A DIVERSITY ANALYSIS OF EARLY-MATURING GROUNDNUT GERMPLASM USING SSR MARKERS

By

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CERTIFICATE

Mr. CHANDA ASHOK KUMAR has satisfactorily prosecuted the course of research and that the thesis entitled **"A DIVERSITY ANALYSIS OF EARLY-MATURING GROUNDNUT GERMPLASM USING SSR MARKERS"** submitted is the result of original research work done and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or any part thereof has not been previously submitted by him for a degree of any university.

(Dr. J. H. CROUCH)
Chairman of the Advisory Committee

Date:

Place:

CERTIFICATE

This is to certify that the thesis entitled **"A DIVERSITY ANALYSIS OF EARLY-MATURING GROUNDNUT GERMPLASM USING SSR MARKERS"** is submitted as a partial fulfillment of the requirements for the degree of **"MASTER OF SCIENCE IN AGRICULTURE"** of the **Acharya N. G. Ranga Agricultural University, Hyderabad**, is a record of the bonafide research work carried out by **Mr. CHANDA ASHOK KUMAR** under my guidance and supervision. The subject of the thesis has been approved by the student's advisory committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigation have been duly acknowledged by the author of the thesis.

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I, **CHANDA ASHOK KUMAR,** here by declare that the thesis entitled " **A DIVERSITY ANALYSIS OF EARLY-MATURING GROUNDNUT GERMPLASM USING SSR MARKERS"** submitted to the **ACHARYA N. G. RANGA AGRICULTURAL UNIVERSITY** for the degree of **MASTER OF SCIENCE IN AGRICULTURE** is the result of original research work done by me. I also declare that any material contained in the thesis has not been published earlier in any manner.

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ABSTRACT

Groundnut (*Arachis hypogaea* L.) is an important crop internationally for both direct human consumption and as an oilseed crop, which is being cultivated in 108 countries of the world. About two thirds of world groundnut production comes from the semi-arid tropic (SAT) regions which are characterized by uncertain rainfall and frequent droughts. Groundnut yields are low and average about 0.8 t/ha in the SAT countries compared to more than 2.6 t/ha of the developed world.

One contribution to increasing yield is development of early maturing, high yielding cultivars that are needed for short growing seasons, multiple cropping systems and which avoid late season droughts. In most breeding programs few sources of early maturity have been used resulting in the narrow genetic base of groundnut cultivars. There is a need for broadening the genetic base to enhance groundnut breeding prospects. The high diversity detected by SSR markers is consistent with the known characteristics – that they are more variable and high expected heterozygosity than the RAPDs, or AFLPs. The high levels of polymorphism associated with SSRs are expected because of unique evolution of these genomic regions: replication slippage rather than mutations, insertions or deletions.

. The present study was initiated to assess diversity using SSR markers among 29 groundnut accessions belonging to two subspecies *fastigiata* and *hypogaea* and three botanical varieties *vulgaris*, *fastigiata*, and *hypogaea*, originating from fifteen countries which include 25 early-maturing and 4 late-maturing accessions. Initially 7 to 10 individual plants from each of ten accessions were assayed for intra-accession variation using 5 SSR primer pairs. These ten accessions include ICG 3540, ICG 4558, ICG 4890, ICG 9427, ICG 11914, ICG 14814, Gangapuri, JL 24, Chico and TMV2. UPGMA clustering of the SSR band profiles revealed significant variation within the accessions. A total of 22 alleles were detected by five primer pairs with an average number of 4.4

alleles per primer pair. The number of alleles ranged from 2 alleles for 2B10 to 8 alleles for 2D12B. To capture this intra-accession diversity in the main study equal amounts of DNA from individual plants were pooled for each accession.

Inter-accession diversity analysis of 29 accessions was performed using 20 SSR primer pairs, which detected a total of 57 alleles with an average of 2.85 alleles per primer pair. The number of alleles per marker ranged from two to five. The PIC values, ranged from 0.53 (17F6) to 0.93 (15C12), with an average of 0.78. The AMOVA analysis indicated 42% of variation in SSR markers used between early and late-maturing accessions. The clustering revealed significant diversity among the 29 accessions used for this study. The different botanical varieties were grouped into 3 different clusters except one accession. The MDS analysis supported the clustering obtained by UPGMA. Clustering also revealed significant diversity among accessions within a particular country. Comparison of genotypic data with phenotypic data for these accessions may project the complete picture of diversity. This analysis will assist groundnut breeding programs aimed at improving early-maturity to maximize the genetic base of their breeding populations.

ABBREVIATIONS

%	: per cent
°C	: degree Celsius
µg	: microgram
µl	: microlitre
µM	: micromolar
AAT	: Asparate Amino Transferase
AFLP	: Amplified Fragment Length Polymorphism
AFLP	: Amplified Fragment Length Polymorphism
AMOVA	: Analysis of Molecular Variance
APS	: Ammonium Persulphate
bp	: base pair
C	: Chloroform
cDNA	: complementary DNA
cM	: centi Morgan
CTAB	: Cetyl Trimethyl Ammonium Bromide
DAF	: DNA Amplification Fingerprinting
DNA	: Deoxyribo Nucleic Acid
dNTP	: deoxy Nucleotide Tri-Phosphate
EDTA	: Ethylene Diamine Tetra Acetic acid
FAO	: Food and Agriculture Organization
g	: gram
IAA	: Iso-amylAlcohol

ISSR	: Inter Simple Sequence Repeat
L	: Litre
M	: Molar
MAB	: Marker-Assisted Breeding
MAS	: Marker-Assisted Selection
MDS	: Multi Dimensional Scaling
mg	: milligram
Mha	: Million hectares
ml	: millilitre
mm	: millimetre
mM	: millimolar
Mt	: Million tonnes
ng	: nanogram
NTSYS	: Numerical Taxonomy and Multivariate Analysis System
PAGE	: Poly Acrylamide Gel Electrophoresis
PCR	: Polymerase Chain Reaction
PHI	: Phospho Hexose Isomerase
PIC	: Polymorphism Information Content
pmol	: picomole
QTL	: QuantitativeTtrait Loci
r	: cophenetic correlation coefficient
RAPD	: Randomly Amplified Polymorphic DNA
RFLP	: Restriction Fragment Length Polymorphism
RNA	: Ribo Nucleic Acid
RNase	: Ribonuclease

RFLP	: Restriction Fragment Length Polymorphism
RNA	: Ribo Nucleic Acid
RNase	: Ribonuclease
rpm	: revolutions per minute
SAHN	: Sequential Agglomerative Hierarchial Non-overlapping
SAT	: Semi-Arid Tropic
SSR	: Simple Sequence Repeat
STMS	: Sequence Tagged Micro-Satellite
t/Ha	: tonnes per hectare
TBE	: Tris Borate EDTA
TE	: Tris-EDTA
TEMED	: N,N.N',N'- Tetra Methyl Ethylene Diamine
UPGMA	: Unweighed Pair Group Method based on Arithmatic Average
UV	: Ultraviolet
v	: volt
v/v	: volume by volume
vol	: volume
W	: Watt
w/v	: weight by volume

CHAPTER I

INTRODUCTION

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INTRODUCTION

Groundnut (*Arachis hypogaea* L) is an important crop internationally for both direct human consumption and as an oilseed crop, grown throughout the tropical and warm temperate regions in an area of 26 41 Mha with a total production of 37 05 Mt (FAO, 2003) Groundnut is cultivated in 108 countries of the world Asia with 60% area produces 68% of world groundnut production followed by Africa with 35% area and 22% production and North-Central America with 3% area and 7% production Groundnut occupies 31 3 % of the total cropped area under oilseeds and accounts for 36 1 % of total oilseeds production in the world Groundnut, the 'king' of oilseeds in India, occupies an area of about 8 0 Mha with a production of 7 5 Mt (FAO, 2003) Groundnut accounts for 45 % of the area and 55 %t of the production of total oilseeds in the country (Varma, 2002). In India groundnut is grown in three seasons i e , rainy (85% area) post rainy (10% area) season groundnut and summer (5% area) The rainy season groundnut, which is grown during the southwest monsoon period (June-November) is spread over the entire country and is generally rainfed

The genus *Arachis* belongs to the family Leguminosaea, tribe *Aeschynomeneae* and subtribe *Stylosanthinae* It is probably originated as a geocarpic form of Stylosanthinaes in Brazil or northeastern Paraguay (Kaprovikas *et al* , 2000) Cultivated groundnut (*Arachis hypogaea* L) can be botanically classified into two subspecies, which mainly differ in their branching pattern subspecies *hypogaea* with alternate branching and subspecies *fastigiata* with sequential branching Each subspecies is again divided into

two botanical varieties; subsp. *hypogaea* into var. *hypogaea* (virginia) and var. *hirsuta* and subsp. *fastigiata* into var. *fastigiata* (valencia), var. *vulgaris* (spanish), var. *peruviana* and var. *aequatoriana*.

Marker-assisted selection (MAS) offers great scope for increasing the efficiency of conventional plant breeding. Molecular markers are especially advantageous for traits with low heritability where traditional selection is difficult, expensive or lack accuracy or precision (Crouch, 2001). The essential requirements for developing MAS breeding programs include (i) availability of polymorphic germplasm with useful characteristics, (ii) identification of flanking markers closely linked on either side of the gene/quantitative loci, (iii) simple robust polymerase chain reaction (PCR)-based marker technology to facilitate rapid and cost effective screening of large breeding populations, and (iv) highly accurate and precise screening techniques for phenotyping of mapping populations. The molecular markers offer many advantages over morphological markers as they are phenotypically neutral, occur throughout the genome, neither influenced by environments nor by pleotropic and epistatic interactions, and expression is not dependent on plant age. Molecular markers also offer savings in time and cost for introgression of genes into cultivars (Tanksley *et al.*, 1989; Melchinger, 1990). The use of DNA markers could speed up this process by three plant generations as they allow the selection of offspring that contain the lowest amounts of the donor genome in every generation (Tanksley *et al.*, 1989). Molecular markers are also useful in the assessment of genetic diversity for (i) the identification and removal of duplicates (ii) production of core collections (iii) evaluating genetic relationships between taxa (iv) selecting diverse parental genotypes required for (a) studying QTLs for complex traits (b) developing mapping populations.

Recent achievements in the development of marker protocols such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR), and simple sequence repeats (SSRs) (also known as microsatellites) have revolutionized the genetic analysis and opened new possibilities in the study of complex traits in the crop plants. SSRs belong to the co-dominant marker class, are easy to manipulate, highly reproducible, and targets hypervariable regions of the genome. They are tandem repeats of DNA sequences of only a few basepairs (1-6 bp) in length (Gupta *et al.*, 1996). Variation in the number of repeated core sequence of nucleotides at a SSR locus among different genotypes provides the basis for polymorphism that can be used in plant genetic studies. SSRs are therefore excellent choice of DNA markers for genetic mapping in plants. Unlike RFLPs, for instance SSR technology is PCR based, requires only nanograms of DNA, and is readily automatable. Unlike RAPDs, SSR markers have proven to be reliable and reproducible. Unlike AFLPs, they are co-dominant and species specific. Moreover, they are both size and sequence specific. SSRs can be used in pedigree analysis to determine kinship among individuals, fingerprinting, forensics, genetic mapping, and phylogenetic analysis (Gupta *et al.*, 1996).

1.1 EXPLOITATION OF MOLECULAR MARKERS IN GROUNDNUT

Knowledge about the groundnut genome is very limited and only in recent years have molecular techniques been used to interpret the genome organization. Extensive variation for morphological and physiological traits has been observed in both wild *Arachis* and cultivated groundnut. Molecular tools such as DNA markers are increasingly

becoming important and useful in groundnut breeding programs. This is necessitated by the presence of polymorphism at the DNA level. Abundant polymorphism in wild *Arachis species* has been observed where as little variation has been reported in cultivated groundnut (*Arachis hypogaea* L.) (Kochert *et al.*, 1991; Halward *et al.*, 1991,1992; Paik-Ro *et al.*, 1992; Stalker *et al.*, 1994; He and Prakash, 1997; Hopkins *et al.*, 1999 and Subramaian *et al.*, 2000). Both RAPD and RFLP markers have been used to monitor introgression of wild *Arachis* chromosome segments into cultivated groundnut (Garcia *et al.*, 1995). A few RAPD (RKN 229, RKN 410, and RKN 440) and RFLP (R2430E, R2545E, and S1137E) markers linked with the root-knot nematode resistance in groundnut are reported (Burow *et al.*, 1996 and Choi *et al.*, 1999). However 19 primer pairs out of 56 designed by He *et al.* (2003), showed polymorphism among the cultivated groundnut genotypes studied. The average number of alleles per locus was 4.25, and up to 14 alleles per locus were found, which suggests that microsatellite DNA markers produce a higher level of DNA polymorphism than other DNA markers in cultivated groundnut (He *et al.*, 2003).

1.2 IMPORTANCE OF EARLY-MATURITY IN GROUNDNUT

About two thirds of world groundnut production comes from the semi-arid tropic (SAT) regions which are characterized by uncertain rainfall and frequent droughts. Groundnut yields are low and average about 0.8 t /ha in the SAT countries compared to more than 2.6 t /ha of the developed world. One contribution to increasing yield is development of early-maturing and high yielding cultivars. Groundnut varieties maturing in less than 100 days are in general considered as early-maturing. Such varieties are preferred for cultivation in drought-prone areas. Earliness reduces duration of crop risk:

allows greater flexibility in planting time within growing seasons; facilitates irrigation water conservation; reduces irrigation expense, and is critically important in areas with short rainy seasons and subsistence farming. Developing early-maturing, high yielding cultivars is one of the main objectives of most groundnut improvement programs. For efficient trait-specific breeding, it is important to have sources of proven value, an efficient procedure of selection in the segregating populations based on proper understanding of inheritance of the trait. In most groundnut breeding programs, few lines (Chico, Gangapuri and JL 24) have been used as sources of early maturity. Further, in advanced breeding programs it is mainly Chico-derived breeding lines/cultivars that have been used in developing breeding populations. This has resulted in narrowing genetic base of cultivars. So the assessment of genetic diversity among the selected early-maturing groundnut germplasm presents the opportunity to addressing the issue for breeding for high yielding and early-maturing varieties whilst maintaining the broadest possible genetic base.

The objectives of the present investigation are

1. Molecular characterization of intra-accession variation of selected groundnut germplasm.
2. Inter-accession diversity analysis of 29 groundnut germplasm lines (25 early-maturing and 4 late-maturing).

CHAPTER II

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

2.1 ORIGIN OF CULTIVATED GROUNDNUT

Archeological evidence from excavations in Peru place the origin of *Arachis hypogaea* at least 3500 years ago (Singh and Simpson, 1994). The cultivated groundnut, *Arachis hypogaea* L. (2n = 4x = 40) was domesticated from its wild progenitor *A. monticola* in South America. Evidence from molecular marker studies of genetic variation suggests that groundnut has a single origin involving a hybridization of the wild species *A. duranensis* Krapovikas and W.C. Gregory (A genome) and *A. ipaensis* Krapovikas and W.C. Gregory (B genome) followed by chromosome duplication (Kochert *et al.*, 1996).

2.2 INHERITANCE OF EARLY-MATURITY IN GROUNDNUT

Upadhyaya and Nigam (1994) studied the inheritance of two components of early-maturity in groundnut: days to first flower from sowing and days to accumulation of 25 flowers from the appearance of first flower, using three groundnut genotypes. Two early-maturing (Chico and Gangapuri) and one late-maturing (M 13) genotypes were crossed in all possible combinations, including reciprocals. The parents, F_1, F_2, F_3 and backcross populations were evaluated for days to first flower from sowing and for days to accumulation of 25 flowers. The data suggested that days to first flower in the crosses studied was governed by a single gene with additive gene action. Chico and Gangapuri possess the same allele for this component of earliness. Three independent genes with

complete dominance at each locus appear to control the days to accumulation of 25 flowers. In crosses between late (M 13) and early (Chico or Gangapuri) parents, a segregation pattern suggesting dominant-recessive epistasis (13 late:3 early) was observed for this component. Segregation in the F_2 generation (1 late:15 early) of both parents (Chico x Gangapuri) indicated that the genes for early accumulation of flowers in these two parents are at different loci.

2.3 EXPLOITING THE POTENTIAL OF GENETIC MARKERS IN GROUNDNUT

2.3.1 Polymorphism

2.3.1.1 Biochemical markers

Identification of up to 17 polymorphic isozymes among wild species suggests that they may have the potential to follow gene introgression in interspecific hybrids and establish phylogenetic relationships in groundnut (Lacks *et al.*, 1991; Lu and Pickersgill, 1993 and Stalker *et al.*, 1994). However, only asparate aminotransferase (AAT), glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH) and phospho hexoseisomerase (PHI) were reported polymorphic in cultivated groundnut (Galgaro and Lopes, 1994; Grieshammer and Wynne, 1990 and Lacks and Stalker, 1993). Low polymorphism shown by isozyme markers in cultivated groundnut reveals their limited utility in genetic enhancement in groundnut.

2.3.1.2 Molecular markers

Both RFLP and PCR-based markers have been used to assess polymorphic variation in cultivated and wild *Arachis* species in groundnut.

2.3.1.2.1 RFLPs (Restriction Fragment Length Polymorphisms)

Halward *et al.* (1991) studied genetic variation among twenty-five unadapted germplasm resources of cultivated groundnuts from South America, Africa and China, where considerable morphological and physiological variability has been reported to exist, two US cultivars (NC4 and Argentine) and wild allotetraploid progenitor of cultivated peanut (*A. monticola* Krap et Rig) following RFLP analysis. They reported high polymorphism among wild *Arachis* species but very little among cultivated groundnut.

Paik-Ro *et al.* (1992) evaluated RFLP variation, using 23 random genomic and cDNA probes, in six groundnut species within the section *Arachis* (*A. hypogaea, A. monticola, A. batizocoi, A. cardenasii, A. duranensis* and *A. glandulifera*). They reported that most of the genomic DNA probes detected the RFLP pattern of a few restriction fragment bands while more than half of the seed cDNA probes hybridized to multiple bands among the accessions of the tetraploid species. However they could not detect any polymorphism within or between *A. hypogaea* and *A. monticola* and interspecific derivatives with the 32 endonuclease-genomic probe combinations and out of seven seed cDNA probes, two multiple band probes showed polymorphism within tetraploid species regardless of endonucleases used.

2.3.1.2.2 RAPDs (Random Amplified Polymorphic DNAs)

Halward *et al.* (1992) evaluated two peanut cultivars, 25 unadapted cultivars of *A. hypogaea*, the wild allotetraplod progenitor of cultivated peanut (*Arachis monticola*), *A. glabrata* (a tetraploid species from section *Rhizomatosae*) and 29 diploid wild species of *Arachis* for polymorphism using primers of arbitrary sequence to amplify segments of genomic DNA. They found no variation in banding pattern among the cultivars and germplasm lines of *A. hypogaea*, where as the wild *Arachis* species were uniquely identified with most primers tested.

Lanham *et al.* (1992) detected 49 polymorphic loci between cultivated *Arachis hypogaea* (TMV 2) and a synthetic amphidiploid $(B \times C)^2$ created from *A. batizocoi* and *A. chacoense* cross. Of these, only SC10-35 and SC10-60 were polymorphic in *Arachis* germplasm.

Bhagwat *et al.* (1997) studied the variation in RAPD profiles between groundnut cultivar Spanish Improved and it's mutants originated by X-ray irradiation. Twelve RAPD primers produced 1182 fragments of which 65 fragments were polymorphic (5.5%) thus giving on average 1.51 polymorphic bands per primer. Primer OPJ 06 yielded high polymorphism among the mutants.

Subramanian *et al.* (2000) evaluated 70 groundnut genotypes representing variability for several morphological, physiological and other characters, for polymorphism employing random amplified polymorphic DNA (RAPD) assay with 48

oligonucleotide primers. Of the 48 oligonucleotide primers, only 7 (14.7%) primers yielded polymorphic amplification products. The total number of bands from 7 primers was 408, of which 27 were polymorphic.

2.3.1.2.3 DAFs (DNA Amplification Fingerprintings)

He and Prakash (1997) used DAF for detecting genetic variation among six divergent genotypes of groundnut from 3 botanical varieties (*hypogaea, fastigiata* and *aequatoriana*). In the DAF study, out of 559 primers of varying types screened, 17 (mostly 10-mers) detected polymorphism producing an average of 3.7 polymorphic bands per primer with a total of 63 polymorphic markers.

2.3.1.2.4 AFLPs (Amplified Fragment Length Polymorphisms)

He and Prakash (1997) used AFLP for detecting genetic variation among six divergent genotypes of groundnut from 3 botanical varieties (*hypogaea, fastigiata* and *aequatoriana*). In the AFLP study, when 64 primer combinations (three selective nucleotides) corresponding to restriction enzymes EcoR I and Mse I were screened, 28 detected polymorphism. On an average 6.7% of bands obtained from these 28 primers were polymorphic resulting in a total of 111 AFLP markers. By AFLP approach 43% of the primer combinations detected polymorphic DNA markers.

2.3.1.2.5 SSRs (Simple Sequence Repeats)

Hopkins *et al.* (1999) identified SSR markers in cultivated peanut and, tested them to discriminate among 22 groundnut accessions. Peanut total genomic DNA libraries were constructed and screened with ^{32}P-labelled dinucleotide repeats, $(GT)_{10}$ and $(CT)_{10}$. DNA sequences were obtained from the SSR-containing clones and, when possible, primer pairs were designed on the basis of DNA sequences flanking the repeat motif. Primer pairs were tested in Polymerase Chain Reaction (PCR) assays using a collection of 22 peanut DNAs, representing both cultivated peanut and wild species. The six SSR markers developed, detected polymorphism among cultivated peanuts. From 2 to 14 DNA fragments were amplified per SSR markers and as a group, the six markers amplified up to 10 putative SSR loci.

He *et al.* (2003) developed 56 SSR markers, out of which 19 could detect polymorphism among 24 cultivated peanut genotypes. The average number of alleles per locus was 4.25. They could identify 14 alleles at PM50 locus when 48 genotypes were surveyed. Using five such highly polymorphic markers they could differentiate 24 cultivated groundnut genotypes.

Ferguson *et al.* (2004) identified and characterized 110 sequence-tagged microsatellite (STMS) markers that revealed genetic variation in a diverse array of 24 peanut accessions, that consisted of 2 accessions from each botanical variety and from, each of three continents, South America, Africa, and Asia. The simple sequence repeats (SSRs) were identified with a probe of two 27,648-clone genomic libraries: one constructed using PstI and the other using Sau3AI/BamHI. The most frequent repeat

motifs identified were ATT and GA, which represented 29% and 28% respectively, of all SSRs identified. These were followed by AT, CTT and GT. Of the amplifiable primers, 81% of ATT and 70.8% of GA repeats were polymorphic in the cultivated peanut test array. The repeat motif AT showed the maximum number of alleles per locus (5.7). Motifs ATT, GT and GA had a mean number of alleles per locus of 4.8, 3.8 and 3.6, respectively.

2.3.2 Gene introgression from wild *Arachis* to cultivated groundnut

Garcia *et al.* (1995) analysed introgression of *A. cardenasii* chromosome segments in to 46 lines derived from a cross between *Arachis hypogaea* and *A. cardenasii*. They used 73 RFLP probes and 70 RAPD primers to detect introgression. Thirty-four RFLP probes and 45 RAPD primers detected *A. cardenasii* segments in one or more introgression lines and the total size of the introgressed segments represented approximately 360 cM of the diploid groundnut genome. They thus demonstrated the utility of molecular markers to tag and enhance the introgession of specific chromosome segments linked with desirable traits from wild *Arachis* to cultivated groundnut. Choi *et al.* (1999) reported RFLP probes R2430E, S11137E and R2545E linked with resistance to nematodes in BC_5F_2 populations of the cross Florunner x TxAG 7.

2.3.3 Genetic linkage map in groundnut

Halward *et al.* (1993) reported first RFLP based genetic linkage map of groundnut using both random genomic and cDNA clones of DNA library constructed using

and 300 cDNA clones of the F_2 populations derived from the interspecific cross between *A. stenosperma* and *A. cardenasii*. Fifteen genomic and 190 cDNA clones revealed polymorphism among the mapping parents. Of the 113 markers analysed for segregation, 117 were distributed into 11 linkage groups with a total map distance of approximately 1063 cM. Burow *et al.* (2001) reported a RFLP based tetraploid genetic linkage map, originating from a cross between Florunner and a synthetic amphidiploid {[(*A. batizocoi* K9484) x (*A. cardenasii* GKP 10017 x *A. digoi* GKP 10602)] ^{4x}}, consisting of 370 RFLP loci spread into 23 linkage groups with a total map distance of 2210 cM.

2.3.4 Application of SSR markers for assessing diversity in other crops

SSR markers have been used successfully for assessing diversity in many crops. Some of them include: Struss *et al.* (1998) estimated genetic diversity among 163 barley genotypes. 15 barley microsatellite markers detected a total of 130 alleles. The number of alleles per microsatellite marker varied from 5 to 15. On an average 8.6 alleles per locus were observed.

Narvel *et al.* (2000) assessed the simple sequence repeat (SSR) diversity of 39 elite soybean genotypes (Elites) and 40 plant introductions (PIs). A total of 397 alleles were detected among the 79 genotypes at 74 SSR marker loci.

Fisher *et al.* (2000) compared the microsatellite genotypes of 83 onion accessions and landraces from living onion collections. As few as four primer pairs were sufficient to assign unique microsatellite patterns to the 83 accessions.

Alvarez *et al.* (2001) used 17 microsatellite loci to study diversity among 31 tomato accessions comprising nine species of the genus *Lycopersicon*. The microsatellite polymorphisms were used to estimate the distribution of diversity throughout the genus to evaluate the efficiency of microsatellites for establishing species relationships in comparison with existing phylogeny reconstructions.

Huang *et al.* (2002) used a set of 24 wheat microsatellite markers, representing at least one marker from each chromosome, for assessment of genetic diversity in 998 accessions of hexaploid bread wheat (*Triticum aestivum* L.) which originated from 68 countries of five continents. A total of 470 alleles were detected with an average allele number of 18.1 per locus.

Meerow *et al.*(2003) studied genetic variation with in *Cocos nucifera* germplasm collections at two locations in South Florida, representing 8 cultivars with SSR microsatellite DNA loci. A total of 67 alleles were detected, with eight the highest number at any one locus.

CHAPTER

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 PLANT MATERIAL

Twenty-nine groundnut accessions belonging to two subspecies *fastigiata* and *hypogaea* and three botanical varieties *vulgaris*, *fastigiata* and *hypogaea*, originating from fifteen countries comprising of landraces, advanced cultivars and breeding material which include 25 early-maturing and four late-maturing accessions were selected for this study (Table1). In each accession 3 to 10 plants were taken.

3.2 METHODS

3.2.1 DNA extraction

Seeds of selected groundnut accessions were sown individually in jiffy pots. Total genomic DNA was isolated from newly expanded leaves by using a modified CTAB method (Saghai-Maroof *et al.*, 1984). DNA was further purified by RNase digestion followed by extraction with phenol/chloroform/iso-amylalcohol and ethanol precipitation. Based on Mace *et al.* (2004), 96 well plate mini DNA extraction was employed.

S.No.	Accession	Sub Spp.	variety	Country of Origin	Maturity	Biological status
1	ICG 3540	*fastigiata*	*vulgaris*	China	Early	Landrace
2	ICG 4558	*fastigiata*	*fastigiata*	India	Early	Landrace
3	ICG 4890	*fastigiata*	*fastigiata*	Argentina	Early	Landrace
4	ICG 9427	*fastigiata*	*vulgaris*	Senegal	Early	Landrace
5	ICG 11914	*fastigiata*	*vulgaris*	Mali	Early	Landrace
6	ICG 14814	*fastigiata*	*vulgaris*	Vietnam	Early	Landrace
7	Gangapuri	*fastigiata*	*fastigiata*	India	Early	Advanced cultivar
8	JL 24	*fastigiata*	*vulgaris*	India	Early	Advanced cultivar
9	Chico	*fastigiata*	*vulgaris*	USA	Early	Advanced cultivar
10	TMV 2	*fastigiata*	*vulgaris*	India	Early	Advanced cultivar
11	ICG 3200	*fastigiata*	*vulgaris*	China	Early	Landrace
12	ICG 3631	*fastigiata*	*vulgaris*	Zaire	Early	Landrace
13	ICG 4729	*fastigiata*	*vulgaris*	China	Early	Landrace
14	ICG 5512	*fastigiata*	*vulgaris*	India	Early	Landrace
15	ICG 5881	*fastigiata*	*vulgaris*	India	Early	Landrace
16	ICG 9930	*fastigiata*	*fastigiata*	Zimbabwe	Early	Landrace
17	ICG 9968	*fastigiata*	*vulgaris*	Sudan	Early	Landrace
18	ICG 13606	*fastigiata*	*vulgaris*	Indonesia	Early	Landrace
19	ICG 14390	*fastigiata*	*vulgaris*	Myanmar	Early	Landrace
20	ICG 14788	*fastigiata*	*vulgaris*	Vietnam	Early	Landrace
21	ICG 972	*hypogaea*	*hypogaea*	India	Late	Breeding material
22	ICG 5560	*fastigiata*	*vulgaris*	India	Early	Landrace
23	ICG 11605	*fastigiata*	*fastigiata*	Bolivia	Early	Landrace
24	ICG 13585	*fastigiata*	*vulgaris*	Indonesia	Early	Landrace
25	ICG 14815	*fastigiata*	*vulgaris*	Vietnam	Early	Landrace
26	NCAc 1107	*hypogaea*	*hypogaea*	USA	Late	Breeding material
27	M 13	*hypogaea*	*hypogaea*	Malawi	Late	Breeding material
28	ICG 13647	*fastigiata*	*vulgaris*	Indonesia	Early	Landrace
29	MK 374	*hypogaea*	*hypogaea*	Nigeria	Late	Advanced cultivar

Table 1. List of twenty-nine groundnut accessions along with subspecies, variety, origin, maturity and biological status.

96 well plate mini DNA extraction

A. Preparation

1. Steel balls (2 per extraction tube), pre-chilled at -20°C for about 30 minutes, were added to the extraction tubes which are kept on ice.
2. 3% CTAB buffer (3%w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-Hcl, pH 8.0, 3% (β-mercaptoethanol) was pre-heated in 65°C water bath before start of sample collection.
3. Two newly expanded leaves were collected from ten days old seedlings and cut in to small pieces. These pieces were transferred to the extraction tubes.

B. Grinding and extraction

1. 450 µl of pre-heated 3% CTAB buffer was added to each extraction tube containing leaf sample.
2. Grinding was carried out using Sigma Geno/Grinder at 500 strokes/min for 2 minutes each.
3. Grinding was repeated until the color of solution becomes pale green and leaf pieces were sufficiently macerated.
4. After grinding, the tube box was fixed in a locking device and incubated at 65°C in a water bath for 10 minutes with occasional manual shaking.

C. Solvent extraction

1. 450 µl of Chloroform:Iso-amyl alcohol (C:IAA=24:1) mixture was added to each tube and the samples were centrifuged at 6200 rpm for 10 minutes.
2. After centrifugation the aqueous layer was transferred to a fresh tube (Approximately 300 µl).

D. Initial DNA precipitation

1. To each tube containing aqueous layer, 0.7 volume (approximately 210 µl) of cold (kept at -20°C) isopropanol was added, then solution was carefully mixed and the tubes were kept at -20°C for 10 minutes.
2. The samples were centrifuged at 6200 rpm for 15 minutes.
3. The supernatant was decanted under a fume-hood and pellets were allowed to air dry (minimum 20 minutes).

E. RNase treatment

1. In order to remove RNA, 200 µl of low salt TE buffer and 30 µg of RNase (stock 10 mg/ml) were added to the each tube containing dry pellet and mixed properly.
2. The solution was incubated at 37°C for 30 minutes.

F. Solvent extraction

1. After incubation, 200 µl of Phenol:Chlorofom:IAA mixture (25:24:1) was added to each tube, carefully mixed and centrifuged at 5000 rpm for 10 minutes.
2. The aqueous layer was transferred to the fresh tubes and this step was repeated with the chloroform:IAA mixture.

G. DNA precipitation

1. To the tubes containing aqueous layer 15 µl (approximately $1/10^{th}$ volume) 3M Sodium acetate and 300 µl (2 vol) 100% ethanol was added and subsequently placed in freezer for 5 minutes.
2. Following incubation, tubes were centrifuged at 6200 rpm for 15 minutes.

H. Ethanol wash

1. After centrifugation, supernatant was carefully decanted and to the pellets 200 µl of 70% ethanol was added followed by centrifugation at 5000 rpm for 5 minutes.

I. Final re-suspension

1. Pellets obtained by carefully decanting the supernatant and allowed to air dry for one hour.
2. Completely dried pellets were resuspended in 100 µl of $T_{10}E_1$ buffer and kept at room temperature to dissolve completely.

Dissolved DNA samples were kept in 4°C.

3.2.2 Pooling the DNA

For main diversity analysis study, equal amounts of DNA from the 7 to 10 plants belonging to same accession were pooled to get representative DNA sample of that particular accession.

3.2.3 Simple sequence repeat (SSR) primers

Twenty SSR primer pairs, specific to cultivated groundnut, developed as a result of collaborative initiatives between University of Georgia, USA and ICRISAT, by Ferguson *et al.* (2004) and 1 primer pair developed by Hopkins *et al.* (1999) were used for this study (Table 2).

SSR marker	SSR repeat family	No. of repeats	Annealing temp. in °C	Allele Size range in bp	Number of alleles
15C12	att	28	60	270-305	5
14 H6	gt	31	59	294-320	4
2D12B	att	16	60	320-370	4
17E 01	att	31	60	330-340	3
7G2	tatc	12	65	255-270	3
2C11	att,cac	17,10	58	205-220	3
18C5	att	23	60	240-265	3
13 E9	att	*	59	340-355	3
LEC 1	at	18	65-55	250-280	3
16G8	att	19	60	250-262	3
2E 06	ga	12	60	263-280	3
3A01	att	22	64	270-295	3
7H6	ctt	12	60	325-330	2
1B09	ga	19	64	280-290	2
8E 12	ttg,att	6,15	59	220-225	2
19 A05	att	12	60	350-360	2
2F05	att	17	58	270-280	2
13A7	att	10	58	300-310	2
14A7	ctt/ctg	*	60	195-205	3
17 F 6	att	9	58	325-330	2
2B10	att	16	58	290-295	2

* data not available

Table 2. List of SSR markers along with their repeat family, no. of repeats, annealing temperature, allele size range in bp and no. of alleles

3.2.4 SSR-PCR

PCR reactions were performed in 10 µl volume using PTC-200 programmable thermal controller (MJ Research, Inc). The reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5-15 ng genomic DNA, 5-15 pmol of each primer (forward and reverse), 0.5-2.5 mM $MgCl_2$, 150-250 µM of each dNTP, and 0.4-0.6 units of Taq DNA polymerase (Bioline). The temperature regime consisted of an initial denaturation step of DNA at 94°C for 2 minutes, followed by 35 cycles: 94°C for 45 seconds, 58-65°C (specific for each primer pair) for 1 minute and 72°C for 1 minute 30 seconds. After the final cycle samples were incubated at 72°C for 10 minutes to ensure complete extension. 2.5 µl of orange dye (1g of orange dye powder was added into 100 ml of solution containing 10 ml of 0.5M EDTA (pH 8.0) + 1 ml of 5M NaCl + 50 ml glycerol + 39 ml distilled water) was added into PCR products prior to agarose and polyacrylamide gel electrophoresis for separating the amplified products.

3.2.5 Agarose gel electrophoresis

The amplified products along with 100 bp ladder (manufacturer) were initially analysed by electrophoresis in 1.2% agarose gels containing ethidium bromide (0.5 µg/ml of gel) and run in 0.5X TBE (Tris Borate EDTA) buffer at a constant voltage (100 v) for 2 hours. The gels were documented under UV illumination using UVi Tech (DOL-008.XD, England) gel documentation system.

3.2.6 Polyacrylamide gel electrophoresis (PAGE)

The PCR products were separated on 6% non-denaturing polyacrylamide gel for better separation of the fragments as PAGE gives a higher resolution than agarose gels. The polyacrylamide gels were prepared with the following recipe:

Component	Quantity
Acrylamide/Bisacrylamide 29:1 (w/w) (Manufacturer)	15 ml
TBE 10X (Tris Borate EDTA Buffer)	7.5ml
Distilled water	52.5ml
TEMED (N,N.N',N'-Tetramethylethylenediamine)	90 µl
10 % Ammonium persulphate (APS)	450 µl

Acrylamide/Bisacrylamide was prepared by dissolving 29.0 g of acrylamide and 1.0 g of bisacrylamide in 100 ml of double distilled water. The solution was then filtered through Whatman No.1 filter paper and stored at 4°C in dark bottles. 10X TBE was prepared by dissolving 109 g of Tris base and 55 g of boric acid one by one in 800 ml of double distilled water containing 40 ml of 0.5M EDTA. The final volume was adjusted to 1.0 L with double distilled water. The solution was then sterilised by autoclaving and stored at 4°C. 10X TBE was diluted 20 times to get 0.5X TBE buffer. Ammonium persulphate (10%) was prepared by dissolving 10 g of ammonium persulphate in 100 ml of double distilled water and stored at 4°C. Bindsilane was prepared by dissolving 1.5 ml bindsilane (manufacturer) in 5 ml of acetic acid and 993.5 ml ethanol and stored at 4°C.

Few drops of repelsilane (manufacturer) was applied to the side of the buffer tank coming in contact with glass plate. Similarly few drops of bindsilane was applied to the glass plate on the side to which gel should stick. Glass plate sandwich was then prepared

by placing spacers (0.4 mm) between the two plates, tightened with clamps and polyacrylamide gel mixture was prepared by mixing correct volumes of all components except TEMED and APS that was added just before pouring the mixture into the gel casting unit. The assembled unit was placed horizontally on a plane surface and the polyacrylamide gel mixture was poured into the glass plates with the help of syringe and then comb was inserted at the top position in reverse direction to form a straight edge of gel. The assembly was left undisturbed for about 30 to 60 minutes for polymerization to occur.

After polymerization, the lower tank and upper reservoir of electrophoresis apparatus was filled with 0.5X TBE buffer. Top of the gel is cleaned with the help of plastic scraper and by aspirating the TBE buffer using a pasture pipette to remove small fragments of gel and tiny bubbles. Finally comb was inserted up to 1 mm into the gel to form wells. The gel was pre-run to warm it for at least 10 minutes at 400v and 9W. 2 µl of PCR product was loaded on to the wells with the help of a multi-channel syringe. After loading of samples, voltage clamps were attached and the gel apparatus was connected to power pack set at 400v and 9W. The gel was run for 3 to 4 hours for migration of DNA fragments to desired resolution.

3.2.7 Silver staining of the polyacrylamide gel

The following reagents were prepared for silver staining the polyacrylamide gel.

CTAB (0.1%)

It was prepared by dissolving 2 g of CTAB in 2 litres of double distilled water.

Liquid ammonia (0.3%)

It was prepared by dissolving 26 ml of 25% liquid ammonia in 2 litres of double distilled water.

Staining solution

2 g of AgNO3 was dissolved in 2 litres of double distilled water and then 8 ml of 1M NaOH solution (40 g of NaOH in 1000 ml of double distilled water) was added that the solution turned brownish and cloudy. Following this, 6 to 8 ml of 25% liquid ammonia was added drop by drop until the solution becomes transparent.

Developer

30 g of sodium carbonate (1.5% Na_2CO_3) was dissolved with intense stirring in 2 litres of double distilled water. 400 µl of formaldehyde was added to the above solution just before transferring the gel into this solution.

Fixer

30 ml of glycerol (1.5%) was added to 2 litres of double distilled water

Silver staining procedure

The gel was first rinsed in water for 3 to 5 minutes, soaked in 0.1% CTAB and gently agitated for 20 minutes and incubated in 0.3% ammonia for 15 minutes. The gel was then incubated in silver staining solution (2 g silver nitrate, 8 ml of 1M NaOH, 6 to 8 ml of 25% liquid ammonia) for about 15 minutes and transferred to 2 litres of distilled water for about 3 seconds. The gel was then developed by gently agitating it in developer solution for about 10 to 15 minutes depending upon

development of bands and then rinsed in 2 litres of distilled water for about 3 seconds. Finally, the gel was placed in fixer solution for about 10 minutes. The gel was dried overnight before scanning.

3.2.8 Scoring amplified products

The amplified fragments were scored as '1' for the presence, '0' for the absence of the alleles from higher to lower molecular weight products and approximate base pair (bp) was determined.

3.2.9 Data analysis

3.2.9.1 Intra-accession variation

Estimates of similarity was based on the Nei and Li's (1979) definition of the similarity: $S_{ij} = 2a/(2a+b+c)$, where S_{ij} is the similarity between two individuals, i and j, a is the number of bands present in both i and j, b is the number of bands present in i and absent in j and c is the number of bands absent in i and present in j. This is also called Dice similarity coefficient (Dice, 1945). Using NTSYS PC software, version 2.1 (Rohlf, 2000), SAHN (Sequential Agglomerative Hierarchial Non-overlapping) (Sneath and Sokal, 1973) clustering was performed using UPGMA (Unweighted Pair-Group Average Method) (Sokal and Michener, 1958). The dendrogram was created with the TREE option of NTSYS.

3.2.9.2 Inter-accession diversity analysis

Estimates of similarity was based on the Nei and Li's (1979) definition of the similarity: $S_{ij} = 2a/(2a+b+c)$, where S_{ij} is the similarity between two individuals, *i* and *j*, a is the number of bands present in both *i* and *j*, *b* is the number of bands present in *i* and absent in *j* and *c* is the number of bands absent in *i* and present in *j*. This is also called Dice similarity coefficient using NTSYS PC software, version 2.1 (Rohlf, 2000). SAHN (Sequential Agglomerative Hierarchial Non-overlapping) clustering was performed using UPGMA (Unweighted Pair-Group Average Method). The dendrogram was created with the TREE option of NTSYS. MDS (Multi Dimensional Scaling) (Kruskal and Wish, 1978)was performed to see whether the observed molecular variation indicated any evidence of clustering among accessions. The goodness of fit of clustering to the data was calculated using COPH and MXCOMP options.

The Polymorphism Information Content (PIC) of each SSR was determined as described by Weir (1990). PIC = $1-\sum P_i^2$, where P_i is the frequency of the i^{th} allele in the examined genotype. PIC values range from 0 (monomorphic SSR) to 1 (highly polymorphic SSR). An Analysis of Molecular Variance (AMOVA) was used to partition genetic variability using Arlequin software, version 2.0 (Schneider *et al.*, 2000) and significance values assigned to variance components based on the random permutation (10,000 times) of individuals assuming no genetic structure.

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

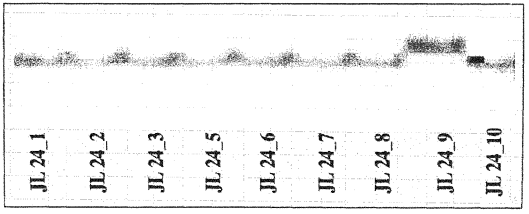
4.1 INTRA-ACCESSION VARIATION

Seven to ten individual plants from each of ICG 3540, ICG 4558, ICG 4890, ICG 9427, ICG 11914, ICG 14814, Gangapuri, JL 24, Chico and TMV2 accessions were evaluated for intra-accession variation using five SSR primer pairs 18C5, 8E12, 7G2, 2D12B and 2B10 All the five primer pairs detected intra-accession variation 18C5 detected intra-accession variation in ICG 3540, ICG 14814 and JL 24 (Figure 1), 7G2 in Gangapuri, JL 24 and TMV 2 (Figure 2), 2B10 in Gangapuri, JL 24 (Figure 3) and TMV2, 8E12 in JL 24 (Figure 4) and 2D12B in ICG 4558, Gangapuri and TMV2 (Figure 5)

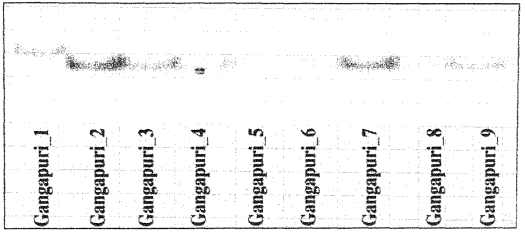
A total of 22 alleles were detected with five primer pairs with an average of 2 2 alleles per primer pair The number of alleles ranged from 2 (2B10) to 8 (2D12B) The band profiles of 86 individuals from ten accessions were compared by Dice similarity coefficient and subjected to UPGMA clustering The UPGMA clustering (Figure 6) revealed significant intra-accession variation Clustering placed individuals of the accession ICG 3540 into two groups, TMV2 into four groups, ICG 9427 into three groups, ICG 14814 into four groups, JL 24 into three groups, ICG 4890 into two groups, ICG 11914 into two groups, Chico into three groups, Gangapuri into two groups and ICG 4558 into four groups

Figure 1. Intra-accession variation as revealed by primer pair 18C5.

Figure 2 Intra-accession variation as revealed by primer pair 7G2.



a. JL 24



b. Gangapuri

Figure 3. Intra-accession variability as revealed by primer pair 2B10.

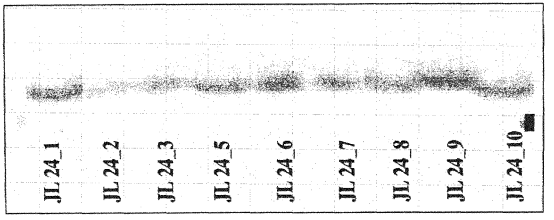


Figure 4. Intra-accession variability as revealed by primer pair 8E12 in JL 24.

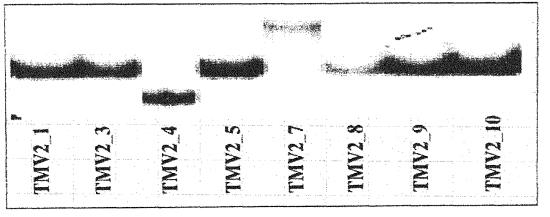
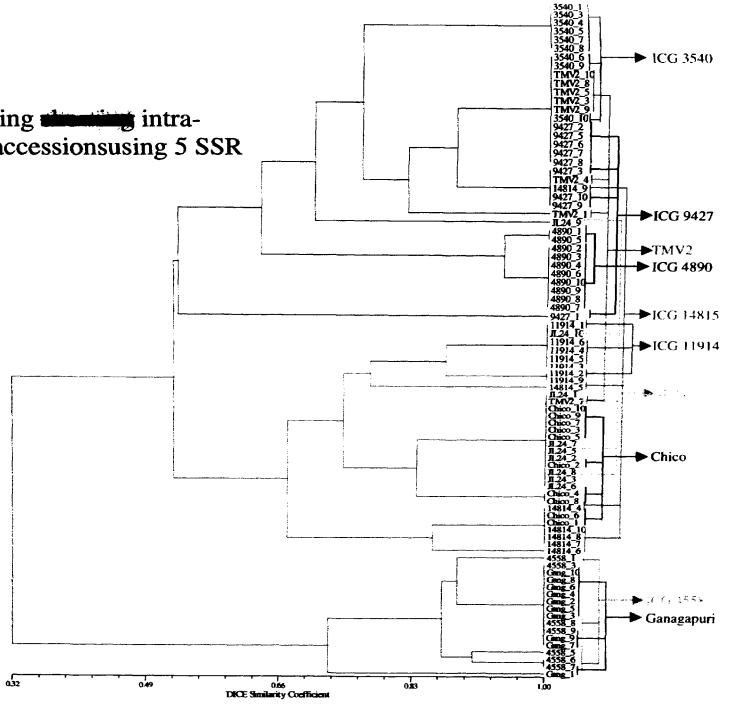


Figure 5. Intra-accession variability as revealed by primer pair 2D12B in TMV2.

PGMA dendrogram showing [redacted] intra-
ccession variation for 10 accessionsusing 5 SSR
rimers

Figure 6.



4.2 INTER-ACCESSION DIVERSITY ANALYSIS

Equal amounts of DNA from 3 to 10 individual plants of an accession was mixed for each of the 29 accessions. A total 20 SSR primer pairs were used to study the diversity among 29 accessions belonging to three botanical varieties *vulgaris*, *fastigiata* and *hypogaea*, and two subspecies *fastigiata* and *vulgaris*. A total of 57 alleles were detected among 29 accessions with average number of 2.85 alleles per primer pair (Table 2). The number of alleles ranged from two for seven primer pairs to five for 15C12. The PIC values (Table 3) calculated for each marker, ranged from 0.53 for 17F6 to 0.93 for 15C12, with an average of 0.78 over all the loci. Inter-accession variability detected with primer pairs 2D12B (Figure 7), LEC 1 (Figure 8), 13E9 (Figure 9) and 7H6 (Figure 10) was shown. A dendrogram (Figure 11) derived from UPGMA cluster analysis based on Dice similarity coefficient for the 29 accessions was constructed. The similarity coefficient for all accessions ranged from 0.3 to 1.0. Because all 29 accessions investigated were from three botanical varieties they grouped into three distinct clusters, except ICG 3631 (var. *vulagris*) clustered with the accessions belonging to var. *fastigiata*. The UPGMA clustering resulted in consistently higher cophenetic correlation score of 0.9, where $r > 0.9$ indicates a very good fit, $r = 0.8$ to 0.9 indicates good fit and $r < 0.8$ indicates a poor fit.

The AMOVA analysis indicated 42% of variation in SSR markers used between early and late maturing accessions. The accessions belonging to different botanical varieties were significantly differentiated based on the Multi Dimensional Scaling (MDS) analysis (Figure 12).

SSR Marker	PIC value
15C12	0.93
14 H6	0.92
2D12B	0.92
17E 01	0.89
7G2	0.88
2C11	0.87
18C5	0.87
13 E9	0.86
LEC 1	0.85
16G8	0.85
2E 06	0.81
3A01	0.8
7H6	0.72
1B09	0.72
8E 12	0.71
19 A05	0.68
2F05	0.65
13A7	0.62
14A7	0.6
17 F 6	0.53

Table 3. List of SSR markers indicating the PIC values

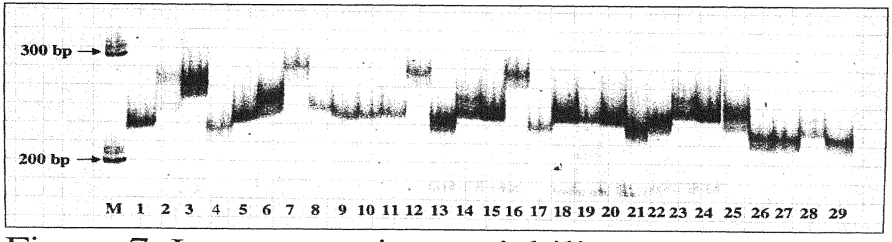


Figure 7. Inter-accession variability as revealed by primer pair 2D12B.
M=MARKER

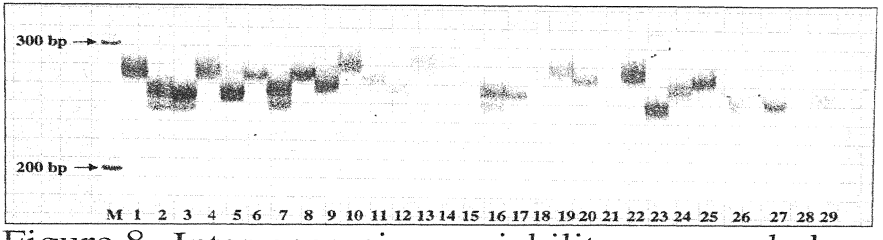


Figure 8. Inter-accession variability as revealed by primer pair LEC1.
Lanes 1-29 represent accessions as listed in Table 1.

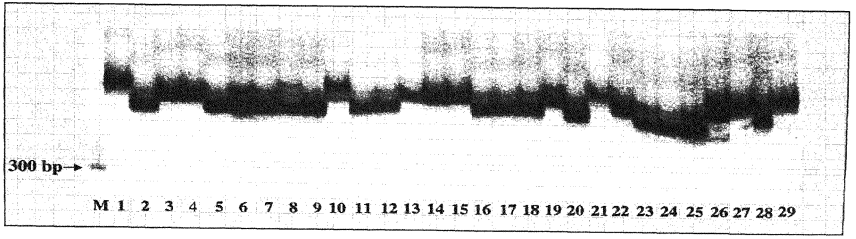


Figure 9. Inter-accession variability as revealed by primer pair 13E9.

M=MARKER

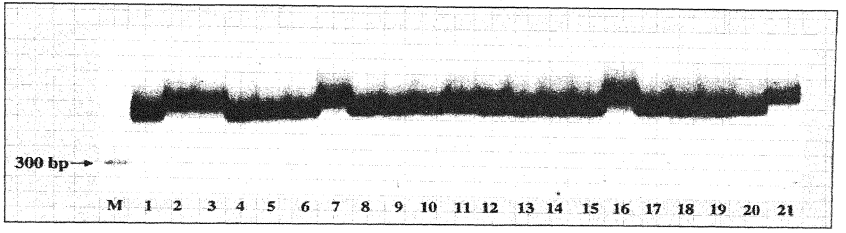
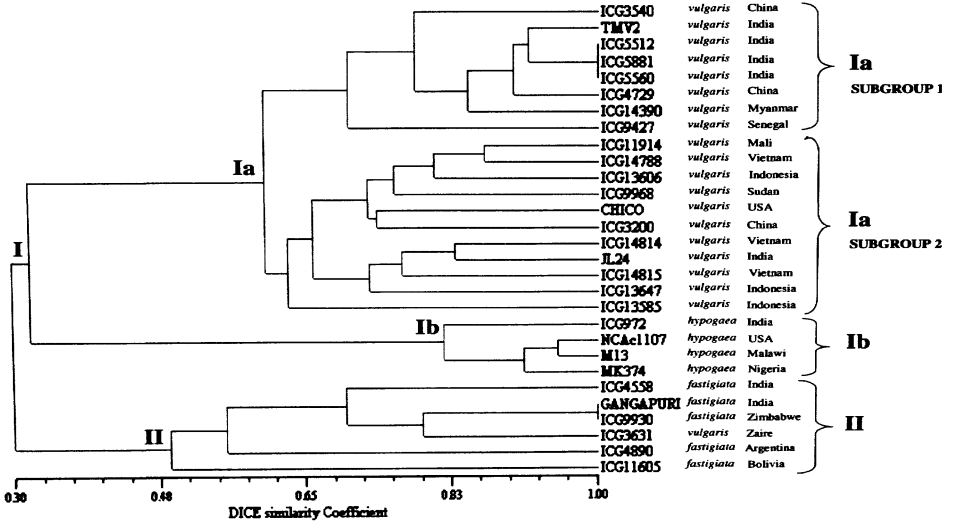


Figure 10. Inter-accession variability as revealed by primer pair 7H6.

Lanes 1-29 represent accessions as listed in Table 1.



Fiure 11. Dendrogram of 29 groundnut accessions using Dice similarity coefficient and UPGMA clustering.

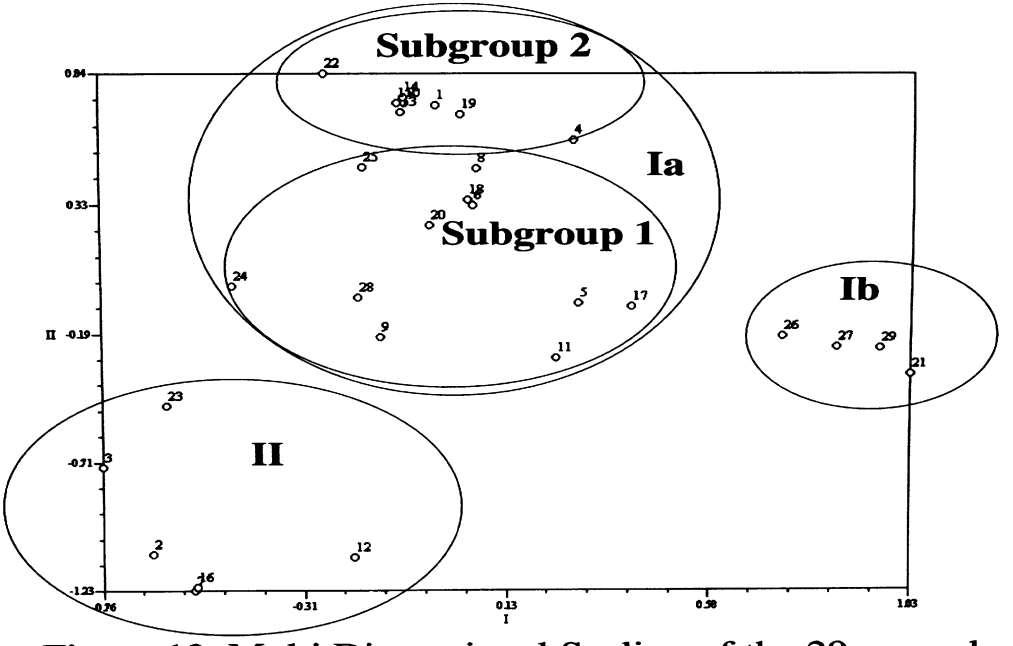


Figure 12. Multi Dimensional Scaling of the 29 groundnut accessions. Clusters labeled as in Figure 11.

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

Efficient use of conserved bio-diversity requires information about the degree and distribution of genetic diversity. Characterization and quantification of genetic diversity requires information about the degree and distribution of genetic diversity. Genetic diversity is the basis for genetic improvement. Knowledge of germplasm diversity has a significant impact on crop plants. The variation in the genetic makeup, in interaction with environment, dictates the observable pattern of diversity shown by multitude of living organisms. The genetic variation within and between species, generated by the process of mutation, sexual reproduction and selection, ensure its capacity in evolutionary change and ecological adaptation.

The cultivated groundnut, *Arachis hypogaea* L. (2n = 4x = 40) was domesticated from its wild progenitor *A. monticola* in South America. Evidence from molecular marker studies of genetic variation suggests that groundnut has a single origin involving a hybridization of the wild species *A. duranensis* Krapovikas and W.C. Gregory (A genome) and *A. ipaensis* Krapovikas and W.C. Gregory (B genome) followed by chromosome duplication (Kochert *et al.*, 1996). Abundant germplasm resources of both the cultivated species and related wild species are available to groundnut breeders; however, most groundnut breeding programs have traditionally relied on crossing of elite breeding lines for developing improved cultivars (Knauft and Gorbet, 1989). As a result, the germplasm base of domesticated groundnut is relatively narrow. Developing early-maturing, high yielding cultivars is one of the main objectives of most groundnut

improvement programs. In general groundnut varieties maturing less than 100 days are considered to be early maturing. Early maturing, high yielding cultivars are needed for short growing season, multiple cropping and to avoid late season droughts. In most breeding programs few sources of early maturity (Chico, Gangapuri and JL 24) have been used resulting in the narrow genetic base of groundnut cultivars. There is a need for broadening the genetic base to enhance groundnut breeding prospects. In view of this, present investigation was undertaken to study diversity among early-maturing groundnut germplasm at molecular level.

5.1 DIVERSITY ANALYSIS

In the present investigation, diversity among 29 accessions of groundnut germplasm, of which 25 are early-maturing and 4 are late-maturing, belonging to two subspecies *fastigiata* and *hypogaea* and three botanical varieties *vulgaris*, *fastigiata* and *hypogaea*, have been studied using SSR markers.

5.1.1 Molecular diversity analysis

Genetic diversity generated by selection, mutation and sexual reproduction, rests on genome changes ranging from single basepair to rearrangements of the entire chromosomes. These nucleotide level changes are reflected in phenotypic differences among individuals, at increasingly high levels of cellular organization, ranging from variation in amino acid sequence of proteins to morphological, physiological, chemical and behavioral characteristics. Traditionally, characterization and identification of cultivars has been performed according to morphological and physiological traits.

However, such traits are not always available for analysis, nor do they remain independent of changing environmental conditions. The deployment of environmental neutral biochemical markers, the isozymes, protein electrophoresis, and molecular markers have circumvented these limitations by focusing directly on the variation at level of genes, the DNA itself. The high resolution of molecular markers make them a valuable tool for a variety of purposes, such as fingerprinting, facilitating appropriate choice of parents for breeding programs, analyzing quantitative traits, location and detection of QTLs, genetic mapping, MAS, gene transfer, studying evolutionary pathways and for assessing genetic diversity of plant germplasm.

5.1.2 Diversity analysis using SSR markers

The high diversity obtained with SSR is consistent with the known characteristics – that they are more variable and high expected heterozygosity than the RAPDs, or AFLPs (Powell *et al.*, 1996). The high levels of polymorphism associated with SSRs are expected because of unique evolution of these genomic regions: replication slippage (Tautz *et al.*, 1986 and Tautz and Renz, 1984) rather than mutations, insertions or deletions.

5.1.3 Intra-accession variation

Seven to ten individual plants each of ICG 3540, ICG 4558, ICG 4890, ICG 9427, ICG 11914, ICG 14814, Gangapuri, JL 24, Chico and TMV2 accessions were evaluated for intra-accession variation using five SSR primer pairs 18C5, 8E12, 7G2, 2D12B and 2B10. All the five primer pairs detected intra-accession variation. 18C5 detected intra-

accession variation in ICG 3540, ICG 14814 and JL 24 (Figure 1); 7G2 in Gangapuri, JL 24 and TMV 2 (Figure 2); 2B10 in Gangapuri, JL 24 (Figure 3) and TMV2; 8E12 in JL 24 (Figure 4) and 2D12B in ICG 4558, Gangapuri and TMV2 (Figure 5).

A total of 22 alleles were detected with five primer pairs with an average of 2.2 alleles per primer pair. The number of alleles ranged from 2 alleles for 2B10 to 8 alleles for 2D12B.The band profiles of 86 individuals from ten accessions were compared by Dice similarity coefficient and subjected to UPGMA clustering (Figure 6). The similarity coefficient ranged from 0.12 to 1.00. ICG 4890 showed the least variability, with two groups of individuals ICG 4890_1, ICG 4890_5 and ICG 4890_2, ICG 4890_3, ICG 4890_4, ICG 4890_6, ICG 4890_7, ICG 4890_8, ICG 4890_9, ICG 4890_10. In contrast ICG 4558 formed 4 groups with ICG 4558_5; ICG 4558_6, and ICG 4558_7; ICG 4558_3, ICG 4558_8, and ICG 4558_9; ICG 4558_1. All the individuals of ICG 4558 clustered together at a similarity coefficient of 0.78. Among all the 10 accessions TMV2 showed much intra-accession diversity forming four groups, all clustering at a similarity coefficient of 0.52.

Small number of individuals or single plant may not be fully representative of that particular accession. On the other hand, the use of more individuals from each accession and pooling the template DNA from several individuals therefore offers an attractive strategy. It is possible that pooling of template DNA may lead to the poor amplification of certain markers such that they become absent from the collective band profile. Several factors could contribute to the production of undetected markers of this kind, including template DNA concentration and purity, primer selection, amplicon size, PCR conditions, and the sensitivity of the product detection. Thus, dilution of template DNA could have

variable effect on the appearance of different markers in pooled band profiles. On a practical basis, problems in the accuracy and reliability of DNA quantification and variation in the purity of extracted DNA may also affect the likelihood of rare band amplification (Gilbert *et al.*, 1999).

5.1.4 Inter-accession diversity analysis (Germplasm diversity)

Equal amounts of DNA from individual plants of an accession were pooled for each accession and SSR profiles were generated. 20 SSR primer pairs (Table 2) were used to study the diversity among 29 accessions belonging to two subspecies *fastigiata* and *vulgaris* and three botanical varieties *vulgaris*, *fastigiata* and *hypogaea*.

A total of 57 alleles were detected among 29 accessions with average number of 2.85 alleles per primer pair. The number of alleles ranged from 2for seven primer pairs to 5 for 15C12 (Table 2).

The cluster analysis enabled the grouping of all the accessions used in the present study into two major clusters I and II (Figure 11). The 23 accessions in cluster I were further grouped into two sub-clusters Ia comprising 19 accessions belonging to botanical var. *vulgaris* and Ib comprising 4 accessions belonging to botanical var. *hypogaea*. Sub-cluster Ia further formed two subgroups 1 and 2 comprising 8 and 11 accessions respectively. The cluster II consists of 6 accessions of which, 5 belong to botanical var. *fastigiata* and 1 belongs to botanical var. *vulgaris*. There is no much significant grouping pattern of the accessions originating from a same country. For example, TMV2, ICG 5512, ICG 5881, ICG 5560 and JL 24 accessions are originated from India and belong to

botanical var. *vulgaris*. But, ICCG 5512, ICG 5881, and ICG 5560 clustered at a similarity coefficient of 1.0 and joined with TMV2 at a similarity coefficient of 0.91. The accessions originated from India clustered at a similarity coefficient of 0.59. All *hypogaea* accessions clustered at a similarity coefficient of 0.82, *fastigiata* at 0.5 and *vulgaris* at 0.59. This suggests that even the accessions originating from a country have significant diversity. The interesting result of this study is that the accessions of a particular variety were clustered together (except ICG 3631). This has been reported by Raina *et al.* (1996) using RAPDs and ISSRs. The accessions belonging to different botanical varieties were significantly differentiated based on the Multi Dimensional Scaling (MDS) analysis (Figure 12).

Earlier investigations in groundnut using molecular marker techniques such as RFLP, PCR based marker analyses such as RAPDs have concluded that there is little demonstrable polymorphism in cultivated groundnut (Kochert *et al.*, 1991; Halward *et al.*, 1991,1992 and Paik-Ro *et al.*, 1992). There can be several reasons why little polymorphism is detected at the DNA sequence level using the commonest molecular techniques between accessions of a species even in the presence of a significant level of variation at the morphological level. The major factors include: (i) most studies (Kochert *et al.*, 1991; Halward etal., 1991,1992 and Paik-Ro *et al.*, 1992) have used very few accessions to detect molecular polymorphism. (ii) earlier studies were confined to cultigens originating in the USA (Kochert *et al.*, 1991) which have their origin in narrow genetic base introduced since the mid-sixteenth century predominantly from Africa and some from Central America. Subsequent studies (Halward *et al.*, 1992) have attempted to include genotypes originating in a wider range of groundnut regions but even they are predominantly from secondary centre of origin in South America. (iii) the number of

enzyme probe combinations used in RFLPs; primer used in RAPDs; and clones or DNA sequences used in PCR analysis are very important when one is dealing with a segmental polyploid especially like groundnut. Unfortunately the number of enzymes and probe combinations, primers, and clones used in groundnut molecular marker studies have been very limited. (iv) Use of multiple restriction enzymes is required to identify most polymorphic restriction enzyme and probe combinations as each enzyme can reveal variation at different restriction sites at or around the region at which the probe is hybridised (Singh *et al.*, 1998).

Arachis hypogaea has been divided into two subspecies, six botanical varieties, two agronomic types, and numerous cultivar groups and varieties that have wide geographic distribution and considerable ecological and morphological variation. The broad adaptation of *A. hypogaea* implies a large number of diverse populations within the species and, correspondingly, a large probability that nuclear DNA mutations occur. This results in diversification and genetic polymorphism in growth habit, vigor, and stem, leaf, seed, and pod characteristics and a physiological polymorphism in maturation period, water-use efficiency, and translocation of photosynthates to seeds through partitioning. The evolution of six botanical varieties in seven agroclimatic zones, for example demonstrates significant levels of variation in response to the selection pressures in these zones (Singh *et al.*, 1998). Given this, it is, therefore, not surprising to find significant levels of polymorphism among 29 accessions of *A. hypogaea* using SSR markers.

The PIC values (Table. 3) calculated for each marker, ranged from 0.53 for 17F6 to 0.93 for 15C12, with an average of 0.78 over all the loci. The UPGMA clustering

resulted in consistently higher cophenetic correlation score of 0.9, where $r > 0.9$ indicates a very good fit, $r = 0.8 - 0.9$ indicates good fit and $r < 0.8$ indicates a poor fit.

The AMOVA analysis detected 42% of variation in SSR markers used between early and late maturing accessions. This may be attributed to physiological polymorphism in maturation period, water-use efficiency, and translocation of photosynthates to seeds thorough partitioning between early and late maturing types.

The SSRs have already been used in *A. hypogaea*, to assess molecular polymorphism. Hopkins *et al.* (1999) used six polymorphic SSR primer pairs with 19 cultivated groundnut accessions among which 17 unique genotypes were generated. He *et al.* (2003) reported that they could differentiate 24 cultivated groundnut genotypes using 5 highly polymorphic markers. Ferguson *et al.* (2004) identified and characterised 110 STMS markers that revealed genetic variation in a diverse array of 24 peanut landraces, that consisted of two accessions from each botanical variety, and from each of three continents, South America, Africa and Asia.

The high levels of polymorphism obtained in this study may be due to (a) using SSR markers (b) prescreening and use of polymorphic SSR markers. The information about diversity among the early maturing germplasm obtained from the present study will assist groundnut breeding programs aimed at improving early-maturity to maximize the genetic base of their breeding populations.

In most groundnut breeding programs, few lines (Chico, Gangapuri and JL 24) have been used as sources of early-maturity. Further in advanced breeding programs it is

mainly chico-derived breeding lines/cultivars that have been used in developing breeding populations. From this study the accessions like ICG 11914, ICG 14788, ICG 13606, ICG 9968, and ICG 3200, which are similar to Chico, can be used for developing breeding lines.

Our results indicate that sufficient polymorphism exists with in the species and it can be exploited for developing genetic linkage map of cultivated groundnut using sufficient SSR markers, which facilitates increased breeding efficiency through marker-assisted selection.

CHAPTER VI

SUMMARY

CHAPTER VI

SUMMARY

Groundnut (*Arachis hypogaea* L.) is an important crop internationally for both direct human consumption and as an oilseed crop, which is being cultivated in 108 countries of the world. About two thirds of world groundnut production comes from the semi-arid tropic (SAT) regions which are characterized by uncertain rainfall and frequent droughts. Groundnut yields are low and average about 0.8 t /ha in the SAT countries compared to more than 2.6 t /ha of the developed world. One contribution to increasing yield is development of early maturing and high yielding cultivars that are needed for short growing seasons, multiple cropping systems and which avoid late season droughts. In most breeding programs few sources of early maturity have been used resulting in the narrow genetic base of groundnut cultivars. There is a need for broadening the genetic base to enhance groundnut breeding prospects.

The present study was initiated to assess diversity using SSR markers among 29 groundnut accessions belonging to two subspecies *fastigiata* and *vulgaris and* three botanical varieties *vulgaris*, *fastigiata*, and *hypogaea*, originating from fifteen countries, which include 25 early-maturing and four late-maturing accessions. Initially intra-accession variation among ten accessions of ICG 3540, ICG 4558, ICG 4890, ICG 9427, ICG 11914, ICG 14814, Gangapuri, JL 24, Chico, and TMV2, was assayed using 5 SSR primer pairs. UPGMA clustering of the SSR band profiles revealed significant variation within the accessions. This suggests that small number of individuals or single plant may not be fully representative of that particular accession. So, use of more individuals from

each accession and pooling the template DNA from several individuals therefore offers an attractive strategy provided we can detect all the constituent bands in the composite profile. A total of 22 alleles were detected by five primer pairs with an average number of 4.4 alleles per primer pair. The number of alleles ranged from 2 (2B10) to 8 (2D12B). To capture this intra-accession diversity in main study, equal amounts of DNA from individual plants were pooled for each accession. Inter-accession diversity analysis of 29 accessions was performed using 20 SSR primer pairs, which detected a total of 57 alleles with an average of 2.85 alleles per primer pair. The number of alleles ranged from two to five. The PIC values, ranged from 0.53 (17F6) to 0.93 (15C12), with an average of 0.78. The AMOVA analysis detected 42% of variation at molecular level between early and late-maturing accessions. The clustering revealed significant diversity among the 29 accessions used for this study. The different botanical varieties were grouped into 3 different clusters except one accession. The MDS analysis supported the clustering obtained by UPGMA. Clustering also revealed that significant diversity among accessions originated from a particular country. Further comparison of genotypic data with phenotypic data for these accessions may project the complete picture of diversity. This analysis will assist groundnut breeding programs aimed at improving early-maturity to maximize the genetic base of their breeding populations.

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