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Molecular characterization of cultivated cowpea (*Vigna unguiculata* L. Walp) using simple sequence repeats markers

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Forty eight accessions of cultivated cowpea were assessed using 12 simple sequence repeat (SSR) markers. The unweighted pair group method with arithmetic mean (UPGMA) dendrogram constructed revealed three main clusters when truncated at 65% similarity coefficient. All accessions showed high genetic variation except four which are genetically similar at 100% similarity coefficient. A two dimensional principal coordinate analysis (PCA) also revealed high genetic relationships among the accessions used. The polymorphism information content (PIC) revealed that the number of alleles per locus ranged from 2 to 5 with a total of 37 alleles generated from the SSR primers. The PIC value ranged from 0.075 to 0.603 with a mean value of 0.344 from a total of 4.467. There was no significant correlation between the repeat number and the allele numbers (r=0.21) so also between repeat numbers and PIC (r=0.11). The PIC also revealed the comparative genetic diversity from three sub-regions in Africa; West Africa, North East and Central Africa and Southern Africa with thirteen accessions each. West African accessions were the most diverse with a PIC value of 4.4310, showing the greatest genetic diversity and most likely the center of origin of cultivated cowpea.

Key words: Cowpea, dendrogram, genetic diversity, polymorphism, simple sequence repeat (SSR) markers.

INTRODUCTION

A detailed study of the variation of a crop, both morphologically and genetically, in relation to the geographical distribution of such variation could help in speculating on the origin of such plant. Crops that have been cultivated for a long time revealed an area with intense variation or great diversity, since in that area there would have been time for large numbers of mutations and gene recombination to take place, as a result of interbreeding among different varieties (Padulosi et al., 2007; Padulosi et al., 2009; Feleke et al., 2006). It is generally observed that a very large numbers of varieties or high variation of the species is found towards the center of the distribution area of the crop, and this is accompanied by a corresponding thinning out of the variability towards the periphery

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(Kuruma et al., 2012; Pasquet, 2000; Feleke et al., 2006).

The arrival of cowpea in West Africa and the development of the cowpea / cereals farming system probably date back from 3 to 4 000 years. Wild cowpea *(subsp. dekindtiana*) could have been gathered as folder to feed cattle and later domesticated as early as 4000 BC in West Africa. During the process of domestication and selection of cowpea from its wild progenitor, characters lost and gained included seed dormancy together with a reduction of pod dehiscence on one hand and an increase in pod and seed size on the other (Adewale et al., 2011; Ogunkanmi et al., 2006; Ogunkanmi et al., 2007).

The selection of cowpea as a pulse as well as folder might have resulted in the establishment of the cultigroup *unguiculata* (Ibrahima et al., 2013; Pasquet, 2000; Kuruma et al., 2008). Selection for types with long peduncle for fibre as well as for folder or seed has resulted in the cultigroup *Textilis* (Ibrahima et al., 2013). Once the cultigroup *unguiculata* was established in West Africa, diversity developed and accumulated through mutation.

Through centuries of cultivation, short day cowpea cultivars became adapted to the cereal farming system, while day neutral cultivars later evolved from these short day cultivars and became adapted to the yam based farming system in the humid zones of West Africa (Manggoel and Uguru, 2011; Ogunkanmi et al., 2007). Through West Africa the cultigroup *unguiculata* was introduced to East Africa, was brought to Europe, there it was known to the Romans about 2300 BC, and to India about 2200 BC (Padulosi et al., 2009). The cowpea underwent further diversification in India and Southeast Asia, producing the cultigroup *Biflora* for its grain and for use as a cover crop, and the cultigroup *Sesquipedalis* with its long pods used as a vegetable (Manggoel and Uguru, 2011; Ogunkanmi et al., 2008) cowpea was probably brought to the Americas during the 17th century by the Spanish and Portuguese traders.

A simple and precise technique for measuring the overall genetic diversity of a crop is not yet available, and no single approach can be considered the best for measuring diversity (Amin et al., 2010; Charcosset and Moreau, 2004; Kuruma et al., 2008). The classification of cultivated crop plants and the determination of their interrelationships require morphological traits together with sophisticated analyses such as the molecular studies as many of the morphological characters commonly used are prone to environmental influences, thereby reducing the fine resolution require ascertaining phylogenetic relationships (Kuruma et al., 2008).

The number of morphological attributes that can be scored is generally limited due to environmental influence hence DNA markers have therefore been used extensively to study relationships within and between crop species as they provide a larger number of characters which are unaffected by environmental influence and consequently can provide unambiguous character state assignments (Aaron et al., 2010; Ibrahim et al., 2007).

Plant systematist have therefore cautioned that whenever possible, systematic/evolutionary relationships and genetic diversity levels should be assessed by more than one class of genetic markers such as morphological together with isozymes and/or DNA based markers (Pasquet, 2000). Molecular markers are therefore being used in many aspects of plant genetics and breeding (Andargie et al., 2011; Moalafi et al., 2010), taxonomy, variability of populations and mating systems. They are based on differences in DNA sequences between individual and they generally detect more polymorphisms than morphological and protein-based markers and constitute a new generation of genetic markers (Badiane et al., 2012; Prasanthi et al., 2012).

Among others for example, restriction fragment length polymorphism (RFLP) markers have been used to construct genetic linkage maps in cowpea (Fatokun et al., 1993b) and to study the taxonomic relationships in the genus *Vigna* (Fatokun et al., 1993a).

However the use of RFLP in germplasm studies is limited by several factors, for example they require relatively large amounts of DNA for the assay, they are time consuming and labour intensive. The microsatellites markers (SSR) on the other hand have many advantages over classical RFLP and RAPD since they require minute amounts of DNA and are relatively cheap and time saving. (Andargie et al., 2011; Aaron et al., 2010; Kuruma et al., 2012)

Microsatellites are stretches of DNA, consisting of tandemly repeating mono-, di-, tri-, tetra-, or penta- nucleotides units, that are arranged throughout the genomes of most eukaryotic species (Kuruma et al., 2012; Badiane et al., 2012; Kuruma et al., 2008). The uniqueness and value of microsatellites arises from their multi-allelic nature, co-dominant transmission, ease of detection by PCR, high information content, ease of genotyping and its relative abundance in genome. They are good for tracing pedigrees, because they represent single loci and avoid the problems associated with multiple banding patterns (multiplex) obtained with other marker system.

The objectives of this work however are to assess the level of diversity within cultivated cowpea and to determine to probable center of origin of cultivated cowpea in Africa using microsatellites markers.

MATERIALS AND METHODS

Plant materials and DNA extraction

Forty eight cultivated cowpea were selected for DNA analysis (Table 1). Two seeds from each accession were sown in pots containing good loamy soil and placed on the floor in a screen house at International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. After two weeks of planting newly opened fresh young leaves were picked from each accession for DNA extraction. 0.3 g fresh leaf sample was ground into fine powder and DNA extracted according to the procedure described by (Dellaporta et al., 1983). The DNA was diluted in 0.1 × TE (1mM Tris 0.1 mM EDTA, pH 8.0) to

Table 1. Origin and accession number of Cowpea lines used for fingerprinting.

Code number	Tvu no	Origin	Region	Cultivars group
1	8130	Ghana		Unguiculata
2	6939	Niger		Unguiculata
3	8001	Nigeria		Unguiculata
4	8510	B. Faso		Unguiculata
5	14532	Mali		Unguiculata
6	8082	CotedVoire		Unguiculata
7	1177	Uganda	W. Africa	Unguiculata
8	8049	Nigeria		Unguiculata
9	11412	Gambia		Unguiculata
10	14818	Senegal		Unguiculata
11	15206	Congo		Unguiculata
12	10843	Cameroon		Unguiculata
13	8650	Togo		Unguiculata
14	7146	Ethiopia		Unguiculata
15	11954	Sudan		Unguiculata
16	9700	Egypt		Unguiculata
17	13484	Kenya		Unguiculata
18	15267	Chad		Unguiculata
19	15247	Chad		Unguiculata
20	13826	CAR	NE and C Africa	Unguiculata
21	13850	CAR		Unguiculata
22	11980	Sudan		Unguiculata
23	9548	Egypt		Unguiculata
24	16029	Somalia		Unguiculata
25	13439	Kenya		Unguiculata
26	13830	CAR		Unguiculata
27	11773	Malawi		Unguiculata
28	11774	Malawi		Unguiculata
29	15388	Zimbabwe		Unguiculata
30	14895	Botswana		Unguiculata
31	15047	Zambia		Unguiculata
32	988	S Africa		Unguiculata
33	15443	Swaziland	Southern Africa	Unguiculata
34	15077	Zambia		Unguiculata
35	1995	S Africa		Unguiculata
36	16098	Zimbabwe		Unguiculata
37	15433	Swaziland		Unguiculata
38	15055	Botswana		Unguiculata
39	15429	Lesotho		Unguiculata
40	3658	China		Cylindrical
41	3657	China		Cylindrical
42	21	Philippine		Sesquipedalis
43	22	Philippine	Asia	Sesquipedalis
44	3655	China		Sesquipedalis
45	1498	India		Sesquipedalis
46	3653	China		Sesquipedalis
47	3656	N. Caledonia		Sesquipedalis
48	3652	Australia	Australia	Sesquipedalis

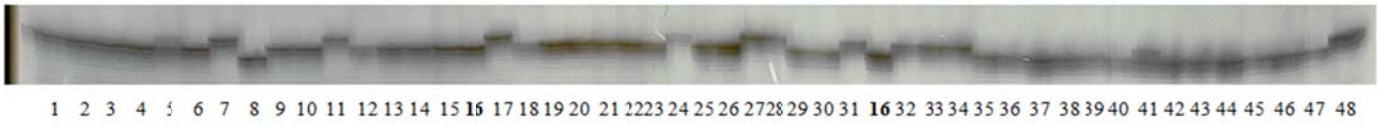

Figure 1. A gel photomicrograph showing the bands of SSR VM 74 with forty-eight cowpea lines. Note sample 16 was loaded twice at different places to serve as control.

10 ng/µL concentration.

Primer screening

A total of 120 SSR primers were screened and optimized for polymerphism and annealing temperature (Tm) using two accessions to ensure optimal primer performance.

Optimal PCR amplification across the two accessions was achieved with the range between 54 and 64°C annealing temperature. Thirteen primers that showed good and clear polymorphism with the PCR products were therefore used for this study.

PCR amplification

A 20 µL reaction volume containing 2.0 µL of 10x buffer, 4.0 µL of 10 ng/µL template DNA, 2.0 µL $MgCl_2$, 1.6 µL mixture of 10 mM dNTPs (dATP, dCTP, dGTP and dTTP), 9.2 µL of ultra pure water, 1.0 µL of SSR primers and 0.2 µL Taq (promega) was loaded in Perkin Elmer Mj cycler for DNA amplification. The PCR reaction was carried out with a profile of 18 cycles at 94°C for 1 min initial denaturing and extension at 72°C for 1 min. Annealing temperatures were progressively decreased by 0.5°C every cycle from 64 to 54°C. The reactions continued for 30 additional cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and ended with a 10 min extension at 72°C after about 3 hours. 2.0 µL of PCR products was loaded in 3% agarose gel to check for polymorphism before running those that showed polymorphism on polyacrylamide gel electro-phoresis.

Polyacrylamide gel electrophoresis of PCR products and data analysis

PCR products were separated on a sequencing gel containing 70 ml freshly prepared 6% polyacrylamide solution, 350 µL ammonium persulphite (APS) and 35 µL TEMED. The gel was run at constant power of 50 W, 2500 V and 60 mA for 3 h. The gel was later fixed, stained, and developed using silver staining kit (Promega corp. Madison WI). Fragments that were clearly resolved on gels (Figure 1) were scored as 1 or 0 that is, present or absent respectively on all the forty eight accessions of cultivated cowpea. The bands that could not be confidently scored were regarded as missing data. Clearly resolved DNA bands were amplified by 12 SSR primers and used for the clustering analysis.

Data analysis

Pairwise distance (similarity) matrices were computed using sequential, hierarchical and nested (SAHN) clustering option of the NTSYS-pc version 2.02j software package (Rohlf, 1993). The program generated a dendrogram, which grouped the test lines on the basis of Nei genetic distance using unweighted pair group using mathematical average (UPGMA) cluster analysis.

The polymorphism information content (PIC) provides an estimate of the discriminatory power of locus or loci, by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values were calculated by the algorithm: PIC = $1-\Sigma^n Pi^2$ where *i* starts from 1 and Pi^2 = frequency of the ith alleles (Ott, 1999). PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles each in equal and low frequency). The two dimension Principal Component Analysis (PCA) programmes of the Statistical Analysis System software package (SAS) version 8.2 was used. Only the distributions of the accessions along the first two principal components were considered in this paper.

RESULTS

A dendrogram (Figure 2) for the 48 cowpea lines was constructed by the UPGMA on the basis of the genetic similarity and Jaccard's Coefficient. The population clustering exhibits three main clusters when truncated at 65% similarity coefficient. The accession numbers, origin, codes, region and the cultivars name are given in Table 1. Codes 14, 16, 30 and 37-formed accessions from cluster three, two of which originated from Southern Africa (Botswana-30 and Swaziland-37) and the other two (14 and 16) from North East Africa (Ethiopia and Egypt), respectively.

The second cluster contained five accessions; two (9 and 12) were from Gambia and Cameroon, two (18 and 24) from Chad and Somalia and one (40) *Cylindrical* from China respectively. The first cluster is large and contained the remaining accessions including the seven *sesquipedalis* from Asia and the two *cylindrical*. The dendrogram distinguished all accessions except four (11 and 41) and (20 and 29) which are genetically similar.

A two dimension principal coordinate analysis did not detect significant sub group among the forty eight lines, they scattered randomly in the coordinates irrespective of the geographical location (Figure 3). However, West African accessions (Code 1-13) distributed more widely than others an evidence for West Africa great diversity of cultivated cowpea.

The PIC revealed that the number of alleles per locus ranged from 2 to 5 with a total of 37 from 12 primers (Table 2). The mean number of allele per locus was 2.92. Four primers (VM 39, VM 98, VM 27 and VM 78) had least number (2) of alleles each. The PIC value for the 12 SSR primers ranged from 0.075 to 0.603 with a mean value of 0.344 from a total of 4.467. There was no significant correlation between the repeat number and the allele numbers (r=0.21) so also between repeat numbers and PIC (r=0.11). Twelve primers that amplified clear polymorphic bands on the polyacrylamide gels were used

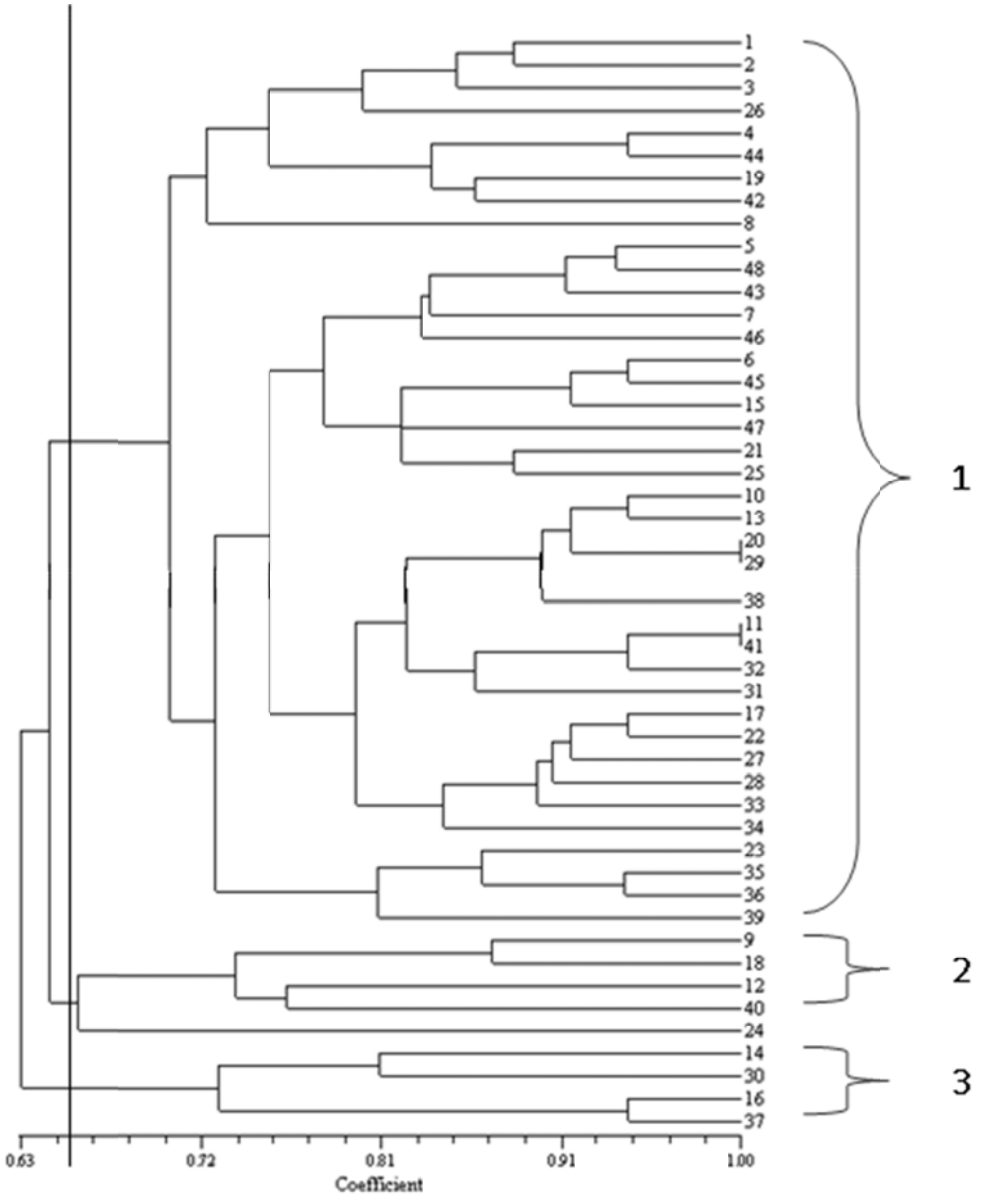

Figure 2. Dendrogram showing the distribution of Forty-eight cowpea accessions with twelve SSR primers.

to analyze the forty eight cowpea lines. These primers, their repeat type, repeat number, allele number and polymerphism information content were listed in Table 2. The information on their polymorphic ability is summarized with a bar chart (Figure 4).

Table 3, reveals the genetic diversity within three sub-region in Africa; West Africa, North East and central Africa and Southern Africa with 13 accessions each. The PIC from the three regions revealed PIC from West African accessions to be 4.4310, Southern Africa have 3.9539 while North East and Central Africa with 3.9872, this is another evidence of great diversity from West Africa region.

DISCUSSION

The use of genetic diversity - on farm through field experimentation or in sophisticated gene transfer procedures remains arguably the best route to secure our food and that of our children. The genetic diversity contained in different varieties provides farmers with options to develop, through selection and breeding new and more productive crops that are resistant to pests, diseases and stress. It refers to the variation at the level of individual genes (polymorphism), and provides a mechanism for populations to adapt to their ever-changing environment. The more variation, the better the chance that at least some

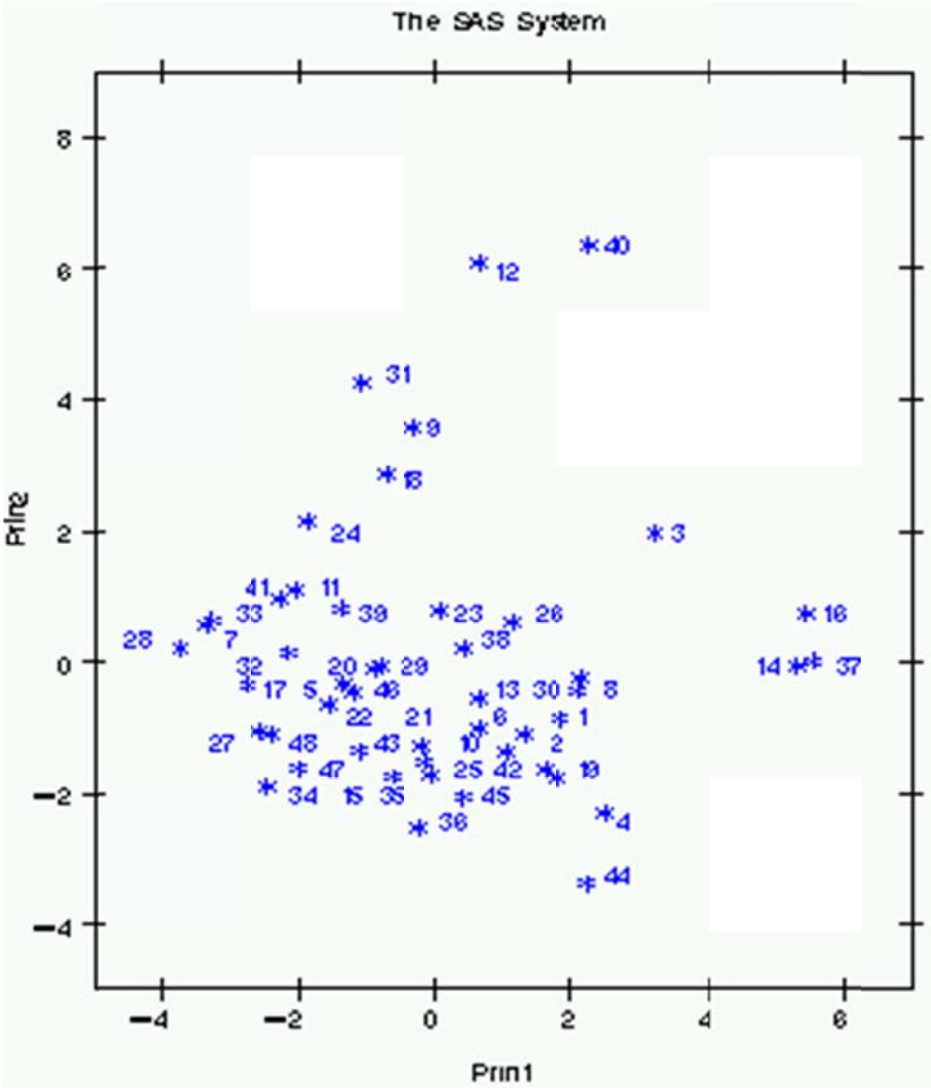

Figure 3. Principal component analysis for the 48 accessions of cowpea using twelve SSR primers.

Table 2. Number of alleles and polymorphism information content (PIC) of the cowpea microsatellite primers.

Primers	Repeat sequence	Repeat number	Number of alleles	PIC
Vm 98	AC /CT	9	2	0.323
Vm 9	AG/CT	21	4	0.519
Vm 37	AG/CT	13	4	0.557
Vm 27	AG/CT	14	2	0.172
Vm 39	AC/TG	13	2	0.376
Vm 22	AG/CT	12	3	0.226
Vm 36	AG/CT	13	4	0.603
Vm 71	AG/CT	12	3	0.480
Vm 74	AC/TG	8	3	0.386
Vm 78	AG/CT	5	2	0.075
Vm 35	AC/TG	11	5	0.502
Vm 34	AG/CT	14	3	0.248
Total			37	4.467

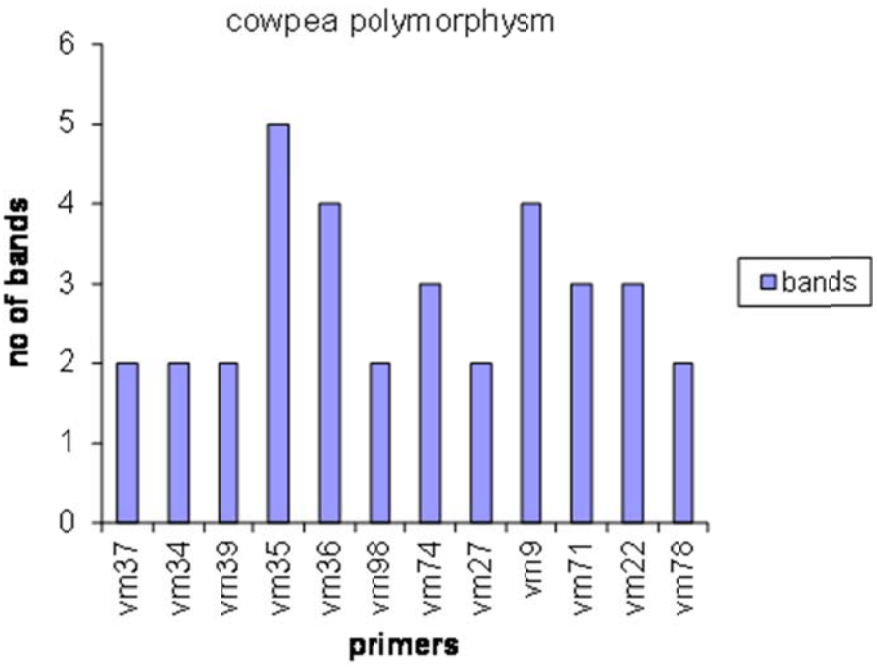

Figure 4. Bar chart showing the number of alleles detected by each primer.

Table 3. Polymorphism Information Content (PIC) of cultivated cowpea from three regions in Africa (North East and Central Africa, West Africa and Southern Africa).

Primer	Diversity Index		
	NE and CA (13)	WA (13)	SA (13)
Vm 37	0.2387	0.3228	0.3820
Vm 34	0.2387	0.3228	0.2387
Vm 39	0.4351	0.382	0.2387
Vm 35	0.4816	0.4714	0.5370
Vm 36	0.3609	0.7264	0.5127
Vm 98	0.3228	0.4297	0.2387
Vm 74	0.4360	0.2387	0.435
Vm 27	0.2387	0.1417	0.1417
Vm 9	0.5229	0.3820	0.5960
Vm 71	0.4731	0.4350	0.3609
Vm 22	0.0000	0.3398	0.2725
Vm 78	0.2387	0.2387	0.0000
Total	3.9872	4.4310	3.9539
Mean	0.3323	0.3693	0.3295

of the individuals will have an allelic variant that is suited for the new environment, and will produce progeny with the variant that will in turn reproduce and continue the population into subsequent generations.

Pasquet (2000), Ng (1995) and Aaron et al. (2010) reported that the area of maximum diversity of cultivated cowpea is found in West Africa in an area encompassing the savannah region of Nigeria, southern Niger, part of Burkina Faso, Northern Benin, Togo and the Northwestern part of Cameroon. In this study, the West African accessions distributed more widely than accessions from other regions, indicating more diverse accessions from that region than others, and may likely be the center of diversity.

Supporting this is the PIC values from the three regions. It revealed the genetic diversity within three sub- regions in Africa; West Africa, North East and central Africa and

Southern Africa with 13 accessions each. The PIC from the three regions varies with accession from West Africa having the highest PIC value of 4.4310, North East and central Africa having PIC of 3.9872 and Southern Africa with PIC of 3.9539. This suggests that genetic variation among lines from WA based on micro-satellite analysis is higher when compared with that observed among accessions from other regions. According to Padulosi et al. (2007), Padulosi et al. (2009) and Ogunkanmi et al. (2007) an area with intense variation may probably be the one where the crop must have been cultivated for a long time as a result of interbreeding and introgression among different varieties. This result is in agreement with the work of (Ogunkanmi et al., 2006) where he postulated West Africa as the center of origin of cowpea based on morphological data.

The 12 microsatellite markers used in this study detected 37 alleles among the 48 cowpea accessions with marker VM 27 detected the smallest number of alleles. In (Li et al., 2001) VM 27 was also reported to detect the lowest number of alleles among 90 cultivated cowpea lines and one wild cross compatible relative. The number of alleles detected in yard long bean ranges from 2 to 7 (Ogunkanmi et al., 2006) tomato 1 to 5 (Broun and Tanksley, 1996), Maize 2 to 11 (Senior and Heun, 1993), Barley 3 to 37 (Becker and Heun, 1995), and wild cowpea 4 to 13 (Ogunkanmi et al., 2008) as against 2 to 5 in this study. This suggests that genetic diversity in vegetable cowpea lines based on microsatellite analysis is higher and have higher genetic base when compared with that observed among cultivated cowpea lines used in this study. It is interesting to note that VM 39 which detected the highest number of alleles in the work of (Ogunkanmi et al., 2006), now showed the least number of alleles as in VM 27 above. The ability to use the same SSR primers in different plant species depends on the extent to which primer sites flanking SSRs are conserved between related taxa and the stability of the SSR over evolutionary time. The high discriminating power of SSRs is also an important factor in the analysis of variation in the gene pool of crops. Wayne et al. (1996) and Fatokun et al. (1999), in their study with rice established that 28% of the allelic variability was lost during the process of cultivar development from landraces. This is evident from the understanding of domestication process involved in the evolution of crop plants. Allelic variance are lost or reduced as plants are domesticated and hence narrow genetic base.

However, the high level of similarity among two pairs of accessions as detected by microsatellite markers (Figure 2) may be due to seed mix up during the process of labeling or handling in the gene bank. It could also be that the similar accessions came from same plant stand and subsequently found their way to the gene bank hence they are given different identification numbers.

To this end, microsatellites markers have been proved to be highly informative and provide an efficient and accurate means of detecting genetic variation in cowpea.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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