Cluster Analysis And Association Between Simple Sequence Repeat Markers With Qualitative Trait In Some Nigerian *Achishuru* Cowpea Landraces

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Abstract: Assessment of the genetic diversity in *Achishuru* cowpea landrace is critical to the development of new and improved cultivars with desirable agronomic traits; 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 20 *Achishuru* cowpea landraces were collected with the aim of assessing genetic diversity of the landraces through qualitative and quantitative characterization and also to determine the association between the qualitative trait and any of the markers. Morphological data was taken in a completely randomized block design. Landraces were characterized based on 10 quantitative and 13 qualitative traits. Cluster analysis shows that group I consist of 10 landraces with similar earliness to maturity, cluster II consist of 6 landraces with similar days to grain filling, clusters III and IV consist of 2 landraces each. For the qualitative traits, cluster I consist of 7 landraces whose members had glabrescent hairs, pronounced twinning tendency and indeterminate in growth. The 6 simple sequence repeat markers were used to amplify the SSR regions of the DNA samples through the Polymerase Chain Reaction. The number of alleles per SSR primer varies between 3 to 6 with a mean of 4.30. The allele frequency ranged from 0.75 to 0.32 with a mean of 0.53. The highest polymorphic information content value was 0.55 for the primer VM31 and the lowest was for the primer VM68 with a mean value of 0.39. No SSR marker was suspected to be associated with any qualitative trait except for twinning tendency with VM39 ($P<0.05$)

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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 (Badiane *et al*., 2012). Major diversity of cowpea is found in Asia and Africa but the precise origin of cowpea has been a matter of speculation and discussionfor many years (Arumuganathan, K. and Earle, E.D. 1991). Food and Agriculture Organization estimated that 5.4 million metric tons 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). As a leguminous crop, it fixes about

and so the local name *Achishuru* meaning "feed with ease" (Diwan *et al*., 1992). As important as this *Achishuru* type of cowpea, not much is known about the genetic architecture of these groups (Akkaya *et al*., 1992). to assess their genetic potential, there is therefore the need to assess 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 (Afikwe, W.T. and Takama, O.O. 2011). 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 (Asare *et al*., 2010). In this study simple sequence repeat molecular markers were used to ascertain their possibly association with some qualitative traits.

Materials And Methods

Description of Experimental Materials

The following variables were measured and used for quantitative characterization of the 20 *Achishuru* cowpea landraces.

Days to emergence: Number of days taken from sowing to the day the di-cotyledons appears above the soil surface.

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

Petiole length: Taken at 50% 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 3 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 dicotyledonous has emerged to when the plant has opened flow-through solution of 98% at appedorff tube. The 500 µl of wash buffer II was added to the column and centrifuged for 3 minutes at a maximum speed of 14000 rpm. The collection tubes were emptied and the purification columns were placed back into the tubes and re-spin for one minute at 14000rpm the collections were discarded with the flow through solution, To elude the genomic DNA, 100 µl of elution buffer was added to the centre of the column membrane, and incubated for 5 minutes at room temperature and later centrifuged for one minute at 10000 rpm, the second elution step was done using another 100 µl of elution buffer using a different elution appendorf tubes. The purified DNA was stored at -20^{o}C.

DNA quality confirmation of the 20 *Achishuru* Landraces

In order to verify that the quality of the DNA fits the desired PCR standard, 0.8% solution of agarose was prepared by melting 0.7 g agarose in 100ml of 1xTBE buffer in a microwave for approximately 5 minutes and was allowed to cool for 4 minutes and 1 µl 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. The 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 120 volt for 60 min. The gel was later exposed to UV light using a UV transilluminator and photographed with a digital camera.

Amplification of SSR loci of the 20 *Achishuru* cowpea Landraces

The DNA extracted from the twenty *Achishuru* cowpea landraces were subjected to PCR which was carried out in a MJ Research PTC-200 Peltiel thermocycler DNA Engine®. Each 23 µl reaction mixture contained 12.5 µl *taq* master mix with standard buffer containing 20Mm Tris-HCl (pH 8.9 at 25^{o}C), 1.8 mM $mgcl_2$, 22mM NH_4Cl, 22mM Kcl, 0.2mm dNTPs, 5% glycerol, 0.06% IGEPAL® CA-(Figure 2) cluster II comprised Classification of the *Achishuru* landraces based on 10 quantitative traits is shown in (Figure 2) cluster II comprised of 10 landraces which include, Kaura14, Smallbro, Kaura05, Baki10, EEKD15, ExtraE17, Jamaa04, Baki10, Largerh08, and ExtraE16. The landraces in this cluster have common quantitative characteristics of similar Earliness to maturity of (65 days), early flowering of (36 days), similar 100 g seed weight of (13.8 g) and a similar grain yield of (15.1) on the averaged. Cluster I comprised of 6 landraces which include: Variegated11, Kaura20, FariEE13, Largefl03, Kaura18 and Largerhom. Members of this cluster had a common characteristic of the highest plant height of (110 cm) on the average, highest similar days to maturity of (76 days) and similar days to grain filling of (22 days). The 2 landraces in Cluster III had the highest days to maturity of (142 days), days to flowering (96 days), and petiole length of (4.5cm) on the averaged. The two landraces in Cluster IV are Wild06 and Mada07; these landraces are distinctly short plants in terms of height (30.2 cm), days to maturity (60 days), and petiole length (2.3).

SSR Statistical Analysis

The allelic data obtained from the most polymorphic primers after the electrophoresis were scored based on the co dominant nature of SSR markers, and were later analysed with the PowerMarker® software (Table 1). The number of alleles per SSR primer varies between 3 to 6 with a mean of 4.30. The allele frequency ranged from 0.75 to 0.32 with a mean of 0.53. The highest polymorphic information content value was 0.55 for the primer VM31 and the lowest was for the primer VM68 with a mean value of 0.39. VM70, VM68 and VM31 were analyzed; VM39, VM35 and VM36 were monomorphic with some few unclear bands which lead to their exclusion from the analysis.

Association of Single Sequence Repeats with the Qualitative Traits

The result of this study, disagree with Afikwe, W.T. and Takama, O.O. (2011)

70 – 2 40 kg per ha of nitrogen per year (Nwosu *et al*., 2012). The largest production is in the moist and dry Savannas of Sub-Saharan Africa (SSA), where it is intensively grown as an intercrop with other cereal crops like millet, sorghum and maize as well as rice fallows (Ishiyaku *et al*, 2010). 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 (Mishra *et al*.,2002). The landraces are sown and harvested just when all other food reserves have been depleted (June-August) er.

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

100g seed weight: Each of the harvested landraces was counted manually to a 100g 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 landrace and weight was recorded in grams.

Qualitative Traits Characterization of the 20 *Achishuru* Cowpea Landraces

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 20 *Achishuru* landraces, extraction was done from 2 weeks old young *Achishuru* seedlings using the Gene JetPlant Genomic DNA Purification mini kit® obtained from Thermo Fisher Scientific USA. Fresh leaves of the 20 samples of 100 g each where ground in liquid nitrogen and immediately transferred to a 1.5 ml microfuge tubes, 350 µl of lyses buffer A was pipette into the appendorf tube which was vortexed for 20 seconds. The 50 µl of lysis buffer B and 20 µl of Rnase were added to each of the microfuge and vortexed for one minute, Centrifugation was done for 5 min at 14,000rpm, 50 µl of the supernatant was transferred into a clear microfuge tube with the addition of 400 µl of plant gDNA binding solution and 400 µl of 96% ethanol. The 700 µl of the prepared mixture was transferred into a spin column and centrifuged for one minute at 8000 rpm. The flow through solution was discarded and the remaining solution was applied to the same column and centrifuged for 1 min at 8000rpm, 500 µl of wash buffer I was added to the column and centrifuged for 10000 rpm and the flow through solution was discarded and the column was replaced into the collection of1 µl of wash through solution using the 7.5 µl flow 630, 0.05% Tween ®20, 25 units/ml and *taq* DNA polymerase, 1 µl of forward and reverse primers of the genomic DNA template, all obtained from inqaba Biotech South Africa, 2 µl final volumes with nuclease-free water (Fermentas Inc.) was added up to a final volume of 23 µl. 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 20 *Achishuru* Landraces

Amplified SSR loci of the 20 *Achishuru* landraces were subjected to electrophoresis by melting 1.5 g agarose powder in 100 ml of 1xTBE buffer in a microwave for approximately 2 min and were allowed to cool for 4 minutes and 1 µl of ethidium bromide was added and stirred. The gel was cast using a supplied tray and a comb, 100 bp DNA ladder (New England) was used to estimate the size of the PCR products of the 20 samples; the electric power pack of the agarose assembly was turn on at 120 v for 60 min. 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 SSR markers and some qualitative traits, a non-parametric test of Spearman's rank-order correlation was used (Oyewole *et al*., 2014).

$$R = 1 - \frac{6\sum d^2}{n^3 - n}$$

Result And Discussion

Classifications of Similar Quantitative Characteristics of the 20 *Achishuru* Cowpea Landraces

Table 1 PowerMarker® Summary Statistics for the Selected 3 SSR Markers

SSR Marker	Allele Freq	No of alleles	PIC
VM31	0.49	3.00	0.55
VM68	0.32	6.00	0.23
VM70	0.75	4.00	0.41
Mean	0.53	4.30	0.39

who reported a primer pair, VM68, which detected a microsatellite allele, that was present only in Nigerian cowpea accessions with late flowering. And that this microsatellite might possibly be useful as a marker associated with late-flowering. 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 6 SSR was found to be associated with late flowering in this study. VM39 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 2).

Classical methods for characterizing genetic diversity in plants include the use of morpho-agronomic traits to establish genetic relationships among commercial cultivars, landraces and wild (Asare *et al*., 2010). 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 (Franco *et al.*, 2013). Earliness to physiological maturity is the most desired agronomic trait to farmers (Nwosu *et al*., 2012). This explains why the only landrace in cluster IV is widely cultivated by farmers because of its extra earliness. 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 (Badiane *et al*., 2012). Those with non-twining tendency are likely to be the cowpeas types which are characteristically erect or decumbent (Barkley *et al*., 2007 Brunel, D. 2012; Badiane *et al*., 2012)).

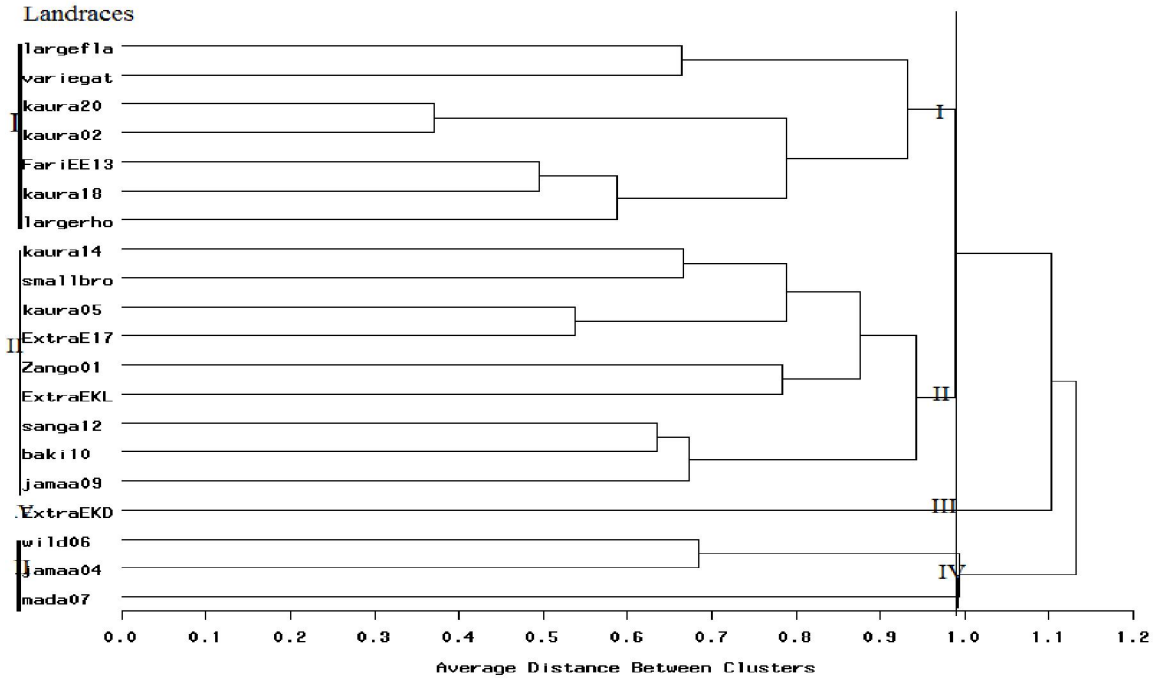

Figure 1 Dendrogram of the 20 *Achishuru* Cowpea Landraces Based on 12 Qualitative Traits

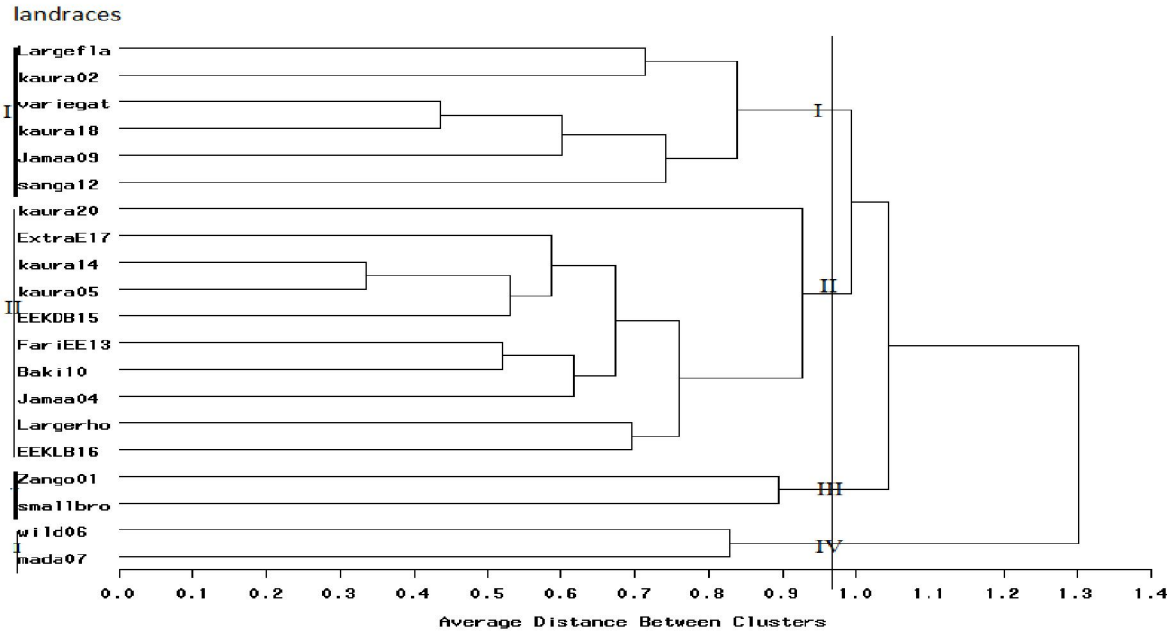

Figure 2 Dendrogram of the 20 *Achishuru* Cowpea Landraces Based on 10 Quantitative Traits

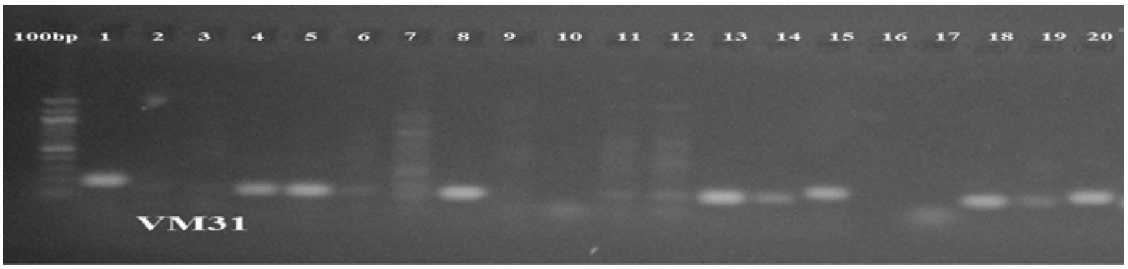

Figure 3 Polymorphism of the SSR Amplified by VM31 in 20 *Achishuru* Cowpea Landrace Shown on Ethidium Stained Agarose Gel.

Genetic diversity plays an important role in the success of any breeding program (Cholastova, T. and Knotova, D. 2012). Knowledge of genetic diversity in available germplasm and genotypes is very useful for plant improvement all over the world, promoting the efficient use of genetic variations in breeding programs through supporting proper selection of cross combination among large sets of parental genotypes (Franco *et al*., 2013). According to various studies, some morphological traits are mainly used as markers including pod per plants, seed per pod and seed size which affect potential yield of cowpea (Grubben *et al.,* 2014). The The yield of the morphological traits obtained in this study under screenhouse as compared to field conditions agrees with Kameswara *et al*., (2010) findings on the influence of environment on yield. 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). No SSR marker was suspected to be associated with any qualitative trait except for twinning tendency and VM39 ($P<0.05$), this may be due to the fewer number of loci detected as compared to Anas, Y. T. (2004) and Afikwe, W.T. and Takama, O.O. (2011) who reported 27 primer pair, and found out that VM68, was associated with late flowering (64 days and above) in some Nigeria cowpea landraces. The extent of genomic similarity between two species determines the transferability and use of molecular markers from one species to other related species (Adetiloye *et al*., 2013). In case of SSR markers, it depends upon the extent of conserved primer binding sites flanking the SSR loci (Grubben *et al.,* 2014). Earlier, cross-species transferability of SSR markers among legumes was studied by many workers including Diouf, D. and Hil, G.H. (2004) who reported transferability of adzuki bean primers to be 100% in blackgram. High proportion of SSR markers (86-92%) were transferred in blackgram from closely related species belonging to *Vigna* genus (Zannou *et al*., 2008), their findings agreed with this work since the primers used in this study were meant for cowpea but the transferability of the primers were detected with some Beans types of the landraces as clear bands were detected for those samples.

	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
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

Summary And Conclusion

Characterization of the *Achishuru* cowpea landraces have shown huge amount of potential genetic resource. Landraces that flower at the same time have been identified as synchronization of flowering is vital in hybridization, landraces with high and early to extra early maturity landraces have been discovered. No SSR marker was suspected to be associated with any qualitative trait under study except for

Acknowledgments

The authors would like to show appreciation to all Biotechnology Laboratory Technical Staff of the Department of Plant Science Faculty of Agriculture, Ahmadu Bello University, Zaria for their support to the success of this work. twinning tendency and VM 39. VM31 revealed the highest discriminatory power of a PIC value of 0.55 among the primers used. The 6 SSR markers were used to screen 20 *Achishuru* landraces in this study; however, higher number of SSR markers can be employed for further cowpea/*Achushuri* research to detect higher polymorphism among landraces and as a step also towards discovering more SSR markers associated with the qualitative trait.

Conflict Of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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