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Studies on Molecular Variation in Commercially Cultivated Groundnuts (*Arachis hypogaea* L.) Using SSR Markers

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Abstract:
Groundnut is an important cash and food crop cultivated throughout the tropics and sub-tropics. In Ghana, the crop is mostly cultivated in the three northern regions. Genetic variation is very important for improvement of the crop. Molecular variation in most commercially cultivated groundnut in most countries including Ghana is not well documented. This study was conducted to find molecular diversity among cultivated groundnut using SSR markers for further improvement. All 13 SSR markers were polymorphic with 76.9% of them showing PIC values above 0.50. Clustering analysis showed genetic similarities from 60% to 90% across the 20 genotypes. Molecular variation therefore exists in the commercially cultivated groundnuts. The 13 markers will be very useful in groundnut improvement programmes in Ghana and elsewhere. Early maturing genotypes 55-437, Shitaochi, Kumawu and Konkoma can be crossed with distantly related late maturing genotypes ICG7878, GK7, Nkatiesari, Otuhia and Summnut22 for further studies.

Keywords: *Diversity, groundnut, molecular, subspecies*

1. Introduction

Groundnut (*Arachis hypogaea* L.) is a major legume crop produced in most countries including Ghana. Ghana produced about 465,103 tonnes of unshelled groundnuts on about 356,780 ha of land (FAOSTAT, 2013). There are two subspecies of cultivated groundnut, viz. *Arachis hypogaea* subspecies *hypogaea* and *Arachis hypogaea* subspecies *fastigiata* (Knauft and Gorbet, 1989). Generally, the *fastigiata* subspecies have erect stems, sequential branching and flowering habit, and bear flowers on the main stem while the hypogaea subspecies are prostate (spreading), have alternate branching and flowering pattern, bear no flowers on the main stem and have darker green leaves (Krapovickas and Gregory, 1994). The crop is a very rich source of plant protein, unsaturated oil, minerals and vitamins (Asibuo et al., 2008). It also fixes atmospheric nitrogen to contribute to soil fertility improvement (Dupriez and DeLeener, 1988) and used as animal feed. Unfortunately, yields of groundnut in Ghana and many developing countries are very low partly due to unimproved cultivars. Variation is the basis for crop improvement. Dwivedi et al., (2001) maintained that knowledge about genetic diversity is very useful for organization of germplasm for conservation, identification of cultivars, selection of ideal parents for hybridization and for predicting favourable heterotic combinations in crop improvement programmes. Diversity in groundnut can be studied using molecular, chemical and morphological means (Dwivedi et al., 2001). Unfortunately, diversity in cultivated groundnut is generally low (He and Prakash, 2001; Selvarajet al. 2009). Young et al. (1996) reported that the low level of variation in groundnut is as a result of its origin from a single polyploidization event that took place on an evolutionary time scale. Morphological classification of groundnut germplasm has been found to be unreliable in most cases due to high environmental influence and need to be complemented with molecular variation. Identification of variation at the DNA level is described as more reliable (Shoba et al., 2010). Many genetic markers viz. RFLP (Halward et al., 1991), AFLP (He and Prakash, 2001), RAPDs (Dwivedi et al.,2001), ISSRs (Raina et al. 2001) and more recently SSRs (Mace et al., 2006; Asibuo et al., 2009; Shoba et al., 2010) have been employed to find genetic variation among cultivated genotypes.

Unfortunately, molecular variation in most released groundnut varieties in most countries including Ghana is not well documented. This study therefore aims at identifying molecular diversity among commercially cultivated groundnuts for further improvement and conservation.

2. Materials and Methods

2.1. Genetic Materials

Eighteen commercially cultivated groundnut varieties (9 each of *hypogaea* and *fastigiata* subspecies) collected from the gene bank of Crop Research Institute of Council for Scientific and Industrial Research (CRI-CSIR), Fumesua-Kumasi, Ghana and additional two varieties (1each of *hypogaea* and *fastigiata* subspecies) from ICRISAT, Niger were used for the study. Information about the varieties is provided in Table 1.

2.2. Plant Genomic DNA Extraction

Genomic DNA of twenty cultivated genotypes of groundnut was extracted from newly expanded leaves using the CTAB method modified by Takrama (2000) in the biotechnology laboratory of the Department of Crop and Soil Sciences, Faculty of Agriculture, Kwame Nkrumah University of Science and Technology, Kumasi. About 20mg of fresh leaf sample was grinded in 2.0ml microtubes to fine powder with liquid Nitrogen. Eight hundred microliters (800µl) of 2% CTAB with 0.1 % of mercaptoethanol was added. The sample was incubated in a sand bath at 65ºC for 30min with intermittent vortexing. The sample was cooled at room temperature and equal volume (800 µl) of chloroform isoamyl alcohol (24:1) was added. It was mixed by several inversions of the tube and centrifuged at 14000rpm for 15min.The aqueous phase of the sample was transferred into a clean 1.5ml tube. Nucleic acids were precipitated by adding two thirds volume of ice cold isopropanol (400µl) and shaken gently and stored at -20°C overnight. It was centrifuged at 14000 rpm for 5min to pellet nucleic acids. The isopropanol was decanted and pellet washed with 500µl of washing buffer on a rocking surface for 15min and centrifuged at 6000rpm for 4min. The washing buffer was decanted and pellet washed in 400µl (80%) ethanol and then centrifuged at 6000rpm for 4min. The ethanol was decanted and pellet dried at 37°C for 10 min. DNA was suspended in 50µl 1X TE buffer and centrifuged at high speed for 30sec to remove all insolubles. The quality of the DNA was checked using 0.8% Agarose gel electrophoresis and bands compared to a DNA standard.

Variety	Sub-species	Days to maturity	Average No. of seeds/pod	Source
1CG7878	*hypogaea*	120	2	ICRISAT, Niger
55-437	*fastigiata*	90	2	ICRISAT, Niger
Obolo (ICGV 97049)	*fastagiata*	105-110	2	CSIR-CRI, Ghana
Oboshie (ICGV 98412)	*fastagiata*	105-110	2	CSIR-CRI, Ghana
Yenyawoso (ICGX SM 87057)	*fastagiata*	90	2	CSIR-CRI, Ghana
Bremawuo	*fastagiata*	90	3	CSIR-CRI, Ghana
Kumawu	*fastagiata*	90	2	CSIR-CRI, Ghana
Konkoma	*fastagiata*	90	3	CSIR-CRI, Ghana
Jenkaar	*hypogaea*	110-120	2	CSIR-CRI, Ghana
Adepa	*hypogaea*	110-120	2	CSIR-CRI, Ghana
Nkosour	*hypogaea*	110-120	2	CSIR-CRI, Ghana
Azivivi	*hypogaea*	110	2	CSIR-CRI, Ghana
Shitaochi	*fastagiata*	86-90	2	CSIR-CRI, Ghana
Nkatekokoo	*fastagiata*	86-90	3	CSIR-CRI, Ghana
Behenase	*fastagiata*	90	3	CSIR-CRI, Ghana
Manipinta	*hypogaea*	110-120	2	CSIR-CRI, Ghana
Otuhia (ICGV 88709)	*hypogaea*	110-115	2	CSIR-CRI, Ghana
GK7	*hypogaea*	110-120	2	CSIR-CRI, Ghana
Nkatiesari (SARGV 88002)	*hypogaea*	110	2	CSIR-CRI, Ghana
Sumnut22	*hypogaea*	110-120	2	CSIR-CRI, Ghana

Table 1: Characteristics and sources of groundnut genotypes used for the study

2.3. Polymerase Chain Reaction and Electrophoresis

DNA of each genotype was primed using selected polymorphic SSR marker primers described by Shoba et al. (2012) and Mace et al. (2006). The primers were purchased from Metabion International AG, Germany. Information on the markers used is provided in Table 2.

The polymerase chain reaction mixtures (20µl) were made up of template DNA (2.0µl), PCR water (6.4µl),KAPA 3G buffer (10.0µl), Primer (F&R) (1.2µl), DNA Taq polymerase (0.16µl), PCR Enhancer (0.2µl), $MgCl_2$ (0.04µl). Amplification was performed in 0.2ml (each tube) thin walled PCR plates (96wells/plate) in a thermal cycler (Applied Biosystems). The PCR cycling parameters are summarized in table 3. KAPA loading dye was added to PCR products before electrophoresis. The amplified products were analyzed

using 2% Agarose gel electrophoresis. KAPA universal ladder was used to assess the base pair difference between the DNA of twenty genotypes of groundnut. Electrophoresis was carried out at a constant power of 120V for 2 hours. DNA bands were photographed under uv-light (Trans-illuminator).

SSR primers	Forward sequence (5'-3')	Reverse sequence (5'-3')	TA(°C)
PM 384	GGCGTGCCAATAGAGGTTTA	TGAAAACCAACAAGTTTAGTCTCTCT	55
pPGPseq5D5	AAAAGAAAGACCTTCCCCGA	GCAGGTAATCTGCCGTGATT	52
PM 375	CGGCAACAGTTTTGATGGTT	GAAAAATATGCCGCCGTTG	55
PMc588	CCATTTTGGACCCCTCAAAT	TGAGCAATAGTGACCTTGCATT	60
pPGPseq2B10	AATGCATGAGCTTCCATCAA	AACCCCATCTTAAAATCTTACCAA	51
pPGPseq2F5	TGACCAAAGTGATGAAGGGA	AAGTTGTTTGTACATCTGTCATCG	51
pPGSseq13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG	52
pPGSseq17F6	CGTCGGATTTATCTGCCAGT	AGTAGGGGCAAGGGTTGATG	52
PM137	AACCAATTCAACAAACCCAGT	GAAGATGGATGAAAACGATG	50
Ah4-26	TGGAATCTATTGCTCATCGGCTCTG	CTCACCCATCATCATCGTCACATT	60
PM343	AGAAACGAGGAGCTCGACAA	GCTCATTTTGATGGAATGAGAG	52
PM377	ACGCTCACATGTTTGCTTTG	GCTCGATTTGATTTGGGTGA	58
PM3	GAAAGAAATTATACACTCCAATTATGC	CGGCATGACAGCTCTATGTT	55

Table 2: Sequences and annealing temperatures of polymorphic SSR markers used
TA=Annealing Temperature

Step	Temperature	Duration
1. Initial Denaturation	95^0C	5mins
2. Denaturation	95^0C	6 sec
3. Annealing	51^0C-55^0C (Table 2)	5 sec
4. Extension	72^0C	20 sec
5. Final Extension	72^0C	20 sec
Total Cycles	40 cycles	

Table 3: PCR cycling parameters

2.4. Analysis of Molecular Diversity

DNA bands were scored for power marker analysis. Summary statistics of each SSR marker was calculated using power marker software (3.25). Each DNA amplicon size was also considered as a unique characteristic and scored as present (1) or absent (0) for cluster analysis. A dendogram which illustrates genetic relationship among the cultivated groundnut varieties was constructed using Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm clustering using SM similarity coefficient in NTSYS software (2.2). Sequential and Hierarchial Nested (SAHN) option was employed (Rohlf, 2000).

3. Results and Discussion

Summary statistics of the thirteen SSR markers used is presented in Table 4. Allelic frequency revealed by the markers across the twenty genotypes of groundnut ranged from 0.23 to 0.84 with an average of 0.46. Primer pPGPseq2B10 andpPGPseq17F6 had the lowest allelic frequency and PM137 the highest. However, primer pPGPseq17F6 recorded the highest gene diversity and polymorphic information content (PIC) values whiles PM137 recorded the lowest. PMc588 had the highest heterozygosity value and PM377 the lowest. Most (76.9%) of the SSR primers had PIC values above 0.50.

Marker	Allele Frequency	Allele No.	Gene Diversity	Heterozygosity	PIC
pPGPseq2F5	0.28	9.00	0.82	0.75	0.79
pPGPseq2B10	0.23	10.00	0.86	0.80	0.84
pPGPseq13A7	0.39	3.00	0.65	0.05	0.58
pPGPseq17F6	0.23	12.00	0.89	0.95	0.88
PM384	0.59	4.00	0.57	0.06	0.51
PM375	0.25	6.00	0.80	0.64	0.77
pPGPseq5D5	0.45	6.00	0.70	0.80	0.66
PMc588	0.50	3.00	0.63	1.00	0.55
PM137	0.84	2.00	0.26	0.31	0.23
Ah4-26	0.77	3.00	0.37	0.47	0.32

PM343	0.44	4.00	0.65	0.89	0.59
PM377	0.69	3.00	0.46	0.00	0.40
PM3	0.32	5.00	0.73	0.82	0.68
Mean	0.46	5.38	0.65	0.58	0.60

Table 4: Allele frequency, allele number, gene diversity, heterozygosity and PIC values of SSR markers

Genetic similarity values range from 60% to 90%. Clustering analysis put the genotypes into one group at 60% similarity. They were however put into six well separated groups at 75% similarity (Fig. 1). The first cluster comprises of three genotypes: ICG7878, Oboshie and Obolo. Oboshie and Obolo are *fastigiata* varieties released in Ghana for commercial production in 2012 whiles genotype ICG7878 is a *hypogaea* subspecies (a leaf spot resistant variety) selected directly from germplasm collections and released in Mali in 2002 as 'WaliyarTiga' (ICRISAT, 2012). The second cluster is made up of five *fastigiata* genotypes *viz.* 55-437, Yenyawoso, Bremawuo, Kumawu and Konkoma. All genotypes are commercially cultivated in Ghana except 55-437 which is a selection from a population from South America and collected from ICRISAT, Niamey, Niger. The third cluster consists of four *hypogaea* varieties grown in Ghana: Jenkaar, Nkosour, Azivivi and Adepa. Within this cluster Nkosour and Azivivi were grouped separately at 90% similarity. The fourth cluster is made of only Manipinta, a *hypogaea* subspecies with variegated seed coat. Fifth cluster contains four Ghanaian commercially cultivated *hypogaea* varieties: Otuhia, GK7, Nkatiesari and Sumnut22. GK7 and Nkatiesari were separately grouped at 90% similarity within this cluster. Finally, the sixth cluster is made up of three *fastigiata* varieties Shitaochi, Nkatekokoo and Behenase. Nkatekokoo and Behenase have red seed coat and are grouped in this cluster. DNA banding profiles of primers pPGPseq17F6 and pPGPseq2F5 showing genetic diversity across the twenty groundnut genotypes are presented in Figure 2 and 3.

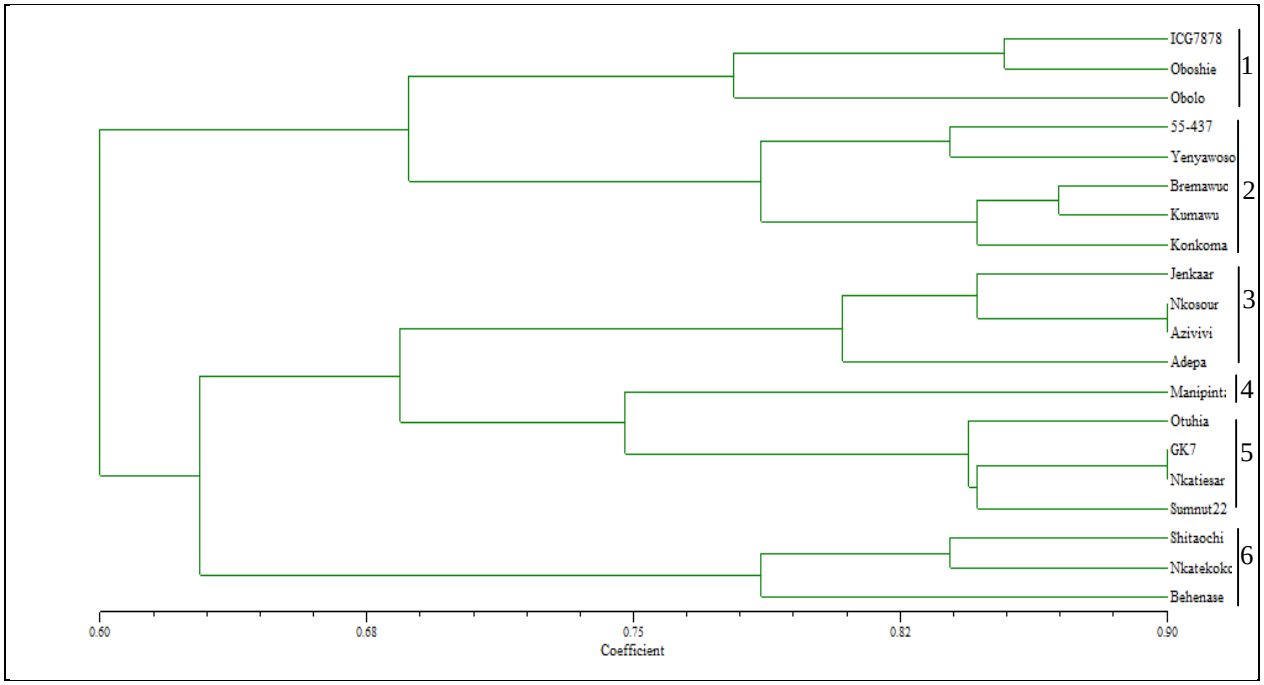

Figure1: A dendogram showing genetic relationship among twenty genotypes of groundnut based on SM coefficient

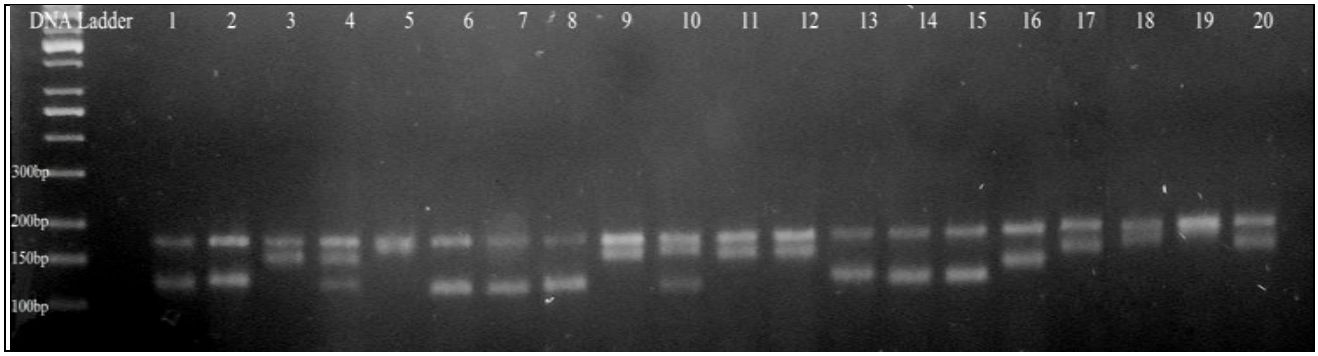

Figure 2: Primer pPGseq17F6 showing genetic diversity in groundnut

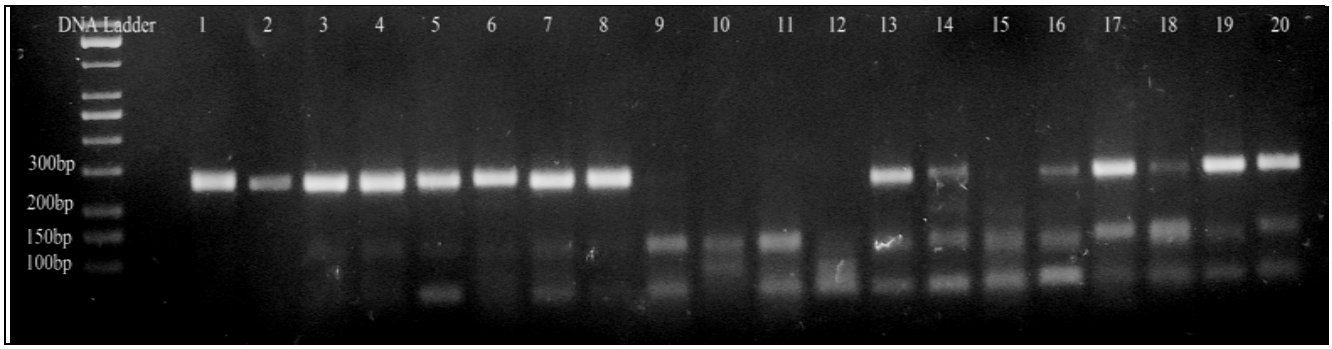

Figure 3: Primer pPGseq2F5 showing genetic diversity in groundnut

Note: 1-ICG7878, 2-55-437, 3-Obolo, 4-Oboshie, 5-Yenyawoso, 6-Bremawuo, 7-Kumawu, 8-Konkoma, 9-Jenkaar, 10-Adepa, 11-Nkosour, 12-Azivivi, 13-Shitaochi, 14-Nkatekokoo, 15-Behenase, 16-Manipinta, 17-Otuhia, 18-GK7, 19-Nkatiesari, 20-Sumnut22

Availability of genetic variation is very vital for crop improvement (Dwivedi et al. 2001). Markers with high PIC values are described as highly polymorphic and thus detect higher level of genetic variation in an organism. Hildebrand et al. (1992) asserted that PIC value is an important primary data which determines informativeness of a genetic marker and that a PIC value of 0.70 and above is highly informative whiles a value of 0.44 is moderately informative. PIC is a statistic that measures the usefulness of a genetic marker for linkage analysis (Elston, 2005; Shete et al., 2000). Therefore, a greater proportion (76.9%) of the markers used in this study have moderate to high informativeness for linkage analysis in groundnut. The considerable high level of polymorphism revealed by these SSR markers for groundnut could be attributed to the use of pre-screened markers. Similarly, high polymorphic percent of 87.5 % and 100% has been reported for primers PM 384 and PM 375 respectively (Tang et al., 2007).

Different levels of polymorphism exist in cultivated groundnut (Dwivedi et al. 2001; Mace et al. 2006; Shoba et al. 2010). Low level of genetic diversity in the groundnut gene pool compared to other crops has been reported (He and Prakash, 2001; Selvaraj et al. 2009). However, SSRs have been able to detect a relatively higher level of variation (Mace et al., 2006). Mace et al. (2006) found up to 56% diversity in cultivated groundnut with SSR markers. Shoba et al. (2010) reported 0.54 to 1.00 genetic similarities in groundnut. Cluster analysis in this study showed that most *hypogaea* subspecies were separated from *fastigiata* ones at the molecular level. This indicates that common genes confer morphological similarities in groundnut. Similarly, Asibuo et al. (2009) reported that cluster analysis located lines in the assigned specific botanical groups of groundnut which conformed to available morphological classification. However, botanical grouping had earlier been reported as poor indicator of genetic diversity (Mace et al., 2006).

Clustering of some varieties with similar reactions to leaf spot disease into the same group in this study possibly suggest a common gene controlling resistance in these genotypes. In a related genetic diversity studies, Dwivedi et al. (2001) found genotypes that reacted similarly to leaf spot disease in the same cluster. Bera et al. (2014) recently reported clustering of groundnut genotypes with the same reaction to groundnut bud necrosis disease into one group.

The genetic dissimilarity (10% to 40%) found in this study shows that molecular variation exists among the commercially cultivated groundnut. The varieties can therefore be further improved. The thirteen SSR markers will be very useful in further molecular studies in commercially cultivated groundnut. Early maturing but leaf spot susceptible genotypes 55-437 (from Niger, ICRISAT), Shitaochi, Kumawu and Konkoma (landraces from Ghana) can be crossed with distantly related late maturing but leaf spot resistant genotypes ICG7878, GK7, Nkatiesari, Otuhia and Summnut22.

4. Acknowledgement

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5. References

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