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STUDIES ON THE NUTRITIONAL QUALITY, INHERITANCE OF FRESH SEED DORMANCY, MICROSATELLITES DIVERSITY AND CONSTRUCTION OF GENETIC LINKAGE MAP OF CULTIVATED GROUNDNUT (*Arachis hypogaea* L.).

Thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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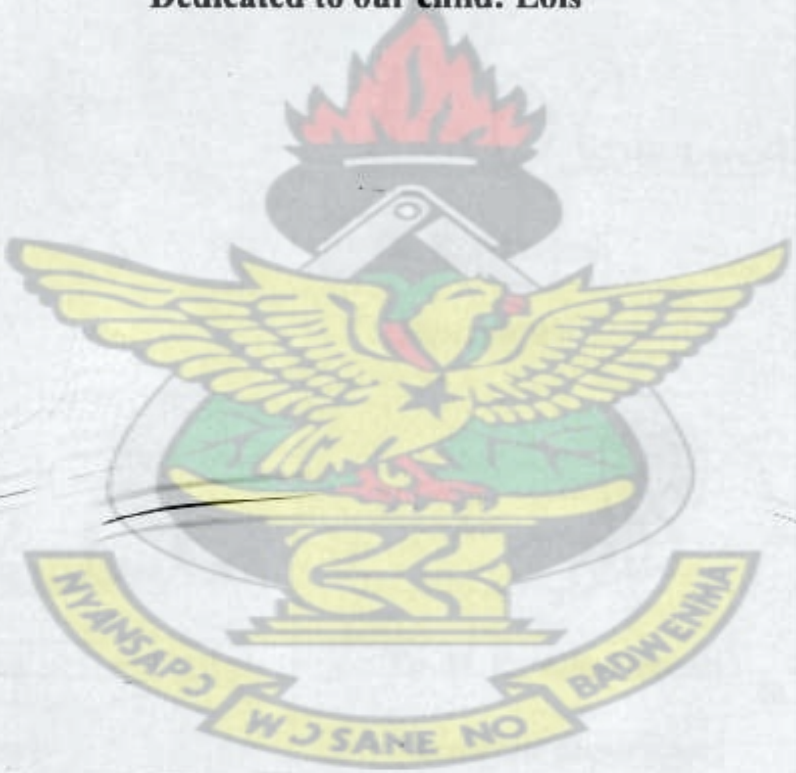
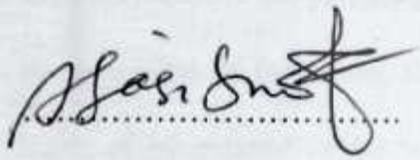
Dedicated to our child: Lois



DECLARATION

This thesis is a presentation of my original research work and it has not been submitted anywhere for any award. Wherever contributions of others are involved, they have been acknowledged.



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Abstract

The aims of this study were to investigate the nutritional quality of groundnut landraces from Ghana; to find additional evidence on the genetics of fresh seed dormancy in the cultivated groundnut; develop microsatellite markers and use them for diversity studies and construction of genetic linkage map of cultivated groundnut. There was significant differences in nutritional qualities among the 20 accessions. Two of the accessions had high oil contents above 54% and 2 accessions oleic/linoleic acid ratio above 3.4, which is an indication of their long shelf life. Fresh seed dormancy studies established that, the mode of inheritance of the trait is monogenic, that is, a single dominant gene should be present for the trait to be expressed. The development of microsatellite markers through the enrichment procedure resulted in the development of 3 polymorphic primers. The diversity studies using 22 botanical varieties of groundnut revealed some level of polymorphism, with the highest number of alleles being 5. The dendogram drawn after scoring the bands generated by the primers clustered the accessions into their respective botanical groups as reflected in the morphological classification. The low level of polymorphism at the DNA level in cultivated groundnut greatly reduced the informativeness of the genetic linkage map that was generated from a cross between 2 cultivated groundnut genotypes with a map distance of 669.5 cM. Only 24 out of the over 600 primers tested were polymorphic between the two parental lines and only 5 primers could be scored from PCR products run on polyacrylamide gel and subsequently used for map construction. Efforts should be directed towards broadening the genetic base of the cultivated groundnut.

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LISTS OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
ai	active ingredient
AT	adenine thymine
BC_1	backcross F_1
Bp	base pair
BF	boron fluoride
^oC	degrees celsius
Ca	calcium
CGIAR	Consultative Group on International Agricultural Research
CSIR	Council for Scientific and Industrial Research
Cu	copper
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotidetriphosphate
EDTA	ethylene diamine tetraacetic acid
EST	expressed sequenced tags
FAME	fatty acid methyl esters
FAO	Food and Agriculture Organization
F_1	first filial generation
F_2	second filial generation
Fe	iron
GC	gas chromatograph
GA	guanine adenine

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GT	guanine thymine
*Hae*III	*Haemophilus aegptus*III
IARCs	International Agriculture Research Centres
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IITA	International Institute of Tropical Agriculture
L	linoleic
LB	luria broth
LOD	logarithm of the odds (to the base 10)
M	molar
MAS	marker-assisted selection
Mg	magnesium
mM	millimolar
mg	milligram
$MgCl_2$	magnesium chloride
Mn	manganese
Na	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
O	oleic
O/L	oleic/linoleic
PAC	pre-harvest aflatoxin contamination
PCR	polymerase chain reaction

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pH	hydrogen ion concentration
PM	peanut microsatellites
Pmol	picomolar
Ppm	parts per million
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
r^2	co-efficient of determination
RFLP	restriction fragment length polymorphism
RILS	recombinant inbred lines
*Rsa*I	*Rhodopseudomonas sphaeroides*
S	saturated
SDS	sodium dodecyl sulphate
SSC	saline-sodium citrate
SSR	simple sequence repeat
TBE	tris-borate-EDTA
TE	Tris- ethylene diamine tetraacetic acid
TSF	total saturated fatty acids Polyunsaturated P
TLCF	total long chain saturated fatty acids
TSWV	tomato spotted wilt virus
χ^2	chi-square
µg	microgram
µl	microlitre
USA	United States of America

KNUST

v/v	volume/volume
w/v	weight/volume
Zn	zinc

KNUST

TABLE OF CONTENTS

KNUST

KNUST

KNUST

KNUST

KNUST

List of Tables

KNUST

KNUST

KNUST

List of figures

KNUST

List of plates

KNUST

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Origin

Groundnut (*Arachis hypogaea* L.), an annual leguminous plant, is one of the world's major oilseed and protein-rich crops. The crop is believed to have originated in the area of Southern Bolivia to Northern Argentina in South America (Stalker and Simpson, 1995). Not much is known about the domestication of groundnut, but the earliest historical evidence is found in the tombs of Peru (Hammons, 1982). However, Gregory *et al.* (1980) contend that, groundnut domestication must have started far away from Peru since none of the related wild species occur there, and the centre of diversity of the genus *Arachis* include parts of Western Brazil, Bolivia, Paraguay, and Northern Argentina. It is widely believed that the Portuguese carried groundnut to Africa from Brazil during the sixteenth century and the Spanish to the Philippines from where it spread to other countries in Asia (Purseglove, 1968). According to Hammons (1982), groundnut reached North America from Africa through the slave trade route between 1707 and 1725.

1.2 Economic and nutritional importance

Groundnut is unique because the plant and its products have a wide range of uses in the daily life of humans, livestock, industry as well as soil fertility regeneration. The seed is utilised in various forms including roasted, boiled, raw, ground or paste. Roasting groundnut with salt is a very common practice throughout the world. Slightly over half of the groundnut production is crushed into oil for human consumption or industrial uses

(FAO, 2004). Approximately one-third of world production is used in the confectionery products (FAO, 2004). The plant helps to enrich the soil and the vines serve as excellent fodder for cattle. The nuts, in addition to being the source of edible oil, are useful in soups, stews and paste.

From a nutritional standpoint, groundnut contains many essential vitamins and minerals necessary for good health. Groundnut seed contains 44 to 56% oil and 22 to 30% protein on a dry seed basis and is a rich source of minerals (phosphorus, calcium, magnesium, and potassium) and vitamins (E, K, and B group) (Savage and Keenan, 1994). Groundnut oil contains about 80% unsaturated fatty acids (Ahmed and Young, 1982) and are beneficial in lowering blood cholesterol levels. The objective of the study was to determine the variability in nutritional composition of groundnut varieties in Ghana

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1.3 Inheritance of fresh seed dormancy

There have been few studies on the inheritance of fresh seed dormancy in groundnut. These studies have drawn contradictory conclusions. Lin and Lin (1971) reported monogenic control, whereas John *et al.* (1948) and Nautiyal *et al.* (1994) indicated that the character may be quantitatively inherited. Lin and Lin (1971) reported complete dominance of dormant over non-dormant seed, whereas Ramachandran *et al.* (1967) observed partial dominance. Khalfaoui (1991), concluded that dormancy is a quantitatively inherited trait and additive, dominance and digenic epistasis effects were involved in its genetic control. Few fastigiates have been developed with fresh seed dormancy (Upadhyaya *et al.*, 1997). This study sought to determine the genetics of fresh seed dormancy in groundnut.

1.4 Development of groundnut microsatellites

Groundnut is unique because the numerous diversity exhibited by the genotypes at the various morphological, physiological and agronomic traits are not reflected at the DNA level. The paucity of polymorphism in groundnut at the molecular level has led to genetic studies in the crop lagging behind compared with the progress made in other crops.

Recent studies using novel DNA techniques like amplified fragment length polymorphism (AFLP) and microsatellites (or simple sequence repeats (SSRs)) have revealed differences between groundnut genotypes (He and Prakash, 1997; Hopkins *et al.*, 1999).

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Microsatellites are short tandem repeats (1-6 bases). They have been found in both prokaryotes and eukaryotes. They are: abundant, evenly distributed throughout the genome, co-dominant, highly reproducible, highly polymorphic within and between species and easy to assay (Hopkins *et al.*, 1999).

SSR markers have been and are being successfully utilized for many applications in crop genetics and improvement such as gene tagging, marker-assisted selection (MAS), cultivar identification, pedigree verification, genetic diversity and evolutionary studies (Rafalski and Tingey, 1993).

Identification of highly polymorphic markers in groundnut would ensure initiation of studies on mapping, gene cloning and marker-assisted selection. The availability of large numbers of molecular markers is a prerequisite for identifying informative markers for genetic analysis. Few informative microsatellites markers are available for groundnut, because development of SSRs is expensive, labour intensive and time consuming (He *et al.*, 2003). Barriers to gene flow from related diploid species to domesticated groundnut

as a consequence of the polyploidization event (Young *et al.*, 1996), combined with self-pollination (Halward *et al.*, 1991) and use of few elite breeding lines and little exotic germplasm in breeding programmes have resulted in a narrow genetic base (Knauft and Gorbet, 1989; Isleib and Wynne, 1992). The objective of the study was to develop groundnut SSR markers and determine their variability, utility in genome analysis and ability to distinguish between genotypes.

1.5 Use of microsatellites in diversity studies

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Domestication of crop plants led to the selection for desirable plants leading to the extinction of genotypes which were selected against. The rejected genotypes may have some desirable traits which might not be obvious at the present. Selection for specific traits has rendered crop plants more vulnerable to diseases and insect attack and destroying the potential for sustained genetic improvement over a long term (Harlan, 1987). Destruction of habitat has also led to complete loss of plant diversity, species, gene and allelic diversity. To reduce the rapid loss of plant diversity, germplasm collection centres and gene banks were established through the effort of national and international organisations (Consultative Group on International Agricultural Research, 1985).

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) holds more than 14,000 groundnut accessions while United States Department of Agriculture has more than 8000 accessions in gene banks. The large number of accessions in the gene banks makes thorough evaluation and identification of desirable trait difficult. Consequently, the numerous accessions in the gene banks have not been evaluated and

utilized in cultivar development. Only a few established cultivars and elite breeding lines have been utilised in breeding programmes. To overcome the difficulty in evaluating the entire accessions in gene banks, the concept of core collection has been developed to ensure effective evaluation.

A core collection is a subset of accessions from the entire collection and represents most of the available genetic diversity of the species and should be about 10% of the entire collection (Brown, 1989). A core collection for the *A. hypogaea* germplasm has been developed to enhance the utilization of the entire collection. The International Crops Research Institute for the Semi-Arid tropics (ICRISAT) developed a core collection of 1704 from over 14,000 accessions based on morphologic, geographic and taxonomic descriptors (Upadhyaya *et al.*, 2002). Holbrook *et al.* (1993) developed a groundnut core collection of 831 accessions from a total of 7432 US groundnut accessions based on country of origin and measurements of morphological characteristics like plant type, pod type, seed size, testa colour, number of seeds per pod, and average seed weight. A major benefit of having a groundnut core collection has been a great increase in peanut germplasm evaluation work. This has resulted in the identification of numerous sources of resistance to several economically significant pathogens. Few molecular tools have been used to evaluate the diversity of US groundnut core collection. The study determined the usefulness of SSRs in diversity studies among 22 genotypes, representing six botanical varieties from the US core collection.

KNUST

1.6 Construction of genetic linkage map of groundnut

A genetic linkage map of groundnut is important for use in genetic studies and breeding program\mmes geared towards the improvement of the crop. Due to limited number of polymorphism in groundnut, the first genetic map was developed using an F_2 population from a cross between two diploid species in the section *Arachis* (*A. stenosperma* and *A. cardenesii*) (Halward *et al.* 1993). The second genetic map of groundnut was constructed using backcross population (BC_1) of a cross between a synthetic amphidiploid (*A. batizocoi* k9484 X (*A. cardenesii* GKP10017 X *A.doigoi* GKP 10602) and a cultivated groundnut, Florunner. The short fall of these maps are that, recent molecular (Kochert *et al.*, 1996) and cytogenic (Raina and Mukai, 1999) studies have revealed that the diploid species used were unlikely to be the ancestors of groundnut. Also the maps were based on RFLP which has not detected much polymorphism in the cultivated groundnut. Even though these maps are important reference point, they do not wholly reflect the genome of groundnut since the species used are not possible progenitors of groundnut. The availability of markers which can detect polymorphism in cultivated groundnut in recent years calls for the construction of a genetic map of the crop based on a cross between two cultivated groundnut genotypes.

Microsatellites are simple sequence tandem repeats of di-, tri- tetra- or pentanucleotides. They originate from unequal crossing-over or replication errors resulting in the formation of unusual DNA secondary structures such as, hairpins or slipped strands (Pearson and Sinden, 1998). Microsatellites have become one of the most popular molecular markers used with applications in many fields. High polymorphism, high abundance, co-dominant inheritance, ease of scoring and readily transferability are the major features that make

KNUST

microsatellites the most preferred molecular marker (Weber, 1990). They are present in both coding and non-coding regions of DNA and usually characterized by a high degree of length polymorphism (Panaud *et al.*, 1995; Morgante *et al.*, 2002). Simple sequence repeats SSR markers were therefore used in this study to provide mapped loci and identify DNA markers near to target genes for future map-based cloning.

The main objectives of this study were to:

1. determine variability in nutritional composition with respect to protein, oil, fatty acids and minerals,
2. determine the genetics of fresh seed dormancy in the cultivated groundnut,
3. determined the usefulness of SSRs in diversity studies among 22 genotypes, representing six botanical varieties from the US core collection.
4. develop groundnut SSR markers and determine their variability, utility in genome analysis and ability to distinguish between genotypes,
5. provide mapped loci and identify DNA markers near to target genes for future map-based cloning.

KNUST

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Botany

Groundnut is a member of the family *Leguminoseae* (also called *Fabaceae*), sub-family *Papillionaceae*, tribe *Aeschynomeneae*, sub-tribe *Stylosanthinae* and the genus *Arachis* (Holbrook and Stalker, 2003). The genus *Arachis* has been subdivided into 9 sections and 69 species based on morphology, geographic distribution and cross-compatibility (Krapovickas and Gregory, 1994). Most of the species in section *Arachis* are diploids. *Arachis monticola* Krapov. and Rig. and *Arachis hypogaea* L. are the only tetraploids in the genus and have the same chromosome number (2n=4x=40) and are cross-compatible to produce fertile hybrids (Pattee *et al.*, 1998). The diploid species in section *Arachis* can hybridise with the cultivated groundnut (Stalker and Moss, 1987; Stalker and Simpson, 1995), but due to ploidy differences, sterility and embryo abortion results (Pattee *et al.*, 1998).

The cultivated groundnut has been divided into two subspecies that are distinguished by branching pattern and distribution of reproductive nodes along the main and lateral branches. The subspecies *fastigiata* has four botanical varieties comprising *fastigiata*, *vulgaris*, *aequatoriana* and *peruviana*. They have sequential flowering; with the main axis and the lateral branches having flowers in all nodes. Subspecies *hypogaea* has two varieties: *hypogaea* and *hirsuta*; without flowers on the main axis and alternate branching of reproductive and vegetative lateral branches (Krapovickas and Gregory, 1994).

KNUST

Intermediates between the two subspecies exist due to crosses between the two subspecies. Groundnut is a herbaceous annual (about 30-50 cm high). Emergence is intermediate between the epigeal and hypogeal types; the hypocotyls elongate but usually stops before cotyledons emerge above ground. The plant may be erect or prostrate with a well developed taproot, lateral roots and nodules. The leaves are alternate, stipulate, pinnate with four leaflets. The flowers are bisexual, zygomorphic, complete and sessile. The bright yellow flowers are borne in the leaf axils below or above ground; one or more flowers may be present at each node and are usually abundant in the lower nodes. The first flowers appear at 4 to 6 weeks after planting depending on the cultivar and the prevailing temperature and continue over a long period due to the indeterminate nature. Naturally, groundnut is more than 99% self-pollinated crop, but Coffelt (1989) observed cross pollination of over 6% by the action of bees. Eight to 14 days after pollination, the ovary develops to form an elongated gynophore, which grows downward by geotropism into the soil to produce the pod. The pod containing 1 to 5 seeds matures in the soil after 7 to 9 weeks.

KNUST

2.2 World groundnut production

Groundnut is widely grown between latitude 40°N to 40°S. The plant originated from South America but is now grown throughout the tropics, sub-tropics and warm temperate areas in Asia, Africa, Oceania, North and South America and Europe. World production was 37.8 million tons from 23.6 million hectares in 2005 (FAO, 2006). China is the world-leading producer of groundnut with annual production of 15.3 million tons from 5.1 million hectares in 2002. India is the second largest producer of groundnut in the

world with annual production of 7.5 million tons from 8.0 million hectares in 2002. China and India accounted for about 60% of the world production of groundnut. Global production of the crop increased by 1.3 % per annum between 1979 and 1996 (FAO, 1999).

2.3 Nutritional quality of groundnut seed

The fatty acids are long hydrocarbon chains with a carboxyl group at one end of the chain and a methyl group at the other end. The hydrocarbon chain may have no double bond (saturated) or may have one or more double bonds (unsaturated).

KNUST

Monounsaturated fatty acids have one double bond between two carbon atoms and their oils tend to be liquid at room temperature, but become solid when refrigerated. These oils have better oxidation stability, as compared to polyunsaturated oils and therefore are better for overall health (Grundy, 1986). Olive oil, groundnut oil, mustard oil, rice bran oil, canola oil are classified as having high and desirable percentage of monounsaturated acids.

Polyunsaturated fatty acids have two or more double bonds. As there is a bend at each double bond, these fatty acids do not pack together easily and tend to be liquid, even when cold. Polyunsaturated fatty acids are beneficial in lowering cholesterol levels. But these oils have poor oxidation stability and thus, food cooked in these oils does not have a long shelf life. Soybean, safflower, sunflower, corn, cotton oil are all polyunsaturated.

Saturated fatty acids are chains of carbon atoms that have hydrogen filling in every bond. Because of their straight configuration, saturated fatty acids pack together easily and tend

to be solid at room temperature. Palm oil and coconut oil are classified as saturated fats, as they contain a preponderance of saturated fatty acids.

Since fatty acids constitute a major proportion of the weight of groundnut oil (Worthington and Hammons, 1971), the chemical and physical properties of the oil tend to depend to a large extend on the fatty acids in the seed. Twelve fatty acids have been reported in groundnut, but only three are present in amounts exceeding 5%. Oleic (O), linoleic (L), and palmitic fatty acids, together, account for about 90% of the total fat in peanut seed (Ahmed and Young 1982; Dwivedi *et al.*, 1993). The remaining nine fatty acids account for about 10%, each ranging in concentration from 0.02 to 3.59% (Norden *et al.*, 1987).

Fatty acid composition is an important attribute of quality in edible oils. Oil stability, nutritional value and quality are all dependent on the relative proportions of saturated and unsaturated fatty acids that constitute the oil. Generally, Oleic (18:1), with 18 carbon atoms and one double bond and linoleic (18:2), with 18 carbon atoms and two double bonds account for about 80% of the total fat in groundnut seed (Dwivedi *et al.*, 1993). Nutritionally, high linoleic acid content is desirable because it is an essential fatty acid, lowers plasma cholesterol level and lipoprotein and reduces the risk of coronary heart disease and atherogenesis (Jackson *et al.*, 1978). However, polyunsaturated fatty acids are unstable and susceptible to oxidative rancidity and have short shelf-life. Oxidation of the double bonds of fatty acids leads to the production of aldehydes, ketones and other hydrocarbons that cause odours and flavours commonly associated with rancidity. Therefore, oils with high polyunsaturated fatty acids (linoleic and linolenic acids), and especially linolenic acid, limit the value of an oil for cooking unless hydrogenated

KNUST

(Rakow and McGregor, 1973). Oils with high content of monounsaturated fatty acid (oleic acid) are less susceptible to oxidative changes during refining, storage and frying. Such oils can be heated to high temperatures without smoking, leading to faster cooking time and absorption of less oil (Miller *et al.*, 1987). Also, the quality of oil with high monounsaturated fatty acids is retained longer during storage than polyunsaturated fatty acids (Robertson and Thomas, 1976). Food industries and consumers interest in oil crops with high oleic acid and low contents of polyunsaturated fatty acids have increased. Groundnut seed with a high O/L ratio have long product stability and shelf-life (James and Young, 1983; Branch *et al.*, 1990). Large genetic variation for seed size, oil content and fatty acid composition have been reported in groundnut germplasm (Treadwell *et al.*, 1983; Norden *et al.*, 1987; Branch *et al.*, 1990; Dwivedi *et al.*, 1998).

KNUST

2.4 Inheritance of fresh seed dormancy

Groundnut belongs to the family *Fabaceae* (*Leguminosea*) and a member of the genus *Arachis*. The crop has been divided into two subspecies based on morphological characteristics (Krapovickas and Gregory, 1994). Subspecies *hypogaea*, is characterized by the main stem that never bears inflorescences, has lateral branches where two vegetative branches alternate with two inflorescences or reproductive branches regularly; plants have dark green leaves with a prostrate to spreading bunchy habit and are late maturing. Pods are typically two-seeded and seeds show marked dormancy when dry and fresh seeds do not suffer from vivipary when harvesting is delayed. Seeds possess seed dormancy ranging from 30 to 360 days (Gregory *et al.*, 1951; Zade *et al.*, 1986). Some researchers have indicated that, subspecies *hypogaea* resembles the wild types and that

subspecies *fastigiata* might have been derived from ssp. *hypogaea* progenitors (Kaprovickas, 1969). Runner cultivars of ssp *hypogaea* are more closely related to *A. monticola* morphologically than members of ssp *fastigiata* (Korchet *et al.*, 1996).

Subspecies *fastigiata* which includes the Spanish and Valencia market types are characterized by the main stem bearing flowers and without a regular pattern in the sequence of reproductive and vegetative branches; cultivars mature earlier; plants are lighter green with erect growth habit. Seeds lack dormancy when fresh and the pods are concentrated on or near the main stem. Spanish types usually have two-seeded pods. Valencia types typically have pods with 3-6 seeds, with concentration of fruit production on or near the main stem, thicker stems and considerably fewer secondary and tertiary branches than Spanish types. However, cultivars developed from crosses between the two subspecies have led to the loss of some sub specific distinct traits such as branching pattern. Few fastigiates have been developed with fresh seed dormancy (Upadhyaya *et al.*, 1997).

Subspecies *fastigiata* will continue to be grown in tropical countries despite its susceptibility to diseases and lack of fresh seed dormancy, because of their early maturity and their erect nature, which allow cultivars to fit into the cropping systems in most localities. Spanish types are predominantly grown in the semi arid zones of Africa and Asia where the growing season is short with erratic rainfall distribution and the crop is grown in multiple cropping systems (Upadhyaya and Nigam, 1999). Sprouting occurs in the ground if it rains prior to harvesting or harvesting is delayed after pod maturity (Plate 3.1). Sprouting of nuts also occur in the stack on the threshing floor. The sub arid tropics account for about 60% of the world's groundnut production area and the frequent losses

KNUST

due to sprouting can be substantial. Yield loss due to *in situ* germination in bunch varieties has been reported to be between 20-40% (Ramanathan, 1987, Reddy *et al.*, 1985, and Nagajun and Radder, 1983), and also affect seed quality and storability. In Ghana, farmers in the forest areas are compelled to harvest early (sometimes prematurely) and sell fresh and boiled in order to save seeds from germinating in the soil. This is typical in the Kwahu area in the Eastern Region where the rains prolong into the harvesting period.

KNUST

2.5 Causes of seed dormancy in groundnut

Seed dormancy in groundnut has been shown to be affected by several factors. Groundnut has indeterminate flowering pattern and therefore pods of the same plants vary in their maturity. Toole *et al.* (1964) observed that immature groundnut seeds have long dormancy period and the period of dormancy declined as maturity progresses. Removal of seed coat in groundnut has been found to improve seed germination. Toole *et al.* (1964) demonstrated that removal of seed coat resulted in the loss of seed dormancy. Patil (1967) confirmed the results when he found that removing of seed coat after 60 days of flowering slightly improve seed germination. Hammons (1973) however, contends that seed dormancy in groundnut is an inherent property of groundnut seed and does not depend on an impervious or protective seed coat.

Hormonal balance between abscisic acid, which acts as germination inhibitor, and ethylene, which acts as a germination activator, is produced by the embryo through the action of cytokinin during seed imbibition and the release of these chemicals is different for different genotypes (Ketring and Morgan 1971, 1972). Depending on the genetic

constitution, different seed parts (coat, cotyledon and embryo) have been reported to have a role in imparting dormancy (Nautiyal *et al.*, 1994).

2.6 Random amplified polymorphic DNA (RAPDs)

Many studies have utilized random amplified polymorphic DNAs (RAPDs) also known as arbitrarily primed polymerase chain reaction (PCR)) to study phylogeny and systematics in various plants (Welsh and McClelland, 1990; Ajmone-Marsan *et al.*, 1993; Demeke *et al.*, 1997; Asante and Offei, 2003). Such studies have shown that the analysis of RAPDs is useful in revealing systematic relation in plants (Silberstein *et al.*, 1999). Random amplified polymorphic DNAs analysis can be carried out on organisms for which there is little or no information on DNA sequences or genomic organization, thus making it possible to analyse DNA sequence variation for almost any organism as long as relatively pure DNA can be obtained. Short primers (about 10 nucleotides in length) of arbitrary nucleotide sequence are used to amplify segments of genomic DNA that are flanked by the annealed primers. If two individuals contain different DNA genomes, their arbitrarily primed PCR products should display differential banding patterns on agarose or polyacrylamide gels. Such differences can be used as a DNA fingerprint. RAPDs is fast, requires little DNA and is technically uncomplicated (Welsh and McClelland, 1990). Relatively little but high quality DNA is required because it is PCR-based. RAPDs are easy to assay and have low development cost. However, it suffers from reproducibility due to mismatch annealing (Neale and Harry, 1994) and as a dominant marker it cannot distinguish homozygous dominant from heterozygous. Previous work with RAPDs on groundnut did not reveal polymorphism (Halward *et al.*,

KNUST

1992, Stalker and Mozingo, 2001). However, RAPDs detected polymorphism among the wild *Arachis* species (Halward *et al.*, 1992).

2.7 Restriction fragment length polymorphisms (RFLPs)

Restriction fragment length polymorphisms (RFLPs) technique involves the digestion of organellar or nuclear DNA with restriction enzyme. The number and size of fragments is a reflection of the distribution of restriction sites in the DNA. The fragment produced is specific for each target DNA/restriction enzyme combination and can be used as a fingerprint specific for a given target DNA or organism. The digested DNA is fractionated on agarose gel through electrophoresis and transferred out of the gel onto a membrane filter by a process called Southern transfer (Southern, 1976, Livini *et al.*, 1992). Radioactive RFLP probes are then hybridized to the fragments. Location of restriction fragments homologous to the probes is determined by autoradiography, and RFLP data can be scored from developed film.

RFLP markers are highly effective in determining polymorphism and have been used for DNA fingerprint and mapping in many crops (Ajmone-Marsan *et al.*, 1998; Becker *et al.*, 1995). It is co-dominant and highly reproducible. There are several drawbacks to RFLPs that call for alternative marker systems. It requires large amounts of high quality DNA, because it is not PCR-based. The technique is technically demanding, not amenable to automation, high cost per analysis and problems associated with radioactive reagents. Kochert *et al.* (1991), using RFLP detected little or no polymorphism in cultivated groundnut but observed a great deal of polymorphism in diploid species in the genus *Arachis*.

2.8 Amplified fragment length polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a multilocus marker technique developed by Vos *et al.* (1995). The AFLP markers are obtained from genomic fragments detected after selective PCR amplification. The AFLP technique has been used to identify markers linked to disease resistance loci (Becker *et al.*, 1995), to fingerprint DNAs (Vos *et al.*, 1995, He and Prakash, 1997) and to assess relationships between molecular polymorphism and hybrid performance (Ajmone-Marsan *et al.*, 1998). It requires moderate quantity and quality of DNA, it is PCR based, highly reproducible and moderate development cost. The level of polymorphism found in *A. hypogaea* using AFLP was low when compared to other crops (He and Prakash, 1997; Gimenes *et al.*, 2002). Mackill *et al.* (1996), using 14 rice accessions observed 27.8% of the loci to be polymorphic. The percentage of polymorphism detected in groundnut using 6 genotypes from three botanical varieties of groundnut was 6.7 % (He and Prakash, 1997). Similar level of polymorphism was observed by Gimenes *et al.* (2002), when they used AFLP markers to determine the genetic relationships between groundnut accessions.

2.9 Microsatellites or simple sequence repeats

Microsatellite DNA sequences are short, tandem repeating DNA sequences comprising 1-6 base pairs. Microsatellites are found throughout the genome of eukaryotic organisms and are highly polymorphic in populations (Morgante *et al.*, 2002; Gur-Arie *et al.*, 2000) because of their ability for insertion-deletion (in-del) mutation of multiples of the repeating units during replication. Their co-dominant and locus specific nature make

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them ideal for paternity testing and genetic linkage map. The polymorphic nature has made microsatellites the marker of choice for genetic mapping, studying genomic instability in cancer, population genetics, forensic and conservation biology (Shinde *et al.*, 2003). Polymorphism is mainly due to allelic length variation, caused by differences in the number of repeat units between the alleles. A number of factors have been proposed for the variation of repeat units during DNA replication (Kunkel and Behenek, 2000). Detection of genetic variation at a genetic locus is observed by amplifying the alleles by the polymerase chain reaction (PCR) using specific primers flanking the repeating units and resolving it on a denaturing gel electrophoresis (Weber, 1990, Tautz, 1989). Sometimes instead of detecting single bands, which is the size of an allele, stutter or shadow bands are also observed. Various explanations have been given for the presence of stutter bands and how to reduce it have been proposed (Hauge and Litt, 1993, Murray *et al.*, 1993). Sequencing of PCR products has revealed that stutter bands occur due to a change in the repeating units due to slipped strand extension by *Taq* DNA polymerase (Shinde *et al.*, 2003). SSR markers have been and are being successfully utilized for many applications in crop genetics and improvement such as gene tagging, marker-assisted selection (MAS), cultivar identification, pedigree verification, genetic diversity and evolutionary studies. Several evidences have demonstrated that the distribution of SSRs in the genome is non-random. Many reports have demonstrated that a large number of SSRs are located in transcribed regions of genomes (Morgante *et al.*, 2002), however, repeat numbers and total length of SSRs in this region are relatively smaller (Kantety *et al.*, 2002; Thiel *et al.*, 2003). It has been reported that 10% of SSRs identified in primates (Jurka and Pethiyagoda 1995), 15% in rabbit (van Lith and van

Zutphen, 1996) are in the protein-rich genes. In cereals (maize, rice, sorghum, wheat and barley), 1.5-7.5% of expressed sequenced tags (ESTs) consist of SSRs (Kantety *et al.*, 2002; Thiel *et al.*, 2003). These ESTs have a range of functions such as metabolic enzymes, structural and storage proteins, disease signalling and transcription factors, suggesting some roles in plant metabolic and gene evolution (Li *et* al., 2004).

Compared with other markers (AFLPs and RAPDs), SSRs are co-dominant and therefore can distinguish homozygote from heterozygote. This attribute is particularly important in marker-assisted selection and linkage mapping. They are accessible to other research laboratories through published primer sequences (Saghai-Maroof *et al.*, 1994). Comparative studies using RAPDs, RFLPs, AFLPs and SSRs indicated that AFLPs and SSRs were highly reproducible between and within laboratories (Jones *et al.*, 1997; Rafalski and Tingey, 1993). Another study to determine the informativeness and applicability of RAPDs, RFLPs, AFLPs and SSRs in genetic diversity in inbred maize lines revealed that, the number of alleles detected by SSRs were higher in comparison to other methods (Pejic *et al.*, 1998). It has also been documented that when SSRs have been compared to other marker systems, they have revealed the highest level of polymorphism (Wu and Tanskley, 1993; Morgante *et al.*, 1995). Smith *et al.* (1997) also observed that, the average SSR carry two-fold more information than AFLPs and RAPDS, and 40% more than RFLP when the target is the number of alleles per locus. They however found AFLPs to simultaneously detect higher levels of polymorphism at several loci.

Hopkins *et al.* (1999) could only detect 6 polymorphic SSRs in cultivated groundnut when they used 26 primers. Out of 67 SSRs markers developed for *Arachis*, Moretzsohn *et al.* (2004) found only 3 to be polymorphic for cultivated peanut.

Recent work has demonstrated that simple sequence repeats are applicable to the fingerprinting of sub-species of groundnut and botanical varieties as well as mapping studies (Hopkins *et al.*, 1999; He *et al.*, 2003), and have the potential to be used in genetic diversity screening and evaluation of germplasm collections. Simple sequence repeats could provide plant breeders with sources of useful traits, optimized and facilitate the breeding processes. It has the potential to identify and remove duplicates and correct mislabelled accessions. Identification of DNA markers associated with the botanical varieties of groundnut would be useful in genotyping, germplasm management, genetic diversity and evolutionary studies.

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2.10 Utilization of groundnut core collection

The development of core collection has stimulated a great deal of interest in germplasm evaluation in groundnut. A core collection can extensively be evaluated and information derived from them can be applied to the whole collection. Anderson *et al.* (1996) identified 55 accessions with resistance to tomato spotted wilt virus (TSWV), when they screened the US groundnut core collection. Tomato spotted wilt virus is among the greatest yield-reducing viruses affecting groundnut and has recently become a major disease in US peanut-production areas (Culbreath *et al.*, 1999). Isleib *et al.* (1995) found 12 lines had less defoliation than the resistant check when they examined the core collection for early leaf spot (*Cercospora arachidicola* Hori) resistance.

Holbrook *et al.* (2000) examined all accessions in the peanut core collection for reaction to the peanut root-knot nematode (*Meloidogyne arenaria* (Neal) Chitwood race 1). Thirty-six core accessions showed a reduction in root galling, egg-mass rating, egg count per root system, and egg count per gram of root compared with Florunner, the resistant check. Use of the peanut core collection has also resulted in the observation of lines with resistance to pre-harvest aflatoxin contamination (PAC) in the US peanut germplasm collection (Holbrook, 1998).

It is becoming more and more evident that the techniques from molecular biology hold a promise of providing detailed information about the genetic structure of natural population, than what has been achieved in the past (Slatkin, 1987). Molecular markers like RFLP and a number of PCR-based markers are being used extensively for reconstructing phylogenies of various species. The techniques have been found to provide novel information regarding the relationship between closely related species and what sort of genetic variations are associated with species formation (Mohan *et al.*, 1997). Furthermore, these studies hold a great promise for revealing more about the pattern of genetic variation within species (Avise, 1994). Identification of DNA markers associated with the botanical varieties of groundnut would be useful in genotyping, germplasm management, genetic diversity and evolutionary studies.

Different marker based techniques such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), simple sequence repeats (SSRs) and others have been developed and applied to a whole array of crop species including groundnut (Welsh and McClelland, 1990; Becker *et al.*, 1996; Vos *et al.*, 1995; Morgante *et al.*, 2002).

2.11 Morphological markers versus molecular markers

Conventional plant breeding is time consuming and often dependent on environmental factors. Breeders depended on morphological markers for selection in the past, because they were the only markers available. However, most morphological markers have limitations because they have either dominance effects; exist in epistatic relationships, or have deleterious effects on the plant (Tanksley, 1983). In addition, morphological markers are limited in number, some appear late in the developmental stage of the crop, making early scoring impossible and some markers can affect other morphological markers due to pleiotropic gene action (Anderson and Lübberstedt, 2003). Hence, breeders are interested in new technologies that would reduce the time and enhance selection efficiency because many of the marker systems have large number of polymorphism; alternate alleles rarely have deleterious effects at the molecular level; they are often co-dominant, which allows all genotypes to be distinguished in each generation; they seldom segregate in epistatic ratios (Stalker and Mozingo, 2001).

Efficient mapping is defined by the choice of parents for crossing and the size of the population. How the cross is advanced, DNA marker used, the generations used for the DNA and phenotypic analyses are of utmost importance. All these factors can strongly affect the observed recombination rates, and thus, the genetic distances reported in differing populations.

2.12 Selection of parents

Parents selected for the cross should exhibit sufficient level of DNA sequence polymorphism. In the absence of polymorphism, segregation and linkage analysis is

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impossible (Young, 2000). In general, cross pollinated species tend to have high levels of DNA sequence variations (Helentjaris, 1987). Conversely, levels of DNA sequence variation are generally lower in naturally inbreeding species and finding suitable DNA polymorphism may be difficult (Miller and Tanksley, 1990). To overcome the difficulty associated with mapping inbreeding species, parents should be as distantly related as possible, which can be geographical or morphological and in some cases a cross between a cultivar and a related wild species (Young, 2000). In the light of narrow genetic base of cultivated groundnut, low detection of polymorphism with molecular markers and the limited success achieved with a variety of methods attempting to introgress germplasm from wild species into peanut breeding programme (Singh, 1986a, b; Simpson, 1991; Stalker, 1992), the first RFLP map of groundnut was constructed by Halward *et al.* (1993) using F_2 population derived from a cross between two diploid *Arachis* species (*A. stenosperma* and *A. cardenasii*). Due to limited polymorphism exhibited at the DNA level in groundnut, Burrow *et al.* (2001), introduced genetic variability in groundnut by crossing a synthetic amphidiploid from a cross between 3 diploid wild *Arachis* species and used as a donor parent to generate a backcross population of 78 progeny. From this population, a genetic map was constructed from 370 RFLP loci and mapped unto 11 linkage groups. Since the mapping population was derived from wild *Arachis* species, their use in detecting and mapping agronomic traits are limited to some extent and could not wholly represent the genome of the cultivated allotetraploid groundnut. Nevertheless, it possesses important information for revealing the organisation and evolution of the groundnut genome.

2.13 Choice of population

Different kinds of genetic populations can be used to construct genetic linkage maps. F_2 populations derived from selfing F_1 hybrids and backcross populations are the simplest and easy to construct for most plant species (Young, 2000). These populations require few seasons and generations to generate them and therefore saving time, effort and money. However, using F_2 and backcross populations have serious limitation; they are tentative and not permanent, since seeds derived from selfing these individual will not breed true and the same materials would not be available for continuous or cooperative research. This drawback can be addressed to a limited extent by cuttings where possible and the use of tissue culture. This calls for permanent resources for genetic mapping; for it is difficult or impossible to measure characters as part of quantitative trait locus (QTL) mapping in multi-locations and /or over several years with F_2 or backcross populations. Recombinant inbred lines (RILS) derived from F_2 plants through selfing for five or more generations offer an excellent alternative (Burr and Burr, 1991). These lines contain different combinations of DNA segments from the original parents. Recombinant inbred lines overcome the drawback of F_2 and backcross populations because they are homogeneous and abundant and can therefore be used from year to year and in several locations without changes in the genetic constitution of the individual plants. Generation of recombinant inbred lines however, takes several years and it's therefore time-consuming, expensive and completely out-crossing species are much more difficult to map with RI lines because of problems associated with selfing. Doubled haploids can also be used for linkage mapping with several of the advantages observed in RI lines (Huen *et al.*, 1991).

2.14 Population size

The size of the mapping population depends on the goal and is of utmost importance. Since the resolution of the map and the ability to determine the marker order depend to a large extent on population size. Population size may be limited by how much seeds are available, but larger seed numbers offer a better mapping population. According to Young (2000), population size less than 50 individuals generally provide too little mapping resolution to be useful. For high resolution mapping in specific genomic regions and mapping quantitative trait loci (QTL), much larger populations are required. Messeguer *et al.* (1991), constructed a high resolution map around the *Mi* gene of tomato by analysing over 1000 F_2 plants. Alpert and Tanksley (1996), examined more than 3,400 plants to obtain a detailed map around a fruit weight locus of tomato. Menz *et al.* (2002) observed greater resolution and more loci mapped at high logarithm of the odds (to the base 10) (LOD) threshold with higher number of RILS in sorghum as compared to smaller F_2 population.

2.15 Molecular markers

RFLP maps have been constructed for various plants and animals. But according to Tanksley *et al.* (1992), most of the RFLP linkage maps constructed have been of low or moderate density. Additionally, the multiplicity of RFLP loci can make linkage map constructed with RFLP markers ambiguous with respect to locus identity (Cregan *et al.*, 1999), and therefore calling for the definition of a RFLP locus not only by probe and enzyme being used, but also the molecular weight of the segregating fragment(s). Even though RFLP maps are useful tools for many genetic studies, they have inherent

limitations that can be addressed by the development of high-density maps, which have closely spaced marker intervals throughout the genome. Halward *et al.* (1992) observed that, the dominant behaviour of RAPD markers limit their use in the construction of genetic linkage maps due to the difficulty in differentiating the heterozygous individual from the homozygous individual with certainty.

Molecular map of groundnut will help in the understanding and genetic basis and evolution of various morphological traits present in the genotypes and the improvement of the crop. Mapping efforts in groundnut have lagged behind the advances made in other crops because of limited number of polymorphic markers developed. The purpose of this study was to provide mapped loci and identify DNA markers near to target genes for future map-based cloning using F_2 population of cultivated groundnut.

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CHAPTER THREE

3.0 VARIABILITY IN NUTRITIONAL QUALITY OF GROUNDNUT (*Arachis hypogaea* L.) CULTIVARS GROWN IN GHANA

3.1 Introduction

Groundnut is a major annual oilseed crop with annual world production of 37.8 million tons from 23.6 million hectares in 2005 (FAO, 2006). It is estimated that about two-thirds of the total groundnut production was crushed for oil and the remaining one-third utilized in confectionery products during the 1990s (Dwivedi *et al.*, 1993). There has been a gradual shift from using groundnut as oil and meal towards confectionery products (Freeman *et al.*, 1999). FAO (2000) estimated that, about 53% of world groundnut production was used as oil, 32% for confectionery products and the remaining 15% for feed and seed production. The global demand for groundnut oil increased from 2.8 to 4.3 million tons between 1979-81 and 1994-96, even though international groundnut prices were increasing (Freeman *et al.*, 1999). They attributed the increase in demand to population growth, growth in per capita income and urbanization, which calls for greater demand for convenient foods.

The seed of most groundnut cultivars contain about 50% oil (Worthington and Hammons, 1971), and therefore the quality of the oil and groundnut products depend, to a large extend, on the oil fraction. The oil content of groundnut differs in quantity, the relative proportion of fatty acids, geographical location, season and growing conditions (Brown *et al.*, 1975, Holaday and Pearson, 1974 and Young *et al.*, 1974).

Twelve fatty acids have been reported in groundnut, but eight major fatty acids constitute 98% of fatty acids in groundnut (Tai, 1972, and Worthington and Holley, 1967).

Differences in the fatty acid composition have been attributed to several factors. There are wide differences in fatty acid composition due to genotype (Fore *et al* 1953; Holaday and Pearson, 1974 and Worthington *et al.*, 1972) and the level of maturity of the seed (Young *et al.*, 1968). Sound mature kernels have higher proportion of fatty acids than immature and shrivel seeds (Brown *et al.*, 1975). Holaday and Pearson (1974), reported that, geographical area of production had significant effect on fatty acid content. They also found significant interaction between genotype and location, location and year and indicated that temperature after pegging could be the most important factor that affects oil composition. Variation in fatty acid content has also been found due to year, season and location (Norden *et al.*, 1987; Holley and Hammons1968; Worthington *et al.*, 1972). Oleic and linoleic acids constitute approximately 80% of the total fatty acid composition of groundnut oil (Ahmed and Young, 1982). Oleic (O), linoleic (L), and palmitic fatty acids, together, account for about 90% of the total fat in peanut seed (Ahmed and Young 1982; Dwivedi *et al.*, 1993).The variability between oleic acid in groundnut generally ranged from 36% to 67% and linoleic acid from 15% to 43% (Treadwell *et al.*, 1983). However, Norden *et al.* (1987) identified a groundnut line with 80% oleic and 2% linoleic acids. This line has since been used in several breeding programmes to transfer the high oleic acid characteristics to groundnut cultivars to improve their quality and to study the genes responsible for the high oleic acid content. Earlier studies indicated high oleic acid to be controlled by two recessive genes in groundnut (Moore and Knauft, 1989; Knauft *et al.*, 1993). This is at variance with what Tai (1972), found in soybean, which is controlled by the maternal genotype. Due to the high proportion of oleic and linoleic

acids in groundnut seed, the chemistry and quality of groundnut oil depend on their relative proportions.

Oils with high content of oleic acid are less susceptible to oxidative changes during refining, storage and frying. Such oils can be heated to high temperatures without smoking, resulting in faster cooking time and absorption of less oil (Miller *et al.*, 1987). Nutritionally, a high content of linoleic acid is preferable because it is an essential fatty acid and produces hypocholesterolemic effect (Jackson *et al.*, 1978). Polyunsaturated oils have been known to lower total blood cholesterol and low density lipo-protein levels. Lower levels of these substances reduces the risk of coronary heart disease and atherogenesis (The Surgeon General's Report on Nutrition on Health, 1988). However, a study by Grundy (1986), showed that human diets containing high levels of monounsaturated fatty acids were as effective in lowering serum cholesterol levels as low-fat diets.

Iodine value, which represents the number of grams of iodine absorbed by 100 g of fat is an indication of the quantity of unsaturated fatty acids present in a fat and has been used to predict the shelf life of fats and oils (James and Young, 1983). High values depict high content of polyunsaturated fatty acid in the product. The shelf life or stability of groundnut oil is measured by the number of days before the onset of oxidative rancidity, a process that involves the whole groundnut seed, groundnut oil or groundnut product by exposure to heat and air (Fore *et al.*, 1953; Picket and Holley, 1951; Worthington and Hammons, 1971).

Oxygen reacts with the double bonds of unsaturated fatty acids to form products characterized by undesirable flavour and odour. Oxidation of the carbon double bonds

results in the formation of acids, aldehydes, ketones and other hydrocarbons thus producing unpleasant odours and flavours associated with rancidity and lower the quality of the finished product. Linoleic acid which has two double bonds is more susceptible to oxidative rancidity than Oleic acid, a monounsaturated acid and the saturated fatty acids (Fore *et al.*, 1953, Mozingo and Steele, 1982).

The ratio of oleic to linoleic acid (O/L ratio) is also a measure of oil stability (Worthington *et al.*, 1972; Young and Waller, 1972; Worthington and Hammons, 1977). Values of O/L ratio greater than 1.0 are preferred in general commerce. However, Sanders *et al.* (1992) indicated that values less than 1 have been found in world trade. The O/L ratio has been found to be quantitatively inherited (Mason and Matlock 1967; Khan *et al.*, 1974, and Tai and Young, 1975).

Reddy (1988), has indicated that the seed of groundnut contains 25% to 32% protein and the cake (the residue after oil extraction) 46-60% protein. Other workers have found groundnut seeds to contain approximately 12.0-36.4% protein (Hoffpauir, 1953; Sekhon *et al.*, 1970, Derise *et al.*, 1974; Woodroof, 1983). Groundnut protein is increasingly becoming important as food and feed, especially in developing countries, where protein from animal sources are not within the means of the majority of the populace. The seed has several uses as whole seed or processed to make peanut butter, oil, and other products. The cake also has several uses in feed and infant food formulations.

The mineral elements in 100g of defatted flour contains 92-200 mg Ca, 10-343 mg Mg, 1.4-33.3 mg Na, 1150-1450 mg K, 1.5-10.9 mg Fe, 0.6-5.2 mg Cu, 4.7-7.0 mg Zn and 3.1-6.1 mg Mn (Oke, 1967; Derise *et al.*, 1974, Galvaro *et al.*, 1976; Khalil and Chughatai, 1983). Groundnut thus provides considerable amounts of mineral elements to

supplement the dietary requirements of humans and farm animals. Derise *et al.* (1974) noted have that roasting groundnut do not lead to reduction in the levels of mineral elements but rather increases the levels since volatile compounds are lost through heating, except sodium where a small reduction was observed.

Sugars in groundnut seed play important role as precursors in the production of the typical roasted groundnut flavour. Seed sugars provide a source of carbon for the production of flavour compounds (Kochler *et al.*, 1969). Sucrose upon hydrolysis produce fructose and glucose, these products are reducing sugars which upon heating can react with some specific amino acids to form flavour components (Grimm *et al.*, 1996). The flavour of roasted groundnut plays an important role in its acceptance by consumers and other users. Flavour also plays an important role in the acceptability of groundnut products, such as peanut butter. Newell *et al.* (1967) have noted that free amino acids and free sugars are important precursors of groundnut flavour.

Starch is the major carbohydrate found in seeds and stored in two related forms, amylose and amylopectin (Bewley and Black, 1994). Bewley and Black (1994) have indicated that hydrolysis of starch yields free sugars and dextrins which are translocated to the growing axis. Groundnut seeds contain 9.5 to 19.0% total carbohydrates as both soluble and insoluble carbohydrates (Crocker and Barton, 1957, Rao *et al.*, 1965; Oke, 1967, Abdel Rahman, 1982; Woodroof, 1983)

The chemical composition of groundnut seeds has been evaluated in relation to protein level (Young and Hammons, 1973), amino acid composition (Young *et al.*, 1973) and fatty acid composition (Grosso and Guznam, 1993) in some countries. With increasing consumer preference for high quality edible oils in Ghana and the desire to increase

groundnut export to the world market, there is the need to investigate the quality of groundnut cultivars grown in the country and to improve the varieties to meet the market demand. However, studies on the chemical composition of groundnut cultivars grown in Ghana have not been undertaken. The objective of the study was to determine variability in nutritional composition with respect to protein, oil, fatty acids and minerals.

3.2 MATERIALS AND METHODS

3.2.1 Nutritional characteristics of groundnut

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Twenty groundnut cultivars were collected from some agro-ecological zones of Ghana. The 20 cultivars were planted in May and September, 2004 at the Crops Research Institute, Fumesua, Ghana to determine their botanical types. Hundred grams of sound mature kernels were handpicked after drying and I was invited to the International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, India for analyses of protein, oil, fatty acids and minerals. The cultivars, their botanical group, towns and the Regions from where they were collected in Ghana are shown in Table 2.1. The cultivars were grouped into two subspecies: *hypogaea* and *fastigiata*, based on the branching pattern, presence or absence of flowers on main stem and flower arrangement on leaf axils. Cultivars with flowers on main stem, sequential branching and flowering were grouped into subspecies *fastigiata* and those without flowers on the main stem, and having alternative branching pattern and alternate flowering as *hypogaea*.

3.2.2 Oil content

Oil content was determined using a commercial nuclear magnetic resonance spectrometer following the procedure described by Jambunathan *et al.* (1985). All readings were taken

on oven–dried (110 °C 16 h) samples and the values were expressed on a uniform 5% seed moisture content basis.

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Table 3. 1. Groundnut varieties, their subspecies, area and Region of collection.

Variety	Subspecies	Town collected from	Region
Dagomba	*hypogaea*	Kintampo	Brong Ahafo
F-Mix	,,	Nyankpala	Northern
Nkatepa	,,	Kumawu	Ashanti
Manipinta	,,	Fumesua	Ashanti
Sinkazie	,,	Nyankpala	Northern
Kumawu early	,,	Kumawu	Ashanti
Nkate kokoo	,,	Asante Mampong	Ashanti
Baasare	*fastigiata*	Abene	Eastern
Broni nkatee	,,	Asante Mampong	Ashanti
Afu	,,	Abene	Eastern
Nkoranza local	,,	Nkoranza	Brong Ahafo
Atebubu local	,,	Atebubu	Brong Ahafo
Aprewa	,,	Abene	Eastern
Kintampo local	,,	Kintampo	Brong Ahafo
Shitaochi	,,	Fumesua	Ashanti
Broni	,,	Kumawu	Ashanti
Kamaloo	,,	Abene	Eastern
Kofi Nsarko	,,	Kumawu	Ashanti
Kowoka	,,	Abene	Eastern
Broni fufuo	,,	Asante Mampong	Ashanti

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3.2.3 Fatty acid composition

About 300 mg of ground groundnut was weighed into a large glass culture tube and 15 ml petroleum ether was added to it and shaken on a tube rotator for 30 minutes. The contents were centrifuged at 4000 rpm for 5 minutes and 5 ml of the supernatant transferred into a small culture tube and the solvent evaporated under a stream of nitrogen gas. About 1.3 ml of 0.5 N NaOH in methanol was added to the content and heated in boiling water bath for 5 minutes. Two millilitres of boron fluoride (BF) in methanol was added to the cooled tube and heated in boiling water bath for 5 minutes. Two millilitres of saturated NaCl solution was added to the cooled tube and shaken on tube rotator for 10 minutes. Two ml of petroleum ether was added and shaken on tube rotator for 5 minutes and then centrifuged at 4000 rpm for 5 minutes. The supernatant petroleum ether layer was transferred into the automatic sampler vial. Fatty acid methyl esters (FAME) were analyzed in a Shimadzu 9A model gas chromatograph (GC) equipped with a flame-ionization detector. They were separated on a glass column (2.1 m, 3 mm I.D.) packed with 10% Altech CS-10 chromosorb W-AW (80-100 mesh). Flow rate of the carrier gas (helium) was 50 ml min^{-1}. The hydrogen flow was 45 ml min^{-1} and the air flow was 500 ml min^{-1}. The injection port temperature/detector temperatures were 260° C. Column temperature was held at 190° C for 4 minutes initially, and increased at the rate of 10 °C min^{-1} to a final temperature of 250° C where it was held for 2 minutes. About 2 µl of sample was injected for analysis. Peaks were identified by matching their retention times to the reference standard mixture of fatty acids (Nucheck 21A peanut fatty acid composition).

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From the fatty acid estimation, the following quality parameters were determined as described by Mozingo *et al.* (1988):

i. Iodine value (IV) = (% oleic acid) (0.8601)+(% Linoleic acid) (1.7321)+(% eicosenoic acid) (0.7854)

ii. Oleic acid (O)/Linoleic acid (L) ratio = % oleic / % linoleic acid.

iii. Percent total saturated fatty acids (TSF) = % palmitic acid +% lignoceric acid.

iv. Polyunsaturated (P)/saturated (S) ratio = % linoleic acid/ TSF

v. Total long chain saturated fatty acids (%) (TLCF) = % arachidic acid + % behenic acid + % lignoceric acid.

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3.2.4 Protein

The nitrogen content of defatted flour was determined by Kjedahl method (Singh and Jambunathan, 1980) and converted to protein content by using the conversion factor 5.46.

3.2.5 Starch

Starch content of defatted flour was determined by Southgate (1976) and Dubois *et al* (1956) methods. Fifty milligrams of defatted flour was weighed into a 50 ml conical flask. Few drops of ethyl alcohol were added to disperse the flour and 10 ml distilled water was added. The flask was covered with nitrogen free paper and the contents autoclaved for 90 min at 19 lb 126° C, (gelatinizing the starch). The content was cooled and 1 ml 2M sodium acetate buffer added. Twenty-five milligrams of amyloglucosidase and approximately 15 ml distilled water added and the contents incubated after covering with parafilm at 55° C for 2 h in a water bath–shaker (hydrolysis). After incubation, 10 ml aliquot was pipetted and diluted to 100 ml. One ml of the above aliquot was pipetted, 1 ml 5% phenol and 5 ml sulphuric acid were added and mixed well, cooled and the

absorbance read on Spectronic 21 at 490 nm against the blank. Also colour was with 10-15 µg/ml of standard glucose with an interval of 10 µg and the absorbance read. Reagent (water phenol + acid) blank and enzyme + buffer blanks with reagents was run.

3.2.6 Total soluble sugars

Hundred milligrams of defatted flour was weighed into a boiling tube.

Twenty-five milliliters of hot 80% ethanol was added into boiling tube and shaken on a vortex mixer. The material was allowed to settle for 20-30 min. The above was filtered into a beaker through a Whatman No. 41 filter paper. The second and fourth steps were repeated, for complete extraction of sugars for 4 times. The extract was evaporated on a hot sand bath until the ethanol was evaporated. Ten milliliters of water was added, and contents dissolved and transferred into a 100 ml volumetric flask; the contents in the beaker was washed 2 to 3 times and added to the volumetric flask; and made up to 100 ml with water.

1. 1 ml aliquot was taken from the above and 1ml water as blank into a test tube.
2. 1 ml of 5% phenol was added and shaken.
3. 5 ml 96% sulphuric acid was added to and shaken vigorously on a vortex mixer and the tubes cooled in water.
4. The absorbance of the golden yellow colour was at 490 nm against the blank.
5. The standards were run with different concentrations (i.e. 10,20,30,40 and 50 µg of glucose standard) from the working standard, keeping the volume to 1 ml with water; and added reagents as in steps 1 and 2.

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3.2.7 Mineral elements

Defatted groundnut samples (0.5 g) was weighed into a crucible and ashed in a muffle furnace at 600 °C for 4 hours. The ash was cooled and dissolved in dilute HCl (HCl:glass distilled water 1:3, v/v) and a few drops of concentrated nitric acid added. The content in the were boiled on a hot sand bath and allowed to cool and transferred to a 50-ml volumetric flask and the volume made up with glass distilled water. The above solution was used for the estimation of Zn, Cu, Fe and Mn.

For Na, K and Mg, 1 ml of the aliquot was diluted to 25 ml after adding 0.5 ml of a solution containing 50,000 ppm Lanthanum (Lanthanum chloride was added for the suppression of interference).

For Ca, the steps for K, Na and Mg was followed except that instead of using 1 ml for dilution, 5 ml was used. For the compressed air, delivery was set at a minimum of 60 psi (4.2 kg/cm) from a cylinder of compressed air. For acetylene, delivery was set at 10 psi (0.75 kg/cm), using line pressure welding grade.

The solutions were sprayed into atomic absorption spectrophotometer to determine the concentrations of the various elements with suitable standards.

3.3 RESULTS

3.3.1 Variability in Oil and protein content

Significant ($p<0.01$) ($p<0.01$) differences were observed among the 20 cultivars for oil, protein, total soluble sugars and starch (Table 2.2). Oil content ranged from 33.6 to 54.95 %. "Broni fufuo", a Spanish variety had the lowest oil content while "Nkate kokoo",

Virginia type gave the highest oil content. Most of the Virginia groundnut varieties had more oil than the Spanish varieties. The mean oil content of the Virginia varieties was 49.7% while it was 47.3% for the Spanish cultivars.

Protein of defatted portion ranged from 39.65 to 53.45%. "Kamaloo" had the highest level of protein after oil extraction and "Sinkazie" the lowest. The mean protein content of defatted flour of Virginia varieties was 45.3% and 48.6% for the Spanish varieties. Variation in total soluble sugars ranged from 9.20 to 13.30%. The total soluble sugar for Virginia varieties ranged from 9.20 to 13.30% as compared to 9.20 to 12.50% in the Spanish varieties. Starch content of defatted portion of groundnut varied between 26.0 and 38.90%.

Total protein content of whole kernel ranged from 18.92 to 30.53% (Table 2.3). "Sinkazie" gave the lowest and "Broni fufuo" the highest. Total soluble sugar content of whole kernel varied between 4.55 and 7.44%. Starch content of whole kernel ranged from 13.37 to 19.04%. Significant differences ($p<0.01$) were observed among the 20 groundnut varieties for oleic acid (Table 2.4). Spanish varieties generally had lower oleic acid than the Virginia

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Table 3. 2. Percentage of oil, protein, total soluble sugars and starch in defatted flour of 20 groundnut varieties.

Variety	Botanical group	Oil	Protein	Total soluble sugars	Starch
1. Dagomba	*hypogaea*	50.50	46.20	9.20	33.60
2. F-Mix	,,	43.00	43.45	13.05	33.40
3 Nkatepa	,,	49.30	44.90	12.30	33.10
4. Manipinta	,,	48.60	50.15	11.50	26.00
5. Sinkazie	,,	52.30	39.65	13.30	38.90
6. Kumawu early	,,	49.50	46.95	11.30	33.95
7. Nkate kokoo	,,	54.95	45.80	10.15	32.05
8. Baasare	*fastigiata*	49.05	51.05	9.65	32.60
9.Broni nkatee	,,	45.80	51.40	10.50	35.10
10. Afu	,,	46.15	51.45	9.30	30.20
11. Nkoranza local	,,	47.60	49.15	10.90	34.20
12. Atebubu local	,,	52.20	44.25	10.50	35.30
13. Aprewa	,,	48.30	48.10	9.20	32.60
14. Kintampo local	,,	48.75	48.30	9.30	31.40
15. Shitaochi	,,	54.65	44.20	9.60	34.10
16. Broni	,,	46.20	49.25	12.50	33.55
17.Kamaloo	,,	44.25	53.45	10.15	30.60
18. Kofi Nsarko	,,	49.05	43.60	12.20	30.95
19. Kowoka	,,	48.80	52.10	9.30	30.90
20. Broni fufuo	,,	33.60	45.95	12.40	28.60
Mean		48.13	47.47	10.82	32.61
SED		0.46	0.58	0.26	0.73
Cv (%)		1.0	1.2	2.9	2.2
Significance		P<0.01	P<0.01	P<0.01	P<0.01

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varieties. The range for the Virginia varieties was from 44.85 to 63.55% of the groundnut oil; and that of the Spanish was 40.85 and 46.40%. The Virginia varieties on the average had 29% more oleic acid than the Spanish varieties.

The linoleic acid content varied between 17.35 and 36.0%. The Spanish varieties generally had higher linoleic acid than the Virginia varieties. Oleic and linoleic acids together accounted for 77.9% of the total fatty acids in the 20 groundnut varieties analyzed.

The values of oleic/linoleic acid ratio of all the groundnut varieties exceeded 1.0. They varied between 1.14 and 3.66. The Virginia varieties generally had higher O/L ratio ranging from 1.43 to 3.66 and with an average of 2.59; as compared to the Spanish varieties which range from 1.14 to 1.51 and a mean of 1.28. The iodine value ranged from 85.77 to 98.43. The Spanish varieties generally had higher values than the Virginia varieties.

Significant variation ($p<0.01$) in palmitic, stearic, arachidic, eicosnoic, behenic and lignoceric acids were observed among the 20 cultivars (Table 2.5). The content of palmitic acid varied between 9.05 and 12.85%. The Virginia varieties had less palmitic acid, with a mean of 10.15% of the total fatty acid content. The mean of the Spanish varieties was 12.15% which was 19.7% higher than the mean for the Virginia varieties. The stearic acid ranged from 1.75 to 3.65% and had a mean value of 2.93%. The content of arachidic acid ranged from 1.05 to 1.70% and that of eicosnoic acid from 0.77 to 1.50%. Behenic acid ranged from 3.10 to 4.40% and lignoceric acid from 1.15 to 1.95%. The sum of the means of oleic, linoleic and palmitic acid was 89.35%.

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Table 3.3. Percentage of protein, total soluble sugars and starch of whole kernel in 20 groundnut varieties

Variety	Botanical group	Protein	Total soluble sugars	Starch
1. Dagomba	*hypogaea*	22.87	4.55	16.63
2. F-Mix	,,	24.77	7.44	19.04
3 Nkatepa	,,	22.76	6.24	16.78
4. Manipinta	,,	25.78	5.91	13.37
5. Sinkazie	,,	18.92	6.34	18.56
6. Kumawu early	,,	23.71	5.71	17.14
7. Nkate kokoo	,,	20.63	4.58	14.44
8. Baasare	*fastigiata*	26.00	4.92	16.61
9.Broni nkatee	,,	27.86	5.69	19.03
10. Afu	,,	27.71	5.01	16.26
11. Nkoranza local	,,	25.76	5.71	17.92
12. Atebubu local	,,	21.15	5.02	16.87
13. Aprewa	,,	24.87	4.76	16.85
14. Kintampo local	,,	24.75	4.77	16.09
15. Shitaochi	,,	20.09	4.36	15.47
16. Broni	,,	26.50	6.73	18.05
17.Kamaloo	,,	29.80	5.66	17.25
18. Kofi Nsarko	,,	22.21	6.22	15.74
19. Kowoka	,,	26.68	4.76	16.18
20. Broni fufuo	,,	30.53	8.25	18.90
Mean		23.15	5.5	16.86
SED		0.34	0.12	0.4
Cv (%)		1.5	2.2	2.4
Significance		$p<0.01$	$p<0.01$	$p<0.01$

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Table 3 .4. Percent of major fatty acids (oleic and linoleic), oleic/linoleic acid ratio and iodine value of the 20 groundnut Ghanaian varieties.

Variety	Sub-species	Oleic	Linoleic	O/L ratio	Iodine value
1. Dagomba	*hypogaea*	63.55	17.35	3.66	85.77
2. F-Mix	,,	51.95	27.55	1.89	93.58
3 Nkatepa	,,	55.35	24.65	2.25	91.29
4. Manipinta	,,	55.35	24.30	2.79	90.76
5. Sinkazie	,,	57.50	21.80	2.64	88.08
6. Kumawu early	,,	44.85	31.45	1.43	93.67
7. Nkate kokoo	,,	62.90	18.15	3.47	86.44
8. Baasare	*fastigiata*	43.85	34.10	1.29	97.51
9.Broni nkatee	,,	40.85	36.00	1.14	98.43
10. Afu	,,	43.25	33.55	1.29	96.02
11. Nkoranza local	,,	42.25	34.05	1.24	96.00
12. Atebubu local	,,	43.60	33.05	1.32	95.38
13. Aprewa	,,	42.65	34.55	1.23	97.21
14. Kintampo local	,,	43.40	33.35	1.30	95.71
15. Shitaochi	,,	46.40	30.75	1.51	93.77
16. Broni	,,	44.00	32.75	1.34	95.51
17.Kamaloo	,,	42.80	34.60	1.24	97.53
18. Kofi Nsarko	,,	43.30	33.65	1.29	96.23
19. Kowoka	,,	43.25	34.05	1.27	96.81
20. Broni fufuo	,,	42.65	34.40	1.24	97.17
Mean		47.69	30.20	1.72	94.14
SED		0.33	0.29	0.03	0.35
Cv (%)		0.7	0.95	1.91	0.38
Significance		p<0.01	p<0.01	p<0.01	p<0.05

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Table 3.5. Percent of minor fatty acids of the 20 groundnut Ghanaian varieties

Variety	Sub-species	Palmitic	Stearic	Arachidic	Eicosonoic	Behenic	Lignoceric
1. Dagomba	*Hypogaea*	9.05	2.95	1.35	1.35	3.90	1.65
2. F-Mix	,,	10.65	1.75	1.05	1.50	3.70	1.75
3 Nkatepa	,,	10.20	2.25	1.25	1.25	3.35	1.75
4. Manipinta	,,	9.45	2.45	1.40	1.35	3.65	1.70
5. Sinkazie	,,	10.25	2.90	1.55	1.10	3.55	1.40
6. Kumawu early	,,	12.25	3.55	1.65	0.80	3.90	1.45
7. Nkate kokoo	,,	9.20	2.85	1.55	1.15	3.10	1.15
8. Baasare	*Fastigiata*	11.75	2.60	1.45	0.93	3.80	1.50
9.Broni nkatee	,,	12.05	2.50	1.40	1.20	4.40	1.60
10. Afu	,,	12.15	2.95	1.50	0.91	4.15	1.50
11.Nkoranza local	,,	12.85	3.15	1.45	0.88	3.95	1.35
12. Atebubu local	,,	12.40	3.55	1.65	0.81	3.75	1.30
13. Aprewa	,,	12.40	2.85	1.50	0.87	3.80	1.40
14.Kintampo local	,,	12.60	3.65	1.55	0.78	3.20	1.30
15. Shitaochi	,,	12.30	3.65	1.70	0.77	3.35	1.30
16. Broni	,,	11.65	2.90	1.40	1.20	4.35	1.95
17.Kamaloo	,,	11.95	2.75	1.40	1.00	3.90	1.55
18. Kofi Nsarko	,,	11.95	3.40	1.60	0.90	3.90	1.35
19. Kowoka	,,	11.75	3.20	1.65	0.81	3.85	1.45
20. Broni fufuo	,,	12.20	2.65	1.50	1.15	4.00	1.50
Mean		11.45	2.93	1.48	1.03	3.73	1.50
SED		0.12	0.21	0.05	0.40	0.10	0.08
Cv (%)		1.06	5.70	3.63	4.30	2.63	11.79
Significance		p<0.01	p<0.01	p<0.01	p<0.01	p<0.01	p<0.05

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Linear correlation coefficients among the fatty acids are presented in Table 2.6. The correlation of palmitic acid content and that of oleic acid and O/L ratio were significant ($p<0.01$) and negative. There was however, significant positive correlation between palmitic acid and linoleic acid and between oleic acid and O/L ratio. On the other hand, correlation between linoleic acid and O/L ratio; stearic and eicosnoic; oleic and linoleic was significant and negative.

Total saturated fatty acids in the varieties ranged from 17.85 to 22.80% (Table 2.7). With the exception of "Kumawu Early", the Virginia varieties had lower saturated fatty acids than the Spanish varieties. The Virginia varieties had a mean saturated fatty acid of 19.22% compared to 22.08% of the Spanish varieties. The Spanish varieties had 14.88% more saturated fatty acid than the Virginia varieties. The ratio of polyunsaturated fatty acid to saturated fatty acids ranged from 0.97 to 1.64 and the long chain fatty acids ranged from 5.80 to 7.70.

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3.3.2 Variability in soluble sugars, starch and micronutrient content

The content of micronutrients in the varieties are presented in Table 2.8. The quantity of zinc varied between 0 and 6.5 mg/100g; the quantity of copper ranged from 0 to 2.7 mg/100g; iron from 0.2 to 3.7 mg/100g and manganese ranged from 1.5 to 2.9 mg/100g.

The levels of some macronutrients in the 100g of defatted flour are presented in Table 2.9 Potassium ranged from 1180 to 1693 mg. The level of sodium ranged between 19 and 48 mg. The calcium level range from 44 to 134 mg. The quantity of Magnesium ranged from 390 to 456 mg.

Table 3.6 Linear correlation between the fatty acids and Oleic/linoliec acid ratio

	Palmitic	Stearic	Oleic	Linoleic	Arachidic	Eicosenoic	Behenic	Lignoceric	O/L
Palmitic	0.458	-0.946**	0.926**	0.388	-0.731**	0.560**	-0.244	-0.918**	
Stearic		-0.299	0.233	0.844**	-0.806**	-0.119	-0.474	-0.215	
Oleic			-0.996**	-0.288	0.599**	-0.728**	0.068	0.983**	
Linoleic				0.238	-0.562**	0.729**	-0.053	-0.984**	
Arachidic					0.788**	-0.010	0.576**	-0.220	
Eicosenoic						-0.010	0.576**	0.548**	
Behenic							0.307	-0.730**	
Lignoceric								0.018	

** Significant at $p<0.01$.

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Table 3.7 Total saturated fatty acid, polyunsaturated /saturated ratio and long chain fatty acid content of groundnut varieties

Variety	Sub-species	Total saturated fatty acid (%)	Polyunsaturated/ saturated fatty acid ratio	Long chain saturated fatty acid
1. Dagomba	*hypogaea*	17.90	0.97	5.90
2. F-Mix	,,	18.90	1.46	6.50
3 Nkatepa	,,	18.80	1.31	6.35
4. Manipinta	,,	18.65	1.30	6.75
5. Sinkazie	,,	19.65	1.11	6.50
6. Kumawu early	,,	22.80	1.38	7.00
7. Nkate kokoo	,,	17.85	1.02	5.80
8. Baasare	*fastigiata*	21.10	1.62	6.75
9.Broni nkatee	,,	21.95	1.64	7.40
10. Afu	,,	22.25	1.51	7.15
11. Nkoranza local	,,	22.75	1.50	6.75
12. Atebubu local	,,	22.65	1.50	6.70
13. Aprewa	,,	21.95	1.57	6.70
14. Kintampo local	,,	22.30	1.50	6.05
15. Shitaochi	,,	22.30	1.38	6.35
16. Broni	,,	22.25	1.47	7.70
17.Kamaloo	,,	21.55	1.61	6.85
18. Kofi Nsarko	,,	22.20	1.52	6.85
19. Kowoka	,,	21.90	1.56	6.95
20. Broni fufuo	,,	21.85	1.57	7.00
Mean		21.08	1.19	6.7
Cv (%)		1.0	1.5	2.2
SED		1.36	0.02	0.15
Significance		p<0.01	p<0.01	p<0.01

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Table 3.8. Micronutrient content in defatted samples of 20 groundnut varieties (mg/100g)

Variety	Sub-species	Zinc	Copper	Iron	Manganese
1. Dagomba	*hypogaea*	6.1	1.9	2.9	1.9
2. F-Mix	,,	4.4	1.9	2.0	1.9
3 Nkatepa	,,	5.0	2.1	3.5	1.5
4. Manipinta	,,	5.0	2.4	3.0	2.9
5. Sinkazie	,,	5.1	2.1	2.0	2.2
6. Kumawu early	,,	6.2	2.4	3.2	2.2
7. Nkate kokoo	,,	6.5	2.0	2.4	2.1
8. Baasare	*fastigiata*	5.8	2.2	3.6	2.2
9.Broni nkatee	,,	5.4	1.7	3.2	2.1
10. Afu	,,	5.3	1.7	3.3	2.2
11. Nkoranza local	,,	6.0	1.8	3.7	2.4
12. Atebubu local	,,	5.9	1.9	2.6	2.5
13. Aprewa	,,	5.3	1.7	2.7	1.9
14. Kintampo local	,,	0	0	0.2	2.1
15. Shitaochi	,,	5.4	2.7	3.7	2.6
16. Broni	,,	6.3	2.2	3.7	1.9
17.Kamaloo	,,	5.0	1.7	3.3	1.7
18. Kofi Nsarko	,,	4.9	2.1	2.6	1.8
19. Kowoka	,,	4.9	1.8	2.4	1.8
20. Broni fufuo	,,	5.6	2.2	2.6	1.9
Mean		5.2	1.9	2.8	2.1
Cv (%)		2.6	2.7	2.9	1.6
SED		0.3	0.1	0.2	0.07
Significance		p<0.01	p<0.01	p<0.01	p<0.01

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Table 3.9. Content of Potassium, Sodium, Calcium and Magnesium in defatted sample of 20 groundnut varieties (mg/100g).

Variety	Sub-species	Potassium	Sodium	Calcium	Magnesium
1. Dagomba	*hypogaea*	1495	33	118	385
2. F-Mix	,,	1610	31	78	308
3 Nkatepa	,,	1493	26	91	343
4. Manipinta	,,	1360	25	59	354
5. Sinkazie	,,	1693	28	75	388
6. Kumawu early	,,	1349	25	68	373
7. Nkate kokoo	,,	1478	35	88	359
8. Baasare	*fastigiata*	1216	23	55	349
9.Broni nkatee	,,	1286	40	86	349
10. Afu	,,	1183	29	53	325
11. Nkoranza local	,,	1354	19	76	384
12. Atebubu local	,,	1458	24	134	456
13. Aprewa	,,	1180	20	71	390
14. Kintampo local	,,	1193	24	96	420
15. Shitaochi	,,	1374	48	131	405
16. Broni	,,	1188	29	80	325
17.Kamaloo	,,	1306	26	48	320
18. Kofi Nsarko	,,	1313	29	61	411
19. Kowoka	,,	1344	28	44	336
20. Broni fufuo	,,	1310	29	60	390
Mean		1359	29	78	364
Cv(%)		1.1	2.3	3.3	1.1
SED		32	1.5	5.7	9.3
Significance		p<0.01	p<0.01	p<0.01	p<0.01

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3.4 DISCUSSION

The oil content of the cultivars generally did not follow the observation of Savage and Keenan (1994) who found oil content of groundnut cultivars they worked with to be between 44 to 56%. The oil content of five varieties ("Nkate kokoo", "Shitaochi", "Sinkazie", "Atebubu" local and "Dagomba") were above 50%. The high oil content of Virginia varieties observed in this study were similar to the results obtained by Dwivedi *et al.* (1993) who found that Virginia cultivars had higher oil content than Valencia and Spanish lines. The observations suggest a genetic attribute rather than environmental and can be transferred to lines with low oil content through breeding. Grimm *et al.* (1996) also observed that *hirsuta* groundnut varieties contain less oil than Virginia lines. There was a variety, Broni fufuo which had exceptionally low oil content of 33% and high protein content (30%). With the increasing demand for products with less fat and oil, this variety can be used for products which require low oil content such as soups and stews. Cultivars with oil content more than 50% could be used as donors in breeding programmes to improve the oil content of lines with low oil content but those selected have to be tested in several locations to determine the stability of the trait, because oil content has been reported to be highly influenced by locations, seasons and growing conditions (Brown *et al.*, 1975, Holaday and Pearson, 1974 and Young *et al.*, 1974).

Protein of defatted portion ranged from 39.65 to 53.45% and the percentage of protein in the seeds ranged from 18.92 to 30.53%. This agrees with the findings of Savage and Keenan (1974) and Reddy (1988), who observed protein content of groundnut genotypes they screened to be between 22 to 32% and the residue after oil extraction between 46-60%. The high protein content of the cultivars is important, because most people in

Ghana cannot afford protein from animal source. Several tons of soybean meal is imported into Ghana for the poultry and livestock industry every year. Using groundnut meal in animal feeds can reduce the overdependence on soybean meal and therefore save the scarce foreign exchange spent in importing soybean meal. Depending on the requirement of the product, the meal or whole groundnut seed could be use for feed or food preparations. One of the varieties, "Sinkazie" had seed protein content of 18.93 % which was the lowest. Seed protein content of most of the cultivars was higher than cowpea which contains about 24% seed protein (IITA, 1989). The protein content of "Broni fufuo" and "Kamaloo" seeds were the highest and could be utilized for food preparation to meet protein requirements in diets.

Soluble sugars are important in giving the sweet taste in peanut. Pattee *et al.* (1995) found that groundnut varieties with high sweet taste intensities had high free sugar content than those with lower intensities. Free soluble sugars have also been associated with the flavour of groundnut. Koehler *et al.* (1969) indicated that free soluble sugars provide carbon for the production of flavour compounds. The range of free sugar content of 9.2 to 13.3 mg/100g defatted seed was lower than what Grimm *et al.* (1996) observed in *hirsuta* lines which ranged from 14.1-17.9 mg/100g. Oupadissakoon and Young (1984) observed a strong correlation (r^2=0.928) between roasted groundnut flavour and amino acid and free sugar content of raw seed. Since sweet taste and flavour play important role in groundnut acceptance by consumers, groundnut varieties with high content of free sugars should be developed in the country through the introduction of new germplasm to improve local accessions.

The sum of free soluble sugars and starch constitute the total carbohydrate content of the varieties. The level of carbohydrate in the cultivars was comparable to the results of Duke (1981). He indicated that total carbohydrate in groundnut ranged from 6.0–24.9 %. "Broni fufuo" however, had unusually high level of carbohydrate (27%) of dry seed weight.

The oleic and linoleic content accounted for 75.30 to 81.05% of the total fatty acids. The results are consistent with the findings of Ahmed and Young (1982) who found that oleic and linoleic acids constitute approximately 80% of the total fatty acid composition of groundnut. Oleic acid, linoleic acid and palmitic acids constituted on the average 89.35% of the total fatty acid. This agrees with the findings of Ahmed and Young (1982) and Dwivedi *et al* (1993), who found the percentage of the three fatty acids to be about 90% in groundnut. The levels of oleic and linoleic acids follow the range observed by Treadwell *et al*. (1983). They found 36 to 67% oleic acid and 15 to 43% linoleic acid in groundnut varieties they analysed. The levels of oleic acid found in the varieties were generally high. High level of oleic acid implies high oil stability and better shelf life of groundnut seeds and products because oleic acid is monounsaturated fatty acid, therefore less prone to oxidative rancidity. Because unsaturated fatty acids have been found to reduce plasma cholesterol levels (Grundy, 1986), varieties with high oleic acids could be used for products to improve the health of consumers and may be used as parents to improve varieties with lower oleic acid content.

Highly significant negative correlation between oleic acid and linoleic acid observed in this study was in agreement with previous studies (Brown *et al*., 1975; and Mercer *et al*., 1990). This indicates selection for high oleic acid will bring about corresponding increase

in O/L ratio and lower levels of linoleic acid. The relationship between palmitic acid and linoleic acid was positive as observed in an earlier study (Worthington and Hammons, 1971).

All the varieties had appreciable amounts of zinc, copper, iron and manganese, except "Kintampo local" which had no Zinc and copper. The results agree with results of several workers (Oke, 1967; Derise *et al.*, 1974, Galvaro *et al.*, 1976; Khalil and Chughatai, 1983). The amounts of micronutrients in the cultivars were nutritionally significant, because small quantities are needed by the body.

The range of K, Na and Mg in the 20 cultivars was generally higher than the results of other workers (Oke, 1967; Derise *et al.*, 1974; Galvaro *et al.*, 1976; Khalil and Chughatai, 1983). They found the following ranges: 92-200 mg Ca, 10-343 mg Mg, 1.4-33.3 mg Na, 1150-1450 mg K This might be due to the limited quantity of genotypes these researchers studied and which probably came from small geographic areas. Screening of genotypes from more botanical groups and from different locations will give a better picture. The level of calcium was within the range given by the above workers. The study showed that the local landraces had significant genetic variations in nutritional qualities among the accessions and between the subspecies which can be exploited for breeding programmes.

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CHAPTER FOUR

4.0 INHERITANCE OF FRESH SEED DORMANCY IN GROUNDNUT

4.1 Introduction

Groundnut genotypes belonging to Spanish and Valencia (subspecies *fastigiata*) types have short life cycle and lack fresh seed dormancy, whereas those of Virginia (ssp. *hypogaea*) type have long life cycles and fresh seed dormancy (Swain *et al*, 2001). Sprouting occurs in the ground in ssp. *fastigiata* if it rains prior to harvesting or harvesting is delayed after pods mature. Sprouting of nuts also occur in the stack on the threshing floor. Yield loss due to *in situ* germination in bunch varieties has been reported to be between 20-40% in India (Ramanathan, 1987, Reddy *et al*., 1985, and Nagarjun and Radder, 1983), which also affects seed quality and storability. To reduce these losses it is essential to have fresh seed dormancy of few weeks so that farmers who are not able to harvest their crop immediately after maturity will not suffer great losses in Spanish cultivars. A short period of seed dormancy is necessary to reduce losses associated with germination if there is rain at harvest and in the absence of proper care to dry pods quickly after harvest.

There have been a few studies on the inheritance of fresh seed dormancy in groundnut. These studies have drawn contradictory conclusions. Lin and Lin (1971) reported monogenic control, whereas John *et al*. (1948) and Nautiyal *et al*. (1994) indicated that the character may be quantitatively inherited. Lin and Lin (1971) reported complete dominance of dormant over non-dormant seed, whereas Ramachandran *et al*. (1967)

observed partial dominance. Khalfaoui (1991), concluded that dormancy is a quantitatively inherited trait and additive, dominance and digenic epistasis effects were involved in its genetic control. To reduce these losses it is essential to have fresh seed dormancy of not less than two weeks so that farmers who are not able to harvest their crop immediately after maturity will not suffer great losses in Spanish and Valencia cultivars. A short period of seed dormancy is therefore necessary to reduce losses associated with germination if there is rain at harvest and in the absence of proper care to dry pods quickly after harvest. Few fastigiates have been developed with fresh seed dormancy (Upadhyaya *et al.*, 1997). This study was designed to determine the genetics of fresh seed dormancy in groundnut.

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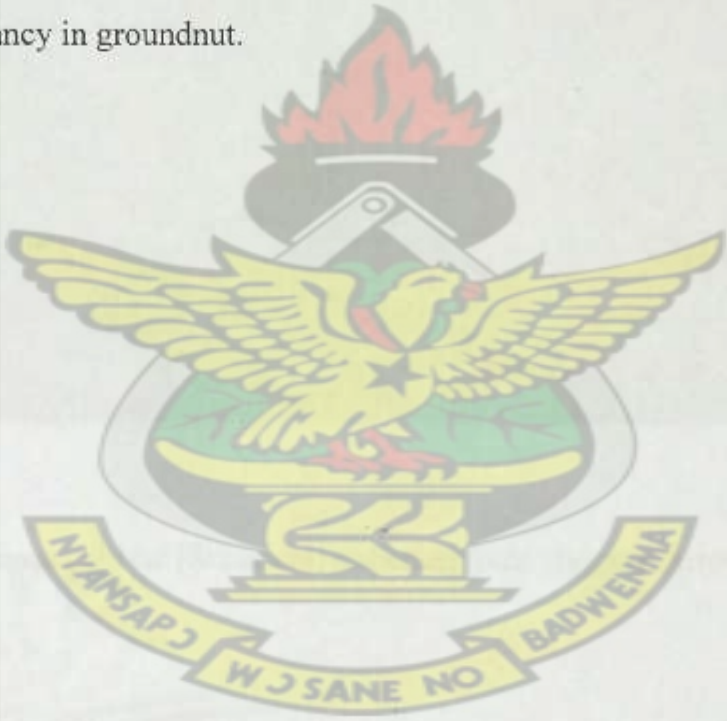

Plate 4.1 Groundnut cultivar (Shitaochi) suffering from vivipary (arrowed).

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4.3 MATERIALS AND METHODS

Four Spanish groundnut genotypes were used in this study. "Shitaochi and Aprewa" (local varieties which suffer from vivipary), were collected from the CSIR-Crops Research Institute, Kumasi, Ghana and ICGV 86158 and ICGV 87378 (lines with fresh seed dormancy) were obtained from the International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, India. Seeds of the four groundnut genotypes were planted on 18th February, 2004, in plastic bucket containing 6 kg of sterilized soil in a lath house at the Crop Science Department, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The seeds were thinned to one plant/pot one week after emergence.

Each of the two local genotypes ("Shitaochi" and "Aprewa") was crossed with the two exotic lines (ICGV 86156 and ICGV 87378) in all possible combination including reciprocals at flowering. There were also reciprocal crosses of ICGV 86158 and ICGV 87378.

The hybrid seeds and seeds of the four parental lines were shelled immediately after harvest. Only mature seeds were shelled and to ensure that the seeds were mature, the presence of black layer in the shell was the criteria used to identify mature seed. Care was taken during shelling to avoid destruction of the testa. Seeds were plated on Petri dishes lined with 9 cm filter paper (Plate 3.3) after sterilization with bavistin (a.i. 0.25g carbendezin/kg seed). Watering was done as and when necessary with distilled water.

Daily records of germinated seeds were taken. Seeds that germinated before 14 days were considered to be non-dormant and those that germinated after 14 days dormant (Uppadhayya and Nigam, 1999). Seeds that did not germinate after 35 days were treated

with 0.05% ethrel to induce germination. Seeds that emerged after 14 days were planted in small plastic buckets containing 1 kg of sterilized soil in a plant house at the CSIR-Crops Research Institute at Fumesua, Kumasi.

Seedlings were transferred to the field and planted on a soil in the Asuansi series, a ferric Acrisol (FAO classification) at Crops Research Institute at Fumesua, Kumasi. The seedlings were planted on ridges 30 cm high, spaced 1.0 m apart, 10 m long and within row spacing of 20cm. Parents of each hybrid were planted on the opposite ends of the same ridge and spaced 2 m apart. Female parents were planted at the first four-metre end and male parents at the other end. Hybrids derived from the parents were planted on ridges immediately following the parents. The arrangement was done in this way to identify and rogue selfed plants within the hybrids and to facilitate easy backcrossing. Supplementary irrigation was given when necessary. The crop was sprayed against aphids with karate (at the rate of 15 g lambda-cyhalothrin per hectare) three weeks after transplanting. Both parents were backcrossed to the F_1 plants to generate 20 backcross populations and some of the F_1s were selfed to produce F_2 populations. The parents were again crossed as in the first season (Table 4.1) to produce fresh F_1 hybrid seeds.

Dormancy of parents, F_1, F_2, and backcrossed generations were assessed in the laboratory by planting freshly harvested seeds on 9 cm filter paper lined in petri dishes and kept moist with distilled water. The number of seeds that germinated was recorded daily for 35 days. To test for the viability of seeds that did not germinate the seeds were treated with 0.05% ethrel solution to stimulate germination. Chi-square test was carried out to test the goodness of fit of the observed to the expected Mendelian ratios.

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4.4 RESULTS

The results indicated that out of 50 freshly harvested seeds of the 4 parents, five seeds of "Aprewa" and four seeds of "Shitaochi" germinated 14 days after incubating in petri dishes. On the other hand, 45 and 47 seeds of ICGV 86158 and ICGV 87378 germinated 14 days after incubation respectively (Table 4.1).

The crosses between the dormant and non-dormant parents produced F_1 seeds which behaved like the dormant parents in terms of the number of days to germination irrespective of the direction of the crosses. Less than 10% of the F_1 seeds behaved like the non-dormant parents and germinated before 14 days (Table 4.1). Crosses between the two dormant parents resulted in more than 90% of the progenies being dormant.

In the F_2 generation, 25% of the progenies behaved like the non-dormant parents and germinated on or before 14 days and 75% germinated after 14 days (Table 4.2). Four seeds from the crosses between two dormant parents germinated before 14 days in the F_2 generation. The backcross progenies with the non-dormant ("Shitaochi" and "Aprewa") parents fitted the expected ratio of 1 dormant: 1 non-dormant (Table 4.3). Backcrosses to the dormant parents produced seeds that were dormant (Table 4.4).

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Table 4.1. Parents and crosses made.

Crosses			Number of seeds Germinated on or before 14 days	Number of seeds germinated after 14 days
Female		Male		
1. Shitaochi	x	ICGV 86158	3	56
2. ICGV 86158	x	Shitaochi	1	72
3. Shitaochi	x	ICGV 87378	2	49
4. ICGV 87378	x	Shitaochi	2	61
5. Aprewa	x	ICGV 86158	0	56
6. ICGV 86158	x	Aprewa	1	73
7. Aprewa	x	ICGV 87378	3	53
8. ICGV 87378	x	Aprewa	0	38
9. ICGV 86158	x	ICGV 87378	2	45
10. ICGV 87378	x	ICGV 86158	0	54
Parents				
Shitaochi			48	2
Aprewa			49	1
ICGV 86158			0	50
ICGV 87378			49	1

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Dormant parents: ICGV86158 and ICGV 87378

Non-dormant parent: Shitaochi and Aprewa

Table 4.2. Goodness of fit for expected ratio of 3 dormant:1 non-dormant seeds in F_2 generations of crosses of ICGV 87378 and ICGV 86158 with Shitaochi and Aprewa.

Cross	Dormant	Non-dormant	Total	χ^2	P (0.05)
Shitaochi x ICGV 87378	129	34	163	0.153	0.695
ICGV 87378 X Shitaochi	107	32	149	0.060	0.806
Shitaochi x ICGV 86158	140	40	180	0.022	0.881
ICGV 86158 x Shitaochi	90	37	127	0.071	0.790
ICGV 87378 x ICGV 86158	163	0	163	-	-
ICGV 86158 x ICGV 87378	133	4	137	-	-
Aprewa x ICGV 87378	126	35	161	0.752	0.385
ICGV 87378 x Aprewa	116	33	149	0.329	0.566
Aprewa x ICGV 86158	103	29	132	0.273	0.602
ICGV 86158 x Aprewa	124	38	162	0.395	0.530

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Dormant parents: ICGV86158 and ICGV 87378

Non-dormant parent: Shitaochi and Aprewa

Table 4.3. Goodness of fit test for expected ratio of 1 dormant:1 non-dormant seeds in backcross F_1 generations of crosses with non-dormant parents

Cross	Dormant	Non-dormant	Total	χ^2	P (0.05)
Shitaochi x (Shitaochi x ICGV 87378)	17	22	39	0.641	0.423
Shitaochi x (ICGV 87378 x Shitaochi)	19	24	43	0.581	0.446
Shitaochi x (Shitaochi x ICGV 86158)	25	17	42	1.523	0.217
Shitaochi x (ICGV 86158 x Shitaochi)	20	17	37	0.243	0.622
Aprewa x (Aprewa x ICGV 87378)	19	18	37	0.027	0.869
Aprewa x (ICGV 87378 x Aprewa)	22	16	38	0.947	0.330
Aprewa x (Aprewa x ICGV 86158)	24	20	44	0.364	0.546
Aprewa x (ICGV 86158 x Aprewa)	18	21	39	0.231	0.631

Dormant parents: ICGV86158 and ICGV 87378

Non-dormant parent: Shitaochi and Aprewa

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Table 4.4. Goodness of fit test for expected ratio of 1 dormant : 1 non-dormant seeds in backcross F_1 generations of crosses with dormant parents

Cross	Dormant	Non-dormant	Total	χ^2	P (0.05)
ICGV 87378 x (Shitaochi x ICGV 87378)	34	0	34	-	-
ICGV 87378 x (ICGV 87378 x Shitaochi)	27	2	29	-	-
ICGV 86158 x (Shitaochi x ICGV 86158)	44	3	47	-	-
ICGV 86158 x (ICGV 86158 x Shitaochi)	46	1	47	-	-
ICGV 87378 x (ICGV 87378 x ICGV 86158)	38	0	38	-	-
ICGV 87378 x (ICGV 86158 x ICGV 87378)	41	0	41	-	-
ICGV 86158 x (ICGV 86158 x ICGV 87378)	32	0	32	-	-
ICGV 86158 x (ICGV 87378 x ICGV 86158)	25	0	25	-	-
ICGV 87378 x (Aprewa x ICGV 87378)	36	4	40	-	-
ICGV 87378 x (ICGV 87378 x Aprewa)	29	2	31	-	-
ICGV 86158 x (Aprewa x ICGV 86158)	38	2	40	-	-
ICGV 86158 x (ICGV 86158 x Aprewa)	35	0	35	-	-

Dormant parents: ICGV86158 and ICGV 87378

Non-dormant parent: Shitaochi and Aprewa

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4.5 DISCUSSION

Less than 10% of the freshly harvested seeds of the non-dormant parents (Shitaochi and Aprewa) germinated after 14 days. However, more than 90% of seeds of the dormant parents germinated after 14 days of incubation. These observations corroborated that Aprewa and shitaochi are non-dormant and ICGV 87378 and ICGV 86158 are dormant. The F_1 progenies generated from crosses between the dormant and non-dormant parents behaved like the dormant parents; more than 90% germinated after 14 days. This shows that at least one dominant allele of the dormant gene must be present to impart dormancy in groundnut seed and therefore dormant characteristics is dominant over non-dormant. Regardless of whether the dormant parent was a male or female, the F_1 progenies were dormant. This observation proves that dormancy is not maternally controlled.

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In the F_2 generations, the ratio of dormant to non-dormant seeds fit the expected ratio of 3:1 in crosses between dormant and non-dormant parents. This confirms the fact that dormancy is controlled by a single allele of the gene for fresh seed dormancy. These results are at variance with the findings of Khalfaoui (1991), who indicated that seed dormancy is controlled by several genes. The results are however, in agreement with the findings of Lin and Lin (1971) and Upadhyaya and Nigam (1999), who observed complete dominance of dormant seed over non- dormant seed.

F_2 progenies of crosses between 2 dormant parents resulted in 4 out of the 300 seeds germinating before 14 days. This is a reflection of no segregation of dormancy. These observations further suggest that the dormant gene controlling seed dormancy in both parents are in the same locus. If the gene for dormancy were in different loci on the chromosome of the dormant parents, there would have been segregation at F_2. Backcross progenies with non-dormant parents fit to an expected 1 dormant: 1 non-dormant.

However, data from the backcross to the dormant parents produced progenies which were dormant. The study showed that seed dormancy is controlled by a single gene with dormancy dominant over non-dormancy.

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CHAPTER FIVE

5.0 DEVELOPMENT OF MICROSATELLITES IN GROUNDNUT USING ENRICHMENT METHOD

5.1 Introduction

Microsatellites or simple sequence repeats (SSRs) have become increasingly popular as a genetic marker in many animal and plant species. Simple sequence repeats are tandem repeating motifs of 1-6 bases that are randomly distributed throughout the genomes of prokaryotic and eukaryotic species (Hamada *et al.*, 1982). They may be classified into three groups: pure, compound and interrupted repeats (Weber, 1990). Microsatellites have been found in higher numbers in mammals and randomly distributed with approximately one microsatellite present in every 15 kilobase of sequence (Tautz, 1989). Van Treuren *et al.* (1997) indicated that, there are more microsatellites in insects than plant species. Two mechanisms are thought to generate SSRs variability, DNA replication error followed by a high efficiency of mismatch repair system (Chambers and MacAvoy, 2000). In the past repetitive DNA sequence was termed "junk" DNA because it was thought to lack any function. Even though the function of SSRs in plant DNA is still not known today, it has become an important tool for researchers. These repeat units have been shown to be highly polymorphic within and between species, a characteristic that has permitted their application as a molecular marker in population genetics (Goldstein *et al.*, 1999), diversity studies (He *et al.*, 2003), genome mapping (Yu *et al.*, 2000), germplasm conservation and for support of intellectual property rights (Smith *et al.*, 1997). Microsatellites are found in both coding and non-coding regions, but differ in their composition and frequency in these regions (Panaud *et al.*, 1996; Morgante *et al.*,

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2002). The highly conserved nature of the flanking sequences of microsatellites allows for primers developed for one species to frequently amplify loci in related species (Roder *et al.*, 1995). The peculiar characteristics of microsatellites, such as presence in all living organisms, high level of polymorphism, co-dominant mode of inheritance and potential for automated analysis make them the best choice for genotyping, mapping and positional cloning of genes (Rakoczy-Trojanowska and Bolibok, 2004; Hopkins *et al.*, 1999; Powell *et al.*, 1996).

Microsatellites are considered as one of the most efficient markers; nevertheless their use is limited because their isolation is quite involving in terms of effort, time and cost. There are two general methods used to develop SSR markers: searching for sequences containing microsatellites in the available data base and constructing and screening the genomic and other libraries with probes complimentary to microsatellite sequences (Rassmann *et al.*, 1991).

The development of microsatellites through data based searching of expressed sequenced tags (EST) is cost-effective, simple, relatively faster and ESTs mined from libraries have been successful in many studies (Cordeiro *et al.*, 2001; Kantety *et al.*, 2002). However, it has some limitations. Data explored from expressed sequence tag do not contain sufficient amount of potential polymorphism, as microsatellites are not only found in expressed regions but also in non-coding regions. Additionally, the method is limited to plants with high economic and scientific interest which are well represented in the database.

Microsatellites developed through library construction can be divided into two groups; non-enrichment libraries and enrichment libraries (Rakoczy-Trojanowska, and Bolibok,

2004). The non-enrichment method has been applied in many instances but several shortfalls have been found, especially with species with large genomes. The most often cited problems are low efficiency for species with low microsatellite frequency and specificity of hybridization as well as the presence of one-side flanks in sequence fragments. Saal and Wricke (1999) observed an efficiency of 10% when they developed SSRs markers from non-enrichment library in rye. Out of 1737 positive clones sequenced in wheat, Roder *et al.* (1995) found less than 2% could amplify fragments with expected length.

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Enrichment method has been successfully applied to plants by several researchers with minor modifications (Prochazka, 1996; He *et al.*, 2003). Compared with the traditional methods (non-enrichment library), enrichment library has high efficiency and ranged from 55% to 100% of the clones containing microsatellites suitable for primer design (Prochazka, 1996; Hamilton *et al.*, 1999).

The most popular method of enriched library construction is selective hybridization of DNA fragments using streptavidin-coated magnetic beads or nylon membrane (He *et al.*, 2003; Edwards *et al.*, 1996). The availability of large numbers of molecular markers is the first step towards identifying informative markers for genetic analysis. Few informative microsatellites markers are available for groundnut, because it is labour intensive, time consuming and the development cost is high. More DNA markers are needed to saturate the existing peanut linkage map and to initiate genetic studies for this plant species. The study was undertaken to develop groundnut SSR markers and determine their variability, utility in genome analysis and ability to distinguish between genotypes.

5.2 MATERIALS AND METHODS

5.2.1 Library construction and screening

Total genomic DNA was extracted from leaves of groundnut line C3424 using MasterPure Plant Leaf DNA Purification kit (Epicentre, Madison, WI). The DNA was digested by three restriction enzymes (*Hae*III, *Rsa*I and *Dra*I). Digested fragments were ligated with corresponding adapters and then amplified using one-base selective primers (*Hae*III-A and *RsaI*-T) by the method described by Vos *et al.* (1995). Three biotinylated SSR probes, $(AT)_{15}$, $(GT)_{15}$, $(GA)_{15}$, which were reported as being rich in other plant species, were separately hybridized with amplified AFLPs. Approximately 200 ng of the pre-amplified AFLP fragments (length range from 100 bp – 600 bp) were added to a single reaction mixture containing 4.2 × SSC (Saline-Sodium Citrate, pH 7.0), 0.07% SDS (sodium dodecyl sulfate), and 10 pmol biotinylated probe. The mixture was incubated at 95°C for 5 minutes and chilled quickly on ice for 2 minutes. It was then kept at the appropriate temperature (depending on the melting temperature of each probe) for one hour to perform annealing, for probe $(GT)_{15}$ at 60°C, $(AT)_{15}$ at 37°C, and $(GA)_{15}$ at 57°C. Meanwhile, Dynabeads M-280 Streptavidin (10 µg/µl) was prepared by gently shaking the vial to obtain a homogenous slurry. About 20 µl of the beads slurry was transferred to a 1.5 ml tube, then washed 4 times with 300 µl bead washing buffer (1 × TE + 100 mM NaCl). The beads were re-suspended with 50 µl of the same buffer, and added into the fragment-probe mix and incubated at room temperature for 30 minutes with constant gentle agitation. After immobilization, the supernatant was removed by applying a magnetic field to precipitate the beads, which attached the SSR containing

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fragments that hybridized to biotinylated probe. The bead-probe-fragment complex was washed three times each for 5 minutes with 400 µl non-stringency washing buffer (1 × TE + 1 M NaCl) at room temperature. The complex was further washed with 400 µl stringency buffer (0.2 × SSC + 0.1% SDS) for three times each for 5 minutes at room temperature. After the final wash, the washing buffer was completely removed, 40 µl of sterile water added, taped gently and incubated at 95°C for 5 minutes. The eluted solution containing single strand, SSR-enriched fragments was cloned into the pCR4-TOPO vector, and the recombinants were transformed to TOP10 Chemically Competent *E. coli* following the instruction of TOPO TA Cloning kit (Invitrogen, San Diego, CA). Transformed cells were incubated at 37°C for 1 hour with vigorous shaking at 200 rpm before culturing on LB medium containing ampicillin antibiotics on LB-agar plates. The plates were cultured at 37°C with vigorous shaking at 200 rmp. Bacterial colonies were carefully scrapped with sterile tooth picks into wells containing 100 µl of Luria Broth agar (1 g trypton, 0.5 g yeast extract, 1g NaCl and 0.7 g agarose in 100ml distilled water) and cultured at 37°C over night. One microlitre of over night cultures were taken into new wells containing 9 µl of sterile water and heated at 95 °C for 10 minutes to release the plasmids and the cloned products from cell, *E. coli*. Polymerase chain reaction was performed using primer M13, 1.9 unit *Taq* polymerase, 0.2 mM of dNTP, 1X buffer. The cloned insert sizes were tested on 0.8 agarose gel electrophoresis at 120 V for 1 hour using 10 µl of PCR products to select those with the right size stained with ethidium bromide (Fig. 4.1). Sequencing was carried out on an ABI PRISM® 377 sequencer using the Big Dye Terminator kit and Beckman Coulter CEQ800 using the CEQDTCS Quick Start kit according to the manufacturer's instructions.

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Fig. 5.1 Cloned products visualized on 0.8% agarose gel stained with ethidium bromide, molecular weight markers are at the extreme ends, AB and CD are 500 base pairs mark.

5.2.2 Primer design

Primers of length 20 to 25 nucleotides were designed when the 5'- and 3'- sequence regions flanking the repeated motifs for trinucleotide repeats greater than six and dinucleotide repeats greater than eight that were detected using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and were synthesized by Sigma-Genosys. Primer selection was based on GC content, melting temperature curve and the absence of a secondary structure.

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5.2.3 PCR amplification

SSR primers were used to amplify the genomic DNA extracted from 2 groundnut lines from diverse origin, C2420 and C2434. PCR reaction mixture consisted of 1 µl/50ng template DNA, 1x PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 250 nM each of forward and reverse primers, and 0.25U *Taq* polymerase in a 10 µl reaction volume. PCR amplifications were carried out in a Perkin-Elmer 9700 thermocycler as 94 °C for 3 minutes for initial denaturation: 94 °C/30s, 65 °C/30s, 72 °C/60 s for two cycles: 94 °C/30 s, 56 °C/30 s, 72 °C/60 s for two cycles: 94 °C/15 s, 55 °C/30 s, 72 °C/60 s for thirty cycles: and 72 °C/10 minutes for final extension (Mellersh and Sampson, 1993). The PCR products were denatured by heating at 94 °C for 3 minutes and immediately placed on ice. Two microlitres of loading buffer (98% formamide, 10 mM EDTA, 0.005% of xylene cyanol FF and 0.005% of bromophenol blue) was added to each tube. PCR products were run on 6% polyacrylamide gels. The gel was pre-run for 20 minutes before loading the samples. Ten microlitres of each sample was loaded per track and electrophoresed on 6% polyacrylamide gels (19:1 acrylamide, 7.5 M urea and 1 X TBE)

for 1 h 30 min at 300 W. After electrophoresis, the glass plates were separated from each other and the gel treated for 10 minutes in fixation solution (7.5% v/v acetic acid) with gentle shaking and then washed in distilled water for 2 minutes. The fixation step was followed with oxidation for 3 minutes (1.5% v/v nitric acid). After incubating in staining solution (0.1% w/v silver nitrate, 750 µl formaldehyde), the gel was washed in distilled water for 10 seconds, and then transferred to cold developing solution (3% w/v sodium carbonate, 3 ml formaldehyde, 250 µl 1X sodium thiosulphate) to develop the silver-stained DNA bands. The development was stopped by using a stop solution (7.5% v/v acetic acid), and followed by detaching the gel from the glass by using sodium hydroxide (4% w/v). The gel was transferred to a 3MM chromatography paper and left at room temperature over- night to dry.

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5.3 RESULTS

5.3.1 Primers

From the 148 clones sequenced, 93 contained microsatellites repeat sequence. From the ninety-three SSR containing clones, primers were designed for 40. The others had flanking sequences that were insufficient for primer design or contained base sequences that constituted the basic motif of the microsatellite, to permit the design of primers. The total number of primers designed, synthesized and tested were 40 (Table 4.1).

5.3.2 Enrichment

The dinucleotide repeats had a higher number of repeats (12 on the average as compared with the trinucleotide (8) repeats. The maximum recorded for the dinucleotide was 22

repeats and that of trinucleotide was 14 repeats. Only insert with dinucleotide and trinucleotide repeats were used for primer design. The most common motif repeats was (GA)n/ (CT)n group.

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Table 5.1 The nucleotide sequences of both forward (F) and reverse (R)) primers

	(5'-3')
PM51-F:	CCAACCCAATCCCACTACAC
PM51-R:	AGACGGACCCACACAAGAAG
PM52-F:	GGTTGGGTTAGCGTTTGAAG
PM52-R:	CCCTGACGATGAGTCCTGAG
PM54-F:	GAAATCGCAGCCTCTACAATG
PM54-R:	TTACACGATTTAGATTCCCGAAA
PM55-F:	GGTTGGGTTAGCGTTTGAAG
PM55-R:	AAAGGCTTAGATCAAGCAGTAAAA
PM56-F:	TGTGTGTGTGTGTGTGTGAGAG
PM56-R:	TGTCCTCTTTCTCCCCTCCT
PM57-F:	TGTACCTGGGTTGGGTTAGC
PM57-R:	CCCCCTCACACACACACAC
PM58-F:	GGCTTAGATCAAGCAGGAAAA
PM58-R:	GGTCGGGTTAGCGTATGAAG
PM59-F:	GGTTGGGTTAGCGTTTGAAG
PM59-R:	TGCACACACAGTCACACACA
PM60-F:	TGTTGATGTTCAAGAGGTGAGAG
PM60-R:	CCTAAATCACCATTACTGTAGCATC
PM61-F:	GGCAACAATAGCCATCAGAAG
PM61-R:	TGTAGAGACGACACCCATGC
PM62-F:	CACAAGCATAACCCAGAGACAG
PM62-R:	TGGTGGTTATATTATATGGATGTGTTT
PM63-F:	CCACCGTACACTAAAATCATTCG
PM63-R:	AGTTATGGCCGATGTGTGTG
PM64-F:	CTCTTCCTCTCTCTCCCTCTC
PM64-R:	CGGTGCTCTCGCAACTTAC
PM66-F:	AGCATGGTTGGCAGTTATGG
PM66-R:	AAAATCATTCGCTCCCTCTTT
PM67-F:	TATATGGATGTGTTTTTGTGAGAGG

PM67-R:	AATTACACACAAGCATAACCCAGA
PM68-F:	GGCAACAATAGCCATCAGAAG
PM68-R:	TCGTTTCTTTTCGTTCTTTCTTTC
PM69-F:	GGCAACAATAGCCATCAGAAG
PM69-R:	TTCGAATTCCACCTTCCAAC
PM70-F:	CAGTTATCGTTTCTTTTCGTTCTTT
PM70-R:	CGGGGAGAGAGAGAGAGAGAG
PM71-F:	TCTGTTGGGTCGGTTAGGAG
PM71-R:	CGGGGAGAGAGAGAGAGAGAG
PM72-F:	TCGTCATACTCAGGACTCATCG
PM72-R:	CGCTCCCCCTTTTCACTAAC
PM74-F:	GGCAACAATAGCCATCAGAAG
PM74-R:	AGGTTCCAATTCCACCTTCC
PM75-F:	GACTCATCGTCAGCACTCTCG
PM75-R:	TTCTTCAACAACAACAGGTGCT
PM76-F:	TGTAGAGACGACACCCATGC
PM76-R:	CGATGGTGATTGGTGAAGTG
PM77-F:	TTCGAATTCCACCTTCCAAC
PM77-R:	AGTTATGGCCGATGTGTGTG
PM78-F:	ACGAGATGAGTCCTGACGAT
PM78-R:	AGGTACCTCTCTCTCTCTCTCTC
PM79-F:	AGTTATGGCCGATGTGTGTG
PM79-R:	CCACCGTACACTAAAATCATTCG
PM80-F:	TGGAGTGCACGAGAGAGAGA
PM80-R:	TTCGAATTCCACCTTCCAAC
PM81-F:	GGAGGAACACTGTAAGACACACA
PM81-R:	ATGACCTTTCCAACCCAAGA
PM82-F:	CCTTCCAACTCCACAAAACG
PM82-R:	ATAGCTCGACGGTGAAGTCG
PM83-F:	GGCAACAATAGCCATCAGAAG

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PM83-R:	TACTGCAACAGCGCGTAGAC
PM84-F:	TGAATGCTAGGCAACCAAAA
PM84-R:	TGTAGAGACGACACCCATGC
PM85-F:	GGCAACAATAGCCATCAGAAG
PM85-R:	AGGTTCCAATTCCACCTTCC
PM86-F:	GAGCTTGCCCTTTGATATGC
PM86-R:	ACCCCTTCCCTTCTCTTCAA
PM87-F:	GGAATTGCTAACTGAGGGAGAG
PM87-R:	CCACCGTACACTAAAATCATTCG
PM88-F:	CGTGGTGCTTGAGTGATTGT
PM88-R:	CCCTAGGGCTCTCTCTCTCTCT
PM89-F:	GTTATGGCCGATGTGTGTGG
PM89-R:	TCCACCGTACACTAAAATCATCC
PM90-F:	GGCAACAATAGCCATCAGAAG
PM90-R:	TGCACTTACACGCCAAATTC
PM91-F:	CCACCGTACACTAAAATCATTCG
PM91-R:	AGTTATGGCCGATGTGTGTG
PM92-F:	ACGCGCCTCTAACATTCATA
PM92-R:	CAGAATTTGGTTGCTACGATG
PM93-F:	CAGCAGCAACAACAACAACA
PM93-R:	GGTGGTAGTGTGAGGCCAGT

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5.4 DISCUSSION

An enriched microsatellite library was created using an enrichment procedure (Edwards *et al.*, 1996) with minor modifications. Out of a total of 148 clones, 93 were sequenced, 40 contained the required size for primer design. This compares favourably with what was obtained in groundnut by previous workers (Hopkins *et al.*, 1999; He *et al.*, 2003). The main factor reducing the number of potentially useful markers was the inadequate size of the flanking regions. This has been a common finding in a number of species (Hopkins *et al.*, 1999; He *et al.*, 2003). The most represented nucleotide sequence in plants are not equally represented, it varies with studies. Earlier reports indicated (AT)n motif to be the most highly represented in plant genomes (Powell *et al.*, 1996), but Ferguson *et al.* (2004) observed ATT and GA to be the dominant motif in groundnut. Differences in the predominant motif may be partly due to the probes used.

There has been reports of higher level of polymorphism for dinucleotide repeats than trinucleotides in groundnut (He *et al.*, 2003), avocardo pear (Ashworth *et al.*, 2004) and in pigeon pea (Odeny *et al.* 2007). Dinucleotide repeats have been reported to reside outside coding regions of genes (Temnykh *et al.*, 2001) and are characterized by higher repeat numbers (Li *et al.*, 2004). However, their use may be limited despite their polymorphism because of the presence of stutter bands in the amplification products which will result in incorrect scoring of alleles (Ashworth *et al.*, 2004, Wang *et al.*, 2005). Conversely trinucleotide repeats are abundant in protein coding regions (Tóth *et al.*, 2000) with relatively shorter repeat units and total length (Thiel *et al.* 2003). Longer repeat units have been found to exhibit higher level of polymorphism (Yu *et al.* 2000, Saghai-Maroof *et al.*, 1994). Some studies have found shorter repeat units not useful in

detecting polymorphism (Yu *et al.* 2000). Budak *et al.* (2003), discarded repeats less than 10 units. However, Odeny *et al.* (2007), found SSRs with four repeats to be highly polymorphic; detecting more than 10 alleles per locus. The number of repeats has been observed to correlate with the mutation rate (Li *et al.*, 2002). This implies that recently evolved microsatellite will be less polymorphic because of fewer occasions for mutation even if the repeat units are long (Budak *et al.* 2003). Cultivated groundnut is the result of recent polyploidization event and hence, a recently evolved species (Halward *et al.*, 1991, 1992).

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Only three of the primers were polymorphic when screened with two groundnut genotypes (C2024 and C3424) with varied backgrounds. The polymorphic primers were used to screen 22 groundnut genotypes from the six botanical varieties and F_2 population. The polymorphic primers can be used by other laboratories when their sequences are published.

CHAPTER SIX

6.0 GENETIC DIVERSITY OF PEANUT CORE COLLECTION FROM THE USA BASED ON SIMPLE SEQUENCE REPEAT MARKERS.

6.1 Introduction

Domestication of crop plants and intentional breeding programmes have led to erosion of genetic materials with its attendant narrowing of genetic variation in crop plants due to continuous selection pressure directed at genes controlling traits of agronomic importance like yield, earliness, fruit size, colour and shape. Selection for specific traits has rendered crop plants more vulnerable to disease and insect attack and destroying the potential for sustained genetic improvement over a long term (Harlan, 1987). The development of germplasm collection centres and gene banks was stimulated by an increasing awareness of the narrow genetic base of advanced agriculture and consequent potential susceptibility to crop failures (National Research Council, 1972). The establishment of the International Agriculture Research Centres (IARCs) and through coordinated multi-national effort has seen tremendous success in collecting and preserving a broad range of diversity of cultivars and wild relatives of the major food crops (Spooner, 1999) According to the State of the World Report (FAO, 1996), 6.1 million *ex situ* germplasm accessions are held world-wide in 1,300 separate collections.

The genetic resources stored in gene banks will be the basis of future crop improvement, however, the large number of accessions and limited resources and experts to thoroughly evaluate the accessions pose a great difficulty in identifying traits of interest.

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) holds more than 14,000 groundnut accessions and the United States have more than 8000

accessions in gene banks. However, this diversity has not been adequately evaluated (Banks, 1976) or extensively used in cultivar development (Knauft and Gorbet 1989), because of the difficulty in knowing which accession to select for cultivar development due to limited knowledge about the accessions. Only a few established cultivars and elite breeding lines have been utilised in breeding programmes. A core collection is a subset of accessions from the entire collection which represent most of the available genetic diversity of the species and should be about 10% of the entire collection with an upper limit of 3000 per species (Brown, 1989). The core collection is thus a representative of the entire collection and smaller group for effective evaluation. A core collection for the *A. hypogaea* germplasm has been developed to enhance the utilization of the entire collection. The International Crops Research Institute for the Semi-Arid tropics (ICRISAT) developed a core collection of 1704 from over 14,000 accessions based on morphological, geographic and taxonomic descriptors (Upadhyaya *et al.*, 2002). The core collection was still large and subsequently a mini core collection of 184 accessions was developed from the core for effective evaluation and utilization (Upadhyaya *et al.*, 2003). Holbrook *et al.* (1993) developed a groundnut core collection of 831 accessions from a total of 7432 US groundnut accessions based on country of origin and measurements of morphological characteristics like plant type, pod type, seed size, testa colour, number of seeds per pod, and average seed weight. This was followed by the use of multivariate analysis on morphological data to cluster the accessions into groups and then randomly sampling 10% from each group. A major benefit of having a peanut core collection has been a great increase in peanut germplasm evaluation work. Work by several groups of researchers (Anderson *et al.*1996; Holbrook *et al.*, 1998; Isleib *et al.*, 1995) has resulted

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in the evaluation of core accessions for 24 characteristics. This has resulted in the identification of numerous sources of resistance to several economically significant pathogens.

6.2 MATERIALS AND METHODS

6.2.1 Plant materials

Twenty-two cultivated groundnut accessions (Table 5.1) representing six botanical varieties were obtained from Tuskegee University, Alabama, USA.

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6.2.2 DNA extraction

Plant genomic DNA was extracted using MasterPure Leaf Purification Kit (Epicenter, Madison, W1). The DNA was tested on 0.8% agarose gel. The quality of DNA concentration was determined by DU640B spectrophotometer (Beckman Coulter, CA). DNA was diluted to 50 ng/µl in sterile water for PCR analysis. PCR reaction was as described in section 4.2.3

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Table 6.1. The accessions used for the detection of DNA polymorphism.

Botanical variety	Plant introduction number
Fastigiata	497517
	494002
	493581
	493536
Aequatoriana	628541
	602357
	497633
	497615
Peruviana	628572
	628572
	628569
	628571
Hirsuta	576616
	576634
Vulgaris	494029
	494053
	497489
	494049
Hypogaea	476093
	475982
	475861
	468213

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Table 6.2. List of groundnut microsatellites markers used, their sequences and repeat motif

Name	Primer 5'-3'	Repeat motif
PM3-F:	GAAAGAAATTATACACTCCAATTATGC	$(GA)_{14}$
PM3-R:	CGGCATGACAGCTCTATGTT	
PM15-F:	CCTTTTCTAACACATTCACACATGA	$GAAA)_3(GA)_8(GA)_9$
PM15-R:	GGCTCCCTTCGATGATGAC	
PM32-F:	AGTGTTGGGTGTGAAAGTGG	$(CT)_{15}$
PM32-R:	GGGACTCGGAACAGTGTTTATC	
PM35-F:	TGTGAAACCAAATCACTTTCATTC	$(GA)_{18}(GAA)_2$
PM35-R:	TGGTGAAAAGAAAGGGGAAA	
PM36-F:	ACTCGCCATAGCCAACAAAC	$(GA)_{18}$
PM36-R:	CATTCCCACAACTCCCACAT	
PM42-F:	ACGGGCCAAGTGAAGTGAT	$(GA)_4AA(GA)_{14}$
PM42-R:	TCTTGCTTCTTTGGTGATTAGC	
PM45-F:	TGAGTTGTGACGGCTTGTGT	$(GA)_{16}$
PM45-R:	GATGCATGTTTAGCACACTTGA	
PM50-F:	CAATTCATGATAGTATTTTATTGGACA	$(GA)_{19}$
PM50-R:	CTTTCTCCTCCCCAATTTGA	
PM53-F:	CCTATCCTATGGGTCACTAGCC	$(AT)_2T(AT)_2$
PM53-R:	GCTTGTGCTCATCTTGAGTTTT	
PM65-F:	GGACGTCTGGCTGCTAGAGA	$(CT)_{12}$
PM65-R:	TCGGCATCAAAACAGTGAGA	
PM137-F:	AACCAATTCAACAAACCCAGT	$(GA)_{19}$
PM137-R:	GAAGATGGATGAAAACGGATG	
PM145-F:	GCTGTAATTAGGATCATTCCACA	$(CT)_{12}(CA)_2(CT)_4(CA)_9$
PM145-R:	CAACGGTTGGATCGATGA	
PM183-F:	TTCTAATGAAAACCGACAAGTTT	$(CT)_{24}$
PM183-R:	CGTGCCAATAGAGTTTTATACGG	
PM188-F:	GGGCTTCACTGCTTTTGATT	$(GA)_8$
PM188-R:	TGCGACTTCTGAGAGGACAA	

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PM200-F:	GCTATGTGGGAAAAATACTGCTT	$(CT)_{22}(CA)_{16}$
PM200-R:	CAGATGTGTGTGTGTGTGTGTG	
PM201-F:	CCTTTATAGAGGACCTTCCCTCTC	$(CT)_{19}$
PM201-R:	GCCTATTTGGTATCGGCTCA	
PM204-F:	TGGGCCTAAACCCAACCTAT	$(GA)_{20}$
PM204-R:	CCACAAACAGTGCAGCAATC	
PM210-F:	CCGCAGATCTTCTCCTGTGT	$(CT)_{25}$
PM210-R:	CCTCCTCATCCTCTAAACTCTGC	
PM238-F:	CTCTCCTCTGCTCTGCACTG	$(CT)_{11}$
PM238-R:	ACAAGAACATGGGGATGAAGA	

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6.2.3 Cluster analysis

Gels were scored for the presence or absence of polymorphic band. Alignment scores and cluster analysis was performed using clustalw programme (http://www.ebi.ac.uk/clustalw)

6.3 RESULTS

The spectrophotometric readings are shown in Table 6.2. The extracted DNA was tested on 0.8% agarose gel. The results showed that all the primers could amplify clear bands in most of the accessions. Six primers could amplify specific bands in particular botanical varieties. The results indicated that few primers could distinguish between botanical varieties and individual accessions within the group. Most of the primers could amplify two or more specific bands for the botanical varieties. Primer PM 343 amplified different size of bands in four botanical varieties and could distinguish the four accessions within three botanical varieties (*equatoriana*, *fastigiata* and *peruviana*) and two accessions in *hirsuta* (Fig. 6.1). Primer PM 42 could identify the accession in *peruviana* (Fig. 6.2). Primer PM 50 was difficult to score because of shadow (stutter) bands. This made distinction between individual accessions within a group very difficult but could distinguish between three botanical varieties (*hypogaea*, *vulgaris* and *hirsuta*.

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Table 6.3. Spectrophotometric readings.

Botanical variety	Plant introduction number	ng/µl	260/280
Fastigiata	497517	1250	1.56
	494002	620	1.40
	493581	745	1.60
	493536	565	1.64
Aequatoriana	628541	690	1.60
	602357	365	1.52
	497633	445	1.37
	497615	715	1.56
Peruviana	628572	225	1.72
	628572	500	1.64
	628569	475	1.48
	628571	670	1.64
Hirsuta	576616	510	1.56
	576634	495	1.64
Vulgaris	494029	455	1.72
	494053	700	1.60
	497489	345	1.58
	494049	375	1.66
Hypogaea	476093	605	1.46
	475982	610	1.64
	475861	835	1.48
	468213	535	1.66

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The alignment scores are presented in Table 6.4. The phylogenetic tree based on SSR data precisely organized the six botanical varieties of the two subspecies into six clusters. Few primers could distinguish all the accessions analysed within a variety

The phylogenetic analysis showed the botanical varieties were formed into two groups that are consistent with two subspecies, *hypogaea* and *fastigiata* (Fig. 6.3). Subspecies *fastigiata* was further divided into two sub-groups; *fastigiata* and *aequatoriana* in one group and *peruviana* and *vulgaris* in another group.

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Fig 6.1. Silver stained acrylamide gel showing PCR products from primer PM 343 tested on 22 groundnut genotypes. The lanes from left to right correspond with the order of arrangement in Table 6.1.

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Fig 6.2. Silver stained acrylamide gel showing PCR products from primer PM 42 tested on 22 groundnut genotypes. The lanes from left to right correspond with the order of arrangement in Table 6.1.

Table 6.4. Similarity indices among 22 peanut accessions based on SSR allelic scores from 20 primer pairs

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1																						
2	.85																					
3	.55	.85																				
4	.70	.75	.85																			
5	.40	.70	.75	.80																		
6	.40	.65	.75	.80	.90																	
7	.40	.50	.65	.70	.80	.90																
8	.60	.65	.55	.55	.75	.80	.90															
9	.30	.65	.55	.60	.65	.55	.50	.45														
10	.35	.50	.55	.45	.55	.50	.50	.50	.80													
11	.50	.55	.55	.60	.65	.65	.35	.50	.60	.90												
12	.25	.60	.50	.50	.40	.45	.40	.40	.80	.55	.55											
13	.35	.45	.35	.30	.25	.25	.25	.35	.20	.15	.40	.35										
14	.30	.40	.30	.25	.20	.20	.20	.30	.45	.35	.40	.35	.95									
15	.25	.35	.45	.45	.50	.55	.50	.45	.60	.70	.45	.75	.45	.50								
16	.36	.47	.42	.47	.47	.52	.52	.47	.57	.68	.47	.73	.42	.47	.89							
17	.20	.45	.45	.45	.55	.55	.45	.40	.60	.65	.60	.75	.40	.25	.80	.84						
18	.15	.40	.45	.45	.55	.65	.40	.45	.50	.60	.50	.65	.25	.40	.80	.78	.90					
19	.25	.25	.35	.25	.30	.30	.55	.30	.25	.30	.20	.35	.15	.20	.45	.21	.55	.55				
20	.20	.25	.25	.25	.25	.35	.25	.30	.25	.25	.30	.15	.20	.15	.35	.36	.30	.35	.85			
21	.20	.25	.25	.25	.25	.35	.30	.30	.20	.25	.30	.20	.15	.20	.15	.36	.30	.35	.75	.90		
22	.20	.20	.25	.15	.20	.15	.15	.30	.35	.20	.25	.30	.15	.20	.10	.31	.35	.35	.60	.70	.80	

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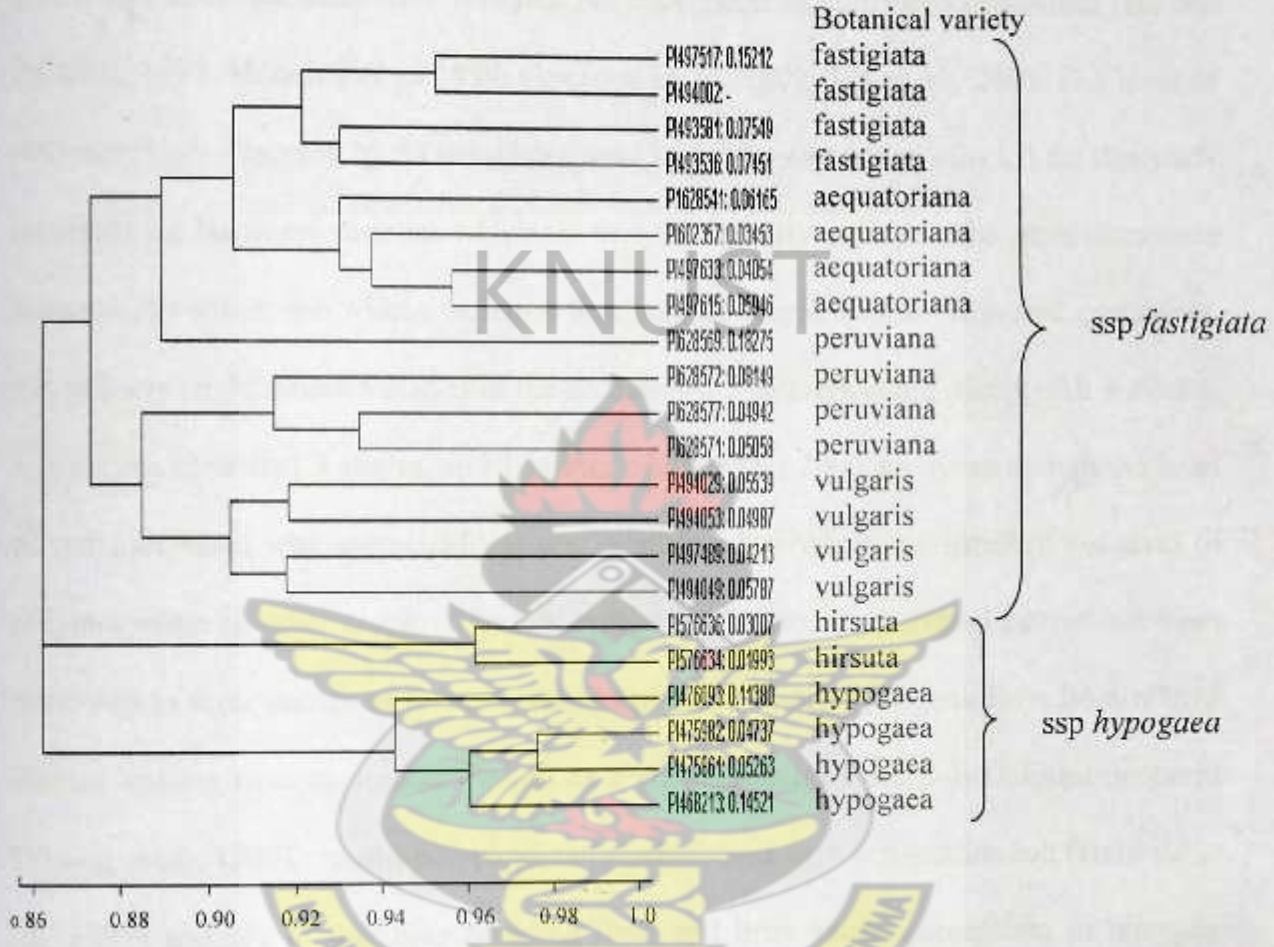

Fig.6.3. Phylogenetic tree computed by the programme CLUSTALW software, displaying the clustering relationship between 22 accessions of groundnut representing six botanical varieties.

6.4 DISCUSSION

Variation in the species *Arachis hypogaea* L. has been studied previously using isozymes, RAPDs and RFLP (Halward *et al.*, 1992; Lacks and Stalker, 1993, Halward, *et al.*, 1991). These studies revealed little variations between cultivars. Similarly, recent studies with AFLP and microsatellites have revealed polymorphism in cultivated groundnut (He and Prakash, 1997, Hopkins *et al* 1999, Gimenes *et al.*, 2002, He *et al.*, 2003 The level of polymorphism observed by these authors were low. The accessions selected for this study represent six botanical varieties which are morphologically variable. The accessions were selected to capture the widest variation that may be found within cultivated groundnut. Six primers could detect 5 alleles in the accessions, 5 primers could distinguish 4 alleles, 10 primers identified 3 alleles, and 1 primer could detect 2 alleles. Even though the level of polymorphism was appreciable, it was relatively low when compared to the level of polymorphism in other crops. The low level of variation in cultivated peanut has been attributed to three causes or to combinations of them: barriers to gene flow from related diploid species to domesticated peanut as a consequence of the polyploidization event (Young *et al.*, 1996), recent polyploidization combined with self-pollination (Halwald *et al.*, 1991) and use of few elite breeding lines and little exotic germplasm in breeding programs, resulting in a narrow genetic base (Knauft and, Gorbet, 1989; Isleib and Wynne 1992).

The phylogenetic tree places *A. hypogaea* accessions at the outermost intra-specific branch, put *fastigiata* and *aequatoriana* in one group, *peruviana* in one group and *hirsuta* and *hypogaea* in another group. Identification of molecular markers associated with only one botanical variety would be very useful. More groundnut SSR markers should be

developed to differentiate specific loci for botanical varieties and accessions. The low level of polymorphism observed in groundnut is due to genetic bottleneck brought about by the polyploidisation event, which prevented gene flow from diploid species in section *Arachis* into the cultivated groundnut (Young *et al.*, 1996). Groundnut is also a self pollinated crop, out- crossing is difficult. The tree obtained from the cluster analysis put the lines in their assigned specific botanical groups in agreement with available morphological classification for groundnut (Kaprovickas and Gregory 1994). The second observation was that, the position of the botanical groups in the clusters did not follow the same sequence as observed by He *et al.* (1997) when they studied diversity within the botanical varieties of groundnut. This observation is not unique, grouping genetically more distant lines in the same cluster have even been reported by Powell *et al.* (1996). The possible reasons for these discrepancies include underlying assumptions in calculating pedigree data (Messmer *et al.*, 1993), genome sampling method (Nei, 1987) and the number of markers or probes employed (Tivang *et al.*, 1994). Pejic *et al.* (1998) observed that to obtain precision in the estimate in RFLP require 30-40 probe-enzyme combination, 40-50 primers of RAPDs, 4-5 enzyme combination in AFLP and 20-30 SSR primers. The phylogenetic tree based on SSR data precisely organized the 22 accessions into six botanical varieties. The molecular data generated using the core collection agrees with the morphological classification of cultivated groundnut and hence, can be used for taxanomic studies.

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CHAPTER SEVEN

7.0 GENETIC LINKAGE MAPPING IN GROUNDNUT BREEDING LINES

7.1 Introduction

A genetic linkage map represents the relative order of genetic markers/genes along a chromosome. The relative distances between the markers are determined by recombination frequencies. Genetic linkage map is essential for mapping of candidate genes, to search for quantitative trait loci (QTL) of agronomically important traits, marker assisted-selection and construction of physical maps. Molecular marker-assisted technology offers alternative short but efficient route to crop improvement by providing a wide range of novel approaches to improving the selection strategies in plant breeding. The main principle of crop improvement is to incorporate one or more desirable genes from a donor parent into the background of an adapted variety. Knowledge of the location of genes and the specific alleles offer the possibility to apply marker-assisted selection in crops. According to Arus and Moreno-Gonzalez (1993), the characteristics of a good marker are: to distinguish between homozygote and heterozygote, thus ensuring more genetic gain per generation than is possible without using the marker; have early expression in the plant, thus saving time waiting for the phenotypic expression of genes; and not having interaction with other markers. Molecular markers also do not require gene expression and are not affected by the environment. Marker assisted selection has been proven to increase selection efficiency, especially for traits with low heritability (Bernardo, 2001).

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Genetic maps are estimated from the recombination rates between loci as a result of crossovers during meiosis (Menz *et al.*, 2002), and serve a number of purposes in basic and applied research. They are a key for chromosome analysis; to clone a gene it is necessary to identify molecular markers closely linked to the gene of interest (Wicking and Williamson, 1991). High-density linkage maps have direct application in plant and animal breeding since virtually any gene of interest will be tightly linked to at least one molecular marker. In breeding programmes such phenomenon can be exploited for marker assisted selection of desirable genes (Burr *et al.*, 1983; Tanksley *et al.*, 1989).

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The principle behind marker-assisted selection and genetic linkage mapping is that, DNA markers are used to identify sequence polymorphisms and monitor the segregation of a DNA sequence among progeny of a cross in order to assist in the selection and/or to construct a linkage map. Backcross and F_2 populations are suitable for DNA-based mapping, but recombinant inbred (Burr and Burr, 1991) and double haploids lines (Huen *et al.*, 1991) offer permanent mapping resources and suited for quantitative trait analysis (Young, 2000). Genetic map is essential for detection, mapping and estimation of gene effects of important agronomic traits, research on the structure, organisation, evolution and function of plant genome (Zhang *et al.*, 2002). Because of the limited variability observed in groundnut, the first RFLP map of the crop was constructed via a cross between two wild diploid *Arachis* species (Halward *et al.*, 1993).

7.2 MATERIALS AND METHODS

7.2.1 Mapping population

The mapping population consisted of F_2 recombinant inbred lines derived from a cross between two distantly related groundnut lines; C2024 and C3424. The population consisted of 80 individuals.

7.2.2 Linkage analysis

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Genomic DNA of the parents and the F_2 mapping population were obtained by courtesy of Dr. He (Tuskegee University, USA). The parents were screened with SSR primers for polymorphism between them (Fig. 6.1). The primers that were found to be polymorphic between the parents were used to evaluate the F_2 mapping population for segregation (Fig. 6.2).

PCR reaction was as described in section 4.2.3.Analyses of the F_2 progeny were scored as A for homozygous parent C2024, and B for homozygous parent C3424 and H for heterozygous. Segregating data were analysed using MAPMAKER 3.0.

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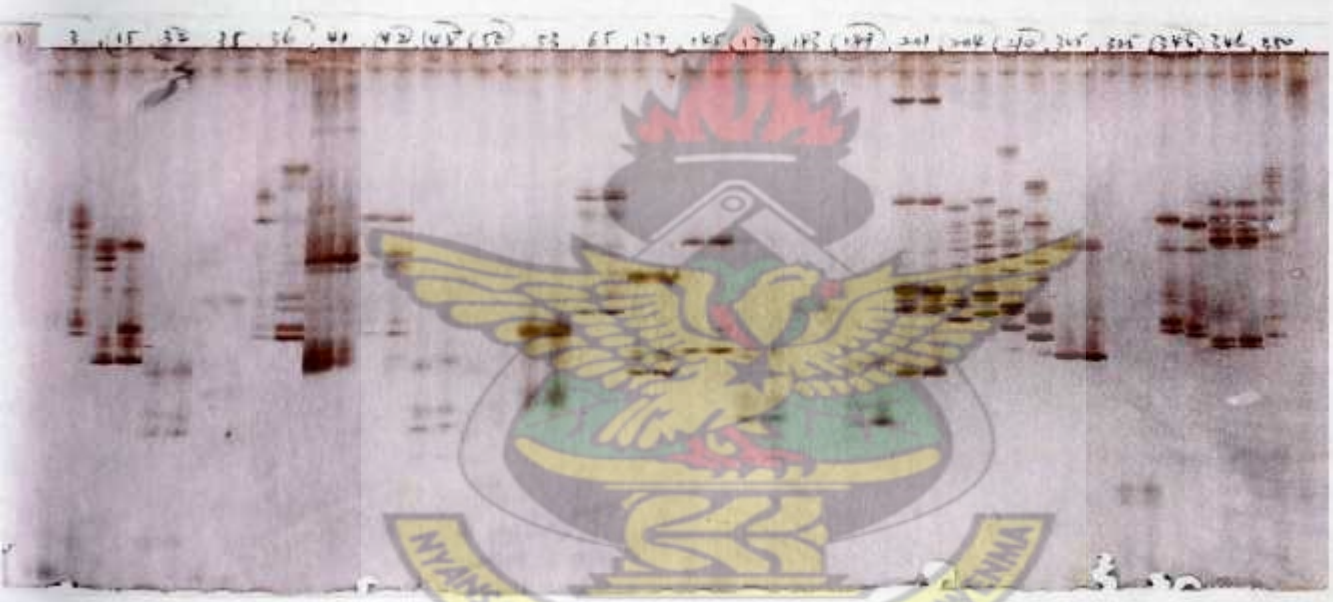

Fig. 6.1 A typical electrophoretic pattern displaying polymorphism in the two parental lines (C2024 and C3424) when tested with SSR primers.

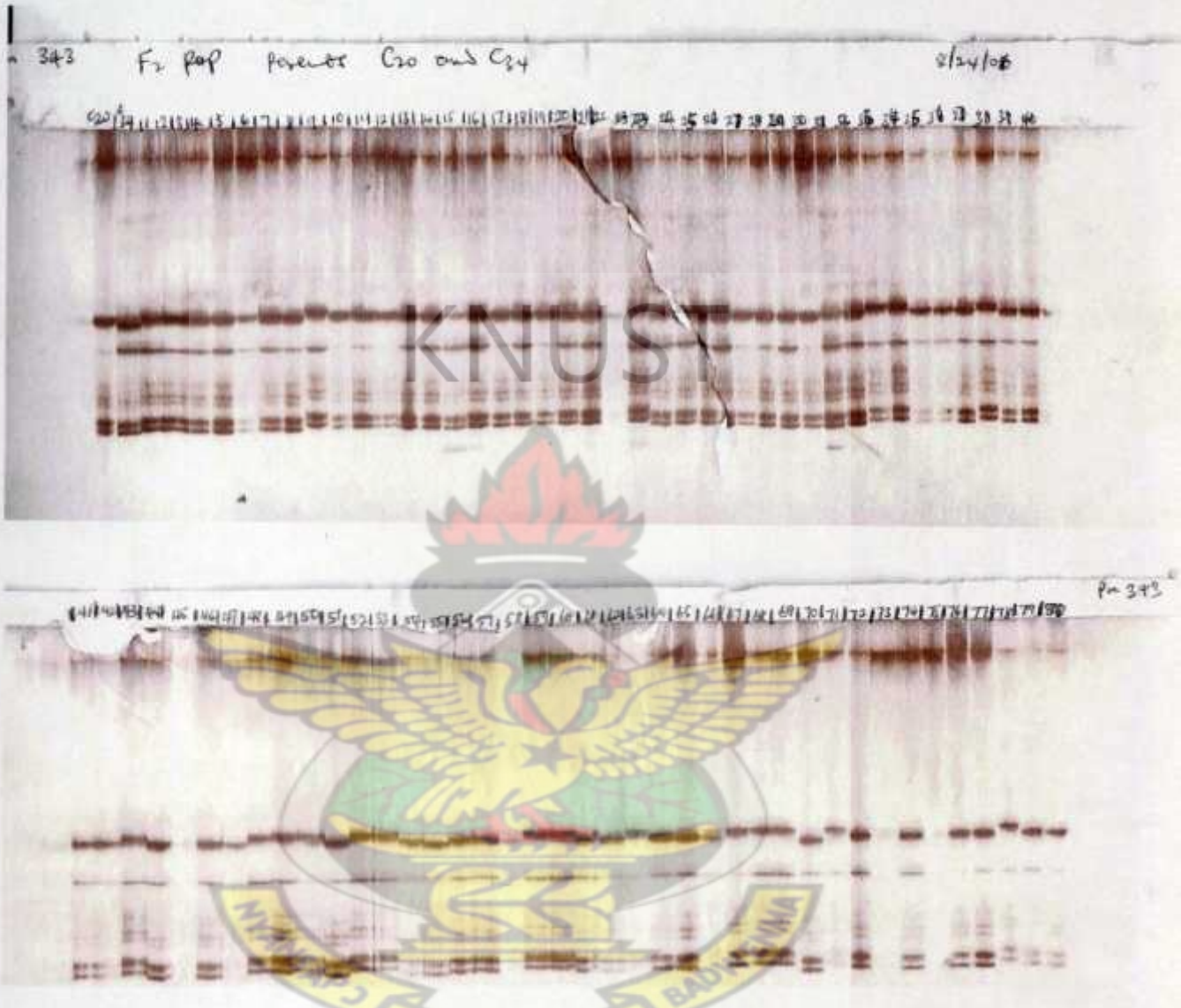

Fig. 7.2. Gel profile of Primer PM 343 used to test the genotypes of the two parents and 80 F_2 population from a cross between C2024 and C3424. (From left: lanes 1 and 2 are the parents followed by the 80 F_2 progenies.

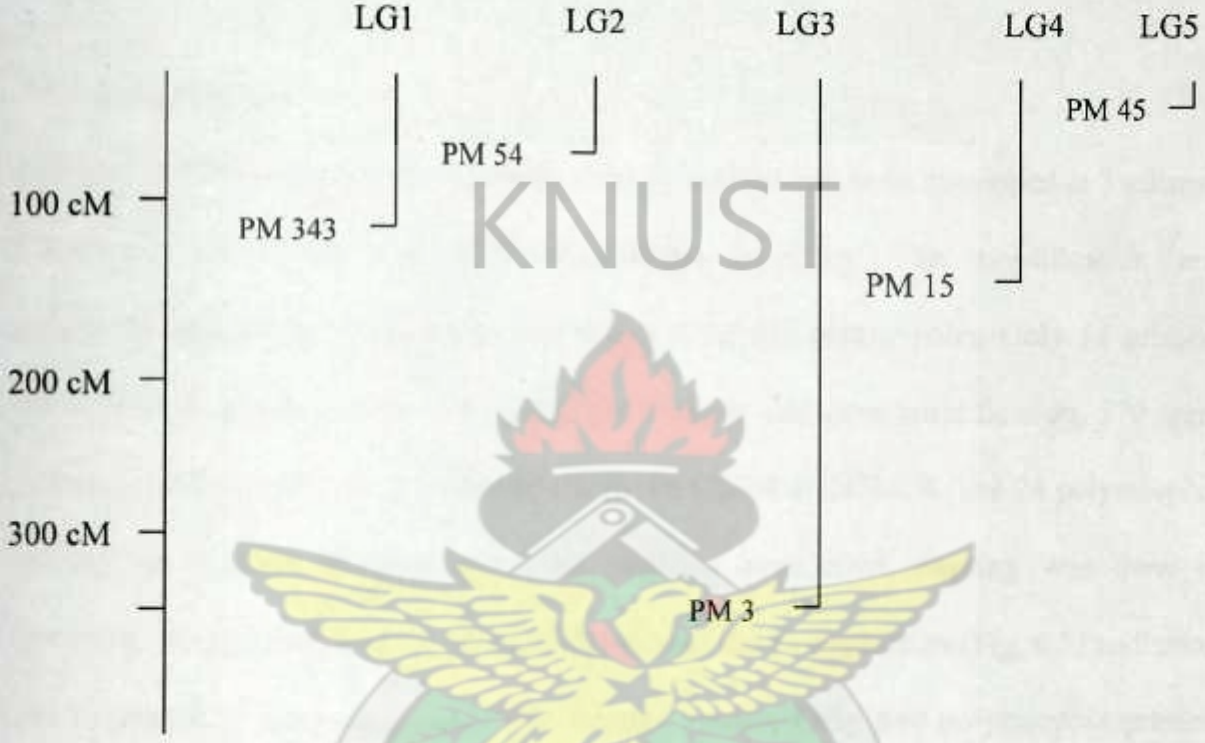

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Fig. 7.3. Genetic linkage map of cultivated groundnut. Kosambi map distance is on the left.

7.3 RESULTS

7.3.1 Mapping population

Groundnut is an allotetraploid and self-pollinated crop, consequently the crop is homozygous. The mapping population consisted of 80 individuals from F_2 population from a cross between C3424 used as female parent and C2024 used as male parent.

7.3.2 SSR markers

Most of the SSR primer pairs originated from groundnut and were developed at Tuskegee University, USA and University of Georgia in USA. The amplification and polymorphism of the two parents were tested using 612 primer pairs. Only 18 primers pairs gave no amplification. Out of the 594 primers that gave amplification, 570 were monomorphic and 24 were polymorphic between C2024 and C3424. The 24 polymorphic primers were used for genotyping the mapping population. Scoring was done to determine the genotypes of the 80 individuals from the F_2 population (Fig. 6.2) and using MAPMAKER 3 software grouped into linkage groups. Only five polymorphic primers could be scored and used for the linkage analysis. The linkage map consisted of 5 linkage groups spanning a distance of 669.5 cM (Fig 6.3)

7.4 DISCUSSION

The maps reported by Halward *et al.* (1993) using F_2 population derived from a cross between two diploid *Arachis* species (*A. stenosperma* and *A. cardenasii*) and Burrow *et al.* (2001), using a synthetic amphidiploid from a cross between 3 diploid wild *Arachis* species and cultivated groundnut involved a backcross population of 78 progeny

constructed with undomesticated groundnut, so they cannot be applied extensively in groundnut genome research, because it could not cover the whole groundnut genome. This study aimed at using cultivated groundnut to construct genetic linkage map. The limited number of polymorphism observed in this study and the difficulty in scoring prevented a dense map from being generated. The limited number of polymorphism in groundnut has been a common finding by many authors. Groundnut has been reported to have narrow genetic base reflected by low levels of isozyme (Halward *et al.*, 1992; Lacks and Stalker, 1993) and DNA polymorphism (Halward *et al.*, 1992, Stalker and Mozingo, 2001) The development of a dense genetic linkage map in cultivated tetraploid groundnut is the first step in understanding the genetic control of important agronomic traits, positioning and tagging genes of interest for marker assisted selection, gene cloning and analysing complex traits (Levi *et al.*, 2001). Agronomic traits are not well evaluated in cultivated groundnut because of lack of polymorphism at the DNA level. SSR markers were chosen because they are handy, PCR based, co-dominant and locus-specific. Yet the limited number of polymorphic SSR markers in groundnut poses a great challenge to the crop's improvement.

The low level of polymorphism observed in this study, in which only 24 primers out of over 600 primers tested could discriminate between the two parental lines from diverse origin, is a reflection of the low level of polymorphism in cultivated groundnut. The results strengthens Halward *et al*'s (1991) observation that cultivated groundnut had a single origin and has since suffered a genetic bottleneck with no introgression from its diploid relatives in the section *Arachis*. Again, the presence of stutter bands made distinction of homozygotes from heterozygotes to be extremely difficult. Even where

primers detected polymorphism between the parents, the sizes of the bands were so close and may have been differed by only a few base pairs (Fig. 6.1). The primary map that was compiled by the MAPMAKER 3 programme had 5 linkage groups, the results of only 5 primers (PM 343, PM 54, PM 3, PM 204 and PM 45) being scored and used for the map construction. The five primers were distributed in 5 linkage groups and were unlinked (Fig. 6.3). The total distance was 669.5cM with log-likehood -93.97. The low level of polymorphic primers developed for groundnut calls for more investment and research in groundnut genomics.

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CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

Information on the nutritional quality of groundnut landraces in Ghana is limiting. This study was undertaken to determine the nutritional quality of 20 groundnut landraces in Ghana. The study showed that the local landraces had significant genetic variations in nutritional qualities among the accessions and between the subspecies. These differences might be exploited to identify accessions with superior qualities to be used as parents for the improvement of the crop. Oil content ranged from 33.60 to 54.95%. "Nkate kokoo" and "kintampo" local had high oil content of 55%. These two varieties compare favourably with improved high oil content groundnut anywhere in the world. These varieties may not only be used as parents to improve the crop but oil processors may opt for these varieties to achieve good oil yields. The oleic/linoleic acid ratio has been used as a parameter for predicting the stability and shelf life of groundnut oil and products; groundnut seed with O/L ratio more than 1.0 is preferable. All the varieties analyzed in this study had values above 1.0. "Dagomba" and "Nkate kokoo" had high O/L ratio of about 3.5; these varieties would be ideal for the development of products that would be utilised over a long period of time, since their products would have longer shelf life. There was high linear correlation between oleic and O/L acid ratio (r^2=0.983) and negative correlation between oleic acid and linoleic acid (r^2=-0.996). This indicates selection for high oleic acid will bring about corresponding increase in O/L ratio and lower levels of linoleic acid.

Groundnut varieties belonging to subspecies *fastigiata* are early maturing and erect in nature. These varieties are popular in Sub-arid tropical regions of Africa and Asia because of their short duration and their ability to fit into the cropping systems. However, these varieties lack dormancy when fresh and germination occur in the field when harvesting is delayed. The mode of inheritance of fresh seed dormancy has been a subject of controversy. Different workers attributed varied gene actions to be responsible for the trait. The study was therefore initiated to find further evidence to the subject. The inheritance of fresh seed dormancy of groundnut was studied under a lath house and field condition using dormant and non-dormant Spanish varieties to understand the mode of inheritance of this trait. Results indicated that less than 10% of the freshly harvested seeds of the non-dormant parents germinated after 14 days; however, more than 90% of the dormant parents germinated after 14 days of incubation. The F_1 progenies generated from crosses between the dormant and non-dormant parents germinated after 14 days and thus, behaved like the dormant parents with the majority of seeds germinating after 14 days. This is in agreement with earlier work (Lin and Lin 1971; Uppadyaya and Nigam 1999). The ratio of the F_2 generation fitted the expected ratio of 3 dormant: 1 non-dormant. Backcross progenies with non-dormant parents fitted to an expected 1 dormant: 1 non-dormant. These observations suggest that fresh seed dormancy is controlled by one allele of the dominant gene. The evidence from this study should lead to the transfer of fresh seed dormancy into early maturing groundnut varieties which are popular in Sub-arid regions of Africa and Asia in order to reduce yield losses associated with *in situ* germination in varieties that lack fresh seed dormancy.

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Groundnut is a unique crop; despite a wealth of diversity at the morphological level, there is little polymorphism in seed storage protein, isozymes and at the DNA-level. The DNA-level variability is so limited that attempts to identify polymorphism among varieties and landraces using restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) markers (Halward *et al.* 1991; Kochert *et al.* 1991) have failed. Among the over 70 simple sequence repeat (SSR) loci evaluated by Hopkins *et al.* (1999), only 6 showed DNA polymorphism among *A. hypogaea* accessions. Microsatellite markers have been used to identify polymorphism in groundnut in recent studies. More polymorphic microsatellites markers should be developed for genetic studies in groundnut. However, microsatellite development is highly expensive, time consuming and labour intensive. Nevertheless, microsatellite markers published in journals and other public databases like genebanks are available to other laboratories through published sequences of primers. Microsatellites primers were develop via the enriched library construction method using streptavidin-coated magnetic beads (He *et al.*, 2004, Edwards *et al.*, 1996). At the end of the study, 148 clones were sequenced, 93 contained microsatellite repeat sequences. From the ninety-three SSR containing clones, primers were designed for 40. The primers were tested on two groundnut lines (C2024 and C3424) with diverse origin. The analysis revealed that only three were polymorphic primers and could discriminate against the two lines. The results agrees with an earlier work by Hopkins *et al.* (1999) who observed among the 70 simple sequence repeat (SSR) loci evaluated in which only 6 showed DNA polymorphism among *A. hypogaea* accessions.

The genetic resources stored in gene banks will be the basis of future crop improvement, however, the large number of accessions and limited resources and experts to thoroughly evaluate the accessions pose a great challenge to identifying traits of interest. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) holds more than 14,000 groundnut accessions and the United States have more than 8000 accessions in gene banks. However, this diversity has not been adequately evaluated (Banks, 1976) or extensively used in cultivar development (Knauft and Gorbet 1989).

A core collection which is a representative of the accessions in the gene banks has been developed to facilitate thorough evaluation. The diversity among the United States of America groundnut core collection was studied using microsatellites markers. In contrast to its apparent wide variations in traits, groundnut genetic variations at molecular level as detected by RAPD, RFLP, and SSR analysis, proved to be unexpectedly low (Halward *et al.*, 1993; Krishna *et al.*, 2004). The 22 accessions selected for this study represented six botanical varieties which were morphologically variable. The accessions were therefore selected to capture the widest variation found within cultivated groundnut. The results indicated that twenty primers were polymorphic; six primers could detect 5 alleles in the accessions, 5 primers could distinguish 4 alleles, 10 primers identified 3 alleles, and 1 primer could detect 2 alleles. Even though the level of polymorphism was appreciable, it was relatively low when compared to the level of polymorphism in other crops. The low level of variation in cultivated peanut has been attributed to three causes or to combinations of them: barriers to gene flow from related diploid species to domesticated peanut as a consequence of the single polyploidization event (Young *et al.*, 1996), that gave rise to the tetraploid, recent polyploidization combined with self-pollination

(Halwald *et al.*, 1991) and use of few elite breeding lines and little exotic germplasm in breeding programmes, resulting in a narrow genetic base (Knauft and, Gorbet, 1989; Isleib and Wynne 1992).

A genetic linkage map represents the relative order of genetic markers/genes along a chromosome. Genetic linkage map is essential for mapping of candidate genes, to search for quantitative trait loci (QTL) of agronomically important traits, marker assisted-selection and construction of physical maps. Genetic map is essential for detection, mapping and estimation of gene effects of important agronomic traits and research on the structure, organisation, evolution and function of plant genome (Zhang *et al.*, 2002). The study aimed at using cultivated groundnut to construct genetic linkage map. Previous studies used wild groundnut species to construct genetic linkage map of groundnut which did not wholly represent the genome. Also these studies used RFLP markers which have detected little polymorphism in cultivated groundnut. The limited number of polymorphism observed in this study (24 primers out of 618 primers were polymorphic) coupled with the difficulty in differentiating homozygotes from heterozygous individuals in the primers that were polymorphic made it difficult to construct a good genetic linkage map. The genetic base of the crop should be broadened by introgression of traits of agronomic importance from wild types into the cultivated groundnut for gene transfer. Introgression from wild diploid species would require identification of the diploid progenitors and their use in developing synthetic tetraploids that could be used as a bridging specific for gene transfer. The limited number of polymorphism in groundnut has been a common finding by many authors. Groundnut has been reported to have narrow genetic base reflected by low levels of isozyme (Halward *et al.*, 1992; Lacks and

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Stalker, 1993) and DNA polymorphism (Halward *et al.*, 1992, Stalker and Mozingo, 2001). The development of a dense genetic linkage map in cultivated tetraploid groundnut is the first step in understanding the genetic control of important agronomic traits, positioning and tagging genes of interest for marker-assisted selection, gene cloning and analysing complex traits (Levi *et al.*, 2001). Agronomic traits are not well evaluated in cultivated groundnut because of lack of polymorphism at the DNA level. SSR markers were chosen because they are handy, PCR based, codominant and locus-specific. Expanding the germplasm base is essential for overcoming the many challenges facing producers, processors, and consumers.

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CONCLUSION

In conclusion, the studies identified local groundnut landraces with varied nutritional qualities which can be exploited for breeding programmes; fresh seed dormancy was found to be controlled by monogenic inheritance; microsatellite primers were developed and used for genetic analysis, some level of polymorphism were detected by the primers, yet the level of polymorphism was low as compared with other crops, more research attention is needed to broaden the genetic base of the crop. Efforts should be directed towards identifying the true progenitors of *A. hypogaea*, to be used in the development of amphidiploids and as a bridging species for transferring genes from the wild into cultivated groundnut.

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