In order to verify that the quality of the DNA fits the desired PCR standard, 0.8% solution of agarose was prepared by melting 0.8g agarose in 100ml of 1xTBE buffer in a microwave for approximately five minutes and was allowed to cool for four minutes and 1ul of ethidium bromide was added and mix. The gel was cast using a supplied tray and a comb, a ratio of 1:5 loading dye to DNA samples was used. 1kb DNA ladder was used to know the various extracted genomic DNA sample sizes; the electric power pack of the agarose assembly was turn on at 120volt for 60 minutes. The gel was later exposed to UV light using a UV transilluminator and photographed with a digital camera.

Amplification of SSR loci of the twenty *Achishuru* cowpea accessions.

The DNA extracted from the twenty *achishuru* cowpea accessions were subjected to PCR which was carried out in a MJ Research PTC-200 Peltiel thermocycler DNA Engine®. Each 23 µl reaction mixture contained 12.5ul *taq* master mix with standard buffer containing 20Mm Tris-HCL (pH 8.9 @ 25^{oc}), 1.8mM $mgcl_2$, 22mM NH_4Cl, 22mM Kcl, 0.2mm dNTPs, 5%.

Lack of association; the minus or plus sign indicate whether the association is negative or positive.

Result

Classifications of Similar Quantitative Characteristics of the Twenty *Achishuru* Cowpea accessions.

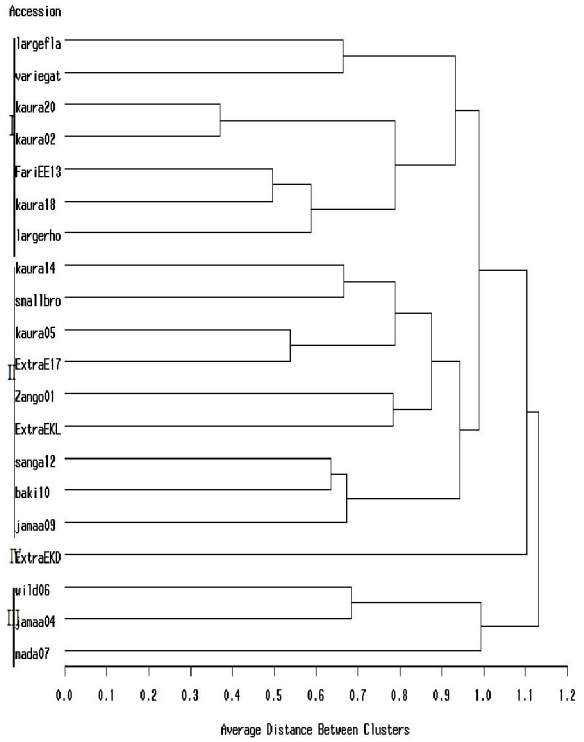

Figure 1. Dendrogram of Twenty *Achishuru* Cowpea Accessions as Revealed by Thirteen Qualitative Traits.

Classification of the *achishuru* accessions based on ten quantitative traits is shown in (Figure 1) CLUSTER I comprised of ten accessions which include, Kaura14, Kaura05 FariEE13, Baki10, EEKD15, ExtraE17, Jamaa04, Baki10, Largerh08, and ExtraE16. All accessions in this cluster have common qualitative characteristics of similar Earliness to maturity of (65days), early flowering of (36days), similar 100g seed weight of (13.8g) and a similar grain yield of (15.1) on the averaged. CLUSTER II comprised of six accessions which include: Variegated11, Kaura18, Jamaa09, Largefl03, Kaura02 and Sanga12. Members of this cluster had a common characteristic of the highest plant height of (110cm) on the average, highest similar days to maturity of (76 days) and similar days to grain filling of (22days). The two accessions in CLUSTER III had the highest days to maturity of (142days), days to flowering (96days), and petiole length of (4.5cm) on the averaged. The two accessions in CLUSTER IV are Zango01 and Smallbr19; these accessions are distinctly short plants in terms of height (30.2cm), days to maturity (60days), and petiole length (7.3cm).

Classifications of Similar Qualitative Characteristics of the Twenty *Achishuru* Cowpea accessions

CLUSTER I consist of seven accessions: Kaura02, FarriEE13, Kaura18, Largerh08.

Discriminated into a single cluster this accession had pigmented tips, and the most extra early maturing accession.

Detection of the Amplification Products of the *Achishuru* SSR Loci through gel electrophoresis.

To detect the SSR regions, polymerase chain reaction was used to investigate the level of genetic diversity among the twenty *achishuru* cowpea accessions. 100bp ladder was used to detect the product size of the SSR in accordance with their molecular sizes. Li *et al* (2001). Amplification of the SSR regions of VM 31 out of the six primers Used is shown in figure 3.

Association between phenotypic traits and the molecular markers is used in the selection process of crop's agronomic traits of interest in breeding since less time is required, as compared to the conventional. In this study simple sequence repeat molecular markers were used to ascertain their possibly association with some qualitative traits.

Material and Methods

Description of Experimental Material Used.

The experimental material used in this experiment where a collection of cowpea local land races of the *achishuru* types collected from farmers, cultivated fields, market places, and two from the wild. The collections were named based on local names, regions of collection, seed size, and time of maturity, seed shape, growing habit, and the seed colour. These accessions were characterized based on different qualitative and quantitative traits following the IBPGR (1993) descriptor for Cowpea. The twenty local cowpea land races were laid out in a completely randomized block design, replicated three times, each plastic pot consist of about 25cm in diameter filled with top soiled sandy loam.

Data Collection of Quantitative Traits of the Twenty *Achishuru* Cowpea Land Races

The following variables where measured and used for quantitative characterization of the twenty *achishuru* cowpea accession.

Days to emergence: Number of days taken from sowing to the day the di-cotyledons appears above the soil surface. from two weeks old young *achishuru* seedlings using the Gene JetPlant Genomic DNA Purification mini kit® obtained from Thermo Fisher Scientific USA. Fresh leaves of the twenty samples of 100g each where grinded in liquid nitrogen and immediately transferred to a 1.5ml microfuge tubes, 350ul of lyses buffer A was pipette into the appendorf tube which was vortexed for 20 seconds. 50ul of lysis buffer B and 20ul of Rnase were added to each of the microfuge and vortex for one minute, incubation of the samples follows for 10 minutes at $65^{oc.}$ 130ul of the precipitation was added to the samples and mixed by inverting the tubes 2-3 times and later incubated on ice for five minutes. Centrifugation was done for 5 minutes at ≥20,000xg (≥14,000rpm) 550ul of the supernatant was transferred to a clear microfuge tube with the addition of 400ul of plant gDNA binding solution and 400ul of 96% ethanol. 700ul of the prepared mixture was transferred to a spin column and centrifuge for one minute at 6000xg (≥8000rpm). The flow through solution was discarded and the remaining solution was applied to the same column and centrifuge for 1 minute at 6000xg (≥8000rpm), 500ul of wash buffer I was added to the column and centrifuge for 10000rpm and the flow through solution was discarded and the column was replaced into the collection tube. 500ul of wash buffer II was added to the column and centrifuge for three minutes at a maximum speed of ≥20000xg (≥14000). The collection tubes were emptied and the purification columns were placed back into the tubes and re-spin for one minute at ≥20000xg (≥14000rpm) the collections were discarded with the flow through solution, glycerol, 0.06% IGEPAL® CA-630, 0.05% Tween®20, 25 units/ml one *taq* DNA polymerase, 1ul of forward and reverse primers, 7.5ul of genomic DNA template, all obtained from inqaba Biotech South Africa. 7.5ul final volume with nuclease-free water (Fermentas Inc.) was added up to a final volume of 23ul. PCR amplification was performed by denaturing DNA at 94°C for 3 min which was followed by 35 cycles each consisting of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, with a final extension at 72°C for 10 min.

Separation of the Amplified SSR Fragments of the Twenty *Achishuru* Accessions.

Amplified SSR loci of the twenty *achishuru* accessions were subjected to electrophoresis by melting 1.5g agarose powder in 100ml of 1xTBE buffer in a microwave for approximately 2 minutes and were allowed to cool for four minutes and 1ul of ethidium bromide was added and stirred. The gel was cast using a supplied tray and a comb, 100bp DNA ladder (New England) was used to estimate the size of the PCR products of the twenty samples; the electric power pack of the agarose assembly was turn on at 120volt for 60 minutes. The gel was later exposed to UV light using a UV transilluminator and photographed with a digital camera.

Determination of Association Between SSR markers and some qualitative traits

To determine the level of association between the six SSR markers and some qualitative traits, a non parametric test was used. Like the Pearson correlation coefficient, the Spearmans rank-order correlation may take on values from -1 to +1. Values close to ±1

indicate a high correlation; values close to zero indicate a

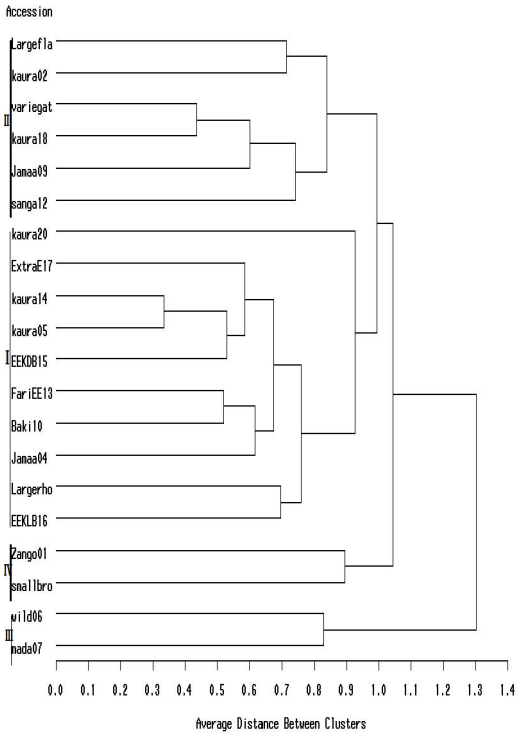

Figure 2 Dendrogram of the Twenty *Achishuru* Cowpea Accessions Based Ten on Quantitative Traits Evaluated in a Screen House.

CLUSTER I consist of seven accessions: Kaura02, FarriEE13, Kaura18, Largerh08, Largefl03, variegated11 and kaura20 had accessions that are characteristically indeterminate in growth, with a pronounced twinning tendency, and are tolerant to cowpea *bruchid* except (Kaura18). CLUSTER II comprised of nine accessions: Kaura05, ExtraE17, Sanga12, Baki10, Kaura14 Smallbr19, Jamaa09, Zango01 and ExtraE16. Accessions in this group have a rhomboid seed shape, early, or extra early maturing; all accessions in this group are susceptible to cowpea *bruchid* except smallbrw19. Wild06 and Jamaa04 and Mada07 are in CLUSTER III. These three accessions are late maturing, and also have glabrescent hairs. ExtraE17 was.

The Level of Association Between SSR Markers and Some Qualitative Traits of the Twenty *Achishuru* Cowpea Landraces.

The level of association between the SSR markers and some qualitative traits is shown in table 3. No significant association ($p>0.05$) was established using the Spearmans rank correlation coefficient between the SSR marker data and the qualitative traits except for twinning tendency and VM 39 ($p<0.05$) which was shown to be significant. Shown in table 3.

Discussion

Classification of Similar Quantitative Characteristics of the Twenty *Achishuru* Cowpea Accessions.

Traditionally, diversity is estimated by measuring variation in phenotypic or qualitative traits such as flower colour, growth habit, or quantitative agronomic traits such as yield potential and stress tolerance (Kameswara, 2004). However, this approach is often limited and expression of quantitative traits is subject to strong environmental influence (Kameswara, *et al.*, 2010). The most desirable agronomic characteristic mostly cherished by the local farmers is earliness to maturity of some of the *achishuru* cowpea types. These accessions have been identified in cluster I (figure 1) which comprises of ten accessions; hence, these accessions are candidates for earliness when it comes a choice of a breeding line.

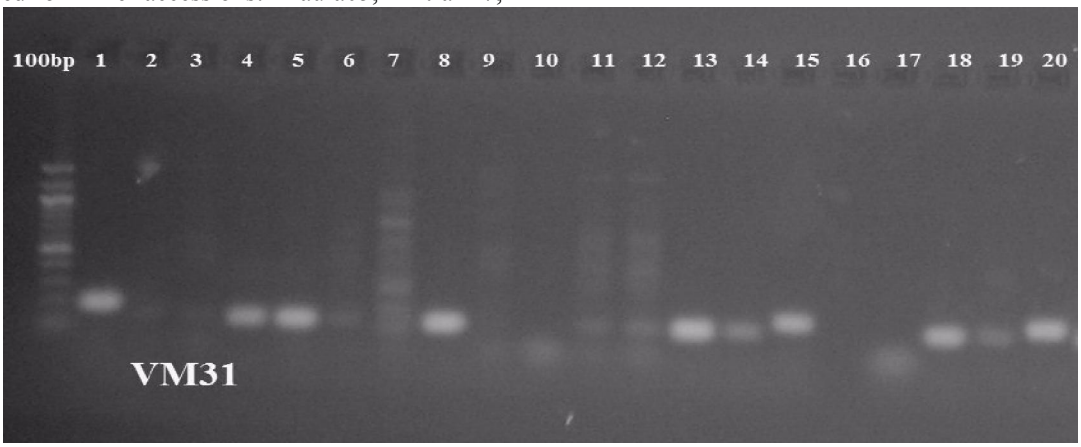

Figure 3: Polymorphism of the SSR Amplified by VM31 in 20 *Achishuru* Cowpea Land Race Accessions Shown on Ethidium Stained Agarose Gel.

Table 3 Association between SSR markers and some qualitative traits of the *achishuru* cowpea.

	SSR Markers					
Qualitative traits	vm31	vm39	vm68	vm35	vm70	vm36
Seed shape	0.02NS	0.10NS	0.28NS	0.29NS	0.16NS	0.12NS
Growth habit	0.10NS	0.37NS	0.29NS	0.30NS	0.33NS	0.17NS
Maturity period	0.30NS	0.12NS	0.23NS	0.21NS	0.22NS	0.05NS
Tolerance to bruchid	0.15NS	0.11NS	0.02NS	0.21NS	0.32NS	0.05NS
Hilum colour	0.06NS	0.24NS	0.04NS	0.12NS	0.17NS	0.15NS
Pod curvature	0.25NS	0.14NS	0.26NS	0.20NS	0.05NS	0.10NS
Texta texture	0.10NS	0.02NS	0.02NS	0.10NS	0.17NS	0.18NS
Twinning tendency	0.11NS	0.41*	0.21NS	0.12NS	0.34NS	0.29NS
Flower colour	0.06NS	0.12NS	0.05NS	0.23NS	0.23NS	0.23NS
Pods attachment	0.12NS	0.23NS	0.12NS	0.25NS	0.22.NS	0.02NS

5% level of significance * 1% level of significance ** NS Not Significant

Synchronization of flowering of the accessions is important breeding information so as to have flowers of different accessions ready for pollination at nearly the same time, an average of 36 days to flower was found in this cluster. Highest similar plant height of averaged 110 cm was found in cluster II comprising of six accessions, this could have been possible since members of this group except kaura02, are of the *phaseolus* types whose vines twines over an external support. Mada06 and Wild07 in cluster III are the most overstayed accessions and when not uprooted, they resume growth and flowering the following year when rain have established. Characterization studies. Molecular markers have been assisting the other marker system like morphological and quantitative data trait for genetic characterization studies. Cowpea *bruchid* is an important storage pest that caused about 95 percent damage to cowpea. Deshpande *et al*. (2011). *Achishuru* cowpea accessions in cluster I are tolerant to cowpea *bruchid* except kaura18, members of this cluster can be considered when breeding for tolerance in cowpea. Accessions in cluster III are not mostly cherished by the farmers because they are very late maturing and a member of the cluster Mada07 is highly susceptible to cowpea *bruchid*. The only accession in cluster IV is widely cultivated by farmers because of its extra earliness. Accessions in cluster II should be discarded when it comes to consideration of tolerance to cowpea *bruchid* except Smallbr19 and Kaura05. Accessions that were early, extra early or late maturing for the qualitative trait were the same for the quantitative trait. Most of the *phaseolus* types of the *achishuru* cowpea have a pronounced twining tendency and are indeterminate in growth this is an important characteristic feature that calls for external support of the vines. Those with non twining tendency are likely to be the cowpeas types and determinate in growth.

Association of Single Sequence Repeats with the Qualitative Traits.

The result of this study, disagree with Afikwe *et al*., (2011) who reported a primer pair, VM68, which detected a microsatellite allele, that was present only in Nigerian cowpea accessions with late flowering.

The accessions in cluster IV do not produce vines during their growth therefore, no external support is required, and hence, less labour is required due to the absence of staking.

Classifications of Similar Qualitative Characteristics of the Twenty *Achishuru* Cowpea Accessions.

Genetic diversity of wild and cultivated cowpeas has been studied in the past, using a variety of approaches including analysis of morphological and physiological traits (Perrino *et al*., 1993; Ehlers and Hall, 1996), molecular markers have been assisting the other marker system like morphological and quantitative data trait for genetic.

And that this microsatellite might possibly be useful as a marker associated with late-flowering. This result disagrees with this finding as no association between VM68 and any of the six SSR was found to be associated with late flowering in this study. VM 39 Was found to be associated with twinning tendency ($P<0.05$) which might be an indication of co-segregation between the marker and the trait (Table 3).

Summary and Conclusion.

Characterization of the *achishuru* cowpea accessions have discovered huge amount of potential genetic resource. Accessions that flower at the same time have been identified as synchronization of flowering is vital in hybridization. Accessions with high yield, *bruchid* tolerant, and earliness to maturity accessions are known. No SSR marker was suspected to be associated with any qualitative trait under study except for twinning tendency and VM 39. Six SSR markers were used to screen twenty *achishuru* accessions in this study; however, higher number of

SSR markers can be employed for further cowpea/*achushuri* research to detect higher polymorphism among accession and as a step also towards discovering SSR markers associated with trait to ease phenotypic selection through marker assistance. Higher number of the markers can also lead to the discovery of QTL.

Landraces of Cowpea Collections Used for This Study.

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