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Molecular Diversity in some Ghanaian Cowpea [*Vigna unguiculata* L. (Walp)] Accessions

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Abstract Cowpea [*Vigna unguiculata* L. (Walp)] is grown mainly for its protein-rich grains and is consumed in various forms in sub-Saharan Africa. Average grain yield in farmers' fields is generally low due to a number of biotic and abiotic stresses. One hundred and six cowpea accessions from Ghana, which had previously been evaluated for seedling drought tolerance, were used for this study. This paper attempts to use three multi-locus PCR-based molecular markers; simple sequence repeats (SSR), inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphisms (REMAP), to analyse genetic diversity in the cowpea accessions. Analysis of the polymorphic bands data indicated that 101 alleles were amplified among 121 cowpea genotypes (83.4%) from 16 SSR primer pairs out of a total of 30 SSR primer pairs. Likewise, a total of 66 (54.5%) polymorphic bands were obtained from IRAP and a total of 114 (94.2%) highly polymorphic bands obtained from REMAP analysis. The outcome indicated the highly polymorphic nature of the DNA markers, as small groups of these molecular markers were found to be able to identify each of the accessions used. Microsatellite markers (SSRs) and retrotransposon-based markers, like IRAP and REMAP, were found to be highly polymorphic and informative, suggesting that genomic fingerprinting has a major role in characterizing populations.

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Abbreviations

IRAP	Inter-retrotransposon amplified polymorphism and
REMAP	Retrotransposon-microsatellite amplified polymorphisms
SSR	Simple Sequence Repeats

Introduction

Cowpea [*Vigna unguiculata (L) Walp*] is an important staple food crop in Ghana and many other parts of the world (Obembe 2008; Timko and Singh 2008). The crop provides strong support to the livelihood of small-scale farmers through its contributions to their nutritional security, income generation and soil fertility enhancement. Worldwide about 6.5 million metric tons of cowpea are produced annually on about 14.5 million hectares of land (Boukar et al. 2016). The cowpea grains contain on average 25% protein, 53.2 mg/kg iron, 38.1 mg/kg zinc, 826 mg/kg calcium, 1915 mg/kg magnesium, 14,890 mg/kg potassium, and 5055 mg/kg phosphorus (Boukar et al. 2011). The low productivity of cowpea is attributable to numerous abiotic and biotic constraints. The abiotic stress factors comprise drought, low soil fertility and heat, while biotic constraints include insects, diseases, parasitic weeds, and nematodes (Asare et al. 2010). Cowpea is generally considered to be a self-pollinating crop, which makes its genetic base narrow (Sharawy and El-Fiky 2003; Poehlman 2013).

Genetic diversity is known to provide the fundamental insight for biological diversity and selective breeding studies (Glaszmann et al. 2010; Kouam et al. 2012). Limited genetic diversity poses a threat to the survival of species as it limits

their ability to respond to changes in climate, pathogen populations and agricultural practices (Manifesto et al. 2001). The source of genetic resources for crop improvement is the available germplasm in genebanks and this needs to be assessed for availability of useful traits for crop improvement (Tan et al. 2012). Cowpea is one of the most researched crops at the genebank of the Council for Scientific and Industrial Research – Plant Genetic Resources Research Institute (CSIR – PGRRI) in Ghana (Egbadzor et al. 2014). These cowpea accessions were collected from different geographical areas of Ghana about three decades ago and were mostly characterized based on morphological (Bennett-Lartey 1992), seed protein (Oppong-Konadu et al. 2005), and drought tolerance traits (Otwe et al. 2011).

Knowledge of genetic distance and diversity at the molecular level among germplasm is important for characterization and identification of gene flow among populations. While numerous studies have evaluated molecular diversity of common bean (Biswas et al. 2010; Zargar et al. 2014; Zargar et al. 2016), a number of reports related to cowpea genetics have focused on linkage maps and genome analysis (Sharp et al. 2000; Gale et al. 2001; Menz et al. 2002). Information on molecular diversity of cowpea is still limited and data on molecular variation at the DNA level of Ghanaian cowpea is lacking. However, simple sequence repeat (SSR) markers have been used to evaluate genetic diversity and phylogenetic relationships of cowpea genotypes (Asare et al. 2010; Badiane et al. 2012). It is critical, for the purposes of efficiency, that the best available tool for genetic diversity assessment is deployed.

Currently, the use of molecular markers for predicting hybrid performance, gene discovery, genetic diversity, molecular breeding and population genetics has become the method of choice and has revolutionized molecular analysis (Abdollahi Mandoulakani et al. 2015). A major step forward in genetic identification is the discovery that about 30–90% of the genome of virtually all the species is constituted by the regions of repetitive DNA, which can be highly polymorphic in nature (Kalendar et al. 2011). Molecular markers have many advantages over phenotypic characters as they are unaffected by the environment.

DNA-based markers, such as simple sequence repeats (SSRs), have been and are still being utilized in cultivar development, quality control of seed production, measurement of genetic diversity for conservation management, varietal identification and intellectual property protection (Smith et al. 1997). The simplicity of the banding pattern and the multi-allelic nature of SSR loci may be extremely useful in interpreting segregation data (Badiane et al. 2012). Inter-retrotransposon amplified polymorphism (IRAP) and Retrotransposon microsatellite amplified polymorphism (REMAP) are potential markers due to the abundance of retrotransposons (RTNs) in eukaryotic genomes and their ability to create new copies (Abdollahi Mandoulakani et al. 2015). IRAP uses PCR primers designed as an outward direction from the conserved sequences of long terminal repeats (LTR). REMAP, on the other hand, resembles IRAP but uses one LTR primer and a primer specific to a nearby microsatellite. The major challenge with the use of IRAP and REMAP markers is that sequence of the RTNs is required for primer design (Kalendar 2011). It is extremely important to study the genetic composition of the germplasm using molecular markers in order to enhance the understanding of the genetic variations among the existing cultivars, for effective planning of crosses and breeding for the trait of interest. Therefore, this paper examines the diversity of an extensive range of cowpea germplasm sampled from Ghana. Diversity analysis using three multi-locus PCR-based molecular markers (SSR, IRAP and REMAP) were performed. The objective was to establish the pattern of diversity, discriminating capacity and the effectiveness of SSR, IRAP and REMAP marker techniques in the cowpea germplasm used.

Results

The SSR, IRAP and REMAP reactions amplified DNA sequences from cowpea accessions with high reliability. Many primer pairs identified extensive polymorphisms between the accessions analysed. Table 1 shows the details of the 16 primers used for the SSR analysis. Analysis of the data generated from the scoring of the polymorphic bands indicated that 101 alleles were amplified among 121 cowpea genotypes (83.4%) from 16 SSR primer pairs.

Patterns of Diversity of the SSR, IRAP and REMAP Markers

The level of polymorphisms exhibited by the SSR markers was revealed through the number of amplified alleles per primer pair. They varied from 4 for VM 26, 37 and 38 to 11 for VM 28, with the mean number of alleles being 6.31 (Table 1). The allele frequencies for all the primers were generally below 0.95 indicating that they were all polymorphic in character. Gene diversity was high, ranging from 0.59 in VM 19 to 0.77 in VM 28 (Table 1). The sizes of amplified alleles ranged from 110 to 310 bp depending upon their respective expected product sizes as shown in Table 1 and Fig. 1 (a and b). The 16 SSR markers were able to produce polymorphic bands in most of the 106 accessions from Ghana and 15 others from Nigeria and the United Kingdom used for the study. Indeed, the three most polymorphic loci were VM17, VM35, and VM36 with clear polymorphic patterns. The IRAP analysis also produced an appreciable level of polymorphisms (54.5%). All the six IRAP primer combinations (Table 2) generated multiple fragments of defined sizes from the genomic DNA of all the

Table 1 Details of SSR primers with various parameters revealing the discriminatory power of each primer

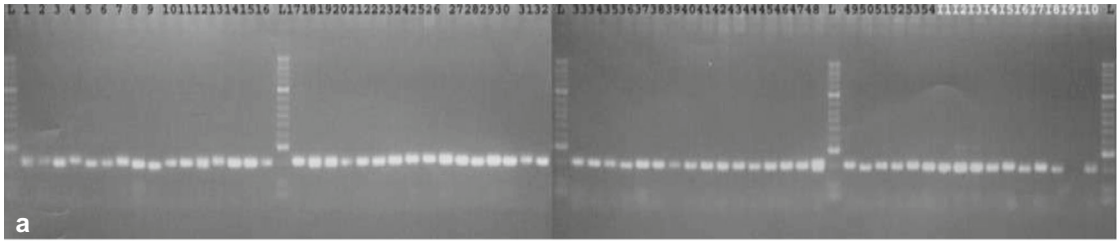
Primer code	Primer sequence	Allele size range (bp)	No. of alleles	Allele frequency	Gene diversity	H_0	PIC
VM 17	5'GGC CTA TAA ATT AAC CCA GTC T 5'TGT GTC TTT GAG TTT TTG TTC TAC	130–170	8	0.35	0.74	0.11	0.70
VM 19	**5'TAT TCA TGC GCC GTG ACA CTA 5'TCG TGG CAC CCC CTA TC**	240–260	7	0.57	0.59	0.07	0.53
VM 22	5'GCGGGT AGT GTA TAC AAT TTG 5'GTA CTG TTC CAT GGA AGA TCT	210–240	6	0.46	0.65	0.07	0.59
VM 26	**5'GCC ATC AGA CAC ATA TCA CTG 5'TGT GGC ATT GAG GGT AGC**	290–320	4	0.45	0.64	0.07	0.57
VM 27	5'GTC CAA AGC AAA TGA GTC AA 5'TGA ATG ACA ATG AGG GTG C	240–310	7	0.50	0.67	0.09	0.62
VM 28	**5'GAA TGA GAG AAG TTA CGG TG 5'GAG CAC GAT AAT ATT TGG AG**	210–310	11	0.34	0.77	0.11	0.74
VM 30	5'CTC TTT CGC GTT CCA CAC TT 5'GCA ATG GGT TGT GGT CTG TG	130–160	5	0.38	0.69	0.06	0.63
VM 31	**5'CGC TCT TCG TTG ATG GTT ATG 5'GTG TTC TAG AGG GTG TGA TGG TA**	170–240	8	0.45	0.71	0.12	0.67
VM 35	5'GGT CAA TAG AAT AAT GGA AAG TGT 5'ATG GCT GAA ATA GGT GTC TGA	120–190	7	0.51	0.66	0.11	0.61
VM 36	**5'ACT TTC TGT TTT ACT CGA CAA CTC 5'GTC GCT GGG GGT GGC TTA TT**	130–200	8	0.39	0.69	0.14	0.63
VM 37	5'TGT CCG CGT TCT ATA AAT CAG C 5'CGA GGA TGA AGT AAC AGA TGA TC	270–300	4	0.48	0.64	0.05	0.57
VM 38	**5'AAT GGG AAA AGA AAG GGA AGC 5'TCG TGG CAT GCA GTG TCA G**	130–160	4	0.43	0.63	0.05	0.56
VM 39	5'GAT GGT TGT AAT GGG AGA GTC 5'AAA AGG ATG AAA TTA GGA GAG CA	170–230	7	0.55	0.63	0.07	0.59
VM 40	**5'TAT TAC GAG AGG CTA TTT ATT GCA 5'CTC TAA CAC CTC AAG TTA GTG ATC**	180–210	4	0.47	0.60	0.03	0.53
VM 68	5'CAA GGC ATG GAA AGA AGT AAG AT 5'TCG AAG CAA CAA ATG GTC ACA C	270–310	5	0.52	0.61	0.07	0.54
VM 70	**5'AAA ATC GGG GAA GGA AAC C 5'GAA GGC AAA ATA CAT GGA GTC AC**	260–310	6	0.51	0.64	0.06	0.59
MEAN			6.31	0.46	0.66	0.08	0.60

NB: H_0 = Observed Heterozygosity; PIC = Polymorphic information content

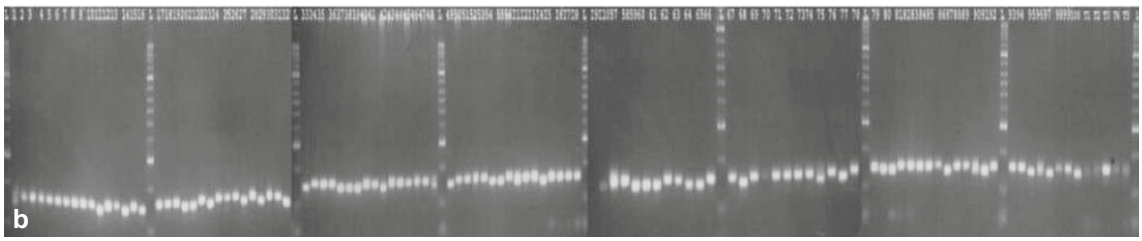
cowpea accessions (Fig. 2 a–d). On average, single *Cicer* and LTR primers yielded 8 to 14 polymorphic bands, with the highest and the smallest number of bands obtained with the Cicer/Cicer combinations (14 bands) and 3′ LTR/3′ LTR (8 bands), respectively (Table 3). The product sizes ranged from 100 bp to about 2.5 kbp as shown in Fig. 2 (a–d). The REMAP analysis was performed with 8 primer combinations, generating multiple fragments of defined sizes from the genomic DNA of all cowpea accessions. A total of 114 (94.2%) highly polymorphic bands were obtained (Table 3) which were reproducible. The primer combinations that amplified the highest and lowest number of bands were Cicer/BT-CTG (18 bands) and TY-2R/BT-CTG (10 bands), respectively. The product sizes ranged from 100 bp to about 3 kbp as shown in Fig. 3 (a–d).

Discriminating Capacity of the SSR, IRAP and REMAP Markers

The polymorphic information content (PIC) calculated helped to assess the discriminating power of each of the markers used in the study. The PIC of SSR markers ranged from 0.53 for VM 19 and 40 to 0.74 for VM 28, with an average of 0.60. PIC values positively correlated ($r = 0.65$) with the number of amplified alleles per primer. The observed heterozygosity (H_0) calculated for each SSR locus ranged from 0.03 to 0.14 with the mean being 0.08 (Table 1). The lowest H_0 value was recorded for VM 40 while the highest value was for VM 36. These low observed heterozygosity values were significant since they tend to substantiate the homozygous nature of most of the accessions and the fact that cowpea is largely self-pollinated.



Amplified PCR products patterns for VM 31



Amplified PCR products patterns for VM 36

Fig. 1 PCR amplification products of microsatellite loci for VM 31 (**a**) and VM 36 (**b**) on genomic DNA of cowpea accessions. L = marker; 1, 2, 3, 4, ……. Represent the cowpea lines

Table 2 Summary of primers used for IRAP and REMAP analysis

Name	Retrotransposon source and orientation	Primer sequence	Accession position
LTR 6149	BARE-1 (Forward)	CTC GCT CGC CCA CTA CAT CAA CCG CGT TTA TT	Z17327 1993–2012
LTR 6150	BARE-1 (Reverse)	CTG GTT CGG CCC ATG TCT ATG TAT CCA CAC ATG TA	Z17327 418–439
3′ LTR	BARE-1 (Forward)	TGT TTC CCA TGC GAC GTT CCC CAA CA	Z17327 2112–2138
5′ LTR1	BARE-1 (Reverse)	TTG CCT CTA GGG CAT ATT TCC AAC A	Z17327 1–26
5′ LTR2	BARE-1 (Reverse)	ATC ATT GCC TCT AGG GCA TAA TTC	Z17327 314–338 7417–7441
SUKKULA	SUKKULA (Forward)	GAT AGG GTC GCA TCT TGG GCG TGA C	AY054376 4301–4326
NIKITA	Nikita (Forward)	CGC ATT TGT TCA AGC CTA AAC C	AY078073 AY078074 AY078075 1–22
CICER IRAP	CICER	ACT TTG GCW WAA AAG YCT CCG AGC C	147I5228 14715227 AJ411814.1 16–41, 58–83
BT-GAC	SSR-GAC [RICH]	GAG AGA GAG AGA GAG AGA C	Randomly designed
BT-CTG	SSR-CTG [RICH]	CTC TCT CTC TCT CTC TCT G	Randomly designed
MUSA Ty2R	SSR-MUSA	GCA TGT CGT CAN CAT ANA RC	Randomly designed

Nucleotide degeneracy: R = A + G; Y = C + T; W = A + T; N = A + G + C + T

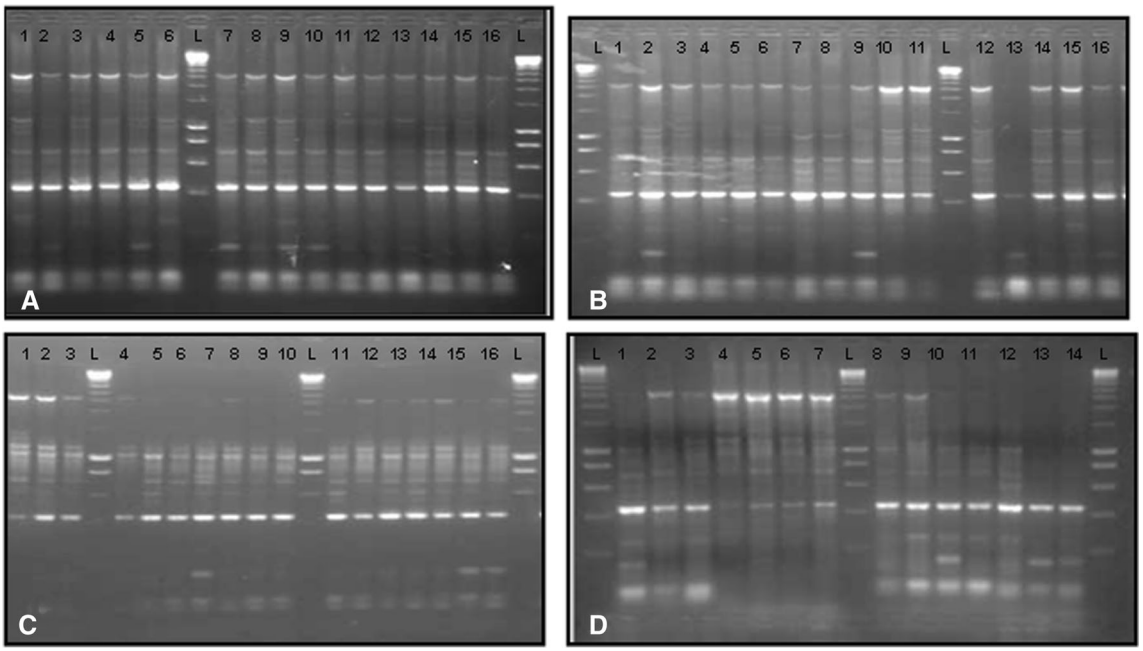


Fig. 2 **a**–**d** Polymorphism patterns from sixteen cowpea accessions by IRAP. L = marker; 1, 2, 3, ……16 represent cowpea lines. **a** Primer combination Cicer + Cicer, **b** Primer combination Nikita + LTR6149, **c** Primer combination Nikita + 3'LTR, **d** Primer combination Nikita + Nikita

Effectiveness of SSR, IRAP and REMAP Markers

The phylogenetic tree generated from the scores of SSR markers and calculated genetic distances (Fig. 4), generally, agreed with the nature of the lines used for the study. The cowpea varieties acquired from Leicester, the United Kingdom, clustered as an outgroup together with other accessions from IITA, Nigeria. Among the 61 accessions used to construct the phylogenetic tree, there were no strong significant groupings, indicating that the diversity represented by these SSR marker alleles was widely distributed both geographically and across the taxa. However, broad groupings, mostly with similarities in both locality and taxon, were evident in the trees. The phylogenetic tree generated from the scores of IRAP (Fig. 5) markers also showed the same pattern as shown by the SSR tree by separating the Leicester cowpea outgroup from the accessions from Ghana and Nigeria, generally. Thus, the results obtained reflected the trend and effectiveness of the three markers used for the study.

Table 3 Details of IRAP and REMAP primer combinations with various degrees of Polymorphisms within the Cowpea accessions

IRAP		REMAP	
Primer combination	Polymorphic bands	Primer combination	Polymorphic bands
Cicer + Cicer	14	Cicer + BT-GAC	16
Nikita +3′ LTR	10	Cicer + BT-CTG	18
Nikita + LTR 6149	12	Cicer + Musa Ty2R	14
3′ LTR + 3′ LTR	8	Nikita + BT-GAC	14
Nikita + Nikita	12	Nikita + BT-CTG	16
3′ LTR + LTR 6149	10	Musa Ty2R + BT-GAC	10
-	-	Nikita + Musa Ty2R	12
-	-	Cicer + VM 35	14
Total no. of polymorphic bands	66	Total No. of Polymorphic bands	114
Mean	11.0		14.25

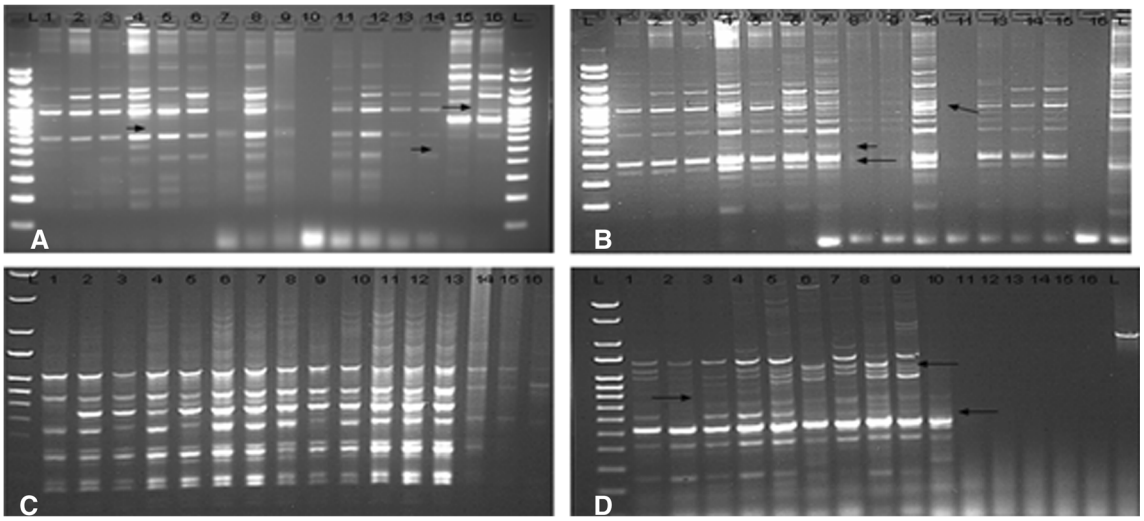


Fig. 3 Polymorphism patterns based on primer combination (**a**–**d**). **a** Primer combination Cicer + BT-GAC; **b** Primer combination Cicer + BT-CTG; **c** Primer combination TY-2R + BT- GAC; **d** Primer combination TY-2R + BT-CTG. The arrowed represents the unique bands identified for future analysis. L = marker; 1, 2, 3, ……16 represent cowpea lines

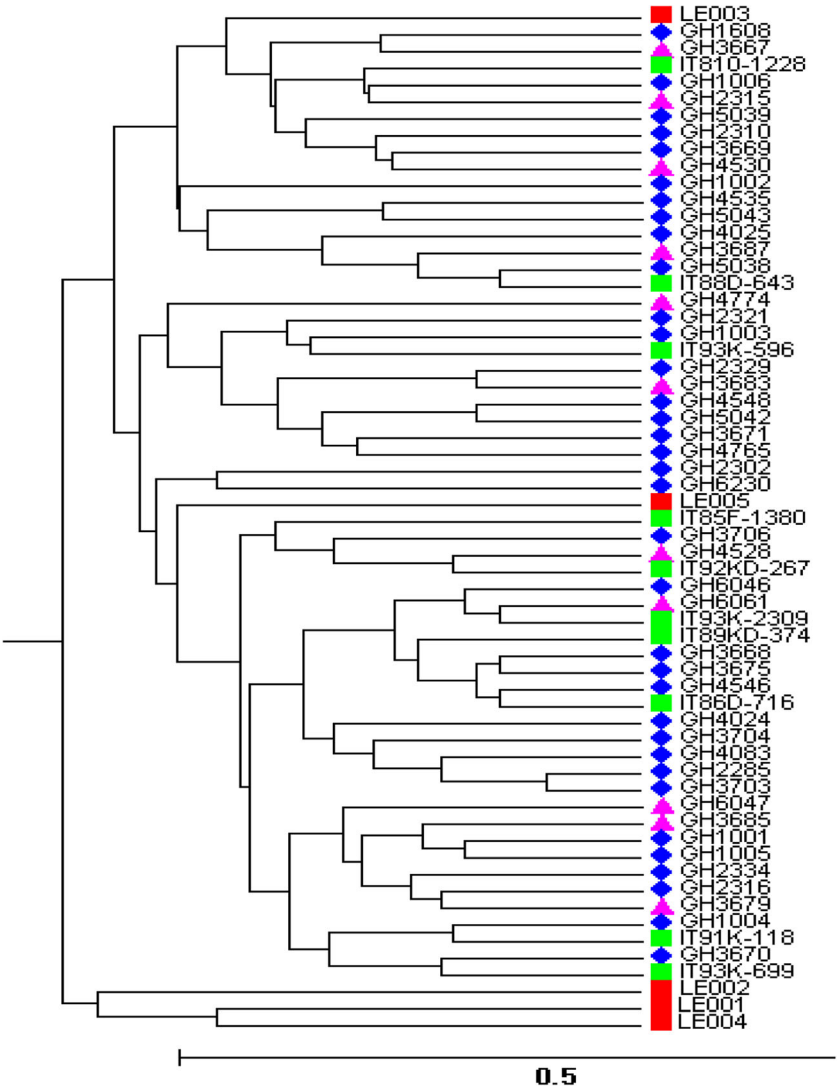


Fig. 4 Phylogenetic relationship among 61 cowpea lines revealed by cluster analysis (UPGMA) based on genetic similarity (Nei et al. 1998) using 16 microsatellite polymorphic primers. ♦= Drought Tolerant; ▲= Drought Susceptible; ■ = Leicester Out-group; ■ = IITA

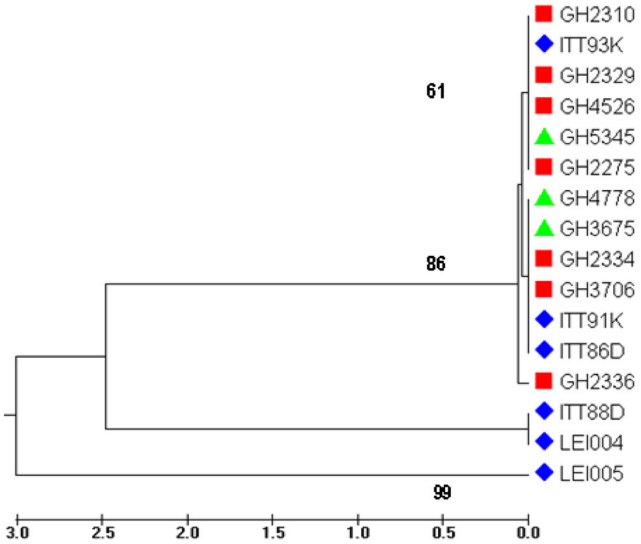


Fig. 5 Phylogenetic model of the IRAP data based on six primer combinations for 16 cowpea accessions using UPGMA clustering method. The percentage values for groups represent 1000 bootstrap cycles. ■ = Drought Tolerant; ▲ = Drought Susceptible; ♦ = IITA and Leicester out-groups

Discussion

Knowledge of genetic variation has important implications for the conservation of genetic resources and breeding programs. The relative genetic diversity can be estimated using various approaches including pedigree information, morphological and molecular markers. Morphological markers are influenced by prevailing environmental conditions (Otwe et al. 2011). DNA-based markers offer consistent results regardless of cropping conditions, type, and age of sampled tissue (Schulman et al. 2012). These characteristics of DNA-based markers make them suitable for cowpea research. In the present study the genetic relationships between 121 cowpea lines using multi-locus DNA markers, SSR, IRAP and REMAP has been determined.

Considering the patterns of diversity of the markers used for this study, the results showed that SSR markers were generally highly polymorphic. Microsatellite markers have shown a high level of polymorphism in many important crops, including rice (Islam et al. 2012; Sajib et al. 2012; Singh et al. 2016), maize (Molin et al. 2013; Salami et al. 2016), sorghum (Galyuon et al. 2016), soybean (Chauhan et al. 2015), common bean (Zargar et al. 2016), and tomato (Zhou et al. 2015). The SSR markers could distinguish cowpea accessions used in the study to a large extent. Twenty-two out of the thirty microsatellite primer pairs used in the study could successfully amplify DNA from the cowpea accessions (73.3%), and sixteen of these primer pairs were polymorphic (72.7%). SSR primers detected 4 to 13 alleles among 48 wild lines of cowpeas with an average of 7.5 alleles per primer (Li et al. 2001). Similarly, sixteen SSR primers generated a range of 5 to 12 allele fragments with an average of 8.2 alleles per primer combination among cowpea genotypes (Sawadogo et al. 2010). The results from our study were in agreement with these recent reports as the number of alleles ranged from 4 to 11 with an average number of alleles being 6.3 (Table 1). Similarly, 25 informative SSR primers were used to analyze Ghanaian cowpea germplasm and it yielded 1 to 6 alleles per primer pair with a mean of 3.8 alleles (Asare et al. 2010). The allele frequency for the 16 primers used for the study ranged from 0.34 to 0.57 with a mean frequency of 0.46. This also compares with the average allele frequency reported by Desalegne et al. (2016) of 0.47 and Doumbia et al. (2014) whose allele frequency ranged from 0.15 to 0.45 with a mean of 0.28. Thus, the level of microsatellite polymorphism in cowpea, although relatively high, is much lower than in other crops (Desalegne et al. 2016). One possible reason could be that the materials used in the study were mostly from the Ghanaian open market or directly from farmers and, thus, had a relatively narrow genetic base (Kuruma et al. 2008; Doumbia et al. 2014). Another possible reason for the low level of microsatellite polymorphism is that the cultivated cowpea is relatively low in genetic diversity compared with other crops (Xiong et al. 2016).

The results of our study also supported the fact that IRAP and REMAP techniques had the ability to detect high levels of polymorphism. The degree of polymorphism of the IRAP and REMAP products in cowpea was high (Table 3) which were similar to those observed in barley (Kalendar et al. 2011), olive (Ergun and Yilmaz-Gokdogan 2016) and rice (Yuzbasioglu et al. 2016). Retrotransposons can potentially integrate into either orientation, enabling the finding of members of a retrotransposon family as head-to-head, head-to-tail and tail-to-tail (Schulman 2007). As indicated in Table 3, all the primer combinations for IRAP gave quite a high degree of polymorphism with the least from the 3′ LTR/3′ LTR combination whose orientation was tail-to-tail. This may probably suggest that the integration level of copia-retrotransposons in tail-to-tail orientation in cowpea is lower than the other two orientations (head-to-tail and head-to-head), but needs further analysis using other combinations of primers.

Our results also gave some insight into the discriminatory potential of the three-multi locus markers used. Polymorphic information content (PIC) measures the discriminatory ability of a locus. Data reported by Kuruma et al. (2008) showed polymorphic information content (PIC) ranging between 0.09 and 0.87 with a mean of 0.34. The mean PIC value (0.60) of the SSR recorded in this study compared favourably with results obtained by Sajib et al. (2012), of mean PIC of 0.48 in some rice genotypes. The observed heterozygosity (H_0) calculated for the 16 SSR loci for our study also ranged from 0.03 to 0.14 with the mean of 0.08. The cowpea crop is known to be generally a self-pollinating plant, which these results seem to ascertain. It also probably indicates that the

accessions used could mostly be homozygous in nature. Genetic diversity of cultivated cowpea and its wild species have been extensively investigated in legume crops (Badiane et al. 2012; Abdollahi Mandoulakani et al. 2015). It has been suggested that cowpea was only domesticated once (Asare et al. 2010), unlike *P. vulgaris* (Singh et al. 2016) or rice (Sweeney and McCOUCH 2007). The low genetic diversity in cultivated cowpea, therefore, may be attributed again to its narrow genetic base. Studies in Azuki bean had demonstrated that genetic diversity was low and less within the cultigens as compared to their wild relatives, where the genetic diversity was high (Xu et al. 2008). The current SSR study has demonstrated that microsatellite markers might be conserved among *Vigna* species; hence, could provide a simple approach to assaying the introduction of such genetic material.

The results of the cluster analysis of cowpea accessions in both the SSR and IRAP techniques (Figs. 2 and 5) were indicative of the fact that the markers selected for the study were efficient in identifying the genetic variability within the collection. In the microsatellite phylogenetic tree (Fig. 2), sixteen Ghanaian accessions were observed to have clustered below a branch point at the lower part of the tree and all these accessions have been determined previously to be drought-tolerant (Otwe et al. 2011). Above this group of clusters were 11 drought-susceptible and 18 drought-tolerant Ghanaian accessions. Although the bootstrap values of these branch points were low and not conventionally significant, the results of the cluster analysis were notable in showing that a group of accessions defined by several branch points was all drought-tolerant, while other accessions included a mixture of susceptible and tolerant genotypes. Within the top half of the tree, both tolerant and susceptible lines were observed, but there was little structure evident as some of the most closely related pairs of accessions included both susceptible and drought-tolerant lines. Similarly, the phylogenetic model of the IRAP data, based on six primer combinations for 16 cowpea accessions (Fig. 5), showed interesting clustering results, indicating very low genetic distances between the groups and non-significant bootstrap values. Both the SSR phylogenetic and IRAP trees could cluster most of the Leicester lines as the outgroup and, therefore, were different from the African lines. However, the Leicester lines in both situations did not represent much diversity. It is obvious from our study and as reported by other researchers (Biswas et al. 2010; Abdollahi Mandoulakani et al. 2015) that measured relative genetic distances among the studied lines, as well as the techniques used, failed to correlate with the source and drought tolerant nature of the lines. The results obtained with the use of IRAP and REMAP techniques have proven to be generally reliable molecular markers with great potential to be used in genome assessments for fingerprinting, mapping and diversity studies. Therefore, multi-locus markers of cowpea could be used in germplasm conservation and analysis, not only for breeding lines and cultivars but also for the wild cowpea species and other *Vigna* species. In addition, these multi-locus markers could be used for comparative genome analysis between the different *Vigna* species.

Conclusion

In this paper we have presented data to support a brief glimpse into the ability of SSR, IRAP and REMAP marker techniques to establish the pattern of diversity, their discriminating capacity and their general effectiveness in determining structure. We could not establish significant associations between the molecular markers, though the results obtained have established a substantial pattern of diversity, some discriminating capacity and the general effectiveness of SSR, IRAP and REMAP marker techniques in determining molecular diversity in the Ghanaian cowpea. We believe that this type of research has contributed in shaping our focus on diversity studies in cowpea. Work is in progress to increase the element specific primer combinations, which have been designed from cowpea genome and inter-primer binding sequences (iPBS). The multi-locus PCR-based markers have potential to be an effective tool for diversity analysis in cowpea, which may be useful in identifying promising candidates for interspecific hybridization programmes and marker-assisted selection (MAS).

Materials and Methods

Plant Material

Plant material consisted 106 cowpea [*Vigna unguiculata* L. (Walp)] genotypes collected from all agro-ecological regions of Ghana, 10 genotypes from Nigeria and 5 genotypes from the United Kingdom were used for the study. For the molecular diversity analysis, fresh young leaves of 20-day-old seedlings were harvested, wrapped in aluminium foil and flash-frozen in liquid nitrogen and the genomic DNA extracted from these young leaves following a modified cetyltrimethylammonium bromide (CTAB) method (Gale et al. 2001). The quality and the concentration of the DNA were determined using a spectrophotometer and electrophoresis in a 2.0% (*w*/*v*) agarose gel.

SSR Primers and Reactions

Thirty SSR primer pairs that previously showed clear polymorphisms in cowpea (Li et al. 2001) were used in this study. SSR assays were performed according to (Colebatch et al. 2002) with minor modifications. The reactions were performed using a Touchdown amplification procedure, depending upon the annealing temperature™ of the primers used.

The general amplification cycle consisted of 18 cycles of 94 °C for 1 min (denaturing) and 72 °C for 1 min (extension). The annealing temperatures (30 s) were progressively decreased by 0.5 °C every cycle from 64 to 55 °C. The PCR reaction then continued for 30 additional cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The reaction ended with a 10-min extension at 72 °C. Amplification products were initially separated on 2% agarose gel in 1× TAE buffer stained with 0.5 μg/ml ethidium bromide and those found to be polymorphic were subsequently separated on 6% denaturing Polyacrylamide gels, and the oligonucleotide products visualized by silver-staining.

IRAP Primers and Reactions

The IRAP amplification reaction was performed according to the protocol described by (Kalendar et al. 1999). The primer sequences, retrotransposon source, and orientation are shown in Table 2. An additional degenerate IRAP primer, designed from a multiple sequence alignment of chickpea (*Cicer*) retrotransposon sources, was also used and is shown in Table 2. The inter-retroelement amplified polymorphism (IRAP) PCR was performed in a 25 μl reaction mixture containing 50 ng DNA, 10X PCR buffer (Promega cat. No. M1861), 2 mM $MgCl_2$, 5 pmol of each primer, 200 μM dNTP mix, 1 U Taq polymerase (Promega, cat. No.1861). The PCR programme consisted of 95 °C for 2 min for initial denaturation followed by 30 cycles of 95 °C for 1 min, annealing at a specified T_a depending on the specific primer combination used for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. The PCR products were then electrophoresed on 3% agarose gel (*w*/*v*) and the bands detected by 0.5 μg/ml ethidium bromide staining.

REMAP Primers and Reactions

The primers used for the IRAP amplification were combined with SSR primers (BT-GAC, BT-CTG, and Musa Ty2R) producing eight LTR-SSR primer combinations, as shown in Table 2, in the REMAP experiments. REMAP amplifications were performed in a final volume of 25 μl, containing 50 ng DNA, 10× PCR buffer (Bioline/York Bio), 2.5 mM $Mgcl_2$, 0.25 μM dNTPs (Bioline), 0.4 μM of each primer and 0.5 U of Taq polymerase (Bioline/York Bio) in a T-Gradient Thermocycler (Biometra, Goettingen) 96-well plate. The amplification programme consisted of an initial denaturation cycle at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 52 °C for 2 min, and 72 °C for 2 min for denaturation, annealing, and extension, respectively. A final extension step was performed at 72 °C for 10 min. The amplification products were separated on 3% agarose gel stained with ethidium bromide.

SSR Data Scoring and Analysis

DNA fragments for the SSR markers were scored visually both from the agarose gel as well as from the scanned images. For each gel, the distance travelled by each marker size of the DNA ladder was measured using a ruler in Adobe Photoshop Elements 2.0. The PowerMarker software package version 3.25 (Liu and Muse 2005) was used to analyse the data obtained and to calculate similarity coefficients among the genotypes. For clarity purposes, a phylogenetic tree was constructed from the similarity coefficient distance matrix of 61 randomly selected cowpea lines using the UPGMA method, instead of the 121 cowpea lines. To investigate the discriminatory power of each SSR primer, the polymorphic information content (PIC) was calculated. The observed heterozygosity (H_0) for each primer set was also obtained.

IRAP and REMAP Data Scoring and Analysis

The amplified fragments for both IRAP and REMAP were scored independently as 1 and 0 for presence and absence at each position, respectively, and the obtained binary data were used for the analysis. The genetic similarity between individual pairs of genotypes was analysed by using the MEGA version 4 (Tamura et al. 2004). The average similarity for all genotype pairs was used as a cut-off value for defining the clusters. The statistical stability of the clusters was also estimated by a bootstrap analysis with 1000 replications, using the MEGA version 4 (Tamura et al. 2004).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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