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Full Length Research Paper

Microsatellite association with seed protein content and flowering time in Nigerian cowpea cultivars

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Cowpea microsatellites were evaluated for their potential to be used as markers associated with seed protein content and plant flowering time. Seeds with high and low protein content as well as plants with early and late flowering time were selected from 104 Nigerian cowpea accessions. Fifteen (15) microsatellite primer pairs were tested for amplification of microsatellite sequences using PCR and seven pairs amplified a single-locus. Sequence analysis showed a high level of diversity between the analysed genotypes. Although, none of the microsatellites allowed clustering for protein content, one primer pair, VM68, detected a (AG)12-repeat microsatellite allele, which was present only in plants with late flowering. This microsatellite might possibly be useful as a marker associated with late-flowering time.

Key words: Cowpea, microsatellite, protein content, flowering time.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is a protein-rich grain legume and an inexpensive dietary protein source in the diet of people in sub-Saharan Africa. Drought is a major production constraint in the drier zones of northern Nigeria and Niger Republic, harbouring the largest area under cowpea cultivation in the world (Singh et al., 1997; Carsky et al., 2001). Early flowering has been recognized as an important trait to escape late season drought (Turner et al., 2001; Mitra, 2001). Plants with an escape strategy can complete their entire life cycle within a relatively short time period when water is available and they can survive dry conditions as seeds (Ludlow, 1989). Although considerable progress has been made in developing early maturing, high-yielding cowpea cultivars and selection of appropriate parents for crossing based on morphological traits is still a major challenge.

There is growing interest to use DNA microsatellites, also called simple sequence repeats (SSRs), for the selection of superior cowpea material. Microsatellites are short sequences of nucleotides 1 to 5 base pairs in length, tandemly repeated and flanked by unique sequences (Scribner and Pearce, 2000). They have several advantages, such as high level of polymorphisms, locus specificity, co-dominance, reproducibility, ease of use through polymerase chain reaction (PCR) and random distribution throughout the genome (Powell et al., 1996). In cowpea, a number of microsatellites has been identified and used to study relationships in cowpea (Li et al., 2001; Diouf and Hilu, 2005; Asare et al., 2010). By sequencing and analyzing the gene-rich,

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Abbreviations: SSRs, simple sequence repeats (SSRs); **IITA,** International Institute of Tropical Agriculture.

hypo-methylated portion of the cowpea genome, Timko et al. (2008) found that approximately 12% of all gene-space sequence reads in cowpea contain an identifiable simple-sequence repeat providing a dataset for micro-satellite design. Gupta and Gopalakrishna (2010) recently identified a total of 1071 microsatellites by screening 15 740 cowpea unigene sequences. However, to our know-ledge, study to associate one of the identified microsate-llites to a specific trait in cowpea has only been carried out by Li and Timko (2009). They identified a cowpea microsatellite marker (SSR-1) co-segregating with resis-tance to *Striga gesnerioides* (striga) race 3 (SG3).

The objective of this study was to screen the existing gene-bank containing Nigerian cowpea accessions for seed protein content and plant flowering time and to evaluate existing cowpea microsatellites for their possible association with one of these traits. Here, we report the genetic diversity analysis of cowpea accessions for seed protein content and flowering time and the identification of a $(AG)_{12}$ repeat microsatellite being possibly asso-ciated with late flowering time.

MATERIALS AND METHODS

Plant material

Seed material of two sub-populations of cowpea accessions were obtained from the Genetic Resources Unit of IITA, Ibadan, Nigeria. The first sub-group (Table 2) was selected from 104 Nigerian cowpea accessions which were pre-screened for their seed protein content. The accessions were sorted in descending order of their protein content and five accessions each with highest and lowest protein content were selected for the experiments. The second subpopulation (Table 4) was selected based on flowering time when grown in a greenhouse at the Biotechnology Research and Development Centre (BRDC) of the Ebonyi State University, Abakaliki, Nigeria. Seven early flowering cowpea accessions (34 to 37 days) and seven late flowering accessions (64 days and more) were selected for the experiment.

Protein determination

Protein content of seeds from all 104 accessions were determined by the method described by Bradford (1976) using a commercial protein determination kit (BioRad, UK).

Flowering time

The flowering time was determined by recording the mean number of days to first appearance of flowers in 3 plants for each genotype.

DNA extraction and amplification

Plant genomic DNA used in this analysis was extracted from cowpea seed powder using the Zymo research plant/seed DNA extraction kit^{tm} according to the manufacturer's instruction. DNA quality was determined by electrophoresis on a 1% agarose gel stained with ethidium bromide using total genomic DNA aliquots and the DNA concentration determined by a Nanodrop spectrophotometer. The Polymerase chain reactions (PCR) reactions were carried out in a MJ Mini^{Tm} Thermal cycler (Bio-Rad, Germany). Each 25 µl reaction mixture contained 50 ng of genomic DNA template, 2.5 µl of 1 x PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTP mix, 0.5 µl of a 10 mM solution of each of forward and reverse primer pair, 0.3 µl of Taq DNA polymerase (Fermentas, Inc.) and was made up to volume with nuclease-free water (Fermentas Inc.). 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. The PCR products (10 µl from each PCR reaction) were resolved on a 2% agarose gel run at 80 V and 500 Amp for 40 min and visualized by ethidium bromide staining. The remainder of the PCR reaction (15 µl) was purified using the QIAquick® PCR purification kit (QIAGEN, ICI Americas Inc.) according to the Manufacturer's instructions and the DNA was quantified using a Nanodrop spectrophotometer prior to sequencing reaction. Names, sequences and predicted product sizes of the primer pairs employed in this study are listed in Table 1.

DNA sequencing

For DNA sequencing, a PCR reaction was carried out in a 10 µl reaction mixture containing 20 ng of a purified PCR-derived DNA template, 2.1 µl of a 5x PCR buffer (Fermentas, Canada), 0.5 µl of Big Dye and 1 µl of either a forward or reverse primer (10 mM). The PCR reaction consisted of heating to 96°C for 10 s which was followed by 35 cycles each consisting of 55°C for 5 s and 60°C for 4 min, with a final extension time at 72°C for 10 min. The obtained PCR product was cleaned using a NucleoSEQ kit (Macherey-Nagel, Germany) containing a Sephadex solution (Sephadex® G-50, Sigma) (1.33 g in 20 ml of sterile water) according to the manufacturer's instruction. The cleaned DNA was transferred into a 0.5 ml micro-centrifuge tube and dried at 45°C for 20 to 25 min using a vacuum drier (Eppendorf Concentrator 5301, Eppendorf, Germany). DNA sequencing was done using an automated DNA sequencing facility at the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

Data analysis

Numbers of microsatellite repeats were determined from the sequence data of PCR-amplified DNA products and used to determine the number of alleles per microsatellite region amplified with the various primers and the polymorphism information content (PIC) was determined as described by Weir (1996).

RESULTS

A total of 15 microsatellite primer pairs were evaluated including 12 with VM primer codes (Li et al., 2001) and three with the SSR primer codes 6217, 6243 and 6608 (Asare et al., 2010). Names, sequences, repeat types and predicted product sizes of primers with the VM code are listed in Table 1. Four of the primer pairs (VM12, VM13, VM14 and VM19) did not show any amplification.
Three primer pairs (VM5, SSR-6217 and SSR-6608)

Table 1. List of cowpea microsatellite primer pairs, type of microsatellite repeats and expected fragment size of amplification products containing microsatellites.

Primer	Sequence	Repeat	Size (bp)
VM5	5'-AGC GAC GGC AAC AAC GAT-3' 5'-TTC CCT GCA ACA AAA ATA CA-3'	(AG)	188
VM12	5'-TTG TCA GCG AAA TAA GCA GAG A-3' 5'-CAA CAG ACG CAG CCC AAC T-3'	(AG)	157
VM13	5'-CAC CCG TGA TTG CTT GTT G-3' 5'-GTC CCC TCC CTC CCA CTG-3'	(CT)	135
VM14	5'-AAT TCG TGG CAT AGT CAC AAG AGA-3' 5'-ATA AAG GAG GGC ATA GGG AGG TAT-3'	(AG)	144
VM19	5'-TAT TCA TGC GCC GTG ACA CTA-3' 5'-TCG TGG CAC CCC CTA TC-3'	(AC).(AC)	241
VM 31	5'-CGC TCT TCG TTG ATG GTT ATG-3' 5'-GTG TTC TAG AGG GTG TGA TGG TA-3'	(CT)	200
VM 35	5'-GGT CAA TAG AAT AAT GGA AAG TGT-3' 5'-ATG GCT GAA ATA GGT GTC TGA-3'	(AG).(T)	127
VM 36	5'-ACT TTC TGT TTT ACT CGA CAA CTC-3' 5'-GTC GCT GGG GGT GGC TTA TT-3'	(CT)	160
VM 39	5'-GAT GGT TGT AAT GGG AGA GTC-3' 5'-AAA AGG ATG AAA TTA GGA GAG CA-3'	(AC).(AT) (TACA)	212
VM 68	5'-CAA GGC ATG GAA AGA AGT AAG AT-3' 5'-TCG AAG CAA CAA ATG GTC ACA C-3'	(GA)	254
VM 70	5'-AAA ATC GGG GAA GGA AAC C-3' 5'-GAA GGC AAA ATA CAT GGA GTC AC-3'	(AG)	186
VM 71	5'-TCG TGG CAG AGA ATC AAA GAC AC-3' 5'-TGG GTG GAG GCA AAA ACA AAA C-3'	(AG) (AAAG)	225

amplified multiple bands and were not used for further analysis. One primer pair (SSR-6243) amplified a single clear band from all the test samples at a different annealing temperature (58°C), but when the bands were sequenced, no simple sequence repeat motifs were found in the sequences. This primer pair was also not used in the analysis. Seven primer pairs (VM31, VM35, VM36, VM39, VM68 VM70 and VM71) amplified a single and clear band on an agarose gel in all the accessions analyzed and were used in the analysis. An example of the amplification product obtained on an agarose gel by primer pair VM71 is shown in Figure 1. All other primer pairs used also gave a single amplification band on an agarose gel.

Analysis of seed protein content from 104 accessions allowed the selection of five accessions each with the highest and lowest seed protein content (Table 2). When the DNA amplification products derived from genomic DNA were analyzed by direct sequencing, polymorphic simple sequence repeats were found and types and numbers of repeats detected for the lines selected for protein content are shown in Table 3. The seven microsatellite primers detected a total of 23 alleles from plants of the 10 cowpea accessions. The number of

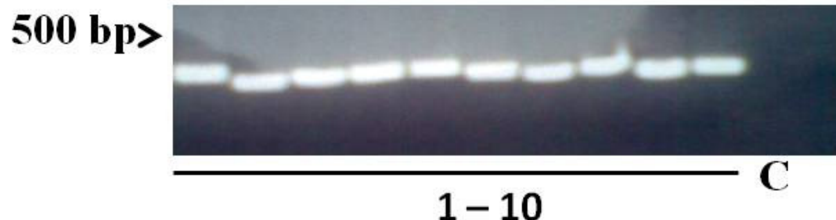

Figure 1. Single-band PCR DNA amplification product using primer pair VM 71 and genomic DNA isolated from seeds of 10 cowpea accessions used for analysis of SSR polymorphism in high and low protein seeds. The lanes are in the order in which the cowpea accessions appear in Table 2. M represents a 100 bp DNA ladder and C represents a negative control without addition of DNA.

Table 2. Seed protein content of cowpea accessions to select accessions with high and low seed protein content. Accessions with high seed protein content underlined and with low seed protein content underlined and in italics.

Accession	Protein mg/100 mg	Accession	Protein mg/100 mg	Accession	Protein mg/100 mg
Tvu-46	8.1	Tvu-4260	6.7	Tvu-8541	8.3
Tvu-160	8.3	Tvu-4408	8.4	Tvu-8546	7.9
Tvu-331	**11.0**	Tvu-4415	8.4	Tvu-8580	9.0
Tvu-442	8.7	Tvu-6318	8.4	Tvu-8586	8.3
Tvu-461	7.3	Tvu-6320	8.0	Tvu-9036	8.5
Tvu-561	6.8	Tvu-6325	6.3	Tvu-9167	5.8
Tvu-702	6.8	Tvu-6674	8.4	Tvu-9176	9.2
Tvu-729	6.9	Tvu-6778	8.0	Tvu-9185	7.4
Tvu-764	8.5	Tvu-6804	7.7	Tvu-9357	6.8
Tvu-839	8.1	Tvu-6815	7.3	Tvu-9769	7.4
Tvu-848	7.0	Tvu-6819	8.1	Tvu-9772	9.3
Tvu-867	7.3	Tvu-6822	8.4	Tvu-9773	8.4
Tvu-930	5.8	Tvu-6830	8.2	Tvu-9774	8.4
Tvu-939	9.0	Tvu-6833	8.5	Tvu-9776	6.2
Tvu-1138	8.6	Tvu-6835	9.2	Tvu-9779	9.7
Tvu-1197	6.8	Tvu-6847	5.7	Tvu-9780	8.2
Tvu-1260	7.1	Tvu-6932	8.3	Tvu-9784	7.8
Tvu-1262	7.7	Tvu-7083	7.6	Tvu-9787	8.7
Tvu-1263	**9.8**	***Tvu-7097***	***4.8***	Tvu-9788	5.9
Tvu-1455	**10.7**	Tvu-7109	8.5	***Tvu-9790***	***5.4***
Tvu-1979	7.9	Tvu-7110	8.3	Tvu-10112	8.9
Tvu-1986	6.7	Tvu-7112	6.4	Tvu-12348	5.9
Tvu-3910	7.2	Tvu-7117	8.5		
Tvu-3919	7.2	**Tvu-7488**	**10.1**		
Tvu-3933	7.1	Tvu-7491	8.2		
Tvu-3960	7.2	Tvu-7531	6.1		
Tvu-4007	8.3	Tvu-7815	8.1		
Tvu-4009	6.7	Tvu-7833	8.1		
Tvu-4015	6.8	Tvu-7838	6.7		
Tvu-4028	8.0	Tvu-7846	5.7		
Tvu-4034	5.7	Tvu-7848	6.2		
Tvu-4044	7.3	Tvu-7853	6.1		
Tvu-4045	8.9	Tvu-7870	6.9		

Table 2.Cont

Tvu-4046	**10.1**	Tvu-7898	7.2
Tvu-4047	8.6	Tvu-7920	8.6
Tvu-4049	8.2	Tvu-7962	5.7
Tvu-4068	7.0	***Tvu-7983***	***5.5***
Tvu-4083	***5.2***	Tvu-7995	8.3
Tvu-4089	8.9	Tvu-8042	7.3
Tvu-4095	***5.0***	Tvu-8164	6.3
Tvu-4100	7.7	Tvu-8387	8.4

Table 3. Protein content of seeds and number of microsatellite repeats amplified with different primer pairs from different cowpea accessions.

Accession	Cultivar/ local variety	Protein content (mg/100 mg FW)	VM31 (CT)	VM35 (AG)	VM36 (CT)	VM39 (AC)	VM68 (GA)	VM70 (AG)	VM71 (AG)
High protein									
331	BANTA 9	11.0±0.01	18	12	14	9	8	16	8
1455	PI 188704	10.8±0.01	18	12	9	14	8	22	9
7488	YANDEV	10.2±0.51	18	12	15	9	12	21	9
4046	KR313	10.1±0.57	19	12	14	13	12	24	9
1263	C 5713-13	9.9±0.09	17	12	9	14	8	20	8
Low protein									
7983	LANGTAN	5.6 ± 0.45	19	12	14	13	15	21	9
9790	O3E	5.4 ± 0.25	18	12	14	13	8	20	8
4083	KR364	5.2 ± 0.21	19	12	15	9	12	22	9
4095	KR376	5.0 ± 0.49	20	12	14	13	12	23	9
7097	GOMBE	4.8 ± 0.09	19	11	14	13	15	21	9
Alleles			4	2	3	3	3	6	2
PIC			0.660	0.180	0.560	0.620	0.640	0.800	0.420

alleles per primer pair varied from two to six with an average of 3.3. Primer pairs VM35 and VM71 identified only two alleles, while primer pairs VM36, VM39 and VM68 each detected three alleles. VM31 detected four alleles, whereas VM70 detected the maximum number, six alleles. The polymorphism information content varied from 0.180 to 0.800 with an average of 0.468. Other repeat types (T) for VM35, (AT) and (TACA) for VM39 and (AAAG) for VM 71 were also detected. These repeats were found to be non-polymorphic among the accessions and were not considered in our analysis.

Analysis of flowering time from 104 accessions allowed selecting seven accessions for either late or early flowering time (Table 4). The identical set of primer pairs used for protein content was also applied for amplification of products from late and early flowering time plants of different accessions. The types and numbers of repeats detected by these primer pairs and their diversity parameters are shown in Table 5. The seven microsatellite primer pairs detected a total of 35 alleles among the plants of the 14 cowpea accessions. The number of alleles per primer pair varied from three to eight with an average of five alleles per primer pair. Primer pairs VM31 and VM39 amplified four alleles each, primer pairs VM36 and VM68 amplified five alleles each, VM70 amplified six and VM71 amplified only three, while VM35 amplified as high as eight alleles. The polymorphism information contents of the primer pairs were relatively high, ranging from 0.613 to 0.807 with an average of 0.703 per primer pair. Primer pairs VM68 detected a microsatellite only present in the late flowering time accessions (64 days and above) and VM71 was also present in late flowering time accessions with the exception of accession 7853 in which it was present but

Table 4. Flowering time of cowpea accessions to select accessions with late and early flowering time. Accessions with longer flowering time underlined and with shorter flowering time underlined and in italics.

Accession	Flowering day	Accession	Flowering day	Accession	Flowering day
Tvu-46	51.0	Tvu-4260	40.3	Tvu-8164	45.0
Tvu-160	**86.0**	Tvu-4408	45.0	Tvu-8387	42.0
Tvu-331	50.0	Tvu-4415	41.3	Tvu-8541	45.6
Tvu-442	46.0	Tvu-6318	37.6	Tvu-8546	41.3
Tvu-461	44.6	***Tvu-6320***	***36.6***	Tvu-8580	47.6
Tvu-561	***34.3***	Tvu-6325	39.6	Tvu-8586	53.3
Tvu-702	41.3	Tvu-6674	46.3	Tvu-9036	47.6
Tvu-729	46.0	Tvu-6778	38.3	Tvu-9167	34.3
Tvu-764	37.3	Tvu-6804	40.6	Tvu-9176	56.3
Tvu-839	39.0	Tvu-6815	53.0	Tvu-9185	42.3
Tvu-848	34.0	Tvu-6819	43.6	TVu-9357	47.3
Tvu-867	39.0	***Tvu-6822***	***34.0***	Tvu-9769	40.3
Tvu-930	52.0	Tvu-6830	55.6	***Tvu-9772***	***37.0***
Tvu-939	53.3	Tvu-6833	42.0	***Tvu-9773***	***37.0***
Tvu-1138	56.0	Tvu-6835	54.6	***Tvu-9774***	***34.6***
Tvu-1197	44.3	Tvu-6847	45.0	**Tvu-9776**	***34.3***
Tvu-1260	44.6	Tvu-6932	52.3	Tvu-9779	37.3
Tvu-1262	47.0	Tvu-7083	55.6	Tvu-9780	49.3
Tvu-1263	54.0	Tvu-7097	39.0	Tvu-9784	37.0
Tvu-1455	39.3	Tvu-7109	41.6	Tvu-9787	44.3
Tvu-3910	42.0	Tvu-7110	38.6	Tvu-9788	45.6
Tvu-3919	43.6	Tvu-7112	40.6	Tvu-9790	44.3
Tvu-3933	39.6	Tvu-7117	46.6	Tvu-10112	49.0
Tvu-3960	46.0	Tvu-7488	37.6	Tvu-11979	45.3
Tvu-4007	43.6	Tvu-7491	49.0	Tvu-11986	60.3
Tvu-4009	42.3	Tvu-7531	46.3	Tvu-12348	46.3
Tvu-4015	43.0	Tvu-7815	42.3		
Tvu-4028	42.3	Tvu-7833	42.0		
Tvu-4034	41.6	Tvu-7838	37.3		
Tvu-4044	**69.3**	Tvu-7846	35.6		
Tvu-4045	**66.3**	Tvu-7848	45.0		
Tvu-4046	51.6	**Tvu-7853**	**63.6**		
Tvu-4047	**70.0**	Tvu-7870	56.3		
Tvu-4049	37.3	Tvu-7898	39.3		
Tvu-4068	37.0	Tvu-7920	53.0		
Tvu-4083	**64.6**	Tvu-7962	44.3		
Tvu-4089	44.3	Tvu-7983	44.0		
Tvu-4095	**64.0**	Tvu-7995	41.3		
Tvu-4100	43.6	Tvu-8042	37.3		

with a single mismatch.

Both seed protein content as well as flowering time differed significantly between accessions with two-times higher seed protein content or flowering time in the high protein and late flowering accessions when compared, there should be no return after accessions. However, no correlation was found between the two traits (r = 0.079).

DISCUSSION

In this study 104 Nigerian cowpea accessions from the IITA cowpea gene-bank have been characterized for diversity in seed protein content and flowering time. No significant correlation between the two traits was found. This information will narrow the gap between available gene-bank data for Nigerian accessions regarding these

Table 5. Flowering time and number of microsatellite repeats amplified with different primer pairs from different cowpea accessions.

Accession	Cultivar/ local variety	Flowering time (day)	VM31 (CT)	VM35 (AG)	VM36 (CT)	VM39 (AC)	VM68 (GA)	VM70 (AG)	VM71 (AG)
Long									
160	OKOBO	86	19	27	15	8	12	17	9
4047	KR313	70	20	12	15	9	12	18	9
4044	KR312	69	18	21	15	9	12	18	9
4045	KR312	67	19	22	15	9	12	18	9
4083	KR364	65	19	12	16	9	12	21	9
7853	OGBOMBO	64	18	23	14	13	12	22	8
4095	KR376	64	20	12	15	13	12	22	9
Short									
6320	S Tvu	37	18	11	15	14	8	20	8
9772	O11D	37	17	29	15	9	16	20	8
9773	O3J	37	18	12	15	13	14	21	8
9776	O42E	34	17	12	10	8	8	19	10
9774	O1E	34	18	12	10	13	14	19	10
561	56-4	34	19	11	13	14	16	20	8
6822	3949	34	20	13	14	13	15	20	8
Alleles			4	8	5	4	5	6	3
PIC			0.724	0.766	0.623	0.705	0.685	0.807	0.613

two important cowpea traits, that is, seed protein content as a protein source and flowering time to provide drought tolerance and end-user information. Gupta et al. (2010) recently screened different cowpea genotypes for total seed protein content and reported values ranging from 22.4 to 27.9%. In the Nigerian accessions used in this study, we found the protein content of the highest seed protein content lines to be 2 to 2.5-times lower than this, demonstrating the significant genotypic and phenotypic differences between cowpea accessions.

We also evaluated existing microsatellites in this study for their potential association with seed protein content and flowering time. Microsatellite analysis detected a high degree of polymorphisms among the tested cowpea accessions. However, the overall degree of polymerphism, with three to eight alleles per primer pair, is rather low but confirms previous cowpea results (Li et al., 2001). A low degree of genetic polymorphisms appears to be inherent in cultivated cowpea and might have resulted from both the domestication process and the inherent self-pollination mechanism (Asare et al., 2010).

A considerable number of microsatellites have been isolated from cowpea, but have not yet been evaluated for a possible association with important agronomic traits in order to demonstrate their usefulness in cowpea breeding. To our knowledge, only Li and Timko (2009) recently identified a microsatellite associated with striga resistance. In this study, we tested for an association between seven known microsatellites (Li et al., 2001) and two important cowpea traits, namely protein content and flowering time.

Although, the selected microsatellites significantly differentiated cowpea accessions, they did not allow clustering of accessions with respect to their seed protein content. However, one primer pair, VM68, amplified a $(AG)_{12}$ repeat exclusively from the late flowering accessions and was not found within any of the early flowering accessions tested. We, therefore, suggest that the identified microsatellite might be associated with late flowering and could be an important marker distinguishing late flowering from early flowering genotypes. However, further detailed studies have to demonstrate if this microsatellite is indeed directly associated with late flowering. A recent data base search to determine if the identified microsatellite might possibly match sequences of known *FT/TFL Arabidopsis* gene sequences did not yield a positive result.

In summary, in many cowpea gene-banks, particular in Africa, only a fraction of the conserved germplasm has actually being characterized in greater detail. This study has contributed to the characterization in more detail of two important cowpea traits in the Nigerian cowpea

accessions available in the IITA gene-bank. This study also confirmed that the overall degree of polymorphism is rather low in cowpea. A microsatellite has been identified that might be associated with late flowering. The confirmation of this association will assist in selection procedures that were tracking the late flowering trait and would likely increase the overall efficiency and effectiveness of any cowpea improvement program for this trait. We are therefore, currently screening a greater number of cowpea accessions to confirm that the identified $(AG)_{12}$ microsatellite is associated with this flowering trait. Crosses will be made between late and early flowering plants to establish a segregating F_2 plant populations and all late flowering plants will be screened for the existence of the $(AG)_{12}$ repeat microsatellite.

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