DEVELOPMENT OF A GENETIC LINKAGE MAP AND QUANTITATIVE TRAIT LOCI (QTL) ANALYSIS IN COWPEA (*Vigna unguiculata* (L.) Walp)

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DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

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"I dedicate this work to my beloved parents and family for their unconditional support, understanding and love throughout this entire long journey."

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ABBREVIATIONS AND ACRONYMS

A- Alcohol

AFLP- Amplified Fragment Length Polymorphism

Al- Aldehyde

ANOVA- Analysis of variance

BAC-Bacterial artificial chromosome

BC-Back cross

bp-base pair

Bt-*Bacillus thuringiensis*

CB-California black-eyed

CCMV-Cowpea Chlorotic Mottle Virus

cDNA-Complementary deoxyribonucleic acid

CGKB-Cowpea genomics knowledge base

CIM-Composite interval mapping

cM-Centi morgan

CTAB-CetylTrimethyl Ammonium Bromide

DNA-Deoxyribonucleic acid

dNTPs-Deoxyribonucleic acid

DRTs-Domestication related traits

EDTA- Ethylenediaminetetraacetic acid

E-Esters

EST-Expressed sequence tags

FC-Flower color

GC-Gas chromatography

GM-Genetically modified

ICIPE-International Center of Insect Physiology and Ecology

K-Ketone

kbp-Kilo base pair

KU-Kenyatta University

LG-Linkage group

LOD-Logarithm of odds

M-Miscellaneous

MAS-Marker assisted selection

MS-Mass spectrometry

PAGE-Polyacrylamide gel electrophoresis

PCR-Polymerase chain reaction

PP-Pod pigmentation

PPO-Pod position

QTL-Quantitative trait loci

Ra-Root architecture

RAPD- Random Amplified Polymorphism DNA

RFLP-Restriction Fragment Length Polymorphism

RILs-Recombinant inbred lines

RPM-Revolution per minute

SNP-Single Nucleotide Polymorphism

SSLP-Simple Sequence Length Polymorphism

SSR-Simple Sequence Repeat

STR-Short Tandem Repeats

SW-Seed weight

TE-Tris-EDTA

TBE-Tris borate EDTA

USA-United states of America

VNTR-Variable number of tandem repeats

YAC-Yeast artificial chromosome

ABSTRACT

Cowpea is a diploid plant species which contributes significantly to food security in developing countries, especially in Africa. This research project was carried out in view of the upcoming introduction of Bt cowpea in Africa which is likely to alter the equilibrium existing within the cowpea taxa. The objectives of this study were to develop viable microsatellite markers and construct the SSR based linkage map, identify quantitative trait loci that regulate yield, domestication related traits as well as flower scent and identify the volatile compounds that attract pollinators to cowpea flowers. In order to achieve these goals 159 F_7 recombinant inbred lines including the two parents and 206 markers (202 SSRs and 4 morphological) were used. The first SSR based linkage map of cowpea was constructed that spans a genetic distance of 2991cM. QTL for seed weight (SW), domestication related traits (DRT), flower scent/aroma were mapped in all 159 F_7 plants and the two parents 524B x 219-01. Six QTL associated with 74 % of the phenotypic variance were detected for SW on chromosomes 1, 2, 3 and 10. Both the 524B and 219-01 alleles increased SW at six of the QTL on chromosomes 1, 2, 3 and 10. For domestication related traits, nine QTL (four for testa size and five for pod fiber thickness layer) explaining 54.5 and 47.9 % of the phenotypic variance, respectively were on chromosomes 1, 2, 4, 6, 7 and 10. The 524B allele increased DRTs at three-fourth of all QTL. QTL for SW and DRTs were clustered on chromosomes 1 and 10. Association of SW and DRTs QTL may be the cause of the significant phenotype and genotypic correlation detected between the two traits. The test of linkage vs pleiotropy for SW and DRT QTL on chromosomes 1 and 10 suggested pleiotropy. For flower scent/aroma, 63 QTL were detected on chromosomes 1, 2, 3, 4, 5, 6, 7, 8, and 10. In addition, a total of twenty-two different volatiles were identified by the GS-MS technique. Clustering of QTL were observed on chromosomes 1, 2, and 4 mainly, suggesting that it can occur either due to the presence of a single locus with pleiotropic effects on several volatiles or as a result of tightly linked different loci. Such loci may encode transcription factors that co-ordinately regulate genes, or they may encode enzymes that catalyse limiting steps in single pathways. It is anticipated that this resource will have an important impact towards the development of marker assisted selection systems for the cowpea breeding community, and for future genetic studies in cowpea.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background information

Cowpea [*Vigna unguiculata (L.) Walp,*] a tropical grain legume originated in Africa and is composed of wild and cultivated forms with the wild form only encountered in Africa (Pasquet, 1999). It is widely grown in Africa, Latin America, Southeast Asia and in the southern United States (Singh, 2005; Timko *et al.*, 2007a). It is estimated that Cowpea's annual production is more than 3 million tons worldwide with West and Central Africa accounting for over 64% of the 12.5 million hectares of the cultivated areas, followed by central and South America (19%), Asia (10%), East and Southern Africa (6%) (Singh *et al.*, 1997). The potential of cowpea is limited by numerous factors, but field and storage insect pests are the most severe constraints, at such a scale that a couple of insecticide sprays usually multiply the yield tenfold. However, most African farmers don't have access to them. Conventional breeding has made some progress towards developing and deploying insect-resistant cultivars, but the gene pool of cowpea lacks adequate sources of resistance for certain insect pests, including pod borers, weevils, pod bugs and thrips. Therefore, molecular biology seems to be the only way to introduce novel insect resistance traits that will help solve this otherwise intractable problem.

Cowpea plays an important role in the livelihood of millions of relatively poor people in the less developed countries in the tropics. It is used as a source of food, animal feed and cash (Quin, 1997). However, like many other crops, cowpea is susceptible to a wide variety of pests and pathogens that attack the legume at all stages of its growth and potentially reduces its production. In order to control cowpea pests and disease yields, the use of insecticides has been the most

commonly adapted method; but most farmers, cannot afford them due to their low income, (Saxena and Kidiavai, 1997), can't have the necessary equipment, don't know how to apply them safely and effectively and they are hazardous (Saxena *et al.*, 1989). Due to the deleterious effects of insecticides to man, the environment and livestock, alternative strategies for the insect pest management are being developed like that of habitat management by the use of mixed cropping system (Saxena and Kidiavai, 1997); on this aspect Dissemond and Hindorf (1990) revealed that insect pest population was lower in sorghum/cowpea/maize intercrops than in pure cowpea strands. The other strategy is the biological control method through biotechnology, i.e. the genetic transformation and developing of resistant/tolerant cowpea cultivars.

Today, a large number of DNA based methods are available for characterization of population variability, evaluation of genetic diversity as well as determination of genetic relationships within or among animal and plant populations. Some of these DNA based methods are random amplified polymorphism DNA (RAPD) (Laity *et al.*, 2003; Fana *et al.*, 2004), amplified fragment length polymorphism (AFLP) (Coulibaly *et al.*, 2002), restriction fragment length polymorphism (RFLP) (Menéndez *et al.*, 1997) and simple sequence repeat (SSR) (Li *et al.*, 2001) markers. The genetic linkage map of the wild and cultivated cowpea can be developed using these molecular markers. Microsatellites also known as simple sequence repeat are becoming the preferred markers for genome analysis because of their co-dominant nature as well as their reproducibility. In a recent study, Li *et al.* (2001) used forty-six microsatellite DNA markers to facilitate significant progress in the development of the cowpea genome.

A major finding in genetic diversity studies in cowpea is that a genetic bottleneck is induced by domestication in spite of substantial variation in seed color, seed coat patterns, plant type, pod type and seed size among cultivated cowpeas (Panella and Gepts, 1992; Vaillancourt *et al.*, 1993; Panella *et al.*, 1993). The total genetic diversity in cultivated cowpea reported in these studies was lower than that reported in many other crops (Doebley, 1989). Since the first traits were described in cowpea (Harland, 1919), many morphological and disease resistance loci have been identified (Fery, 1985). However, prior to 1993 only few reports of genetic linkage map in cowpea were reported in the literature. A cowpea linkage map was developed from a cross between an improved cultivar and a putative wild progenitor type (*Vigna unguiculata* var. spontanea (NI963)) from Senegal. This cowpea map consisted of 87 random genomic and five cDNA RFLPs, five RAPDs, and two morphological loci/locus clusters arranged in ten linkage groups (Fatokun *et al.*, 1992). Another cowpea linkage map was also developed from a cross between two agronomically contrasting breeding lines, "IT84S-2049" and"524B". This cowpea map consisted of 181 loci, comprising 133 RAPDs, 19 RFLPs, 25 AFLPs, three morphological/classical-markers, and a biochemical marker (dehydrin) (Menendez *et al.*, 1997). Ouédraogo *et al* (2002a) also constructed a cowpea linkage map based on the segregation of various molecular markers and biological resistance traits in a population of 94 recombinant inbred lines (RILs) derived from the cross between 'IT84S-2049' and '524B'. This cowpea map consisted of 11 linkage groups (LGs) spanning a total of 2670 cM, with an average distance of 6.43 cM between markers.

Constructions of genetic maps based on narrow/related crosses have the disadvantage of identifying loci that may be polymorphic only between less divergent genotypes. For this reason

molecular maps based on crosses involving wild progenitors have a greater impact in breeding programs that exploits interspecific variation within cultivated and the wild forms. A genetic linkage map constructed from a cross between the cultivated and the wild gene pool would, therefore, be desirable and is also used in order to introduce desirable characters from wild relatives into cultivars. The low level of polymorphism at the isozyme level within the cultivated cowpea revealed by previous studies (Fatokun *et al.*, 1993), in addition to their low number, precludes the use of that type of marker in any cowpea mapping study. Although RFLP markers remain extremely useful, they have failed to detect enough polymorphism in intraspecific crosses of crops with low genetic diversity (Foolad *et al.*, 1993). Alternative molecular markers showing higher level of polymorphisms among closely related genotypes include microsatellites (Akkaya *et al.*, 1995), RAPDs (Williams *et al.*, 1993), minisatellites (Sonnante *et al.*, 1994) and AFLPs (Vos *et al.*, 1995).

The rapid development of biotechnology has greatly promoted the research and development of genetically modified (GM) crops worldwide. Consequently, a large number of transgenes conferring diverse traits have been successfully transferred into crop varieties through the transgenic biotechnology (Repellin *et al.*, 2001; Lu and Snow, 2005; Lee *et al.*, 2006; Zhao *et al.*, 2007). These traits include high protein contents and unique nutritional compounds (Gura, 1999; Hasler, 2000; Ye *et al.*, 2000), disease and insect resistance (Datta *et al.*, 1998; Huang *et al.*, 2005; Bock, 2007), virus resistance (Shepherd *et al.*, 2007), herbicide resistance (Lutz *et al.*, 2001; Toyama *et al.*, 2003), and salt and drought tolerance (Bahieldin *et al.*, 2005; Tang *et al.*, 2006). Likewise, in the process of research and development of GM cowpea, beneficial traits with unique functions have been transferred into this crop by genetic engineering. The great

success in transgenic biotechnology has had a tremendous impact on the world crop production and cultivation patterns of agricultural species such as cotton, soybean, canola, and maize (James, 2007).

The commercial production of GM crops with various agronomically beneficial traits provides great opportunities for world's food security by enhanced efficiency of crop production. However, the extensive environmental release and cultivation of GM crop varieties have also aroused enormous biosafety concerns and debates worldwide (Stewart *et al.*, 2000; Ellstrand, 2001, 2003), including food and health safety (Cromwell *et al.*, 2005; Hothorn and Oberdoerfer, 2006; Marshall, 2007), environmental safety (Conner *et al.*, 2003; Sanvido *et al.*, 2007), as well as socio-economical and ethic concerns (Finucane and Holup, 2005; Aerni, 2007; Einsele, 2007).

Among the environmental biosafety issues, transgene escape from a GM crop variety to its non-GM crop counterparts or wild relatives has aroused tremendous debates worldwide (Ellstrand *et al.*, 1999; Ellstrand, 2001, 2003; Lu and Snow, 2005). This is because transgene escape can easily happen via gene flow that may result in potential ecological consequences if significant amount of transgenes constantly move to non-GM crops and wild relative species. This is particularly true when these transgenes can bring evolutionary selective advantages or disadvantages to crop varieties or wild populations.

Gene flow can take place either through seed dispersal or pollen flow assisted by pollinators. Results of assessing the risk of transgene dissemination associated with the introduction of genetically modified crops in Africa using cowpea as a model show that hybrids between wild

and domesticated cowpea (as well as their progeny) are fit, and most importantly they can easily take advantage of their inherent protection against insects to boost their seed production.

A report from a floral biology study (Pasquet, unpublished data) suggests a way of preventing gene flow. The peak of bee activity is in the morning which is related to sunrise time while time of cowpea flower opening fluctuates little during the year. Therefore, if domesticated cowpea flowers open late, much later than the peak of bee activity, or do not open at all, gene flow can be greatly reduced. The normal bee activity also suggests another way to prevent gene flow, through nectar aroma; bees seem to detect the aroma level of the flowers and do not visit empty flowers so changing flower aroma could be a second way to prevent gene flow.

Considering its importance cowpea improvement has not received a high priority, however a considerable number of cultivars have been developed. The main characteristics improved by conventional breeding methods are yield, maturity and disease resistance. However, several important characters like seed size and yield are controlled by polygenes, which can not be easily improved by conventional breeding. Molecular techniques, beginning in the 1980s, have become useful tools in crop improvement programs.

The quantitative trait which shows continuous variation is difficult to make selections by conventional methods. Genes are located at a particular location on a chromosome, called a locus. Quantitative traits are controlled by many regions on the chromosomes, but each such region may have multiple genes, or regulatory elements, simply referred to as quantitative trait loci (QTL). Such QTL can be identified if there are markers associated with them. To identify a

QTL, it is important to map the loci controlling the trait of interest on the chromosome, then identify the markers that are associated with the trait. Once these tasks are done, the markers can be used in selection to improve the trait of interest.

Considering the limitations of RFLP and AFLP, a DNA marker capable of detecting the polymorphism even in the event of multiple alleles at a single locus would be more useful. Simple sequence repeat markers are single locus markers with multiple alleles serving as co-dominant markers (Cregan *et al.*, 1999). SSR markers are dependent on the number of alleles and their frequencies to determine the polymorphism (Cregan *et al.*, 1999). An SSR or microsatellite is a small segment of DNA, usually 2 to 5 bp in length that repeats itself a number of times. Useful SSRs usually repeat the core motif 9-30 times. The regions flanking the microsatellite are generally conserved among genotypes of the same species and polymerase chain reaction (PCR) primers to the flanking regions can be used to amplify SSR DNA fragments (Cregan *et al.*, 1999). Length polymorphisms are created when PCR products from different alleles vary in length as a result of variation in the number of repeat units in the SSR. These can be analyzed by electrophoresis and can resolve contrasting alleles. Currently SSR are regarded as a marker of choice because of the high level of informativeness, co-dominance, wide spread in eukaryotic genomes, and ease of amplification by standard PCR technique.

Despite the importance of cowpea domestication as well as flower scent traits, no QTL have been reported for both traits. Therefore, a need exists to continue construction of a cowpea linkage map using microsatellite markers and utilize this map to locate QTLs for domestication,

aroma and agronomic traits to particular positions on the chromosomes to facilitate future breeding programs to adopt marker assisted selection (MAS) and identify the chemical compounds for the cowpea flower aroma. This research was directed to achieve these objectives.

1.2 Justification of the study

Cultivated and wild plants are not resistant to insect pests. For this reason, scientists have been working to produce genetically modified cowpea plants with insect resistant gene, producing insect specific toxin, such as *Bacillus thuringiensis* (Bt) toxin (Murdock and Shade, 2002). This protein is specific in its activity against Coleoptera and Lepidoptera (Schnepf *et al.*, 1998), especially for Maruca Pod Borer (*Maruca vitrata*), which heavily reduce cowpea productivity in Africa (Machuka *et al.*, 1999). Therefore, with the upcoming introduction of Bt cowpea in Africa, there are possibilities that the Bt gene will be transferred through hybridization and backcross processes from transgenic plants to other cultivars and wild relatives because of the existence of weak genetic barriers within cowpeas (Fatokun, 1991).

1.3 Statment of the problem

Given that cultivated plant species and their putative wild relatives represent an interesting system to study crop evolution, cowpea appears an excellent example in studying wild-domesticated plant relationship. Molecular invesstigations highlight a unique domestication event in *V. Unguiculata* (Panella and Gepts, 1992 ; Vaillancourt *et al.*, 1993 ; Pasquet, 1993b ; 1998 ; 1999 ; 2000 ; Coulibaly *et al.*, 2002 ; Ba *et al.*, Feleke *et al.*, 2006), the domestication

related as well as scent/aroma traits of cowpea is weakly documented. Thus, identification of the quantitative traits of these traits ahead of the release of the genetically modified cowpea is important since the possible dissemination of the inserted gene may interfere with the genetic relationship existing between wild and cultivated cowpea.

It is likely that an introgressed wild plant will be able to take advantage conferred by an insect-resistance gene. This raises a potential concern that an insect resistance transgene can turn wild cowpeas into invasive weed. It is therefore important to construct a cowpea genetic linkage map using a prominent molecular marker and identify those genes that regulate the domestication traits as well as those that are responsible for the production of different volatile compounds in cowpea flowers in order to overcome the problem and solve one of the major concerns related to the introduction of GM cowpea in Africa since gene flow mainly takes place by pollen flow and seed dispersal.

1.4 Research null hypothesis

The research null hypotheses were:

1) Primary domestication traits are clustered like in *Pearl millet*
2) Using a wild parent will give more polymorphism
3) Wild parents that are agronomically inferior will improve agronomically important trait
4) Using SSR will give a regular coverage of the genome unlike clustered AFLP maps
5) Mapping genes controlling the amount of aroma compounds as well as domestication traits and closely linked molecular markers is helpful for marker assisted selection
6) There will be one major gene that is responsible for cowpea flower scent

1.5 OBJECTIVES OF THE STUDY

The general objective of the research was to construct a genetic linkage map of cowpea mainly based on microsatellite markers and identification of QTLs that control yield, domestication traits associated to cowpea and scent of cowpea flowers.

The Specific objectives were to:

i) to construct an SSR based genetic linkage map of cowpea

ii) to identify and map the different quantitative trait loci that regulate yield as well as domestication traits of cowpea,

iii) to identify and map the QTLs that governs scent of cowpea flowers

iv) to identify the aroma compounds that attracts bees to cowpea flowers

CHAPTER TWO

LITERATURE REVIEW

2.1Taxonomy and nomenclature of cowpea

Cowpea (*Vigna unguiculata (L.) Walp*) is one of the 80 species of the genus *Vigna* (Pasquet, 2001). It is a *Dicotyledonea* belonging to the order *Fabales*, family *Fabaceae*, subfamily *Faboideae*, tribe *Phaseoleae*, subtribe *Phaseolinae*, genus *Vigna* (Padulosi and Ng, 1997). In the United States, it is referred to as black-eyed pea, crowder pea, southern pea (Duke, 1981) or black-eyed bean (Miller, 1989); this legume is called niébé in French speaking West Africa.

2.2 Ecology, biology and reproduction of cowpea

Cowpea is a herbaceous legume grown in tropical areas. As a warm season crop, cowpea is well adapted in many areas of the humid tropics and temperate zones. It tolerates heat and dry conditions but is intolerant to frost (Duke, 1981). Cowpea also performs well on a wide variety of soils and soil conditions, but performs best on well-drained sandy loams or sandy soils from highly acidic to neutral; and is less tolerant in alkaline conditions (Duke, 1981). Cowpea germination is rapid at a temperature above 18°C with an optimum at 28°C (Craufurd *et al.*, 1997). Seed germination is epigeal with the cotyledons emerging from the ground. The first two leaves above cotyledons are simple and opposite, the others are alternate, petiolate and trifoliate (Pasquet and Baudoin, 2001). Peduncle that arises from the leaf axial contains commonly two or three pods and sometimes can carry four or more pods. Some cowpea plant gives flowers 30 to 40 days after germinating with a life cycle of 60-240 days (Miller *et al.*, 1989; Duke, 1981). Strongly tap rooted in general, with a strong principal root and many spreading lateral roots on

the soil surface; disposition enables the plant to explore the soil for moistures (Pasquet and Baudoin, 2001). Cowpea's roots have nodules containing nitrogen-fixing bacteria. It can be grown in polyculture, associated with cassava, corn, sorghum or millets (Duke, 1981). Annual cowpea primarily is a self-pollinating plant (Fery, 1985), the open display of flowers above the foliage and the presence of floral nectarines attract insects some of which have been identified as cowpea pollinators. The cultivated cowpea flowers open at the end of the night and close late morning. Some wild cowpea forms are considered to be allogamous, due to a particular arrangement of the anthers and stigma that prevent self-pollen to reach the stigma (Pasquet and Baudoin, 2001). In inbreeding plants (autogamous), the stigmatic surface and the anthers are in contact (Lush, 1979). The low fertility in allogamous flower, which range from 0 to 40% versus 40 to 70% for autogamous flowers, can be compensated for by manual fertilization or insect ripping (Pasquet and Baudoin, 2001).

2.3 Organisation of *Vigna unguiculata*

Vigna unguiculata (L) Walp. is composed of cultivated cowpea (*V. unguiculata* var *unguiculata*), subdivided in five cultigroups or varieties namely *unguiculata, biflora, melanophthalmus, sesquipedalis and textilis* (Pasquet, 1998). Wild gene pool includes annual wild cowpea (*Vigna unguiculata ssp. unguiculata var. spontanea)* and ten wild perennial subspecies divided in two groups according to their breeding system. The perennial allo-autogamous subspecies (*dekindtiana, stenophylla, tenius, alba* and *pubescens*) are the most closely related to the annual forms than do the perennial allogamous subspecies (*pawekiae, burundiensis, letouzeyi, baoulensis* and *aduensis*) (Pasquet and Baudoin, 2001).

The cultivated cowpeas form a genetically coherent group and are closely related to the annual wild cowpea, *ssp. unguiculata var. spontanea (Schweinf) Pasquet*. The morphology and growth habits of this wild legume are very similar to those of cultivated cowpea except that its mature pods contains small seeds (wild-like attribute) and are dehiscent, much smaller than those of cultivated cowpea (Padulosi and Ng, 1997)

2.4 Morphological diversity of cowpea

Cowpea morphologically is very diverse (Pasquet, 1999). From the wild, Cowpea has been selected for various traits, giving several varieties with a lot of differences in their morphology. The cultivated forms of cowpea show a great diversity in their seed and pod while the wild forms present a lot of variability in their floral morphology and vegetative characters (Pasquet, 1993a). Cultivated cowpeas differ from wild forms by having larger seeds and pods, non-dormant seeds and non-dehiscent pods (Lush and Evans, 1981). Cowpea plant can be found erect, semi-erect, prostrate or climbing in their grown areas. Fruits of the cowpea plant are pods that vary in size, color and texture; they can be erect, crescent-shaped or coiled and are usually yellow when ripe but can also be brown or purple in color. The length of the pod, which usually can reach 30 cm, contains 8-20 seeds that vary in size, shape and color. Seed color is determined by the C gene, a dominant gene associated with genes controlling pigments synthesis (Pasquet and Baudoin, 2001). They are very diverse, ranging from white, black, brown, purple, green, and red to various types of mottled seeds. Cowpea stems are smooth or slightly hairy and sometimes tinged with purple. Stigma orientation can be vertical or horizontal (Lush and Wien, 1980). Self-pollinating flowers are arranged in raceme or intermediate inflorescence in alternate pair and can be white, dirty yellow, pink, pale blue or purple in color. Flower length ranges from 21 to 43 mm (Pasquet,

1993a). Wild cowpea has characteristics such as perenniality, hairiness, small size of pods and seeds, pod shattering, outbreeding and bearded stigma (Padulosi and Ng, 1997).

2.5 Genetic diversity of cowpea

Previous studies carried out on cowpea reveal that the crop exhibits important genetic diversities and variabilities (Pasquet, 1999; Li *et al.*, 2001; Laïty *et al.*, 2003). Using allozymes variations to study 199 germplasm accessions of wild and cultivated cowpea, Pasquet (1999) showed that cowpea taxa (ranked as subspecies) could be considered as different species considering the high genetic distances observed between accessions from different taxa.

2.6 Uses of cowpea

Cowpea has several uses. It can be used at all stages of its growth (fresh leaves, peas and pods) as a vegetable crop, several snacks and meal dishes are prepared from its grains (Quin, 1997). Its tender green leaves are an important food source in Africa and are prepared as a potherb like spinach. In industrialized countries, variety types of cowpea green seeds are cooked, canned or frozen to make them ready to serve. Dried mature seeds are also suitable for boiling and canning. With its high protein content (20-25%), cowpea has been referred to as a poor man's meat (Laïty *et al.*, 2003) and is considered as a source of cheap protein in both rural and urban tropical African diet with its protein digestibility higher than that of other legumes (Marconi *et al.*, 1990). Proteins in cowpea seeds are rich in amino acid lysine and tryptophan compared to cereal grains; however, it is deficient in methionine and cystine when compared to animal proteins. Therefore,

cowpea seeds are valued as a nutritional supplement to cereals and an extender to animal proteins.

Cowpea is also used to improve soil fertility (Ogbuinya, 1997). As nitrogen fixing crop through the symbiotic association with the bacteria *Bradyrhizobium ssp,* cowpea contributes to the available N level in the soil and in that case, increasing the yield of cereal crops when growing in rotation. Therefore, cowpea is a versatile crop feeding people, their livestock and the next crop. With its deep roots, cowpea helps to stabilize the soil preventing land deterioration and minimizing soil erosion. The cultivar group textilis with its long floral peduncles is used for fiber production in West Africa. Cowpea plant is also used for medicinal purposes (Padulosi and Ng, 1997).

2.7 Constraints of cowpea production

2.7.1 Abiotic factors

Environmental factors that include soil salinity, extreme temperatures and drought are the major factors that limit agricultural productivity of cowpea. Some plant species have developed various mechanisms to adapt in such stressful conditions (Hirt and Shinozaki, 2003). The result of the environmental effects on plant growth may be the difference of the damage effect or stress caused by the environment and the adaptive response controlled by the plant (Fitter and Hay, 1987). When the environmental factor/stress is dominant, damages may occur and are manifested by the death of all or part of the plant, reduction in the growth rate and productivity. Cowpea is largely cultivated in tropical and semi-arid zones where drought frequently occurs and it may be the most serious environmental agent that is able to limit the cowpea production.

2.7.2 Biotic factors

i) Diseases

A wide range of parasites and pests limits cowpea production and these include bacteria (*Xanthomonas axonopodis pv. vignicola, Clavibacter michiganensis subsp. michiganensis* and *Acidovorax avenae subsp. Citrulli)* (Gitaitis *et al.*, 2004). Fungi in the *Pythium ssp,* such as *Rhyzoctonia solani* and *Phytophtora ssp* mainly attack seeds while *Corynespora cassiicola, Cercospora canescens* attack leaves; *Cladosporium vignae, Choanephora cucurbitarum* attack the pods (Allen, 1983). Cowpea is also attacked by viruses such as CCMV (Cowpea Chlorotic Mottle Virus), Yellow Mosaic Virus, transmitted by white fly (*Bemisia tabaci*) vector and affect both vegetative and reproductive part of the plant (Yousaf *et al.*, 2002). Yellow mosaic virus may cause 14-54% decrease in plant height, 30 to 95% decrease in dry stem weight of cowpea (Yousaf *et al.*, 2002). Cowpea is further attacked by pests throughout its growth cycle like nematodes (*Meloidogyne, Rotylenchus and Pratylenchus*) on the roots (Pasquet & Baudoin, 2001).

ii) Insect pests

Cowpea also suffers from insect pests both in the field as well as in storage (Oghiakhe, 1995). In Africa, no other crop suffers such high yield losses due to a plethora insect pest as cowpea does (Hans, 1996) and these pests constitute the greatest constraint on cowpea production in Africa (Oghiakhe, 1995). One of such pests is cowpea aphid (*Aphid cruciform*) (Nuessly *et al.*, 2004). It feeds by piercing plant tissues and sucking plant juices. Their feeding, especially on the fruiting stem, considerably reduces the quantity of the plant nutrients available for pod and pea development. Other insect pests include the very destructive maruca pod borer *(Maruca vitrata),*

pod sucking bug (*Chlavigralla tementosicollis*) (Hans, 1996; Oghiakhe, 1995), bean fly (*Ophiomyia phaseoli*), leafhoppers (*Empoasca spp*) and cowpea storage weevil (*Callosobruchus maculatus*) (Oghiakhe, 1995); cowpea curculio cause blister like spots on the surface of the pod; leaf feeding beetles that cause irregular shaped holes in the leave. Another most important cowpea insect pest is legume or bean-flower thrips (*Megalurothrips sjostedti*). It is the most destructive, attacking the reproductive structures of the cowpea during plant development (Saxena and Kidiavai, 1997).

2.8 Genetic improvement of cowpea: Bt technology

Grain yield losses in cowpea are particularly due to biotic stresses and especially by insect pests that include Maruca Pod Borer (*Maruca vitrata*), pod sucking bug, aphids, thrips and bruchids. Conventional insecticides may not be the answer to the insect problems because many cowpea growers cannot afford them. Insect resistant traits have been introduced into the cowpea genome (Higgins, 2004). Studies carried out on some main cowpea insect pests indicated that these insects could be controlled by *Bacillus thuringiensis* crystal protein or (Bt) toxin produced by *Bacillus thuringiensis* (Bt), a bacterium occurring naturally in the soil (Lambert and Peferoen, 1992). The Bt toxin act by binding to the membrane of the insect midgut epithelial cell leading to the lyses of the cell and eventually kills the insect (Schnepf *et al.*, 1998).

The Bt technology starts with the identification of the Bt gene producing the desired lethal protein and then follows four steps. (1) The Bt gene is combined with a marker gene with antibiotic resistance characteristic, (2) The combined Bt gene + marker is then inserted into the

plant cell, (3) Plant cells are grown in the presence of antibiotics, (4) Plant cell carrying the Bt and the antibiotic resistance gene grows in the plant and the plant survives from an insect attack.

2.9 Environmental risks linked to the introduction of transgenic or Bt cowpea

Scientists have made possible the development of the genetically modified cowpea called Bt (*Bacillus thuringiensis*) cowpea through biotechnology with a highly effective insect resistant gene (Murdock and Shade, 2002; Higgins, 2004). However, the introduction of this Bt cowpea like other Bt crops is viewed with many perceived risks: (a) they can have a harmful effect on non-target and beneficial insects; (b) after a widespread use they may transform the insect pests that they are intended to control into insect species that are resistant to Bt toxin; (c) accumulation of the transgene through gene flow into the native materials will have the possibility of affecting the genetic diversity of landraces and wild plants; (d) the possible transfer of the insect resistant gene (Bt gene) through pollen flow from the transgenic plant to other cultivars and wild relatives leads to the evolution of more aggressive weeds which are difficult to control (Ellstrand and Hoffman, 1990) (Fig.1); and specially within *Vigna unguiculata* subspecies where genetic barriers are weak (Fatokun, 1991). This happens because genetically engineered plants very often have the potential to spontaneously hybridize with the wild relatives growing in proximity (Ellstrand and Hoffman, 1990; Papa and Gepts, 2003).

2.10 Genetic exchange within cowpeas

Gene flow is the movement of gene among populations or within a population. It has a significant influence on the distribution of the genetic traits (Hamrick, 1989). Gene flow occurs through reproductive means such as cross-pollination or directly through "horizontal gene

transfer" occurring between species like during genetic engineering. Between *Vigna unguiculata* subspecies, reproductive barriers are weak (Fatokun, 1991) and thus, should facilitate gene flow.

Allozyme markers suggest that within *Vigna unguiculata*, gene flow is quite widespread between the wild and the cultivated cowpea, giving a large crop-weed complex well distributed in the entire Sub Saharan Africa (Pasquet, 1999). Nkongolo (2003) working with Malawian cowpea using RAPD markers reported variation among cowpea accessions with variation accounting for 96% sustaining an uncontrolled gene flow. Coulibaly *et al.* (2002) also reported extensive gene flow between wild and cultivated cowpeas when evaluating genetic relationships in 117 accessions of cowpeas (*Vigna unguiculata* (L.) Walp) using amplified fragment length polymorphism (AFLP), with the wild cowpea more diverse than the cultivated.

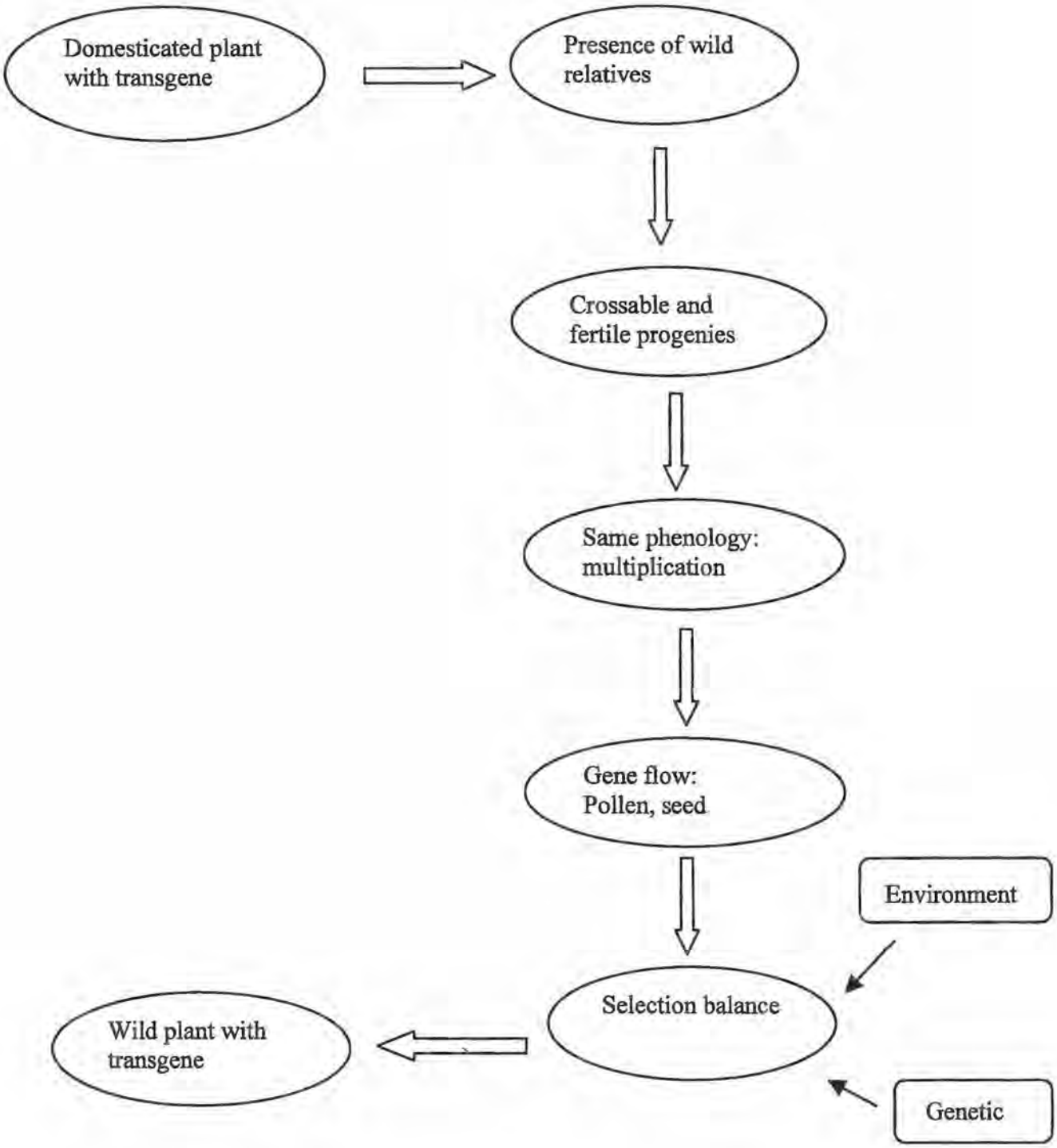


Figure 1. Escape process of transgene from domesticated plants into wild relatives (Gepts & Papa, 2003)

2.11 Methods used in plant gene mapping

2.11.1 Molecular markers

To be an effective genetic marker, the marker locus has to detect variation at different levels. The variation could be a simple heritable phenotype or a difference in the nucleotide sequence (Liu, 1998; Mohan *et al.*, 1997). This detectable and heritable variation at a locus is referred to as a polymorphism and is essential to identify desirable traits. A number of genetic marker systems have been developed for use in different plant species; however, some systems may not be suitable for all purposes. In general, the desirable characteristics of a marker system are to detect a high level of polymorphism, detect specific loci, provide clear, highly heritable genetic information in a short period of time and be easily automated (Liu, 1998). The marker systems available for any species depend on the amount of pre-existing genome information.

The first available molecular markers used were allozymes, protein variants detected by differences in migration on starch gels in an electric field. Since the late 1960s, protein markers were used extensively and were relatively inexpensive to score in large numbers but there was often insufficient protein variation for high-resolution mapping. During the mid 1980s, methods became available to evaluate genetic variation directly at the DNA level and lead to allozymes being replaced with DNA based markers in mapping studies (Tanksley, 1993; Liu, 1998). The advent of molecular DNA technology has made it possible to map and characterize the genes controlling economically important traits in crop species. DNA-based molecular markers are used in genomic analysis and provide the foundation for marker-assisted selection.

There are two basic approaches, hybridization or amplification, used to detect variation in DNA. Detection of variation through random fragment length polymorphisms (RFLPs) is hybridization based, while amplification based technologies use the polymerase chain reaction (PCR) and includes random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs), and microsatellite markers also known as simple sequence repeats (SSRs) (Mohan *et al.*, 1997; Gupta *et al*,. 1999; Liu, 1998). Molecular markers may exhibit either codominance or dominance characters. Codominant markers distinguish between homozygous and heterozygous genotypes while dominant markers are scored as present or absent and cannot distinguish heterozygous from homozygous individuals.

The main application of molecular markers in legumes and other field crops can be divided into three categories; (a) assessment of genetic variability and characterization of germplasm; (b) identification and characterization of genomic regions controlling quantitative traits and (c) marker assisted selection following the identification of specific genomic regions (Ribaut *et al.*, 2002).

2.11.1.1 SSRs (Simple Sequence Repeats)

Microsatellites, or Simple Sequence Repeats (SSRs), are polymorphic loci present in nuclear DNA and organellar DNA that consist of repeating units of 1-6 base pairs in length. They are typically neutral, co-dominant and have wide-ranging applications in the field of genetics, including kinship and population studies. Microsatellites can also be used to study gene dosage (looking for duplications or deletions of a particular genetic region) (Wang *et al.*, 2003).

They are tandemly repeated motifs of 1-6 nucleotides found in all prokaryotic and eukaryotic genomes (Zane *et al.*, 2002). According to Pupko and Graur (1999), any number of tandem repeats of a certain nucleotide combination may be regarded as a microsatellite. These repeats are present in both coding and non-coding regions (Hancock, 1995) and are usually characterized by a high degree of length polymorphism (Zane *et al.*, 2002). Microsatellite loci are inherently unstable with high mutation rates, a phenomenon that is reported to be caused by DNA polymerase slippage and/or unequal recombination (Li *et al.*, 2001). Due to their high mutability, SSRs play a significant role as molecular markers for evolutionary and population genetic studies.

Microsatellites offer several advantages compared to other molecular markers: they are highly reproducible, highly polymorphic, PCR-based and readily portable within a species (Edwards *et al.*, 1996). In a recent study comparing SSRs, RAPDs and AFLPs for the genetic analysis of yeast *(Saccharomyces cerevisiae)* strains, Gallego *et al.* (2005) reported that SSR analysis gave the highest level of information content. Similar results had earlier been reported in soybean (Powell *et al.*, 1996). Microsatellites have also attracted scientific attention because they have been shown to be part of or linked to some genes of agronomic interest (Yu *et al.*, 2000). All these positive attributes coupled with their multi-allelic nature, co-dominant transmission, relative abundance, extensive genome coverage and requirement of only a small amount of template DNA have contributed to the extraordinary increase of interest in SSRs in many organisms (Zane *et al.*, 2002).

According to the quality of the repeat, microsatellites can be classified into three

i) Perfect microsatellites where the sequence consist of a single motif repeated without interruption
ii) Imperfect microsatellites where there is a break within the repeat sequence
iii) Compound microsatellite where the sequence consists of two or more adjacent different repeats

The genomes of higher organisms contain three types of multiple copies of simple repetitive DNA sequences (satellite DNAs, minisatellites, and microsatellites) arranged in arrays of vastly differing size (Hancock, 1995). Microsatellites (Litt and Luty, 1989), also known as simple sequence repeats (SSRs; Tautz *et al.*, 1986), short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs; McDonald and Potts, 1997), are the smallest class of simple repetitive DNA sequences. Some authors (e.g. Hancock, 1995) define microsatellites as 2–8 bp repeats, others (e.g., Goldstein and Schlotterer, 1999) as 1–6 or even 1–5 bp repeats (Schlotterer, 1998). Chambers and MacAvoy (2000) suggested following a strict definition of 2–6 bp repeats, in line with the descriptions of the original authors. Microsatellites are born from regions in which variants of simple repetitive DNA sequence motifs are already over represented (Tautz *et al.*, 1986). It is now well established that the predominant mutation mechanism in microsatellite tracts is 'slipped-strand mispairing' (Levinson and Gutman, 1987). This process has been well described by Eisen (1999). When slipped-strand mispairing occurs within a microsatellite array during DNA synthesis, it can result in the gain or loss of one, or more, repeat units depending on whether the newly synthesized DNA chain loops out or the template chain loops out, respectively. The relative propensity for either chain to loop out seems to depend in part on the

sequences making up the array, and in part on whether the event occurs on the leading (continuous DNA synthesis) or lagging (discontinuous DNA synthesis) strand (Freudenreich *et al.*, 1997). SSR allelic differences are, therefore, the results of variable numbers of repeat units within the microsatellite structure. The repeated sequence is often simple, consisting of two, three or four nucleotides (di-, tri-, and tetranucleotide repeats, respectively). One common example of a microsatellite is a dinucleotide repeat (CA)n, where n refers to the total number of repeats that ranges between 10 and 100. These markers often present high levels of inter- and intra-specific polymorphism, particularly when tandem repeats number is ten or greater (Queller *et al.*, 1993).

PCR reactions for SSRs is run in the presence of forward and reverse primers that anneal at the 5` and 3` ends of the template DNA, respectively. PCR fragments are usually separated on polyacrylamide gels in combination with $AgNO_3$ staining, autoradiography or fluorescent detection systems. Agarose gels (usually 3%) with EtBr can also be used when differences in allele size among samples is larger than 10 bp. However, the establishment of microsatellite primers from scratch for a new species presents a considerable technical challenge. Several protocols have been developed (Bruford *et al.*, 1996; McDonald and Potts, 1997; Hammond *et al.*, 1998; Schlotterer, 1998) and details of the methodologies are reviewed by different authors (e.g., Chambers and MacAvoy, 2000; Zane *et al.*, 2002; Squirrell *et al.*, 2003). A review by Zane *et al.* (2002) describes some of the technical advances that have been made in recent years to facilitate microsatellite development. They cover a range of methods for obtaining sequences rich in microsatellite repeats (some of which can be undertaken in a matter of days), and also

highlight the availability of companies who will undertake the construction of enriched microsatellite libraries as a commercial service.

According to Roder *et al.* (1998) the development of microsatellite markers involves several distinct steps from obtaining the library to developing a working set of primers that can amplify polymorphic microsatellite loci. These include:

(i) Microsatellite library construction,

(ii) Identification of unique microsatellite loci,

(iii) Identifying a suitable area for primer design,

(iv) Obtaining a PCR product,

(v) Evaluation and interpretation of banding patterns,

(vi) Assessing PCR products for polymorphism,

SSR primers are developed by cloning random segments of DNA from the target species. These are inserted into a cloning vector, which is in turn, implanted into *Escherichia coli* bacteria for replication. Colonies are then developed, and screened with single or mixed simple sequence oligonucleotide probes that will hybridize to a microsatellite repeat, if present on the DNA segment. If positive clones for microsatellite are obtained from this procedure, the DNA is sequenced and PCR primers are chosen from sequences flanking such regions to determine a specific locus. This process involves significant trial and error on the part of researchers, as microsatellite repeat sequences must be predicted and primers that are randomly isolated may not display polymorphism (Queller *et al.*, 1993; Jarne and Lagoda, 1996).

The next step is to select the best candidate markers and then to optimize conditions for their amplification. Optimization of microsatellite systems involves a more or less comprehensive survey of PCR conditions for amplification of candidate loci. The objective here is to adequately balance the often conflicting requirements for high specificity and high intensity of amplification products. Thus, the issue of signal strength and purity remains the primary focus. Other considerations include obtaining products from various loci with non-overlapping ranges of allele sizes, which can be amplified with similar efficiency under a standard set of conditions and enables multiplexing for high throughput analysis (Schlotterer, 1998). Microsatellite loci are more common in some organisms than in others, and screening may produce few useful loci in some species (Cooper, 1995). The efficiency of microsatellite marker development depends on the abundance of repeats in the target species and the ease with which these repeats can be developed into informative markers.

During isolation of plant microsatellites, about 30% of the sequenced clones, on average, can be lost due to the absence of unique microsatellites. Of those sequences that contain unique microsatellites, a number of the clones in a library can contain identical sequences (and hence there is a level of redundancy) and/or chimeric sequences (i.e., one of the flanking regions matches that of another clone). At each stage of SSR development, therefore, there is the potential to lose loci, and hence the number of loci that will finally constitute the working primer set will be a fraction of the original number of clones sequenced (Squirrell *et al.*, 2003). The conversion of microsatellite-containing sequences into useful markers can be quite difficult, especially in species with large genomes (Smith and Devey, 1994; Kostia *et al.*, 1995; Roder *et al.*, 1998; Pfeiffer *et al.*, 1997; Song *et al.*, 2002). The low conversion rates of primer pairs to

useful markers in these species are due to the high level of repetitive DNA sequences in their genomes. The recovery rate for useful SSR primers is generally low due to different reasons:

a) The primer may not amplify any PCR product,

b) The primer may produce very complex, weak or nonspecific amplification patterns,

c) The amplification product may not be polymorphic,

Loci containing tri and tetra-nucleotide repeat arrays are preferred rather than dinucleotide arrays because the former frequently give fewer "stutter bands" (multiple near-identical 'ladders' of PCR products which are one or two nucleotides shorter or longer than the full length product; (Hearne *et al.*, 1992; Diwan and Cregan, 1997). Thus, allele sizing is less error prone using tri- and tetra-nucleotide repeats than di-nucleotide repeats (Diwan and Cregan, 1997). However, this idea must be balanced against practical considerations. Di-nucleotide repeat arrays occur much more frequently than tri- or tetra-nucleotide repeat arrays, and it is easier to run combinational screens for them. SSRs are now the marker of choice in most areas of molecular genetics as they are highly polymorphic even between closely related lines, require low amount of DNA, can be easily automated for high throughput screening, can be exchanged between laboratories, and are highly transferable between populations (Gupta *et al.*, 1999). For example, a total of 18,828 SSR sequences have been detected in the rice genome (The Rice Genome Mapping project, 2005), of which only 10 -15% have yet been used, suggesting the high potential available for such marker systems. SSRs are mostly codominant markers, and are indeed excellent for studies of population genetics and mapping (Jarne and Lagoda, 1996; Goldstein and Schlotterer, 1999). The use of fluorescent primers in combination with automatic capillary or gel-based DNA sequencers has

got its way in most advanced laboratories and SSRs are excellent markers for fluorescent techniques, multiplexing and high throughput analysis.

The major constraint of using SSR markers from genomic libraries is the high development cost and effort required to obtain working primers for a given study species. This has restricted their use to only a few of the agriculturally important crops. A more widespread use of genomic SSRs in plants would also be facilitated if such loci were transferable across species. Recently, a new alternative source of SSRs development from expressed sequence tag (EST) databases has been utilized (Kota *et al.*, 2001; Kantety *et al.*, 2002; Michalek *et al.*, 2002). With the availability of large numbers of ESTs and other DNA sequence data, development of EST-based SSR markers through data mining has become a fast, efficient, and relatively inexpensive compared with the development of genomic SSRs (Gupta *et al.*, 2003). This is due to the fact that the time-consuming and expensive processes of generating genomic libraries and sequencing of large numbers of clones for finding the SSR containing DNA regions are not needed in this approach (Eujayl *et al.*, 2004). However, the development of EST SSRs is limited to species for which this type of database exists. Furthermore, the EST-SSR markers have been reported to have lower rate of polymorphism compared to the SSR markers derived from genomic libraries (Cho *et al.*, 2000; Scott *et al.*, 2000; Eujayl *et al.*, 2002; Chabane *et al.*, 2005).

Differences in SSR allele size is often difficult to resolve on agarose gels and high resolutions can be achieved through the use of polyacrylamide gels in combination with $AgNO_3$ staining. The cost of polyacrylamide gels is higher than agarose gels and it is not also as rapid as the latter. The establishment and running cost for an automatic DNA sequencer is not affordable for

researchers at the national research systems and universities in developing countries. The other technical problem with microsatellites is the fact that it is not always possible to compare data produced by different laboratories, due to the eventuality of inconsistencies in allele size calling. Such inconsistencies are mainly due to the large variety of automatic sequencing machines used, each providing different gel migration, fluorescent dyes, allele calling software's, and PCR reaction. For the later, the enzyme used for DNA synthesis (Taq DNA polymerase) catalyses the addition of an extra base (usually an adenine) at the end of the PCR product. The proportion of fragments with this extra base may vary from none to 100%, inducing one base pair size differences and complicating data analysis. Although biochemical treatments after PCR or modification of PCR primers can circumvent this problem (Brownstein *et al.*, 1996; Ginot *et al*,. 1996), they are seldom used.

Microsatellite markers detect high level of genetic polymorphism and this is why they have often been applied to study the genetic variation for a wide range of plant species (Blair *et al.*, 2003; Flandez *et al.*, 2003; Li *et al.*, 2001; Zeigenhagen *et al.*, 1998). By looking at the variation of microsatellites or the differences in the number of repeat units between alleles at a given locus, detected on high resolution gel, inferences can be made about population genetic structure, degree of relatedness and gene flow. The frequent utilization of microsatellites by scientists is due to its advantages: (1) Microsatellite allows the identification of many alleles at a single locus. (2) They are present in all living organism and are evenly distributed all over the genome, coding and non-coding regions (Monika and Hanna, 2004). (3) They are codominant; each allele is expressed so that a heterozygous is distinguished from both homozygous. (4) There is a good chance of obtaining result with a low concentration of partially degraded DNA because of the

small size of microsatellite loci. However, their use is still limited because of the long and laborious steps involving their isolation (Monika and Hanna, 2004).

2.12 Linkage Maps

Several types of DNA markers have been widely used, restriction fragment length polymorphisms (RFLPs) (Botstein *et al.*, 1980), random amplified polymorphic DNAs (RAPDs) (Williams *et al.*, 1990), simple sequence repeats (SSRs or microsatellites) (Litt and Luty, 1989) and amplified fragment length polymorphisms (AFLPs) (Vos *et al.*, 1995). All types of DNA markers detect sequence polymorphisms and monitor the segregation of a DNA sequence among progeny of a genetic cross in order to construct a linkage map. While the theory of linkage mapping is the same for DNA markers as in classical genetic mapping, special considerations must be kept in mind. This is primarily a result of the fact that potentially unlimited numbers of DNA markers can be analyzed in a single mapping population. Backcross and F_2 populations are suitable for DNA-based mapping, but recombinant inbred (Burr and Burr, 1991) and doubled haploid lines (Heun *et al.*, 1991) provide permanent mapping resources. These types of populations are also better suited for analysis of quantitative traits.

2.12.1. Constructing a linkage map with DNA markers

One of the most critical decisions in constructing a linkage map with DNA markers is the mapping population (Collard *et al.*, 2005) (Fig.2). In making this decision, several factors must be kept in mind, the most important of which is the goal of the mapping project. Is the goal simply to generate a framework map to provide a set of mapped loci for the future, or instead, to

identify and orient DNA markers near a target gene for eventual map-based cloning? Perhaps the goal is mapping quantitative trait loci (QTL), or the monitoring of several disease resistance loci in the process of pyramiding them into a single background. Whichever goal is the motivating factor behind mapping, it will have a critical influence on which parents are chosen for crossing, the size of the population, how the cross is advanced, and which generations are used for DNA and phenotypic analysis.

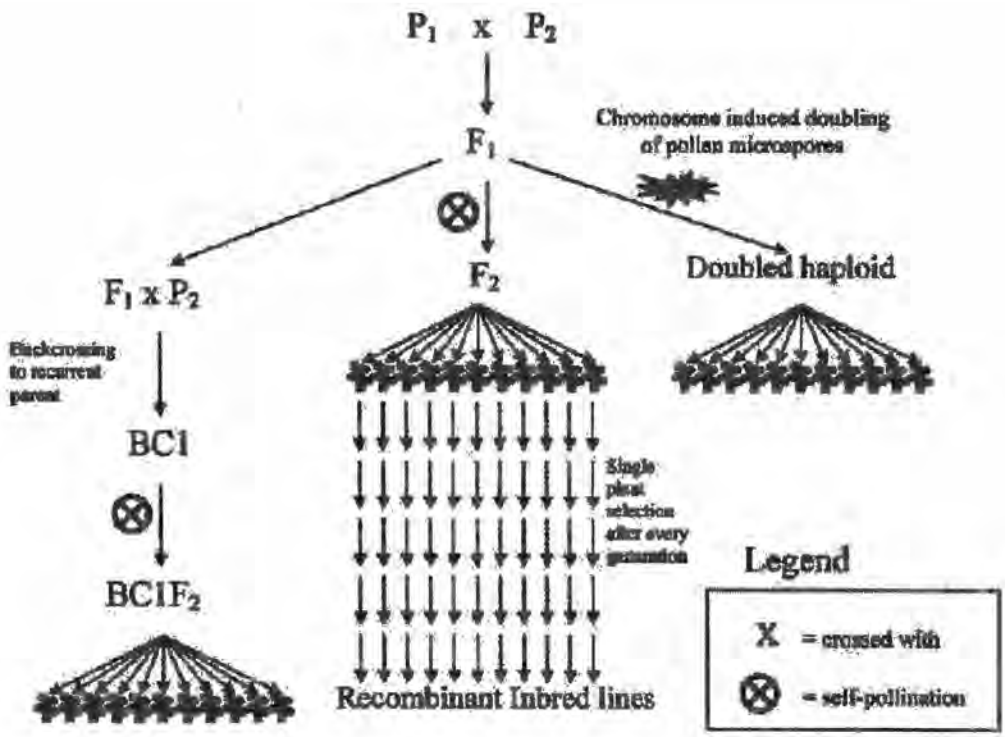


Figure 2 Diagram of the main types of mapping populations for self-pollinating species. (Collard *et al.* 2005)

Once suitable mapping population is established the next factor that needs consideration is the presence of sufficient DNA sequence polymorphisms between parents. This cannot be

overemphasized, for in the absence of DNA polymorphism, segregation analysis and linkage mapping are impossible. Naturally outcrossing species, such as maize, tend to have high levels of DNA polymorphisms and virtually any cross that does not involve related individuals will provide sufficient polymorphism for mapping (Helentjaris *et al.*, 1986). However, levels of DNA sequence variation are generally lower in naturally inbreeding species and finding suitable DNA polymorphisms may be more challenging (Miller and Tanksley, 1990). Sometimes mapping of inbreeding species requires that parents be as distantly related as possible, which can often be inferred from geographical, morphological, or isozyme diversity. In some cases, suitable wide crosses may already be available because a frequent goal in plant breeding in the past has been the introduction of desirable characters from wild relatives into cultivars. Moreover, SSR markers tend to exhibit high levels of polymorphism, even and narrow crosses (Rongwen *et al.*, 1995), providing the possibility of constructing maps in crosses between closely related parents.

Once suitable parents have been chosen, the type of genetic population to use for linkage mapping must be considered. Several different kinds of genetic populations are suitable. The simplest are F_2 populations derived from F_1 hybrids and backcross populations. For most plant species, populations such as these are easy to construct, although sterility in the F_1 hybrid may limit some combinations of parents, particularly in wide crosses (Burr and Burrr, 1991).

The major drawback to F_2 and backcross populations is that they are ephemeral, that is, seed derived from selfing these individuals will not breed true. This limitation can be overcome to a limited extent by cuttings, tissue culture or bulking F_3 plants to provide a constant supply of plant material for DNA isolation. Nevertheless, it is difficult or impossible to measure characters

as part of quantitative trait locus (QTL) mapping in several locations or over several years with F_2 or backcross populations. For these reasons, permanent resources for genetic mapping are essential (Burr *et al.*, 1988).

The best solution to this dilemma is the use of inbred populations that provide a permanent mapping resource. Recombinant inbred (RI) lines derived from individual F_2 plants are an excellent strategy (Burr *et al.*, 1988; Burr and Burr, 1991). RI lines are created by single seed descent from sibling F_2 plants through at least five or more generations. This process leads to lines that each contains a different combination of linkage blocks from the original parents. The differing linkage blocks in each RI line provide a basis for linkage analysis. However, several generations of breeding are required to generate a set of RIs, so this process can be quite time-consuming. Moreover, some regions of the genome tend to stay heterozygous longer than expected from theory (Burr and Burr, 1991) and obligate outcrossing species are much more difficult to map with RIs because of the difficulty in selfing plants.

Nevertheless, in cases where it is feasible, seed from RI lines is predominantly homogeneous and abundant, so the seed can be sent to any lab interested in adding markers to an existing linkage map previously constructed with the RI lines. Moreover, RI lines can be grown in replicated trials, several locations, and over several years making them ideal for QTL mapping. Similar types of inbred populations, such as doubled haploids, can also be used for linkage mapping with many of the same advantages of RI lines (Heun *et al.*, 1991), while recurrent intermated populations have been used for genome-wide high resolution mapping (Liu *et al.*, 1998).

Once an appropriate mapping population has been chosen, the appropriate population size must be determined. Since the resolution of a map and the ability to determine marker order is largely dependent on population size, this is a critical decision. Clearly, population size may be technically limited by how many seeds are available or by the number of DNA samples that can reasonably be prepared. Whenever possible the larger the mapping population the better. Populations less than 50 individuals generally provide too little mapping resolution to be useful. Moreover, if the goal is high resolution mapping in specific genomic regions or mapping QTLs of minor effect, much larger populations will be required. For example, Messeguer *et al.* (1991) examined over 1000 F_2 plants to construct a high resolution map around the *Mi* gene of tomato, Stuber et al. (1987) analyzed over 1800 maize F_2's to find QTLs controlling as little as 1% of the variation in yield components, and Alpert and Tanskley analyzed more than 3,400 individuals to obtain a detailed map around a fruit weight locus (Alpert and Tanksley, 1996).

Fortunately, plants can be grown in a variety of environments and in different locations and still provide starting material for DNA isolation. This is in contrast to phenotypic markers, such as morphological or disease resistance traits, whose expression tend to be highly dependent upon growth conditions.

Several methods for DNA extraction have been developed, beginning with those aimed at RFLP technology (Dellaporta *et al*, 1983; Murray and Thompson, 1984; Tai and Tanksley, 1990). More recently, researchers have moved to polymerase chain reaction (PCR)-based markers, which all require smaller amounts of starting material and simpler extraction technologies than RFLP (Berthomieu and Meyer, 1991; Edwards *et al.*, 1996; Lamalay *et al.*, 1990; Lange *et al.*, 1998;

Luo *et al.*, 1992; Thompson and Henry, 1995; Wang *et al.*, 1993). With these methods, the goals are simplicity, speed, and a small amount of starting material. Simplicity and speed are absolutely essential for processing large numbers of individuals - an obvious necessity when large populations of several hundred, or even thousands, of individuals need to be examined. Small amounts of starting material are advantageous if larger quantities are hard to obtain, such as seeds, seedlings, or physically small plants like *Arabidopsis*.

DNA used for genetic mapping does not need to be highly purified (Edwards *et al*, 1996). As long as an extraction provides DNA in sufficient quantity and quality for restriction enzyme digestion or as a template for PCR, the method is probably satisfactory. Further efforts to purify DNA take time and cut down on the number of samples that can be processed. In general, limits to genetic mapping are more often due to small numbers of individuals in a mapping population (or difficulties with associated phenotypic scoring) than to DNA purity.

2.12.2 Relationships among genetic maps

The most common method to relate DNA marker maps to specific chromosomes is the use of aneuploids, such as monosomics (Helentjaris *et al.*, 1986; Rooney *et al.*, 1994), trisomics (Young *et al.*, 1987), and substitution lines (Sharp *et al.*, 1989). In species where aneuploid lines for each chromosome are available, nucleic acid hybridization with a mapped DNA clone indicates its chromosome location by observing the loss of a band (in the case of nullisomics) or a change in the relative signal on an autoradiogram (McCouch *et al.*, 1988). This type of analysis may require "within lane" standards (such as a second DNA clone of previously determined

chromosome location), so that subtle changes in the relative intensity of a band can be compared between lanes.

Using substitution lines to associate mapped DNA markers to specific chromosomes is similar in concept to aneuploid mapping. In cereal species where this approach is most common, lines with known chromosomes or chromosome arms substituted with homoeologous segments from alien species have been developed. Probing a DNA clone onto a blot containing restriction digested DNA from a complete set of substitution lines easily identifies the chromosome location of that clone (Sharp *et al.*, 1989). This is because the substitution line corresponding to the location of a clone shows a different restriction fragment pattern compared to the other substitution lines.

Distances between DNA markers are now described not only by recombination frequency, but also by actual physical distance. This kind of information will be abundantly clear in *Arabidopsis* and rice through complete physical mapping and eventual genome sequencing (Schmidt *et al.*, 1997; Zhang and Wing, 1997). Even in other more complex plant genomes, positional cloning projects based on yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) libraries are beginning to shed light on genetic to physical relationships. Fine structure mapping of the same genome region using both recombination and physical techniques is the best method to compare different types of maps directly. One general observation has been that the relationship between genetic and physical distance varies dramatically according to location on a chromosome (Ganal *et al.*, 1989). In other studies, large genomic contigs have provided estimates for the ratio between kilobase pairs (kbp) and centimorgans (cM). In one study in *Arabidopsis,* this ratio was estimated at 160 kbp/cM averaged over 1,440 kbp genomic segment

near the top of chromosome V (Thorlby *et al.*, 1997). In tomato, a study of a 610 kbp region found that the ratio changed abruptly from 105-140 kbp/cM to less than 24 kbp/cM (Gorman *et al.*, 1996). Indeed, in the *bronze* locus of maize, the level of recombination has been shown to be more than 100 times greater than the genome as a whole (Dooner and Marinez-Ferez, 1997).

2.12.3. Parallel mapping in the same species

In the most important plant species there are often multiple efforts to construct DNA based genome maps. This has led to the unfortunate situation of having several maps for the same species with little or no information correlating one map to another. Of course this makes it difficult to relate the reported location of a gene on one map to its location on another map. It also means that the maps are less saturated, and therefore less powerful, than they could be.

Even where there is no proprietary barrier to relating maps to one another, there are often practical and theoretical problems. The most obvious is that markers polymorphic in one mapping population may not show variation in a second population. The first genetic maps were based on mapping populations optimized for DNA polymorphisms, often including parents from distinct, but cross-compatible species. As researchers move to more narrow crosses, previously excellent genetic markers will be useless for lack of polymorphism. When this happens it will be difficult to relate genetic map location between populations, except by cloning sequences that flank the original marker (a substantial amount of effort) or by testing adjacent DNA markers in hopes that they show more sequence variation.

A similar problem may be observed when one attempts to relate RAPD markers among different crosses. While there are often several bands observed in the analysis of each RAPD primer, only one of the bands may be polymorphic between two individuals (Williams *et al.*, 1990). If an identical RAPD primer is analyzed in a second population, there is no guarantee that the same band (locus) will be the one that segregates. While any bands that do segregate in the second population will be suitable as markers, it is unlikely that they represent the same locus as the original marker. Similar situations can arise with RFLPs if they correspond to a sequence with multiple loci. Finally, there can be theoretical problems in relating linkage order data from one map to another, since each map is based on a different set of segregating individuals. However, the use of appropriate computer algorithms can potentially overcome this problem (Qui *et al.*, 1996; Stam, 1993).

Simple sequence repeat markers have played a critical role in merging disparate linkage maps (Akkaya *et al.*, 1995; Bell and Ecker, 1994). Because they are nearly always single locus markers, even in complex genomes like the grasses and soybean, SSRs define specific locations in a genome unambiguously. This makes them suitable to tie multiple maps together. Moreover, being PCR-based, the information necessary to map SSR loci can be shared among labs simply by sharing primer sequence data.

2.12.4. Parallel mapping in related taxa

One of the most powerful aspects of genetic mapping with DNA markers, particularly RFLPs, is the fact that markers mapped in one genus or species can often be used to construct parallel maps in related, but genetically incompatible, taxa. For this reason, a new mapping project can often

build on previous mapping work in related organisms. Examples include a potato map constructed with tomato markers (Bonierbale *et al.*, 1988; Gebhardt *et al.*, 1991; Tanksley *et al.*, 1992), sorghum maps constructed with maize markers (Hulbert *et al.*, 1990; Pereira *et al.*, 1994), a turnip map constructed with markers from cabbage (McGrath and Quiros, 1991), and a mungbean map constructed with markers from both soybean and common bean (Menancio-Hautea *et al.*, 1993).

Not only does a pre-existing map provide a set of previously tested DNA markers, it also gives an indication of linkage groups and marker order. In the case of tomato and potato, only five paracentric inversions involving complete chromosome arms differentiate the two maps (Bonierbale *et al.*, 1988; Gebhardt *et al.*, 1991; Tanksley *et al.*, 1992). Similar conservation of linkage order was observed between sorghum and maize (Hulbert *et al.*, 1990; Pereira *et al.*, 1994) and indeed, among most of the grasses (Bennetzen and Freeling, 1993) as well as among legumes (Boutin *et al.*, 1995). In cases like these, markers can be added to a new map in an optimum manner, either by focusing on markers evenly distributed throughout the genome, or by targeting specific regions of interest (Concibido *et al.*, 1996). In some cases, though, DNA clones may hybridize in multiple taxa, yet show little conservation in linkage group or order. Even though the tomato and potato maps are nearly homosequential (syntenic) in marker order, both differ significantly from the linkage map of pepper, despite the fact that all were constructed with the same RFLP markers (Prince *et al.*, 1993).

2.12.5 Targeting specific genomic regions

In most cases, genome mapping is directed toward a comprehensive genetic map covering all chromosomes evenly. This is essential for effective marker-assisted breeding, QTL mapping, and chromosome characterization. However, there are special situations in which specific regions of the genome hold special interest. One example is where the primary goal of a research project is map-based cloning. In this case, markers that are very close to a target gene and suitable as starting points for chromosome walking are needed, so the goal is to generate a high density linkage map around that gene as quickly as possible. While the construction of a complete genome map by conventional means eventually leads to a high density map throughout the genome, special strategies for rapidly targeting specific regions have also been developed (Dapprich *et al.*, 2008).

The first strategy for targeting specific regions was based on near isogenic lines (NILs). Over the years, breeders have utilized recurrent backcross selection to introduce traits of interest from wild relatives into cultivated lines. This process led to the development of pairs of NILs; one, the recurrent parent and the other, a new line resembling the recurrent parent throughout most of its genome except for the region surrounding the selected gene(s). This introgressed region, derived from the donor parent and often highly polymorphic at the DNA sequence level, provides a target for rapidly identifying clones located near the gene of interest (Young *et al.*, 1988; Martin *et al.*, 1991; Paran *et al.*, 1991; Muehlbauer *et al.*, 1991). NILs make it easy to determine the location of a marker relative to the target gene. This is in contrast to typical genetic mapping where it would be necessary to test every clone with a complete mapping population to determine whether it mapped near the gene of interest.

Another, more general strategy makes it possible to target specific genomic regions without the need for developing specialized genotypes, generally known as bulked segregant analysis (Michelmore *et al.*, 1991; Giovanonni *et al.*, 1991). The strategy is to select individuals from a segregating population that are homozygous for a trait of interest and pool their DNA. In the pooled DNA sample, the only genomic region that will be homozygous will be the region encompassing the genomic region of interest, which can then be used as a target for screening DNA markers rapidly. This means that any trait that can be scored in an F_2, backcross, or RI population can now be rapidly targeted with DNA markers (Zhang *et al.*, 1994). Used in conjunction with AFLP markers, it is possible to identify large numbers of DNA markers in a region of interest in a short time.

Moreover, pooled DNA samples can also be generated based on homozygosity for a DNA marker (as opposed to a phenotypic trait). In this way, any genomic region of interest that has been previously mapped in terms of DNA markers can be rapidly targeted with new markers. This may be especially useful in trying to fill in gaps on a genetic map. All that is required is a pooled DNA sample selected on the basis of DNA markers flanking the genomic region of interest (Giovanonni *et al.*, 1991).

2.13. Quantitative Trait Loci (QTL)

Quantitative traits are controlled by a number of genes with small effects, are significantly influenced by the environment, and vary in degree rather than kind (Falconer and MacKay, 1981).These traits are difficult to study because the continuous phenotype distribution does not provide any insight into the genotype of the trait. The lack of discrete phenotypic categories also

does not allow the use of phenotypic ratios or inheritance patterns to describe quantitative traits. Until the 1980's, the study of quantitative traits was limited to means, variances, covariances of relatives, and heritabilities (Tanksley, 1993; Falconer and MacKay, 1981). These statistics allowed a number of parameters to be estimated including the approximate number of loci affecting the trait of interest, the average gene action, and the degree to which the various genes interact to determine the phenotype (Tanksley, 1993). However, it is difficult to determine the magnitude of effect, inheritance, or gene action of any specific locus affecting the trait of interest. As a result, the term quantitative trait locus (QTL) was coined to describe a region of a chromosome that has a significant effect on a quantitative trait.

2.13.1 QTL Analysis

The principles of QTL analysis were developed more than 87 years ago when Sax (1923) reported the first linkage of a trait, seed weight in beans (*Phaseolus vulgaris*), to a major gene for seed pigmentation. Wide scale application of QTL analysis was not possible at the time due to the lack of available genetic markers. The identification of QTL followed 50 years later when the first class of molecular markers (RFLPs) successfully produced complete genetic maps in many crop species (Botstein *et al.*, 1980).

The goal of QTL analysis is to estimate the number, location, and effect of QTL controlling a quantitative trait. QTL analysis is based on the principle of detecting an association between phenotype and the genotype of the marker. QTL can only be identified for traits that segregate between the parents used to develop the mapping population. A number of methods have been developed to model the effects of either single QTL or multiple QTL. The three most common

methods of QTL analysis are: single factor analysis, interval mapping, and composite interval mapping (Liu, 1998; Tanksley, 1993).

i) Single-Factor Analysis

Single-factor analysis (also called single-marker or single-point analysis) refers to the detection of QTL by considering one marker at a time. Differences among genotype means are tested for significance at each marker locus using an analysis of variance (ANOVA), t-test, or linear regression (Liu, 1998). Each marker trait association is performed independent of information from all other markers and does not require a complete genetic map. This is the simplest method to identify QTL; however, the analysis is limited by two factors. Separate estimates are not provided in the analysis for the location of the QTL relative to the marker and its effects. Location of the QTL can be inferred from the markers with the greatest differences between genotype means. It is also possible, two or more adjacent markers could detect the same or different QTL. Secondly, as the distance between the QTL and markers increases, the power to detect QTL decreases due to crossing over events between the marker and the QTL. Single factor analysis is a good method to detect QTL rather than estimate its position and effects. Single-factor analysis was used in the first molecular marker/quantitative genetic studies (Tanksley, 1993; Liu, 1998).

ii) Interval Mapping

To overcome the disadvantages of single factor analysis Lander and Botstein (1989) developed interval mapping (also referred to as simple interval mapping). This method requires a complete

genetic map and like single-factor analysis assumes only a single QTL is present. The location of a QTL is determined relative to adjacent pairs of flanking markers instead of using single markers. Using a maximum likelihood approach, interval mapping evaluates the likelihood that a QTL is located at a specific position. The procedure involves calculating a logarithm of odds (LOD) score, which is equal to the logarithm of the likelihood ratio. The likelihood ratio is a function of the likelihood that the data arose from a linked QTL, divided by the likelihood that the data did not arise from a linked QTL. The conventional threshold for declaring the presence of a QTL is a LOD score of 3.0, which corresponds to odds of 1000:1 (Lander and Botstein, 1989). Significance thresholds are more widely determined using permutation tests (Churchill and Doerge, 1994). The LOD threshold will depend on population size, genome size, marker density, population type, and marker used (Hackett and Broadfoot, 2002). The LOD score is then plotted against genome location and is compared to a genome wide threshold. Whenever the LOD score exceeds the threshold, the presence of a QTL is inferred. The point at which the LOD is maximized (the peak) is used as the estimate of the QTL location. A one-or two-LOD interval around the inferred QTL is used as an estimate for QTL location.

Interval mapping can also be performed using a regression approach, known as regression mapping. A series of regression analyses are performed at all positions between a pair of adjacent markers. A QTL is declared at the position where the residual sums of squares are minimized. Regression mapping is computationally simpler than interval mapping by maximum likelihood (Haley and Knott, 1992; Martinez and Curnow, 1992).

An accurate estimate of the QTL location is not always provided with interval mapping, especially when two or more QTL are present in a small chromosome region. Martinez and Curnow (1992) found that interval mapping could lead to the detection of 'ghost' or non-existent QTL between two pairs of flanking markers. They recommended that information from three or more nearby markers are used to map the QTL. By using linked markers in the analysis, interval mapping can compensate for the recombination between the markers and the QTL, increasing the possibility of statistically detecting the QTL and also providing an unbiased estimate of the QTL effect of the character. Interval mapping was first used on an interspecific backcross of tomato (Paterson *et al.*, 1988) and has subsequently been used in several quantitative trait studies. A number of software packages have implemented interval mapping including MAPMAKER/QTL (Lander and Botstein, 1989) and QTL Cartographer (Basten *et al.*, 1994).

When interval mapping and single-factor analysis are compared, interval mapping gives a more precise estimate of the location and effect of a QTL but does not give an increase in the power to detect QTL and requires a great deal more computational effort than single-factor analysis (Lander and Botstein, 1989; Liu, 1998). Interval mapping should be used when the linked markers are relatively far apart (greater than 20cM) since there are likely to be a number of crossovers between the marker and QTL, which can be compensated for with interval mapping. When the marker density is less than 15cM apart, single-factor analysis and interval mapping are identical. However, when the marker loci are very far apart (greater than 35cM), interval mapping is inefficient in detecting QTL in the interval between the loci (Tanksley, 1993; Knott and Haley, 1992; Heun *et al.*, 1991).

iii) Composite Interval Mapping

In the last two decades, efforts have been made to develop methods to model multiple QTL in an attempt to improve the sensitivity of QTL analysis and separate linked QTL. Utz and Melchinger (1994) found estimates of QTL locations and effects can be biased if the effects of other QTL are not taken into account. Jansen and Zeng independently developed a method, which combines interval mapping and linear regression to reduce the multi-dimensional search for identifying multiple QTL to a one dimensional search (Jansen, 1993; Jansen and Stam, 1994; Zeng, 1993, 1994). Jansen (1993) referred to this method as MQM (multiple QTL mapping) while Zeng called this method composite interval mapping (CIM).

The location of a QTL between a pair of markers is estimated by interval mapping, while the effects of QTL located in other intervals of the genome are accounted for by regression analysis. Additional markers are incorporated as cofactors in the regression to control the effects of QTL in other intervals while improving the power of detecting and estimating QTL effects more precisely (Liu, 1998). The selection of cofactors is determined by regression analysis (forwards, backwards, or stepwise) in QTL mapping software such as QTL Cartographer (Basten *et al.*, 1994).

Forward stepwise regression with backward elimination is a common method of stepwise regression used in QTL Cartographer (Basten *et al.*, 1994). This method ranks the markers for their effect on the quantitative trait as well as determines whether adding or deleting a marker makes a significant difference to the fit of the model. The model tests each marker in turn for its effect on the quantitative trait using linear regression but only adds markers to the model while

the p-value of the partial F-statistic is below a defined threshold, *p (Fin)*. When a step is reached where no more markers can be added, all of the markers are retested to determine whether they are still significant. Each marker is in turn deleted from the model, a p-value is calculated for the partial F-statistic, and if the p-value is greater than a specified level *p (Fout)*, it is deleted. Then in and out value of 0.1 is considered to have low stringency in searching for QTLs while the in and out value of 0.01 are for greater stringency. CIM uses the results of the stepwise regression analysis to estimate the location and effect of the chromosome regions associated with the trait(s) of interest. As in interval mapping, a LOD score is calculated at each locus and is plotted as a function of genome position and is compared to a genome wide threshold. When the LOD curve exceeds the threshold, a QTL is said to be present in that area of the genome. Empirical significance thresholds are usually determined with permutation tests (Churchill and Doerge, 1994).

2.14. Linkage and QTL maps in cowpea

In general, there are few reports on quantitative trait loci (QTL) studies in cowpea. Fatokun *et al.* (1992) reported a linkage map for cowpea including the QTLs for seed weight. This linkage map covered 684cM and consisted of 87 random genomic and five cDNA RFLPs, five RAPDs, and two morphological loci clusters arranged in nine linkage groups. Later Menendez *et al.* (1997) reported the second cowpea map which was developed from F_9 recombinant inbred population derived from a cross between two cultivated genotypes, 'IT 84S-2049' and '524B' based on various molecular markers and biochemical traits. This map contained 181 markers, mostly containing Random Amplified Polymorphic DNA (RAPDs), assigned to twelve linkage groups covering a total of 972cM. Ubi *et al.* (2000) also constructed a linkage map of cowpea using

RAPD markers and a recombinant inbred population derived from the inter-subspecific cross between IT84S-2246-4 and TVNu 110-3A. This map spanned 669.8 cM of the genome and comprised 80 mapped loci (77 RAPD and 3 morphological loci) assembled into 12 linkage groups. This linkage map was used to locate quantitative trait loci (QTLs) for days to flowering, days to maturity, pod length, seed/pod weight percent, leaf length, leaf width, primary leaf length, primary leaf width and the derived traits. The other cowpea genetic map which was constructed by Ouedraogo *et al.* (2002a) was an improvement over the Menendez *et al.* (1997) map based on various molecular (like AFLP and RFLP) and biochemical markers. This map contained 423 markers mostly having 242 Amplified Fragment Length Polymorphisms (AFLPs) together with previously identified 181 RAPDs and RFLPs that are arranged in eleven linkage groups that spanned a total genetic distance of 2670cM. Inaddition to the above ones, Muchero *et al.* (2009) constructed a consensus genetic map of cowpea using EST-derived SNPs and 741 recombinant inbred lines from six mapping populations. 928 SNPs were included into this consensus genetic map and the map spanned a total distance of 680cM with 11 linkage groups. In 2005, Ogundiwin *et al.* reported the mapping of QTL for the nine morphological and agronomic traits in a wild cross-incompatible relative of cowpea. In another study by Omo-Ikerodah *et al.* (2008), QTL that mediate resistance to flower bud thrips in cowpea were mapped. Recently, Muchero *et al.* (2009) reported the mapping of twelve QTLs that are associated with seedling drought tolerance and maturity in cowpea.

2.15. Gene flow agents in cowpea

In insect-pollinated plants, pollen movement, rather than movement of seeds, is generally the main component of gene flow Ennos (1994) and Fenster (1991). The carpenter bee *Xylocopa*

flavorufa (DeGeer) is one of the main cowpea pollinators mainly in a place where wild and domesticated cowpea are found. This large, solitary bee has a very fast and powerful flight (Pasquet *et al.*, 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant materials, floral scent collection, morphological trait characterization and DNA isolation

3.1.1 Plant materials

Recombinant inbred populations consisting of 159 individuals in the F_7 were used as a mapping population in this study. This population was developed by single-seed descent from a cross between a breeding line and a wild type, "524B" and "219-01" respectively. Line 524B is a California black-eyed type that shows resistance to Fusarium wilt and is developed from a cross between California cultivars CB5 and CB3. 219-01 is a unique wild perennial plant from coastal Kenya with a rootstock and outbreeding flower morphology having scented flowers.Generation or development of Recombinant Inbred Lines was done as it is indicated on Figure 3.

For pod, seed coat analysis and for scoring of the 100 mean seed weight (SW), seeds of each recombinant inbred lines and the seed of the parental lines were planted in small plastic pots (2" square x 2-1/2" D) in the green house at the International Center of Insect Physiology and Ecology, Nairobi, Kenya, in late May 2007. Three weeks later, the seedlings for each RIL and the parents were transplanted into a plastic bag of 7.5 cm in diameter and 20 cm in depth. Dried pods were collected from each plant and seeds threshed by hand, and used for scoring the 100 mean seed weight (SW) while younger pods were collected for pod and seed coat analysis.

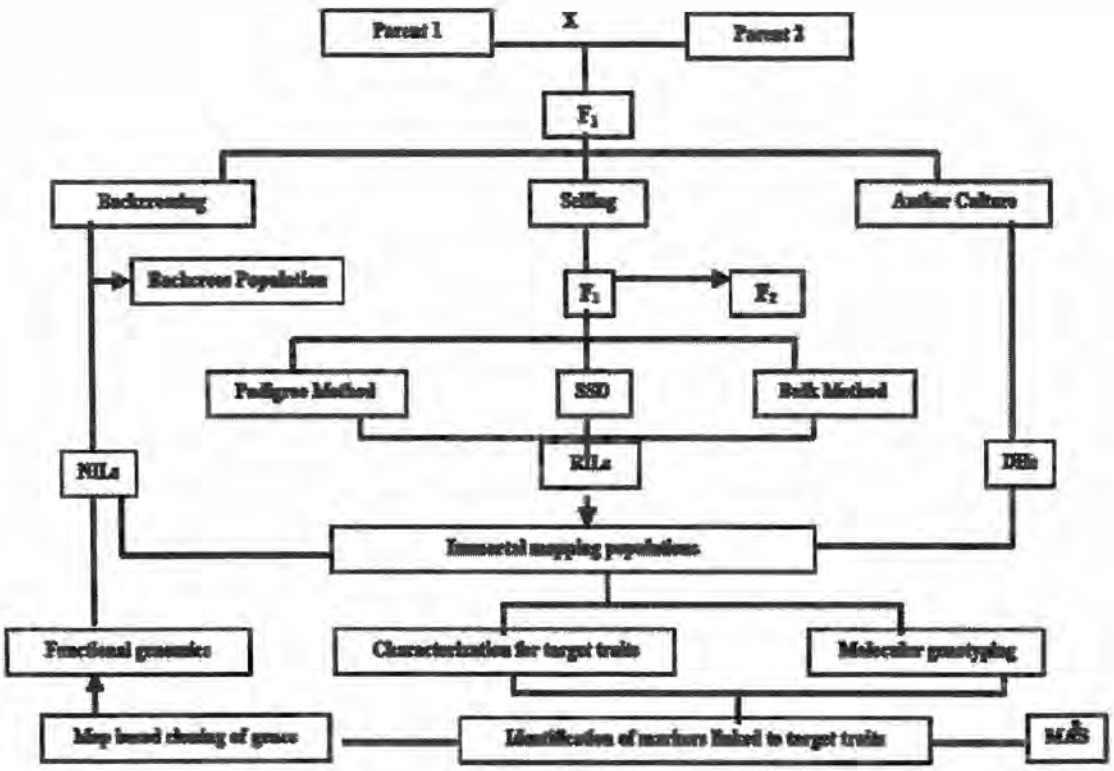


Figure 3 Development of a mapping population

3.1.2 Floral scent compound collection

Floral scent samples were collected from both the parents ('524B' and '219-01') and from all the 159 F_7 recombinant inbred lines. All plants were grown at Muhaka field station (ICIPE) in coastal Kenya. The floral scent was collected by means of head-space adsorption. The inflorescences were covered by polyacetate bags and the scent-containing air in the bags was pumped through, and trapped on cartridges containing adsorbents by a battery operated membrane pump. The cartridges contained 200 mg of a 1:1 by weight mixture of Tenax-TA

(mesh size 20-35; Chrompack, Raritan, New Jersey, USA) and Carbotrap (mesh size 20-40; Supelco, Bellefonte, Pensylvania, USA). Prior to use, the adsorbents were cleaned with 2 ml of methanol, 2 ml of acetone, 2 ml of pentane, and 2 ml of high grade pentane, dried with nitrogen and heated for two minutes at 350^0C with a continuous flow of nitrogen and then cooled with a flow of nitrogen. The cartridges were wrapped in aluminum foil and stored in polyacetate bags prior to use. In parallel with each floral scent samples, a blank sample was collected from the surrounding air. The air-flow through the polyacetate bags was ca.150ml/min. Floral scent was collected for between 3 and 6 hours. The adsorbed scent was extracted with 2ml of high grade hexane into a vial, and 10 μg of methyl stearate was added as an internal standard to all samples before analysis. The eluates were stored at -18^0C. Prior to analysis all scent samples were concentrated down to 100μl at room temperature.

3.1.3 Analysis of morphological traits

The recombinant inbred population comprising of 159 individuals at F_7 generation and two parents were planted in the green houses of the International Center of Insect Physiology and Ecology, Kenya and in the Department of Biology, University of Virginia. All the parental lines and the progenies were scored for the flower color, pod position, pod color and root architecture (Table 1). The data obtained from all the 159 recombinant inbred lines were used for linkage analysis.

Table 1. Morphological traits that were scored in the parental lines and F_7 recombinant inbred lines

Trait	524B	219-01
Flower color	Tinged/White	Purple
Pod color	Green tips	Purple tips
Pod position	Erect	Drooping
Root architecture	Fibrous	Tap-rooted

Flower color was scored as white or purple while pod color was scored based on the pigmentation of the tip part of the pod as green or purple, pod position was scored according to the alignment/orientation of the pod and root architecture was also scored; to score root architecture a set-up was done using a bigger sized Petri dish (150 × 15 mm). All the cowpea seeds were germinated in Petri dishes lined with moist Whatman # 1 filter paper. Once germinated they were transferred into larger Petri dishes lined with a layer of rock wool covered by a nylon mesh. These setups were watered as and when necessary. After eight weeks of the transfer, the root architecture was scored for all the F_7 population. All the morphological trait scoring was done for all the 159 recombinant inbred lines together with the two parents.

3.1.4 Analysis of quantitative traits

All the 159 F_7 individuals were used for phenotyping of yield related trait (seed weight), and domestication related traits (testa size and pod size). Seed weight was recorded by counting and weighing of 100 seeds on a laboratory balance whereas testa and pod size measurement was

carried out by cutting the cross section of a young pod and measuring the size of the cross-section using a microscope with an ocular micrometer after it was stained using malachite blue.

3.1.5 DNA isolation

Total genomic DNA was extracted from freshly harvested leaves of the 159 F_7 individuals and the two parental lines according to a modified CTAB procedure (Mignouna *et al.*, 1998). About 1g of cowpea leaf tissue was frozen in liquid nitrogen and then crushed in 1.5 ml microfuge tubes using a mini-pestle. 400µl of CTAB extraction buffer [2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCL (pH 8.0) and 1% 2-mercaptoethanol] was added to the ground tissue. The mixture was incubated at 65^0C for 20 minutes followed by the addition of 400µl of chloroform: isoamyl alcohol (24:1). The solution was then mixed on a shaker for 15 minutes, centrifuged at 13,000 revolutions per minute (RPM) for 5 minutes and transferred to a new tube containing 400µl of phenol: chloroform (1:1), and mixed gently for 15 minutes followed by a centrifugation at 13,000 RPM for 5 minutes. The supernatant was then transferred to a new tube containing 400µl isopropanol, mixed gently and left at room temperature for 5 minutes. The DNA was pelleted by centrifugation for 3 minutes at 13,000 RPM and then washed 3 times with 70% ethanol and air dried for 10 minutes by inverting the tube. A hundred microliters of 0.1 TE buffer (10mM Tris-HCL pH 8.0, 0.1mM EDTA) was used to re-suspend the pellet followed by the addition of 4ul of RNAse A (10mg/ml) and then incubated for 15 minutes. The pellet was precipitated with 1ml of absolute ethanol for 15 minutes and then centrifuged at 13,000 RPM for 5 minutes to pellet the DNA. The DNA pellet was dried for 2 hours, and then suspended in 50ul sterile distilled water. DNA quality was assessed visually after electrophoresis in 1% (w/v) agarose gels and the concentration was determined from the UV absorbance at 260 nm using a

Beckman-spectrophotometer model DU 640B (USA). The DNA samples were then stored at -20^{0}C until PCR amplification was carried out.

3.2 Primer design and microsatellite analysis

Genespace sequence reads (Timko *et al.*, 2008) obtained from the CGKB database (http://cowpeagenomics.med.virginia.edu/CGKB/) were scanned with SSRIT –Simple sequence repeat identification tool (http://www.gramene.org/) and duplicated sequences were removed using BLAST software (http://www.ncbi.nim.nih.gov/). The primers flanking simple sequence repeats were designed using OLIGOTECH software (version 1.00). The main criteria for designing primers were 20-25 nucleotides long and annealing temperature greater than 55^{0}C.

3.3 Marker polymorphism and analysis

Cowpea derived microsatellite primers were screened for polymorphism between the parental lines and subsequently identified polymorphic primer pairs were used to genotype all the 159 RILs. PCR reactions were conducted with 20 ng of template DNA, 2 µM of each primer, 10 x of PCR buffer [100 mM Tris-HCl (pH 8.5), 500 mM KCl, 15 Mm Mg Cl_2], 3mM each of dNTP, 0.5 units of Taq DNA polymerase (Genescript, USA) in total volume of 25 µL. PCR was performed in eppendorf mastercycler gradient, using the following profile: an initial denaturation at 95^{0}C for 2 min, followed by 38 cycles of 25 sec denaturation at 94^{0}C, 25 sec of annealing at annealing temperature specified for each primer pair (i.e. 55^{0}C), and 45 sec extension at 70^{0}C. The final cycle was followed by a 10-min extension at 72^{0}C. The PCR product was held at 4^{0}C

before analysis. PCR products were resolved on 6% non-denaturing polyacrylamide gels (PAGE) and electrophoresed in 1 x TBE at 290V for 3 hours and also on 2.5% ultra-pure agarose gels.

3.4 Data analysis

3.4.1 Linkage analysis

Genotyped marker data were obtained from visual scoring of the banding patterns and genotyping for identified polymorphic markers was carried out on 159 F_7 RILs. Marker segregation was subjected to the chi-square test to examine distortion from the expected 1:1 segregation. JoinMap 4.0 software was used for linkage analysis and map construction (Van Ooijen, 2006). This is a general-purpose program for linkage analysis and genetic mapping in both inbred and cross-bred populations. The map was constructed using a LOD value of 3.0 and a recombination frequency of 0.30 for all linkage groups. Linkage distance (centimorgans, cM) values were calculated using the Kosambi mapping function (Kosambi, 1944). Designation of the individual SSR markers was based on the standard naming (e.g., SSR-6222) and the primer sequences are listed on the cowpea genomics knowledge base (CGKB) website of the University of Virginia also a table of primer sequences and marker sizes is also given in Table 2.

3.4.2 Analysis of segregation distortion of markers

Marker segregation was subjected to the chi-square test to examine distortion from the expected 1:1 segregation.

$$\chi_c^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

Where, O_i = observed values for each group, and

E_i = expected values for each group

3.4.3 Quantitative trait locus (QTL) analysis

Genotypic and phenotypic data obtained for testa (seed coat) size, pod size and seed weight as well as the genotypic data of 159 F_7 RILs of the mapping population together with phenotypic data obtained by GS-MS for volatile compounds putatively involved in cowpea flower scent were analyzed for mapping QTLs by using the method of composite interval mapping (CIM, Zeng, 1994) in the Qgene version 4.3 (Nelson, 2005). The LOD curves were created by scanning at 2 cM intervals, while a permutation test (1000 resamplings) was performed to determine the critical LOD score appropriate to empirically identify a putative QTL with a genome-wide error at a 0.05 confidence level (Churchill and Doerge, 1994). A minimum LOD score of 2.0 was used for the identification of putative QTLs, and the percentages of total phenotypic variation and additive effects explained by each QTL for the traits of interest were also calculated.

3.4.4 Floral scent compound analysis

The floral scent samples were analyzed by gas chromatography (GC) and mass spectrometry (MS) on an HP5890 connected to an HP5972 mass selective detector (Agilent Technologies, Palo Alto, California, USA). The injector temperature was 220 ^{0}C. A 25 m long fused silica GC column with an inner diameter of 0.25 mm and which is coated with OV-351 at a film density of 0.25 µm was used as a stationary phase (Supelco). The GC was programmed for 5 min at 50 ^{0}C, increased by 8^0/min to that of 230 ^{0}C and held steady for 10 minutes. Helium was used as the

carrier gas. General identification of scent compounds were made by comparing mass spectra and retention times obtained with those of authentic reference compounds and tentative identifications by comparison with spectra in computer libraries.

3.4.5 Quantification of floral scent compounds

To produce calibration (dose-response) curves dilution series for each constituting compound were prepared in redistilled hexane (100 microgram/ml (10^{-4}), 10 microgram/ml (10^{-5}), 1 microgram/ml (10^{-6}), 0.1 microgram/ml (10^{-7}) and to each dilution 500 ng of methyl stearate was added. The calibration samples were analyzed by GC-MS along with the floral scent samples. Ideally a unique ion to quantify against should be selected for each compound. This was possible except for 1-octen-3-ol, where instead the largest ion, i. e. 57, was chosen and the amount reported of this compound may therefore be slightly overestimated because ion 57 often is present in small amounts in background contaminations. All calibration curves and later on quantification of floral scent samples were performed manually. Graphs of the $\log_{10}$ (response factor (calibration ion/internal standard ion)) as a function of $\log_{10}$ (amount of calibration compound) was made for each reference compound. The equation for the best fitted line and its intercept with the y-axis for each reference compound was used to quantify most compounds in the samples. All calibration curves showed a linear relationship with correlation coefficient (r^2) between 0.98 and 0.999. (*Z*)- and (*E*)-Cinnamic aldehyde and methyl (*Z*)- and (*E*)-cinnamate were quantified using their respective (*E*)-isomer, only.

Table 2: Code, primer sequence and predicted fragment length of SSR markers

Code	Left primer	Right primer	Allele size 524B (bp)	Allele size 219-01 (bp)
SSR-6171	agatcccacgctgattatgg	acttgacgcagagccatctt	201	220
SSR-6188	Accaggtgcaatgcttctct	ccacaccctgttccgtactc	198	170
SSR-6924-1	Gatcacctcccacacctcag	tagcagtttcccaccagctt	220	201
SSR-6924-2	Gatcacctcccacacctcag	tagcagtttcccaccagctt	220	201
SSR-6923	Aaccgaatcaacccactcag	agagcgagcaggttcagaaa	230	201
SSR-6922	Gaaggccacacaagagacca	tttgatgttttcatgttcttctgtt	220	240
SSR-6192	Aacgggtcctaaacgaatga	atccttgaactccgtgttgc	170	200
SSR-6204	cgatcacggtttacgactga	gggagtgaagaagcaatgga	220	210
SSR-6210	Agcgtagtgacttttttccagatt	tgtctttctgcactcaaagga	201	154
SSR-6211	Tgtcctcaatttcaataacaagttt	aacagttggtcggatacgaaa	290	230
SSR-6218	Gtggaaggaatgggtccag	aggaaatttgcattcccttgt	298	220
SSR-6222	Agtacgcacggcaaccttag	gtgcaaccctaacgctcttc	180	160
SSR-6225	Ctcaagcttggttgagatgaaa	atatcgggcgcacttttgta	154	134
SSR-6228	Cacgttttcctttcctcacc	tacaatgaaatgggctgcac	198	160
SSR-6240	Ttcaatgtgggaggatgaga	ggttccggattcaatttcc	154	134
SSR-6242	Tgttgactggcagaggttga	ttccacgaatcatcgacaga	190	154
SSR-6243	Gtagggagttggccacgata	caaccgatgtaaaaagtggaca	201	154
SSR-6245	Cgaacatgtttttggtcacg	ctacaaccgcgttagccttc	154	180
SSR-6268	Gcaaagggatcaccaaacat	tcgttcagttgagccac	201	170
SSR-6273-1	Cccccagaacaaatagaaactc	tgaatttgaagaagagatggttg	154	220
SSR-6273-2	Cccccagaacaaatagaaactc	tgaatttgaagaagagatggttg	300	344
SSR-6277	Caccccccgtacacacacac	cacttaaattttcaccaggcatt	134	154
SSR-6280	Gttatcagatctggtcagatgc	gaagaaaccacccgaccat	105	134
SSR-6294	Tggtgcttgtaagaaaaacagaa	ggagagcagaagatgaagtgaa	250	220
SSR-6302	Tggaggcataaaaatgacacct	aagctgattgtggaaccattg	197	190
SSR-6312	Actacaccgatgaaagcaact	ttccaaaatagttcacaactta	220	134
SSR-6313	Acggattcagaattgccatc	gcagatgagttatcttgcagtgtt	180	134
SSR-6314	Tggaggcataaaaatgacacct	tgaagctgattgtggaaccat	198	160
SSR-6323	Caaagggtcatcaggattgg	tttaagcagccaagcagttgt	220	298
SSR-6324	Cagcaggggttgtttcagat	caggttgatgaggaaccaaga	160	201
SSR-6327	Acgaaacgatgttaatgctgatt	aaaaagatttgatgtgatctatgatgtt	260	284
SSR-6331	Tggtgctcaacttcctcactt	ggcactcctccaggtgacta	154	100
SSR-6333	Ctccccctttttcatattcagg	agttctcgaggcggtgaata	210	120
SSR-6921	Tcctcctgattggacctcac	tcctcatcacaatgttcatcatc	201	154
SSR-6345	Aaacatcaaaattaaggataatcaatg	agtagcgtgggtggaatttg	134	100
SSR-6348	Cctcttgctttgcctttgtc	ccccttttatgacatgaagc	154	130
SSR-6353	Tcatgggttaaatttgcttcaa	aaaccatgtggttgttgcac	134	160
SSR-6354	Cgaaaattcacagagatgcag	cagtctaacgaagaactgggcta	290	300
SSR-6360	Ttttcaatcctcccttgtc	tgtagttaaaatcagagacttacagg	154	148
SSR-6362	Taggagcaatggacggagag	gggtccaacgtccacttaaa	125	101
SS4-6920	Tgctttggcaataaaaagtaaa	ataccgaaccgacaatgagc	120	100
SSR-6367	Atcgcggagttacaaggtgt	ttccatgttggtgatgccta	170	150
SSR-6369	Cctcaacacctttggagga	caaatgcacctcctgtgcta	300	270
SSR-6372	Aacaggtgtcagtcatccgtta	attggcatgtcaaacattcg	154	201
SSR-6375-1	Gctcggatatggtcctgaaa	tcagtgtcagcaccatacc	298	317
SSR-6375-2	Gctcggatatggtcctgaaa	tcagtgtcagcaccatacc	201	220
SSR-6376	Ggacacggacacaaatacga	tgatcactacttttcacttttt	200	134
SSR-6395	Caattaatgatcggacaagagtg	gcatgaacactactgtgagcaa	260	298
SSR-6402	Atctccaccacccctttttct	tttaataaaaagttattccaactctc	170	200
SSR-6429	Tttggttcaaaactattgtgatttt	aagacccttgagccacttca	201	220
SSR-6451	Aaagagatacacatgcctaaca	gaccaacagcgactttgagc	154	134
SSR-6465	Ggattttacttcggatgtctttttc	tcatgtgaaagttttaagtgat	220	201
SSR-6466	Cagcttctctgctagcaacaataa	gcaaatttcactttcaacatttca	280	250
SSR-6469	Cataatgtcacagaggtggaaaa	tctttccttcctttttcaccaa	298	270

Code	Left primer	Right primer	Allele size 524B (bp)	Allele size 219-01 (bp)
SSR-6475	Ccagtgctgcaagataaaagg	agcctttggaggttttggt	201	220
SSR-6477	Ccaacaccatgcagatacga	attttctccggaggcattg	201	190
SSR-6513	Catggaaaagatgctgtgga	aacgcaaccaaagccttta	220	201
SSR-6515	Gctccttcctacacacacattc	aaactgtggttcctggtgga	230	270
SSR-6516	Tgtttagatatgaaacacaattt	cctcggattgtttccttctg	270	225
SSR-6519	Caagggtgttgtttcagtgc	tcagatgtcaatttcactgttgac	201	230
SSR-6520	Ccctcttggatctgcaaataa	ttcagtttcatgataggtctcac	130	100
SSR-6891	Ttgttggtcatgttgggatg	aatagattgttagggaaac	270	201
SSR-6895	Gttggcttctgttgtggcat	gttacaccaatgccaaaaac	201	220
SSR-6906	Ggacatttaggattgggtgg	caagaatgtctgaaactaatatgc	220	290
SSR-6909	Gtacctaacaagtatgatgaa	aaacttctccattattgagt	150	175
SSR-6537	Ttgagaggaaggaaagcatg	ggtttttgtttactgtgcta	290	250
SSR-7078	Gatgtgttacagtttttcac	cagatgaactccctgcagct	230	201
SSR-7079	Gcacgggcatgtactgaaaa	gttttggtgatctggacat	210	230
SSR-6547	Aaactgacacttgaacacga	ctcatgcagagttcaagatc	344	298
SSR-6577	Gaacttgataggatcctaga	ttctggtatgcactgaggga	295	280
SSR-6592	Caggcatgcattcatctttccc	gaaattaattaggaaaaataacaagccac	344	275
SSR-6594	Ccccagaaaaaccaggtcc	ctactaccaccaccgtacgtg	405	399
SSR-6597	Gtgtcttctctcatcataacg	ggttaagcttgattaggaatg	345	298
SSR-6603	Gagaacttcacgcacaatag	cgcggtagcatgattgaatttttg	330	301
SSR-6604	Ggaccatcttacataactcaatg	ccacattccaccactctcc	298	315
SSR-6607	Gagagtatcaaatgctgtggc	caatgaactcagacatctcac	320	300
SSR-6609	Gggttcaagtggggaag	cttacattcctccctctccc	150	110
SSR-6611	Gccacctagtccaccaac	aaaattagggtgtagttgtc	220	344
SSR-6612	Gaagtagtttagaagggagaggg	gaatatgaaggaaattgagtttgag	340	298
SSR-6618	Cctctaagttactctctgatgc	ctaaagtctaaactgaatcaccc	298	344
SSR-6623	Gcacttcaaaggagaccacac	ccatattccaaatcatcttaacc	201	220
SSR-6624	Catatcaatcacttctgtgcc	cgaaatccaggcttatccac	344	357
SSR-6626	Gtgtgggatcttattgcttgttc	caaaagcacaaagcaaaaaatcc	344	298
SSR-6639	Ctgtattattttccagtttctccc	gacaagacaggggaacgaac	315	300
SSR-6641	Cataagcaaaaaactctaaactctg	ggcactccaatcagagctg	298	315
SSR-6645	Gggaatcgatgacgccg	cgagatggcgaggtgg	396	305
SSR-6657	Cgccaaaaccccgataacc	gccccctccgactg	200	220
SSR-6662	Gataactaagttgaggtttgg	gtttggatggtaagtgcttaac	344	298
SSR-6663-1	Ccgaattccttcctccaac	gagggaagagaagaagg	300	285
SSR-6663-2	Ccgaattccttcctccaac	gagggaagagaagaagg	280	250
SSR-6666	Cactgtgatgctttctgtcaattg	ccccagtatgcatccaac	295	302
SSR-6673	Gaaaagtgaaccgcagaataacc	cagacattgaagtgagcc	100	134
SSR-6680	Gacagacagacaccaggaaag	gtccatgtggtcttgagcttgtc	344	396
SSR-6682	Cacccgatcatgttttccaaag	gatgtgtggagatgattgaaatg	230	220
SSR-6683	Gcagtgagcacatcgttgac	caggaaaagttggctctcagg	298	344
SSR-6686	Gtgtccttccatttttgatgtg	gacaagaaaagggttccataactg	396	430
SSR-6694-1	Ctagagatgccttaactcgg	cgaaacttagcgtacagaggttc	201	220
SSR-6694-2	Ctagagatgccttaactcgg	cgaaacttagcgtacagaggttc	240	259
SSR-6697	Cactccacttgcaccattgttg	gtgattccattcaagtgtat	220	201
SSR-6698	Ggaattctctacggactagtcatac	ctagcaattgtaccagccgaag	240	260
SSR-6699	Gatatctctctatgcagcaag	ccttggaggggacctaactg	260	298
SSR-6701	Gccctcgccaatgattctgag	gcctttatagaacccagcatacc	298	260
SSR-6705	Gctcacctacgtgtgttcgatc	gcaagtggatgtggtgatctc	230	220
SSR-6914	Gagccggaatacaggatcatg	gatgcaggctataaccgcgg	298	240
SSR-6915	Gcaccatatccctccagctt	gctcatgattcaagtcaaggacc	298	260
SSR-6916-1	Gcccctaaaacctgcaacaac	gttagctagccacgtatgaag	220	201
SSR-6916-2	Gcccctaaaacctgcaacaac	gttagctagccacgtatgaag	270	230
SSR-6917	Ggtcgttttcctctttgtcag	cgtggaagaaggaaaacaaggag	154	190
SSR-6717	Cctcactctgaattgcatac	ctgaatcacccaatttgcttcc	298	270
SSR-6719	Ggatcttgaatctgggatgcc	caaatcaccactaccccacac	240	225

Code	Left primer	Right primer	Allele size 524B (bp)	Allele size 219-01 (bp)
SSR-6720	Tggtgggtggattgctgaca	ggcattccactaagaacatc	201	134
SSR-6918	Gaggtgcatcaatgtatcgc	cctgcaactctaccaatcct	210	134
SSR-6919	Agcaaaagcctccatcactt	agaaaacaggagggagatga	280	290
SSR-6724	caagacccgtacaagaatttg	tttccacggtccactcttcc	201	220
SSR-6726	Tcgcttccagcactcgtatg	gctagcatgagtaggaccac	154	134
SSR-6730	Tgcttcctcgtctgccctcc	gagggcaaagcaaggcgaaa	270	260
SSR-6733-1	Catgtccaagatgtatgtagg	cctgggattgcgggattgtt	220	201
SSR-6733-2	Catgtccaagatgtatgtagg	cctgggattgcgggattgtt	298	317
SSR-6743	Tctctccctctttcatcccc	cttcccgaaacttccttagg	230	210
SSR-6744-1	gacagacagacaccaggaaag	tgagcttgtcgagaccacag	210	220
SSR-6744-2	gacagacagacaccaggaaag	tgagcttgtcgagaccacag	300	310
SSR-6927	cgagaaagctgcggcccttac	cgaagtcgaggattttgatgg	220	201
SSR-6934	Gatgtggtgaattgcatgcaa	gatctcaagatggggtgggaa	320	290
SSR-6935	gtcccagcactccaaccgata	aggagctttgtgagtgtggta	344	298
SSR-6939	Cgcacgttatacactctttct	ccaatgtcaagagcctgcaag	300	344
SSR-6941	ctcttgaccagaaacaggaag	gagcataaggacatgaacaca	170	190
SSR-6944	Cacctgcttctgtactgttaa	ttgtaagttagccgggcagta	290	154
SSR-6947	cagaaatggaatgcaagcagc	agagcataaggacatgaacac	396	380
SSR-6950	Tgacctttgaagatcgagaca	ctatgatcctaccgctgagta	280	344
SSR-6962	Ctcgagctctttcaatgagtt	ctcaatctattggttgtcagg	300	280
SSR-6964	gaagagcggtaaaaccaacaa	atgtcaagagcctgcaaggac	269	298
SSR-6965-1	Gcattcagctacgatgtgttc	ggcactttgtaaaagacaggc	298	280
SSR-6965-2	Gcattcagctacgatgtgttc	ggcactttgtaaaagacaggc	340	300
SSR-6971	Gcgagctctcatcatctatgc	gataatcgcacattgtccatg	344	396
SSR-6973	gttgagaccaaacattgttcgc	gcgtaagcaagttaatctctag	396	344
SSR-6979-1	gaaacgaacctgaaaatagtcggc	gcattcttgatgtgtctcttacct	344	240
SSR-6979-2	gaaacgaacctgaaaatagtcggc	gcattcttgatgtgtctcttacct	506	396
SSR-6979-3	gaaacgaacctgaaaatagtcggc	gcattcttgatgtgtctcttacct	510	400
SSR-6982-1	gcatgagtgtgtaacctgtgac	gacggcacattgaaaccacttg	290	310
SSR-6982-2	gcatgagtgtgtaacctgtgac	gacggcacattgaaaccacttg	340	360
SSR-6982-3	gcatgagtgtgtaacctgtgac	gacggcacattgaaaccacttg	506	526
SSR-6983	gatgatctgcgatgtttgaggc	gatttgctcggtcttttccagg	298	270
SSR-6990	Ggatctgggtatgttttgagc	gacacacgaaggttctggaca	396	344
SSR-6994	Gagtttattcactgcagcatc	gtagggctccaactgatatcc	290	344
SSR-6996	gatatctctctatgcagcaagttc	gtctggcaaaacaacaggtaagac	396	410
SSR-6998	Gaatctctgtggttgctcttc	gtatgcctttatagaacccag	500	402
SSR-6999	gtgacgatgagacaaatgaatc	gttctttcagatgacatgcgct	396	340
SSR-7000-1	gaagcttaatccacagaatctacgc	ggaaactgtttgcacttttatccca	220	201
SSR-7000-2	gaagcttaatccacagaatctacgc	ggaaactgtttgcacttttatccca	240	221
SSR-7000-3	gaagcttaatccacagaatctacgc	ggaaactgtttgcacttttatccca	344	298
SSR-7001	gaatcacataagaggagcacaa	gatgaaaccgacatgaagaagc	298	344
SSR-7004	ggctctgagaaatttatgatcc	gcctgtttttttgttgctattgc	396	506
SSR-7005	gtttgatcctacctggtgccat	gctcatgattcaagtcaaggac	260	298
SSR-7008-1	gaagataccaagatgcccctaaaac	gtatatgttagctagccacgtatga	220	201
SSR-7008-2	gaagataccaagatgcccctaaaac	gtatatgttagctagccacgtatga	300	280
SSR-7009-1	Gccatggttgaaatttgcatc	gacaggccatgaaagcaatac	298	320
SSR-7009-2	Gccatggttgaaatttgcatc	gacaggccatgaaagcaatac	298	320
SSR-7011	Gttgtcacgtgattaattccac	ggtctcccagtaatgcttgtac	240	260
SSR-7013-1	Gaatctgggctcttatgtgttc	gactacacgccatgcatagtac	407	396
SSR-7013-2	Gaatctgggctcttatgtgttc	gactacacgccatgcatagtac	517	506
SSR-7014	gcagcaaagagcttgaatctc	gcgtctgtaaaggaaacaaac	296	270
SSR-7015	gtgtcaaattaatggagcagc	gggtggcttactgaaagttcc	220	201
SSR-7017	Ggcaggtgattttacatctca	gtgttagaagaaactagcacc	330	380
SSR-7025	gtttctctagaggtgcatcaat	gtacctgcaaccacaagtaatg	380	344
SSR-7027-1	Catacgcttccccaattcac	cctctcttccctcaccacaa	270	250
SSR-7027-2	Catacgcttccccaattcac	cctctcttccctcaccacaa	280	260

Code	Left primer	Right primer	Allele size 524B (bp)	Allele size 219-01 (bp)
SSR-7027-3	Catacgcttccccaattcac	cctctcttccctcaccacaa	298	278
SSR-7027-4	Catacgcttccccaattcac	cctctcttccctcaccacaa	330	310
SSR-7028	Tggatgggccttttaatgtg	gggcctccttgagaagcata	298	270
SSR-7040-1	Ccgattgtgcctttatgtcc	cggcttcttccactgatgat	250	240
SSR-7040-2	Ccgattgtgcctttatgtcc	cggcttcttccactgatgat	280	270
SSR-7040-3	Ccgattgtgcctttatgtcc	cggcttcttccactgatgat	344	334
SSR-7040-4	Ccgattgtgcctttatgtcc	cggcttcttccactgatgat	396	386
SSR-7041	Agacgatgcatagccgtagc	cgttttccattgctcattcc	240	260
SSR-7043	Caccactctcctgcactcaa	ggttgggcaaagaggaactc	240	260
SSR-7045-1	Cttggggtgatgatgaaacc	aggggtgaaaagttgtcttgc	290	230
SSR-7045-2	Cttggggtgatgatgaaacc	aggggtgaaaagttgtcttgc	320	260
SSR-7052	Tcagttggagctttgtggcta	cgcgtgcaacaaccaataat	298	270
SSR-7053-1	Tggcaagatctgattggtga	gcgggattctattccagtga	320	270
SSR-7053-2	Tggcaagatctgattggtga	gcgggattctattccagtga	350	298
SSR-7056	Cgctcctcttccttttgttct	agaaaagggcatgttcgttg	250	270
SSR-7060	Aaaaggatttttggggtgga	gagagtggaagccgttgaaa	240	220
SSR-7061	Tgcgcttgaacttctccttt	caccctccattctcaaacca	260	240
SSR-7063	Acctcactgaatctgggctct	aaatgaatggatgcctgagc	290	270
SSR-7067	Attcgccccttttctcactt	gcagaggtggtagcagaagg	280	260
SSR-7068	ctaggaaacaacgggagcaa	gcaactgcattctgcaaaca	298	270
SSR-7069	Cattggaaaaacacgcactg	tggtgacgagagtgcttcag	250	240
SSR-7072	Gggttgtccctggtaaggtt	agtttgtcggtccattctgc	298	320
SS4-7082	Atgttccaaatccacaactac	cgtgttcttcagtcattcatt	201	134
SSR-7101-1	Tctccacctaccagtgtgtc	gaggaatcagtctcaccatt	154	134
SSR-7101-2	Tctccacctaccagtgtgtc	gaggaatcagtctcaccatt	220	201
SSR-7117	Ttcttcgtaacactctcactca	cttctcactctcctcctcttc	380	360
SSR-6856	gcatacatgtgcttaagtgt	cttcgtgggtggttacattc	201	220
SSR-6807	gaactattatacaatcatgcacga	gtagcttacttcaatgattag	240	280
SSR-6859	acaaatgctgttacagagggc	gtagctgcagatgacttcaat	500	480
SSR-6790	acgacgttgtaaaaccttaccttcacctatagac	cattaagttcccattacactggggtcgcctaaggaag	201	220
SSR-6810	gaccatccaagactaagacactga	aaagattatgctctattcacaga	320	360
SSR-6838	agaagatccacgttttgcattga	catagttgatagtgatttgactaa	480	450
SSR-6788	acgacgttgtaaaagtgccattaatttaagtagac	cattaagttcccattacagatgctgagcgatacaag	298	220

CHAPTER FOUR

RESULTS

4.1 Phenotypic observations

4.1.1 Flower color

In the F_7 population 44.03 % of the plants had white flower color and 55.97 % of the remaining plants had purple flowers, following the expected segregation pattern of 1:1 at P =0.05 (Fig.4).

Figure 4 A representative picture of flower color in the F_7 RIL population.

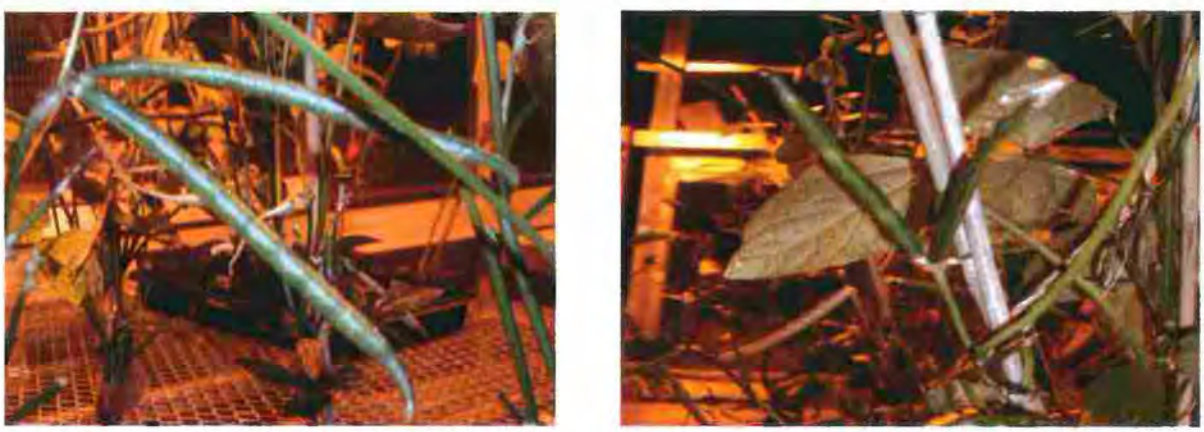
4.1.2 Pod color/pigmentation

Pod color was also segregated according to the expected 1:1 ratio at P = 0.05 for the RILs (i.e. 45.91 % of the plants had green tips while 54.09 % had purple tips) (Fig.5).

Figure 5 A representative picture of pod color in the F_7 RIL population.

4.1.3 Pod position

In the F_7 recombinant inbred lines 54.09 % of the plants had an up-right (erect) pod position/ alignment and 45.91 % of the remaining plants had a drooping pod position, and they follow the expected segregation pattern of 1:1 at P = 0.05 (Fig.6).



(a) (b)

Figure 6 A representative picture of pod orientation (a) drooping and (b) upright pod position.

4.1.4 Root architecture

In the F_7 recombinant inbred lines 50.31 % of the plants had a fibrous root system and 49.69 % of the remaining plants had a tap-rooted system, which also follows the expected 1:1 segregation pattern at P = 0.05 (Fig.7).

(a)

(b)

(c)

Figure 7 A representative picture of scoring for root architecture; (a) Set-up of plants for root architecture scoring; (b) fibrous root system; (c) tap-root system.

4.2 SSR markers

4.2.1 Polymorphism

The parents 524B and 219-01, along with 159 F_7 plants were screened with 912 SSR primer pairs. These primer pairs are available on the cowpea genomics knowledge base (CGKB) website of the Department of Biology, University of Virginia. A total of 639 primer pairs produced amplification products and from these a total of 202 loci showed polymorphism between the parents, were also segregated among the F_7 recombinant inbred lines and generally the level of polymorphism that is shown by this primers is about 31.6% (Example: Figure 8). The size of the amplified fragments was between 100 and 500bp. The actual size of the fragments that are obtained by the respective primer for each parent is shown in Table 2.

Figure 8 Part of the polyacrylamide gel (PAGE) obtained with SSR-6243. Electrophoretic patterns of ethidium bromide-stained amplified DNA from the two parental lines (P1 and P2 i.e. 524B and 219-01 respectively) and the individual recombinant inbreds (lanes 1-30).The size marker (M) is a 1Kb DNA ladder (Invitrogen). The arrows show the polymorphisms.

4.2.2 Segregation distortion

Genotyping data obtained for all the 206 loci were checked for segregation ratio using a chi-square test. A total of 184 loci showed the expected 1:1 segregation ratio ($P < 0.05$) with 1 df and were initially used to establish the LGs. Twenty-two markers (10.67 %) showed distorted segregation ($P \leq 0.05$, chi-square test). Results of linkage analysis revealed that markers with distorted segregation were distributed throughout the genome. Deviation from the expected segregation ratios was observed for markers on six LGs. The number of markers showing segregation distortion varied from 1 to 11 per LG.

4.3 Linkage map

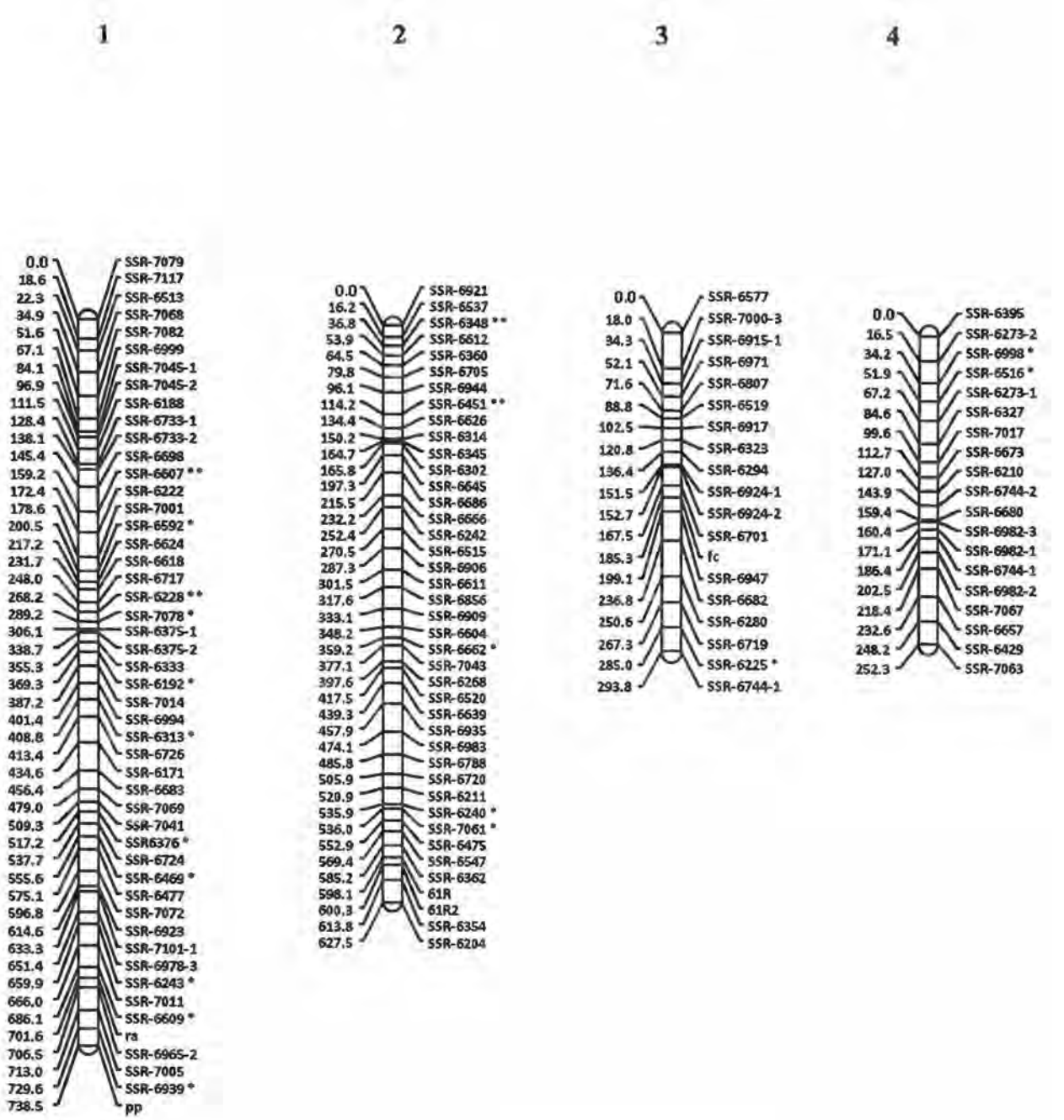
4.3.1 A microsatellite based linkage map

The 202 markers which showed polymorphism were selected and assigned to 11 linkage groups (LG1 – LG11). The number of markers ranged from 5-49 per linkage group, and a linkage group length varied from 57 cM (LG11) to 738 cM (LG1) (Table 3). The linkage map of the F_7 population spans a total genetic distance of 2991 cM, with an average distance of 14.5 cM between markers with no markers remaining unlinked. Markers were randomly distributed on the 11 linkage groups. While three LGs (LG 11, LG 9 and LG 6) had 5, 6 and 7 marker loci respectively, the other LGs contained 11 (LG7), 13 (LG8), 16 (LG10), 19 (LG3 and LG4), 20 (LG5), 41(LG2) and 49 (LG1). Distorted markers were indicated with * ($P \leq 0.05$) or ** ($P \leq 0.01$). The primer pairs detecting more than one locus were identified by numbers -1, - 2 after each primer name (Fig. 9). The size of the amplified bands ranged in between 100 and 500bp.

The distance between the markers on the map also varied greatly across the different linkage groups. The average marker distance was 14.5 cM, with intervals between loci ranging from 1 to 41 cM (Fig. 9). Table 3 provides a summary of SSR marker distribution on different linkage groups showing the number of markers, linkage group size, and the average marker interval per linkage group.

Table 3 Number of markers and linkage group size per linkage group of the F_7 linkage map

Linkage group	Number of markers	Average interval (cM)	Linkage group size (cM)
1	49	15.1	738
2	41	15.3	627
3	19	15.4	294
4	19	13.3	252
5	20	15.1	301
6	7	12.3	86
7	11	14.8	163
8	13	13.5	175
9	6	10.3	62
10	16	14.8	236
11	5	11.4	57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34.9 SSR-7068
51.6 SSR-7082
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84.1 SSR-7045-1
96.9 SSR-7045-2
111.5 SSR-6188
128.4 SSR-6733-1
138.1 SSR-6733-2
145.4 SSR-6698
159.2 SSR-6607 **
172.4 SSR-6222
178.6 SSR-7001
200.5 SSR-6592 *
217.2 SSR-6624
231.7 SSR-6618
248.0 SSR-6717
268.2 SSR-6228 **
289.2 SSR-7078 *
306.1 SSR-6375-1
338.7 SSR-6375-2
355.3 SSR-6333
369.3 SSR-6192 *
387.2 SSR-7014
401.4 SSR-6994
408.8 SSR-6313 *
413.4 SSR-6726
434.6 SSR-6171
456.4 SSR-6683
479.0 SSR-7069
509.3 SSR-7041
517.2 SSR6376 *
537.7 SSR-6724
555.6 SSR-6469 *
575.1 SSR-6477
596.8 SSR-7072
614.6 SSR-6923
633.3 SSR-7101-1
651.4 SSR-6978-3
659.9 SSR-6243 *
666.0 SSR-7011
686.1 SSR-6609 *
701.6 ra
706.5 SSR-6965-2
713.0 SSR-7005
729.6 SSR-6939 *
738.5 pp
0.0 SSR-6921
16.2 SSR-6537
36.8 SSR-6348 **
53.9 SSR-6612
64.5 SSR-6360
79.8 SSR-6705
96.1 SSR-6944
114.2 SSR-6451 **
134.4 SSR-6626
150.2 SSR-6314
164.7 SSR-6345
165.8 SSR-6302
197.3 SSR-6645
215.5 SSR-6686
232.2 SSR-6666
252.4 SSR-6242
270.5 SSR-6515
287.3 SSR-6906
301.5 SSR-6611
317.6 SSR-6856
333.1 SSR-6909
348.2 SSR-6604
359.2 SSR-6662 *
377.1 SSR-7043
397.6 SSR-6268
417.5 SSR-6520
439.3 SSR-6639
457.9 SSR-6935
474.1 SSR-6983
485.8 SSR-6788
505.9 SSR-6720
520.9 SSR-6211
535.9 SSR-6240 *
536.0 SSR-7061 *
552.9 SSR-6475
569.4 SSR-6547
585.2 SSR-6362
598.1 61R
600.3 61R2
613.8 SSR-6354
627.5 SSR-6204
0.0 SSR-6577
18.0 SSR-7000-3
34.3 SSR-6915-1
52.1 SSR-6971
71.6 SSR-6807
88.8 SSR-6519
102.5 SSR-6917
120.8 SSR-6323
136.4 SSR-6294
151.5 SSR-6924-1
152.7 SSR-6924-2
167.5 SSR-6701
185.3 fc
199.1 SSR-6947
236.8 SSR-6682
250.6 SSR-6280
267.3 SSR-6719
285.0 SSR-6225 *
293.8 SSR-6744-1
0.0 SSR-6395
16.5 SSR-6273-2
34.2 SSR-6998 *
51.9 SSR-6516 *
67.2 SSR-6273-1
84.6 SSR-6327
99.6 SSR-7017
112.7 SSR-6673
127.0 SSR-6210
143.9 SSR-6744-2
159.4 SSR-6680
160.4 SSR-6982-3
171.1 SSR-6982-1
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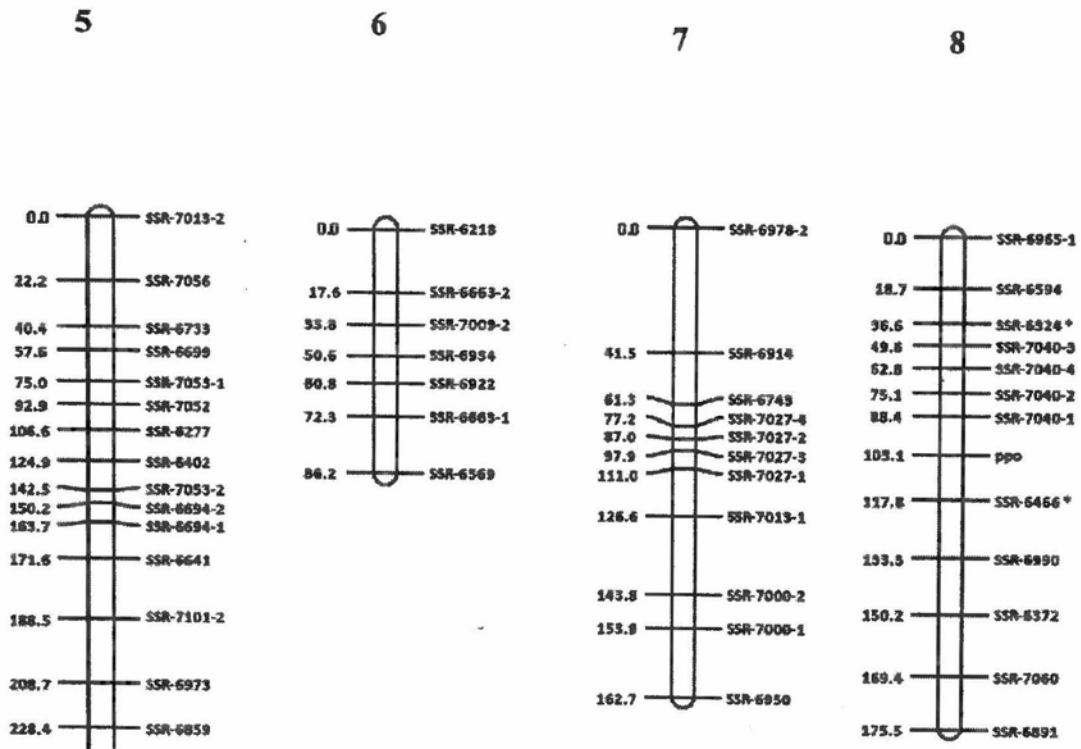
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106.6 SSR-8277
124.9 SSR-8402
142.5 SSR-7053-2
150.2 SSR-8694-2
163.7 SSR-8694-1
171.6 SSR-6641
188.5 SSR-7101-2
208.7 SSR-6973
228.4 SSR-6859
234.5 SSR-6603
251.6 SSR-6790
265.6 SSR-6245
289.5 SSR-6623*
300.8 SSR-6962
0.0 SSR-6218
17.6 SSR-6663-2
33.8 SSR-7009-2
50.6 SSR-6954
60.8 SSR-6922
72.3 SSR-6669-1
86.2 SSR-6569
0.0 SSR-6978-2
41.5 SSR-6914
61.3 SSR-6749
77.2 SSR-7027-4
87.0 SSR-7027-2
97.9 SSR-7027-3
111.0 SSR-7027-1
126.6 SSR-7013-1
143.8 SSR-7000-2
153.9 SSR-7000-1
162.7 SSR-6950
0.0 SSR-6965-1
18.7 SSR-6594
36.6 SSR-6526*
49.6 SSR-7040-3
62.8 SSR-7040-4
75.1 SSR-7040-2
88.4 SSR-7040-1
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117.8 SSR-6466*
133.5 SSR-6990
150.2 SSR-6372
169.4 SSR-7060
175.5 SSR-6891

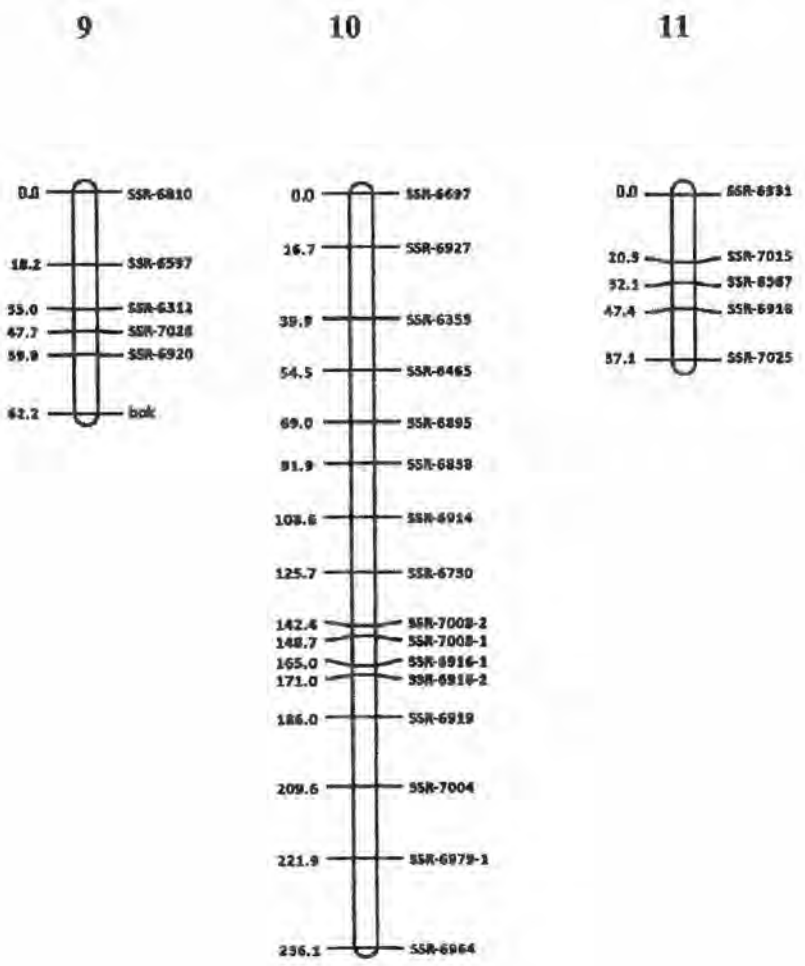


Figure 9 Genetic linkage map of cowpea that comprised the 11 LGs derived from 524B x 219-01 using SSR markers. Distances (in cM) between adjacent markers and the relative marker names are indicated at the left and right side respectively. The asterisks show the x^2 *p* levels of significance (*, 0.05; **, 0.01).

4.3.2 Segregation and mapping of genes controlling morphological traits

The locus for pod alignment/pod position segregates at a 1:1 ratio in the F_7 recombinant inbred population and this locus was mapped on LG8 (Fig. 9). With regard to the flower color the purple and white flowers segregated according to the expected 1:1 ratio in the F_7 population and the fc locus is mapped at LG3. The locus that is responsible for pod color (pod pigmentation) as well as the locus that regulates the root architecture is also segregated at a 1:1 ratio and both the pp and ra locus are mapped on LG1 (Fig.9).

4.4 QTL mapping

4.4.1 Trait phenotyping and QTL analysis

The two parental genotypes of the mapping population, 524B and 219-01, were found to show variation in the different domestication traits as well as yield related trait, seed weight. Therefore all the 159 RILs were phenotyped for the above mentioned traits. Frequency distribution of the phenotypic data suggested multigenic activity (Fig. 11a-c). Results of the QTL analysis are reported in Table 4 and represented graphically in Figure 10. Composite interval mapping revealed the presence of 16 QTLs affecting the three quantitative traits of interest. The number of QTLs mapped for a given trait ranged from 4 to 6 and two of the traits (seed weight and pod size) had more than one QTL on a single linkage group.

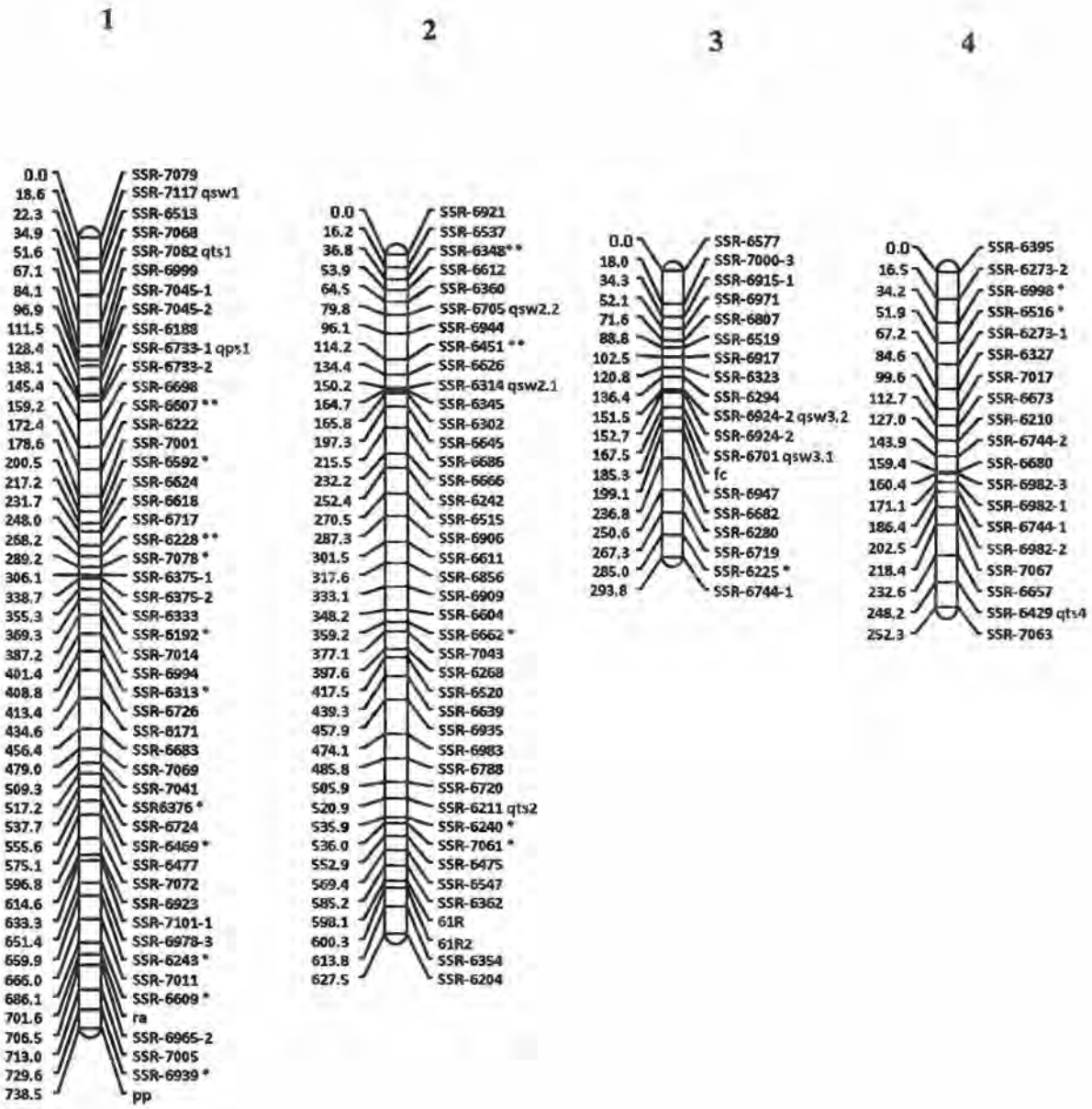
4.4.1.1 QTLs for Seed weight

Composite Interval Mapping (CIM) revealed a total of 6 significant ($P = 0.05$) QTLs affecting seed weight (SW) by the genome–wide analysis with permutation tests. These QTLs were distributed in

four of the 11 linkage groups, and individual QTLs accounted for 8.9-19.1 % of the phenotypic variance observed (Table 4; Fig. 10 and Fig. 12). All together the QTLs for seed weight in those four regions of the chromosome explained 74.4 % of the variation.

Table 4 Quantitative trait loci for domestication related and agronomic traits identified by composite interval mapping (CIM) method

Trait	QTL name	Linkage group	Nearest marker	Position (cM)	Highest LOD score	1-LOD Interval	Phenotypic variation	Additive effect
Seed	qsw1	1	SSR-7117	18.6	3.282	0.0-22.3	19.1	20.756
weight	qsw2.1	2	SSR-6314	150.2	3.127	134.4-164.7	8.9	-17.054
	qsw2.2	2	SSR-6705	79.8	3.794	64.5-96.1	13.8	-8.961
	qsw3.1	3	SSR-6701	167.5	3.241	152.7-185.3	10.1	13.146
	qsw3.2	3	SSR-6924-2	152.7	4.096	151.5-167.5	13.3	13.972
	qsw10	10	SSR-6919	186.0	3.268	171.0-209.6	9.2	-14.658
Pod	qps1	1	SSR-6733-1	128.4	2.581	111.5-138.1	17.2	11.729
size	qps6.1	6	SSR-6663-2	17.6	2.772	0.0-33.8	7.7	-9.004
	qps6.2	6	SSR-6369	86.2	2.289	60.8-86.2	6.4	10.004
	qps10	10	SSR-7008-2	142.4	2.344	125.7-148.7	16.6	13.6969
	qts1	1	SSR-7082	51.6	2.654	34.9-67.1	7.4	9.012
Testa	qts2	2	SSR-6211	520.9	2.008	505.9-535.9	5.6	-6.004
size	qts4	4	SSR-6429	248.2	2.163	232.6-252.3	9.8	12.4782
	qts7	7	SSR-6950	162.7	2.002	143.8-162.7	5.6	-8.002
	qts10	10	SSR-6838	91.9	2.064	69.0-108.6	26.1	16.6286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34.9 SSR-7068
51.6 SSR-7082 qts1
67.1 SSR-6999
84.1 SSR-7045-1
96.9 SSR-7045-2
111.5 SSR-6188
128.4 SSR-6733-1 qps1
138.1 SSR-6733-2
145.4 SSR-6698
159.2 SSR-6607 **
172.4 SSR-6222
178.6 SSR-7001
200.5 SSR-6592 *
217.2 SSR-6624
231.7 SSR-6618
248.0 SSR-6717
258.2 SSR-6228 **
289.2 SSR-7078 *
306.1 SSR-6375-1
338.7 SSR-6375-2
355.3 SSR-6333
369.3 SSR-6192 *
387.2 SSR-7014
401.4 SSR-6994
408.8 SSR-6313 *
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434.6 SSR-8171
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509.3 SSR-7041
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596.8 SSR-7072
614.6 SSR-6923
633.3 SSR-7101-1
651.4 SSR-6978-3
659.9 SSR-6243 *
666.0 SSR-7011
686.1 SSR-6609 *
701.6 ra
706.5 SSR-6965-2
713.0 SSR-7005
729.6 SSR-6939 *
738.5 pp
0.0 SSR-6921
16.2 SSR-6537
36.8 SSR-6348 **
53.9 SSR-6612
64.5 SSR-6360
79.8 SSR-6705 qsw2.2
96.1 SSR-6944
114.2 SSR-6451 **
134.4 SSR-6626
150.2 SSR-6314 qsw2.1
164.7 SSR-6345
165.8 SSR-6302
197.3 SSR-6645
215.5 SSR-6686
232.2 SSR-6666
252.4 SSR-6242
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377.1 SSR-7043
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439.3 SSR-6639
457.9 SSR-6935
474.1 SSR-6983
485.8 SSR-6788
505.9 SSR-6720
520.9 SSR-6211 qts2
535.9 SSR-6240 *
536.0 SSR-7061 *
552.9 SSR-6475
569.4 SSR-6547
585.2 SSR-6362
598.1 61R
600.3 61R2
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627.5 SSR-6204
0.0 SSR-6577
18.0 SSR-7000-3
34.3 SSR-6915-1
52.1 SSR-6971
71.6 SSR-6807
88.8 SSR-6519
102.5 SSR-6917
120.8 SSR-6323
136.4 SSR-6294
151.5 SSR-6924-2 qsw3.2
152.7 SSR-6924-2
167.5 SSR-6701 qsw3.1
185.3 fc
199.1 SSR-6947
236.8 SSR-6682
250.6 SSR-6280
267.3 SSR-6719
285.0 SSR-6225 *
293.8 SSR-6744-1
0.0 SSR-6395
16.5 SSR-6273-2
34.2 SSR-6998 *
51.9 SSR-6516 *
67.2 SSR-6273-1
84.6 SSR-6327
99.6 SSR-7017
112.7 SSR-6673
127.0 SSR-6210
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160.4 SSR-6982-3
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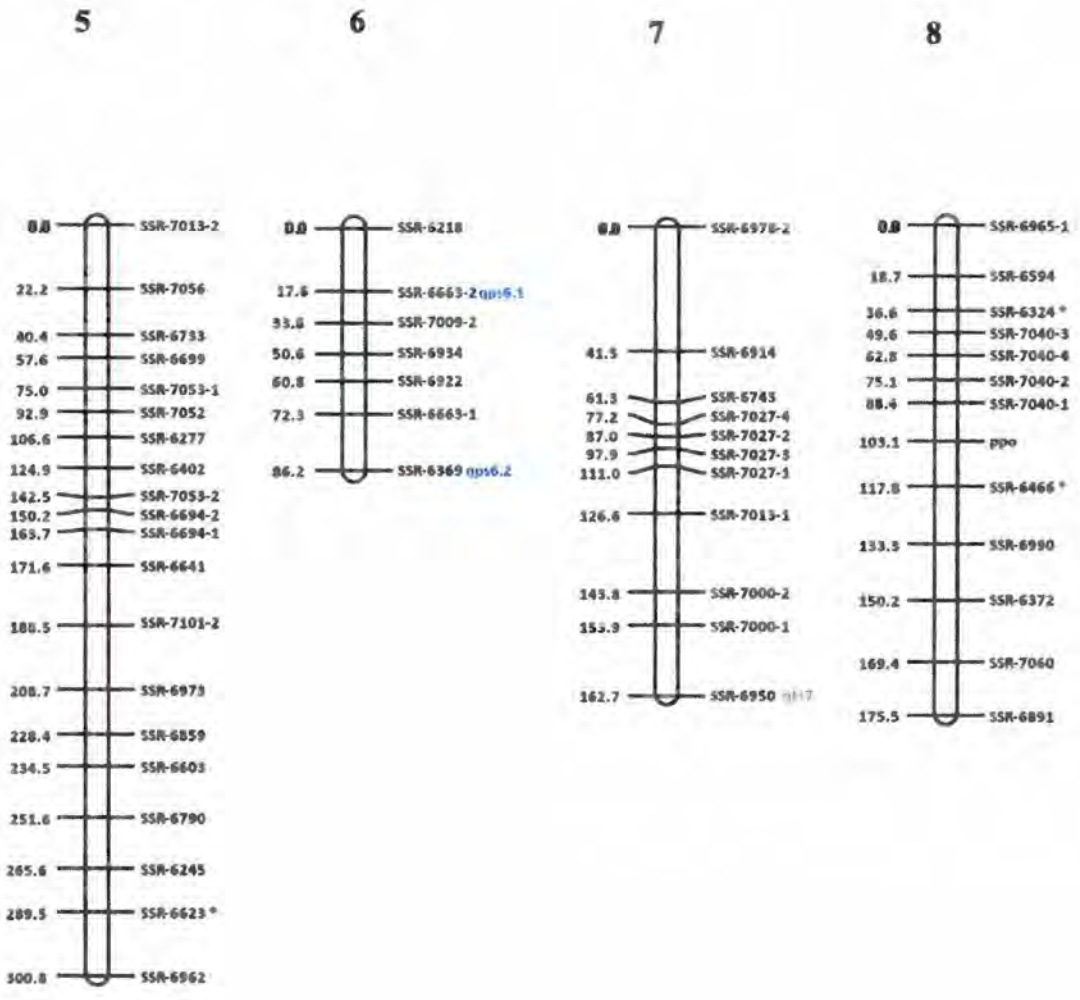
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106.6 SSR-6277
124.9 SSR-6402
142.5 SSR-7053-2
150.2 SSR-6694-2
163.7 SSR-6694-1
171.6 SSR-6641
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208.7 SSR-6973
228.4 SSR-6859
234.5 SSR-6603
251.6 SSR-6790
265.6 SSR-6245
289.5 SSR-6623 *
300.8 SSR-6962
0.0 SSR-6218
17.6 SSR-6663-2 qps6.1
33.6 SSR-7009-2
50.6 SSR-6934
60.8 SSR-6922
72.3 SSR-6663-1
86.2 SSR-6369 qps6.2
0.0 SSR-6978-2
41.5 SSR-6914
61.3 SSR-6743
77.2 SSR-7027-4
87.0 SSR-7027-2
97.9 SSR-7027-3
111.0 SSR-7027-1
126.6 SSR-7013-1
143.8 SSR-7000-2
153.9 SSR-7000-1
162.7 SSR-6950 qpl7
0.0 SSR-6965-1
18.7 SSR-6594
36.6 SSR-6324 *
49.6 SSR-7040-3
62.8 SSR-7040-4
75.1 SSR-7040-2
88.4 SSR-7040-1
103.1 ppo
117.8 SSR-6466 *
133.3 SSR-6990
150.2 SSR-6372
169.4 SSR-7060
175.5 SSR-6891

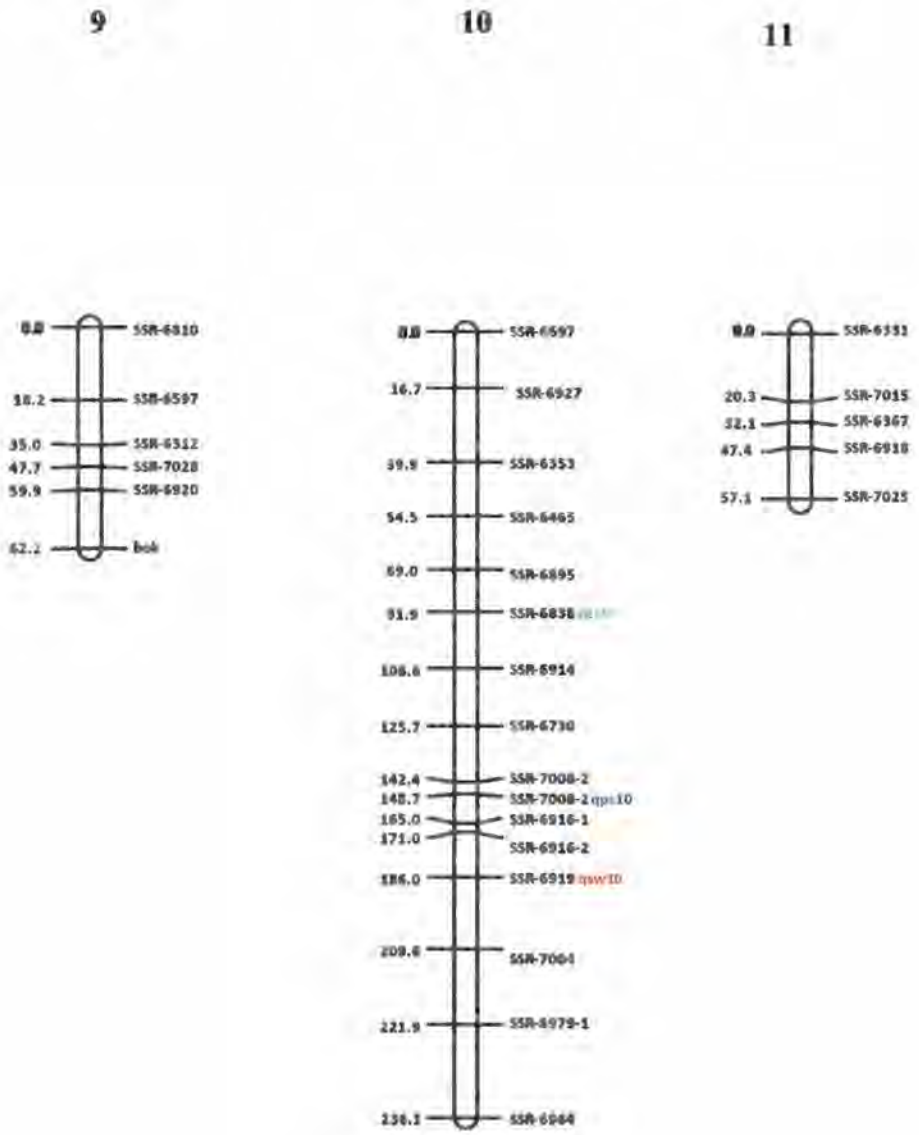


Figure 10 Genetic linkage map of cowpea that comprised the 11 LGs derived from 524B x 219-01 using SSR markers. Distances (in cM) between adjacent markers and the relative marker names are indicated at the left and right side respectively. Detected QTLs are represented by red, green and blue labels for seed weight, testa size and pod size respectively. The asterisks show the x^2 p levels of significance (*, 0.05; **, 0.01).

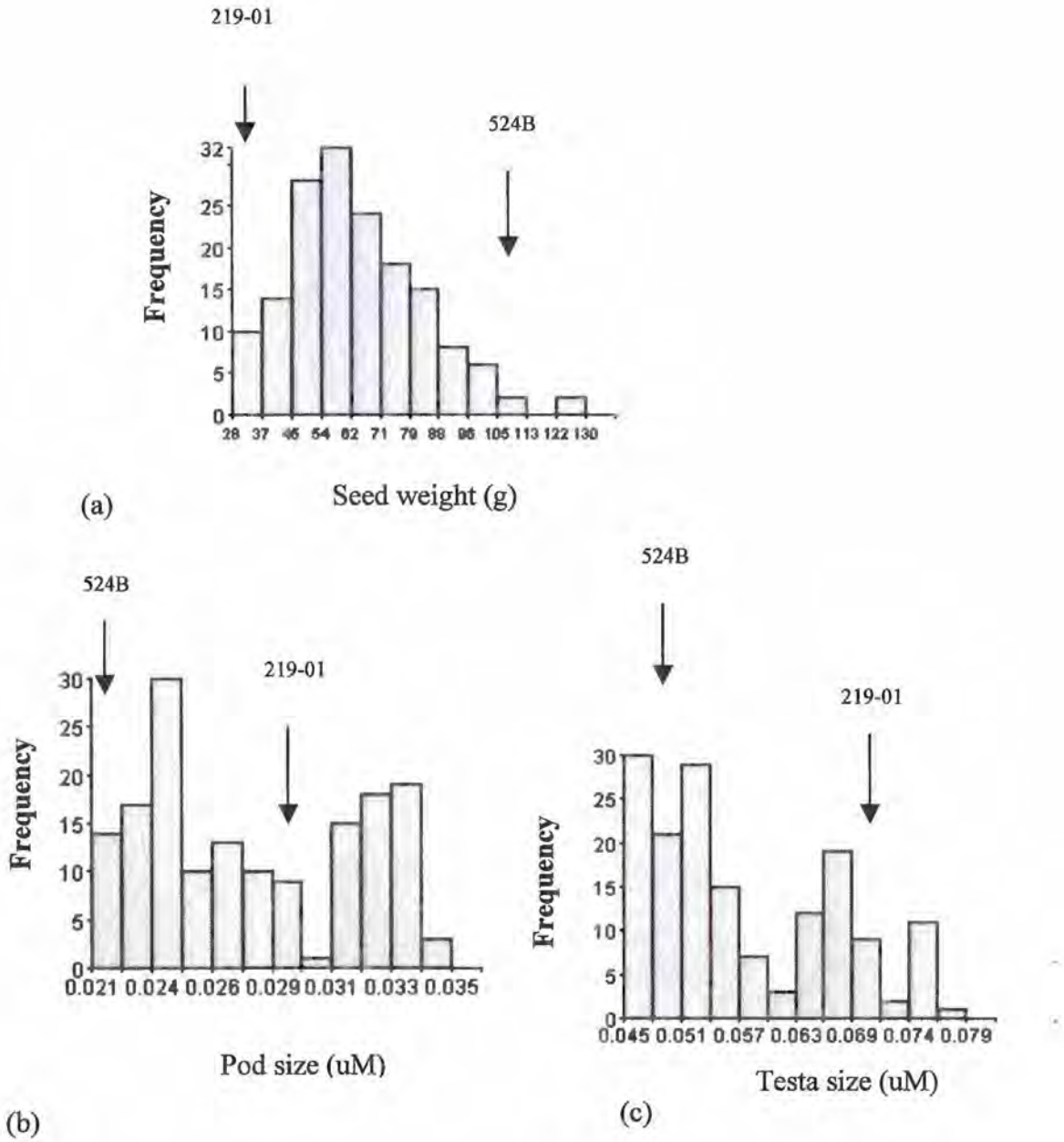
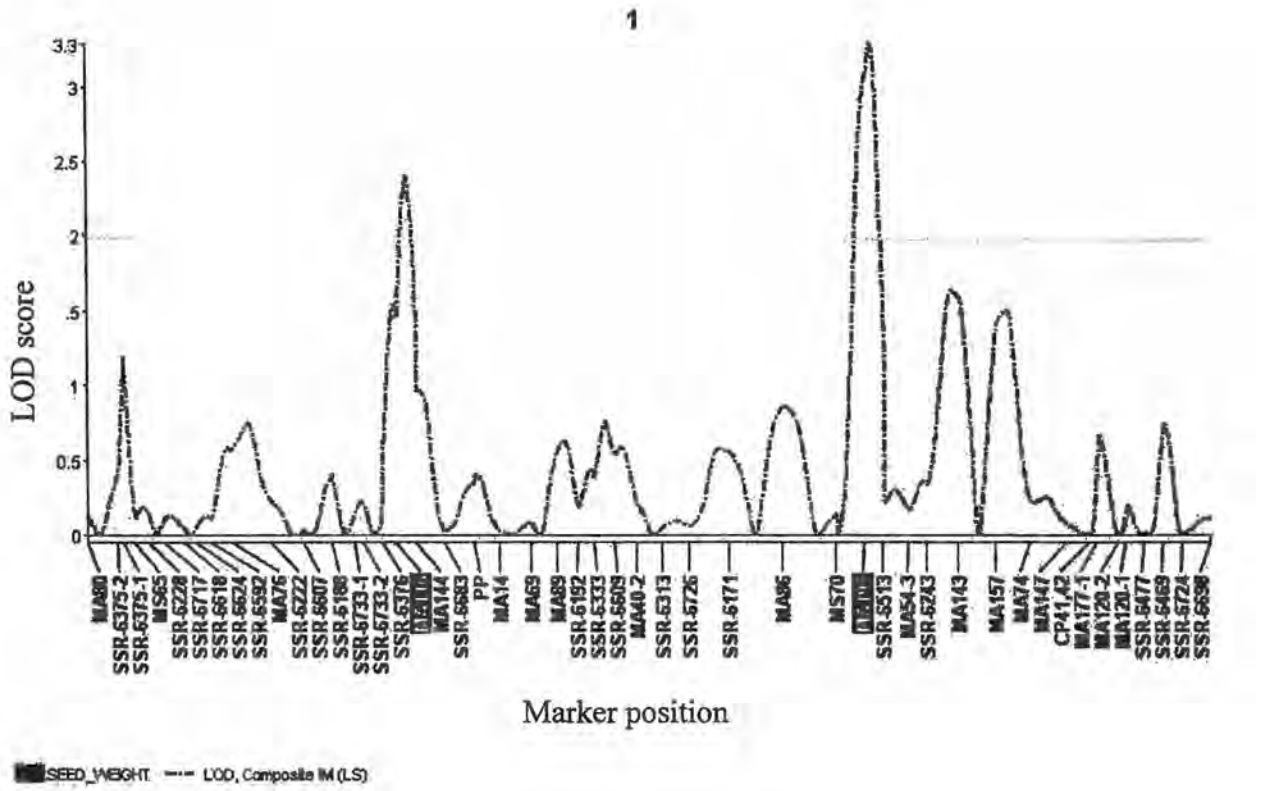


Figure 11 Frequency distribution of yield and domestication related traits measured in '524B' × '219-01' F_7 RILs for a) seed weight; b) pod size and c) testa size.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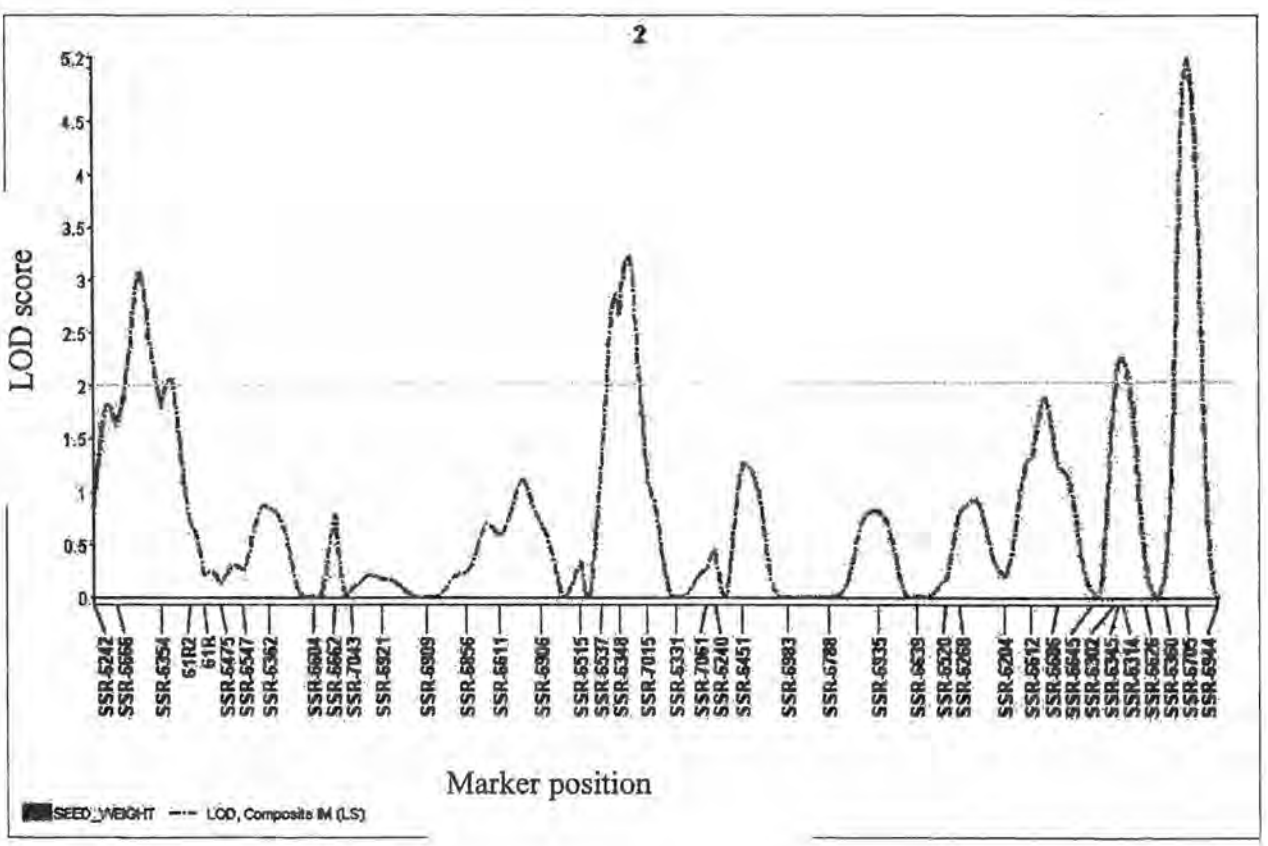
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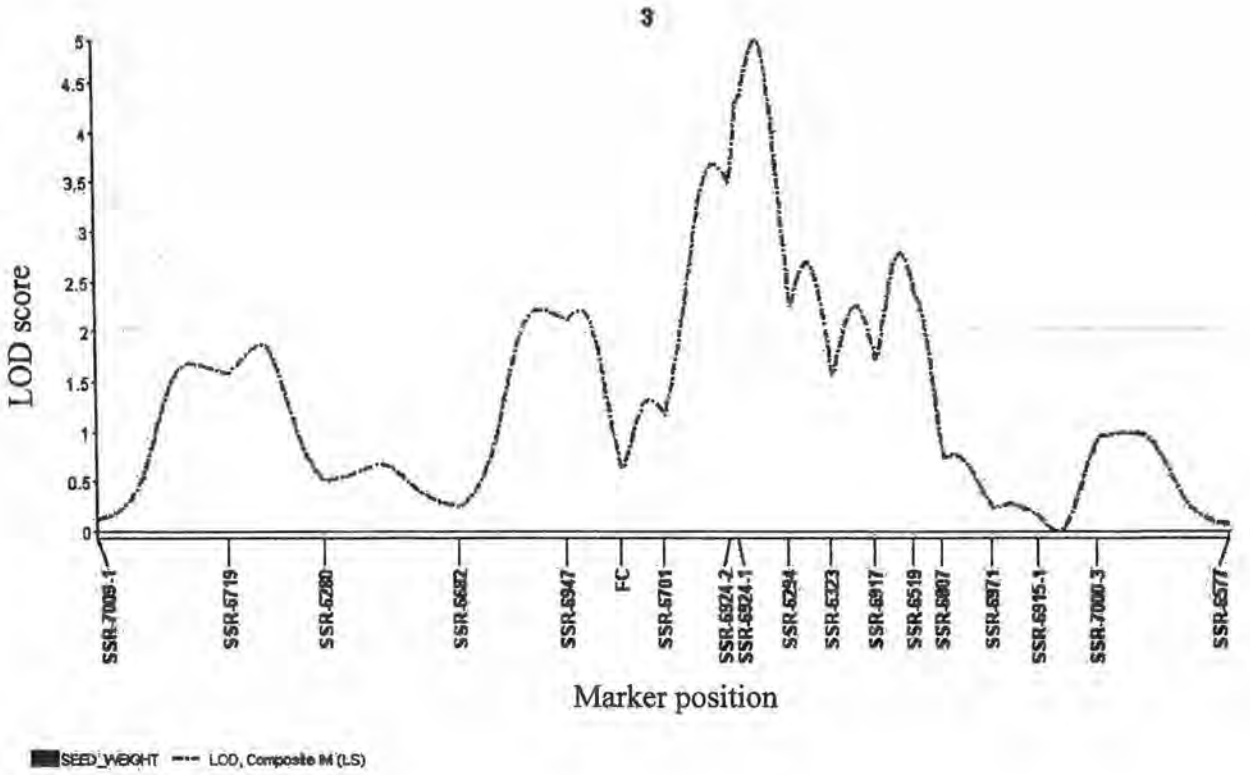


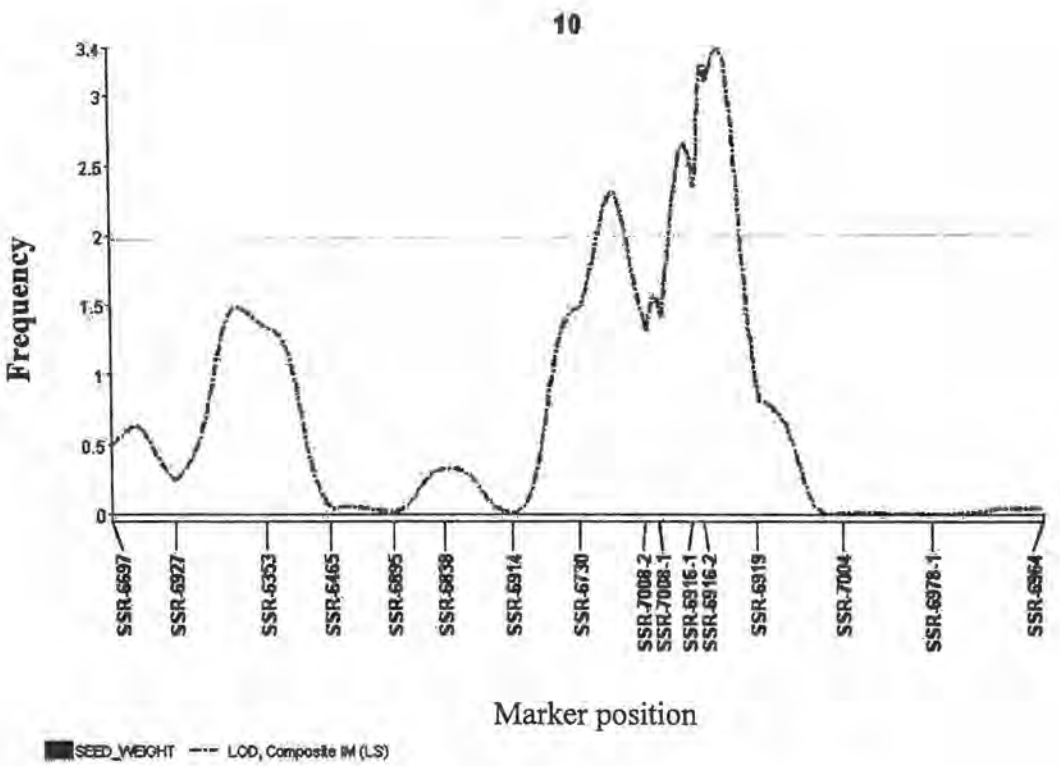
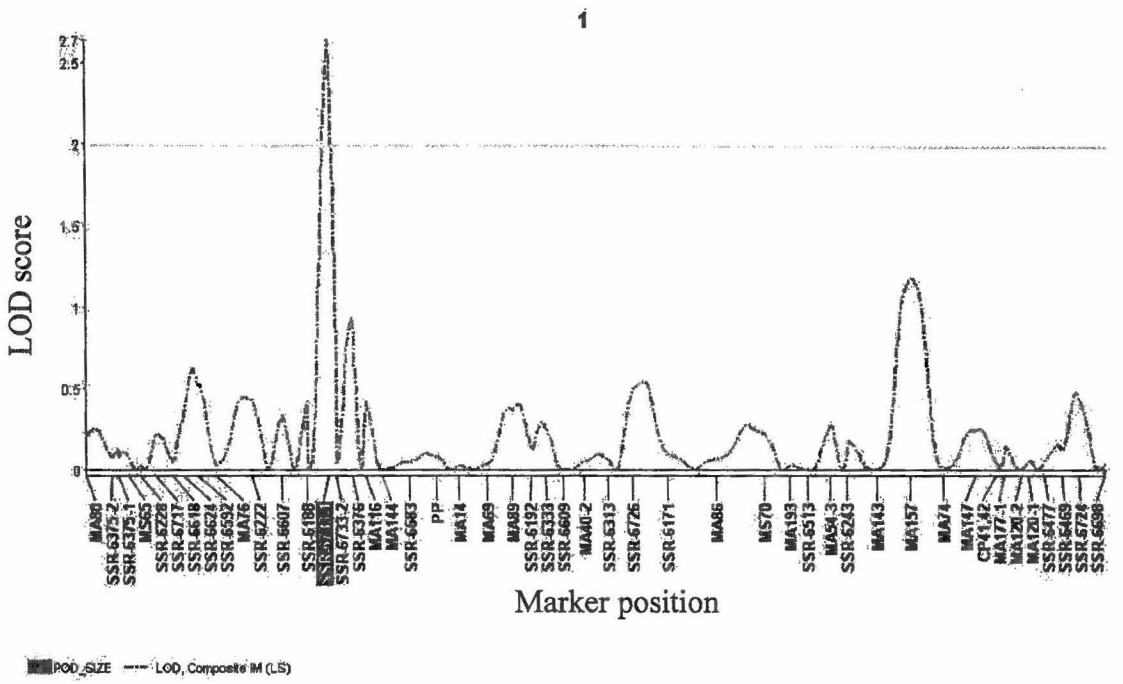


Figure 12 Quantitative trait loci map for seed weight of the F_7 RILs.

4.4.1.2 QTLs for domestication traits

A total of 9 significant (P = 0.05) QTLs affecting DRTs (domestication related traits) by the genome–wide analysis with permutation tests are revealed using Composite Interval Mapping (CIM) (Fig. 10, 13 and 14). These QTLs were distributed in six of the 11 linkage groups, and individual QTLs accounted for 5.6-26.1 % of the phenotypic variance observed (Table 4). Unlike the QTLs that are responsible for SW the alleles which confer a thin testa size and a thick pod size were derived mainly from the cultivated parent (524B). A significant QTL influencing both the domestication traits and yield related trait was detected on linkage groups 1 and 10 (Fig. 10).



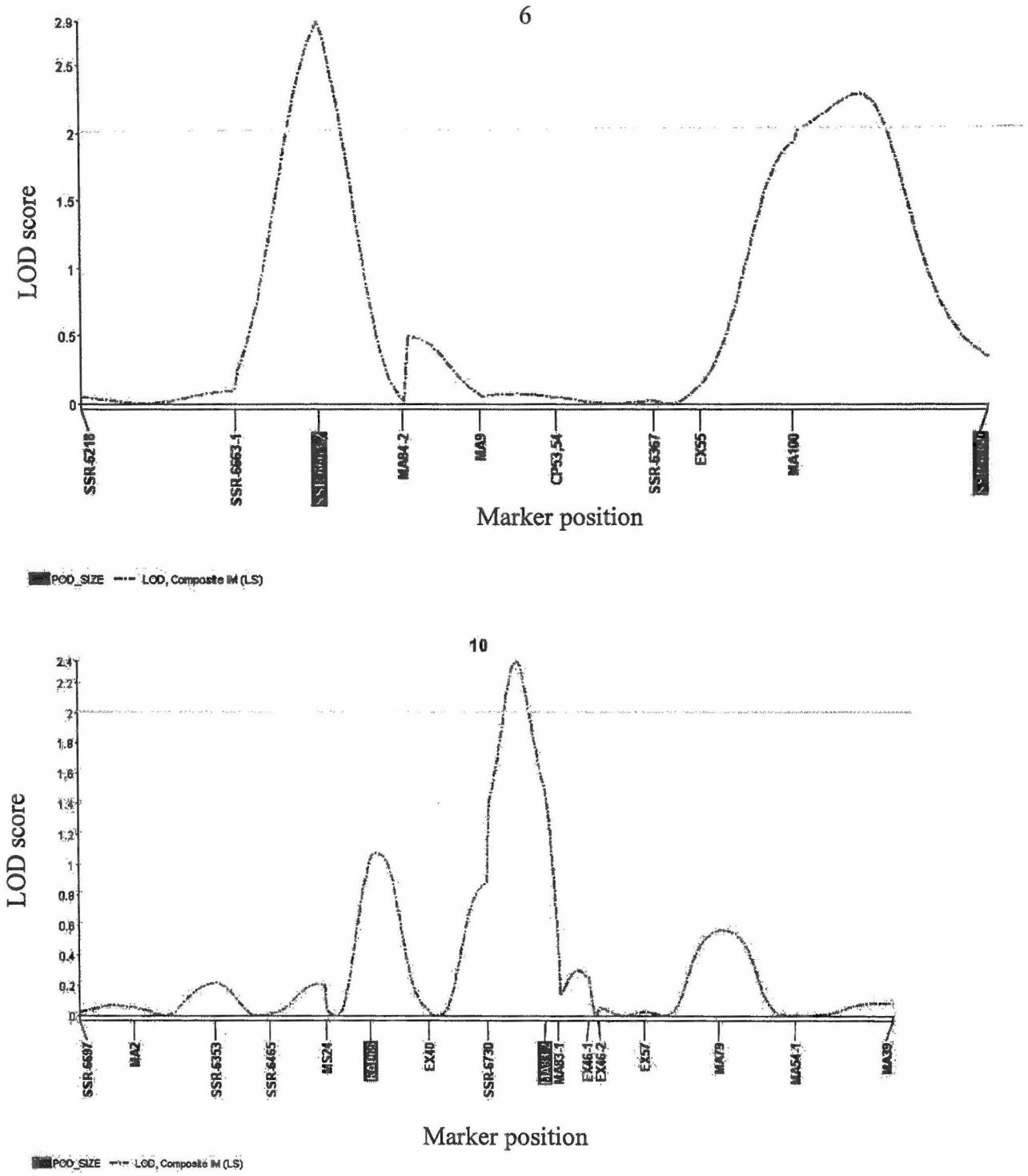
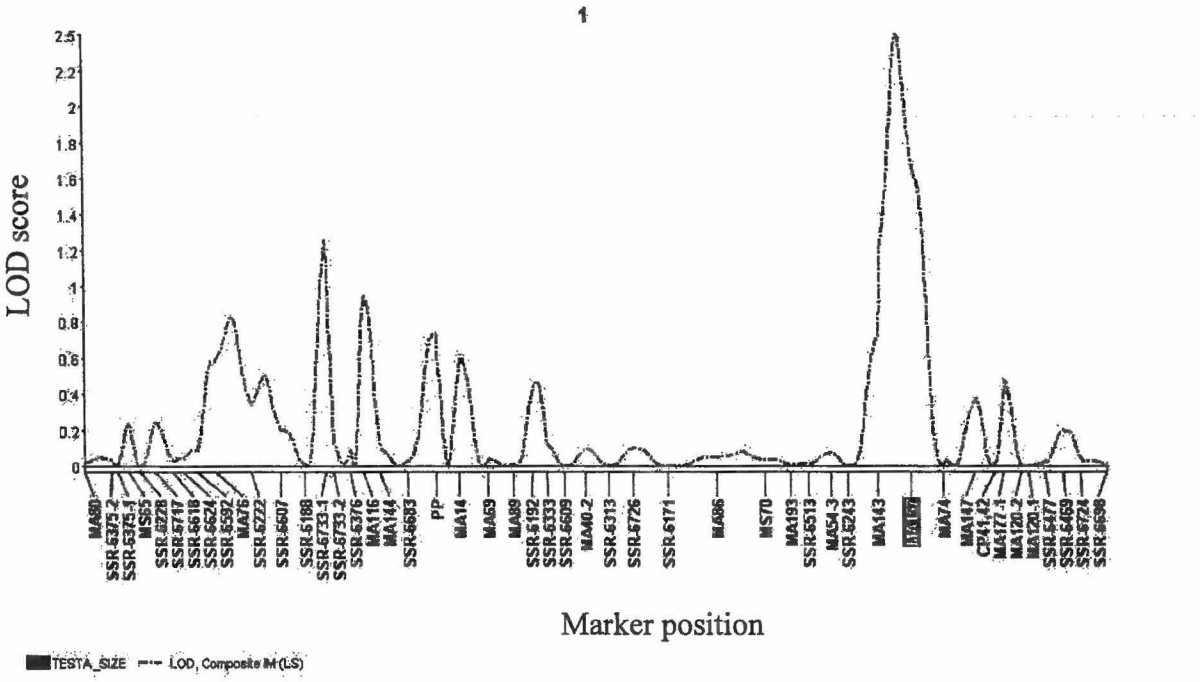


Figure 13 Quantitative trait loci map for pod size of the F_7 RILs.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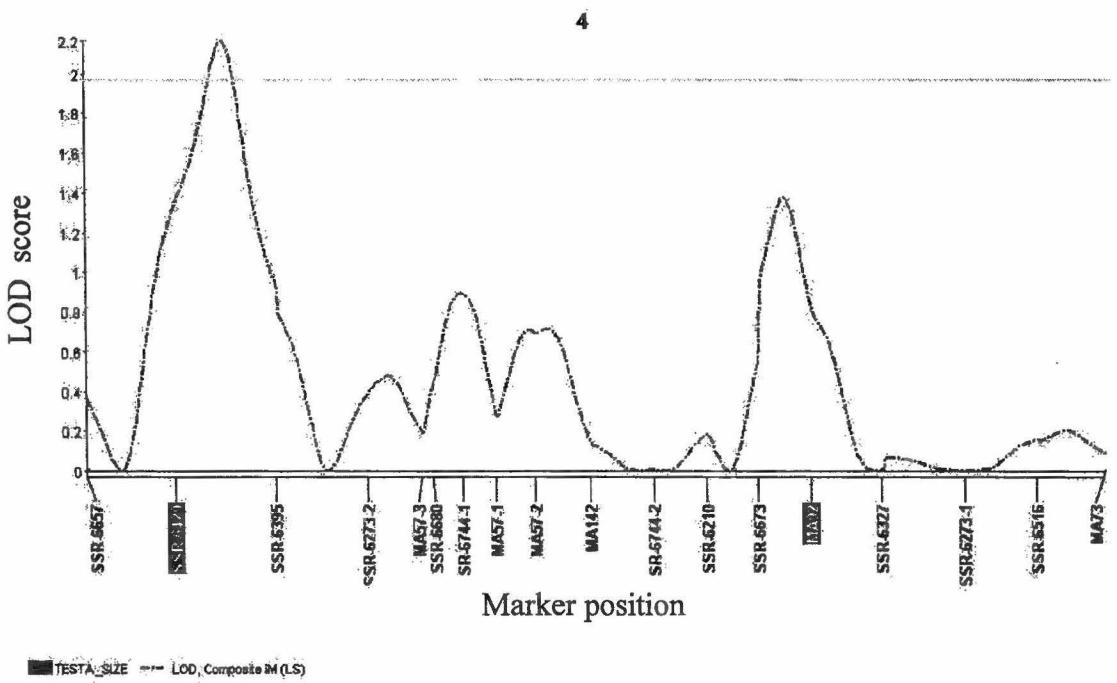
Marker position
TESTA_SIZE
LOD, Composite IM (LS)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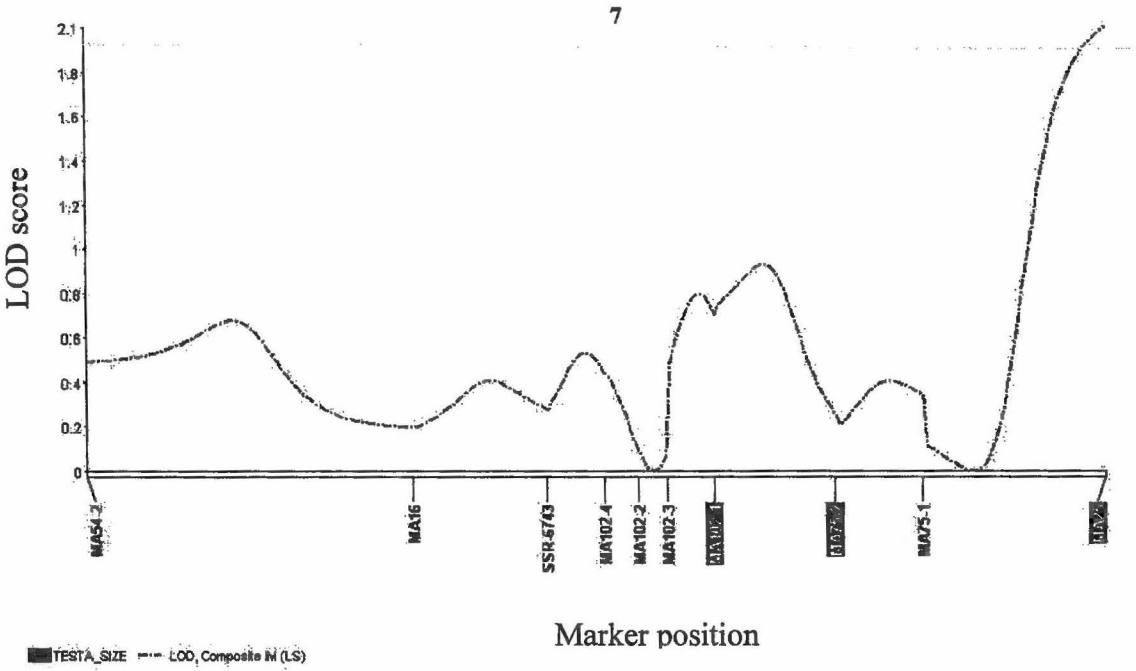
LOD score
Marker position
TESTA_SIZE
LOD, Composite IM (LS)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LOD score
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1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
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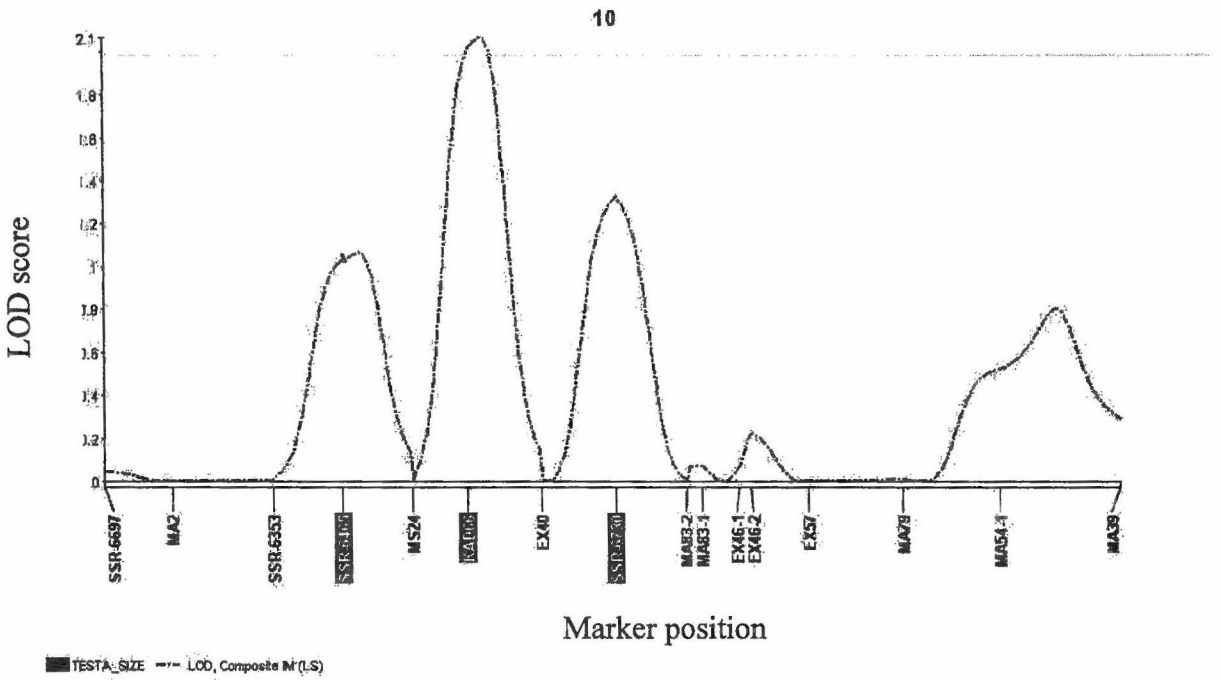
MA16
SSR-6743
MA102-4
MA102-2
MA102-3
MA75-1
Marker position
TESTA_SIZE
LOD, Composite IM (LS)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EX40
MA83-2
MA83-1
EX46-1
EX46-2
EX57
MA78
MA54-1
MA39
Marker position
TESTA_SIZE
LOD, Composite IM (LS)

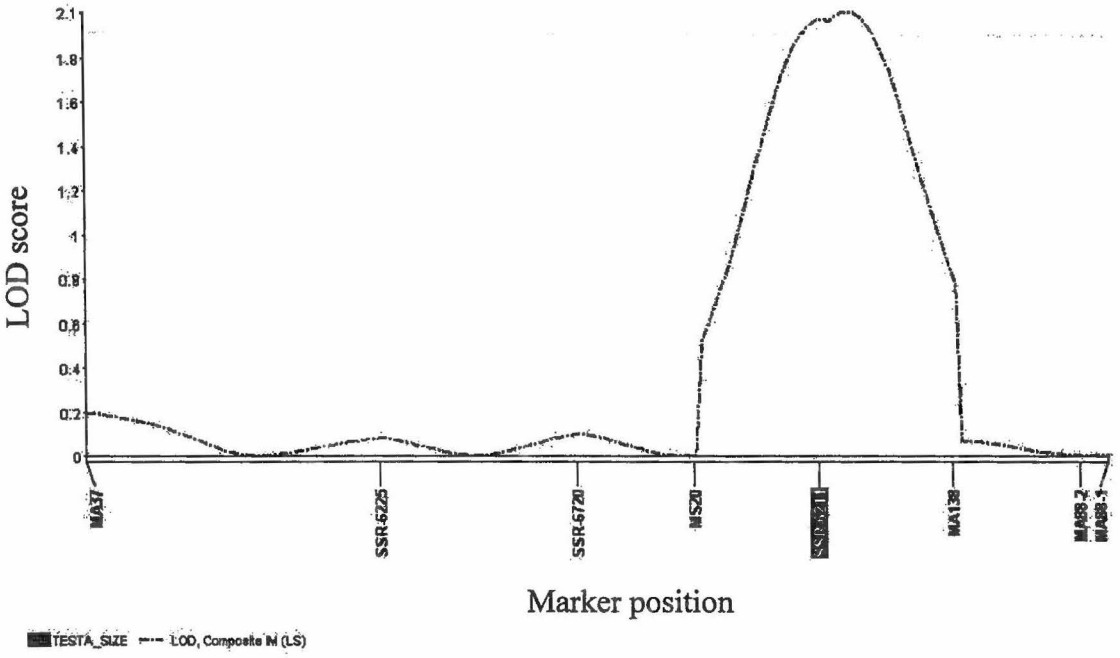


Figure 14 Quantitative trait loci map for testa size of the F_7 RILs.

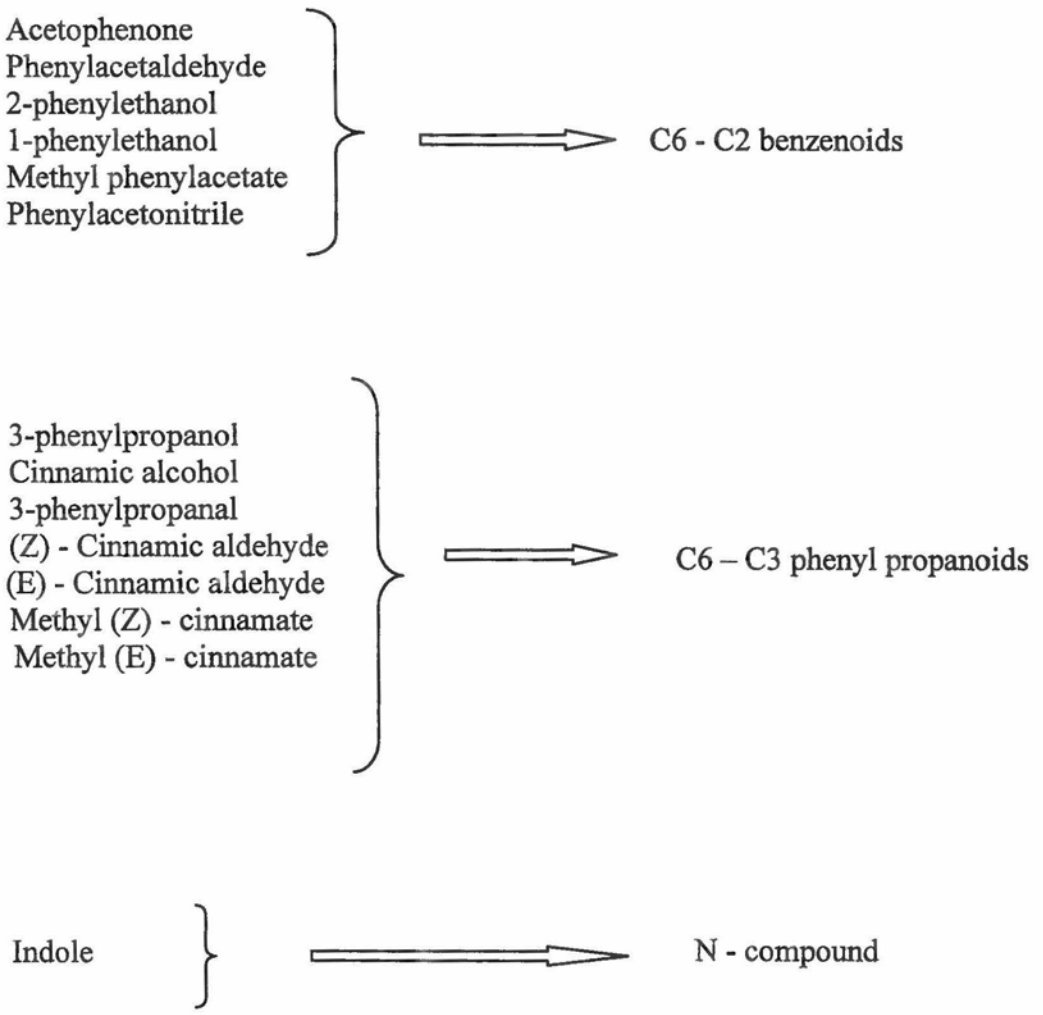
4.4.1.3 Identification and variability of scent compounds in the parental as well as in the F_7 RILs

Gas chromatographic separation produced chromatograms with a considerable number of peaks. Table 5 consists of 23 peaks that were detected in varying quantities in the parental as well as in the F_7 recombinant inbred lines and the way they were categorized was based on the guide for distribution of floral scent compounds by Knudsen *et al.* (2006). The substance identification of these volatiles was carried out by mass spectrometry with those of authentic reference compounds and tentative identifications by comparison with spectra in computer libraries. The identified substances contain 7 alcohols, 6 esters, 2 ketones, 6 aldehydes and 2 (phenylacetonitrile and indole) from other pathways. QTLs were estimated from this data. The descriptive statistics of these 23 volatile compounds are shown in Table 7. In addition,

exemplary frequency distributions of three esters (methyl (E) cinnamate, methyl benzoate and methyl anthranilate), four aldehydes ((E)-cinnamic aldehyde, (Z) - cinnamic aldehyde, 2-aminobenzaldehyde and benzaldehyde), one alcohol (2-phenylethanol) and one ketone (acetophenone) are given in Fig. 17. The frequency distribution of the F_7 RILs of the volatile compounds was not normal, and in most cases it was highly to moderately skewed towards the lower value (Fig. 17). All compounds exhibited a continuous variation in the progeny, which is typical for a polygenic inheritance. The phenotypic distributions of a few traits, i.e. for the concentration of (E) cinnamic aldehyde, approached a bimodal frequency of distribution (Fig.17).

Table 5 Floral scent composition of the two parental '524B & 219-01' and the 159 F_7 RILs. The compounds are ordered in classes, which to some degree reflect their biosynthetic origin (see Knudsen *et al.*, 2006).

Identified Volatile compound	Category
3-Octanone 3-Octanol 1-Octen-3-ol	Fatty acid derivatives
Benzylalcohol 2-Aminobenzaldehyde Benzaldehyde Methyl benzoate Methyl salicylate Methyl anthranilate	C6 - C1 benzennoids


Acetophenone
Phenylacetaldehyde
2-phenylethanol
1-phenylethanol
Methyl phenylacetate
Phenylacetonitrile
C6 - C2 benzenoids
3-phenylpropanol
Cinnamic alcohol
3-phenylpropanal
(Z) - Cinnamic aldehyde
(E) - Cinnamic aldehyde
Methyl (Z) - cinnamate
Methyl (E) - cinnamate
C6 – C3 phenyl propanoids
Indole
N - compound

4.4.1.4 QTLs for scent of the flowers

Sixty-three significant quantitative trait loci that govern scent among the population for the twenty-two chemical compounds using the composite interval mapping (CIM) method were identified. The final results are listed in Table 6 and also graphically presented in Fig. 15. QTLs were found for 22 out of 23 chemical compounds identified in this population. There was no QTL for the presence of phenyl acetaldehyde (AL 6). Generally, one QTL was identified for a specific volatile considering the defined stringent conditions for significance. For most of the volatiles more than one QTL was found. These includes for example, 3-octanone (K1) with five QTLs, with two of them on the same linkage group (LG 2), 3-octanol (A1) with five QTLs, with two of them on the same linkage group (LG 4), benzylalcohol (A3) with eight QTLs, with two of them on the same linkage group (LG 4), acetophenone (K2), (E) cinnamic aldehyde (AL 5) and methyl (Z) cinnamate each with five QTLs respectively (Table 6 and Fig. 16).

Table 6 Quantitative trait loci for scent traits identified by composite interval mapping (CIM) method

Trait	QTL name	Linkage group	Nearest marker	Position (cM)	Highest LOD score	1-LOD Interval	Phenotypic variation	Additive effect
3-Octanone	q3oe1	1	SSR-6592	200.5	2.007	178.6-217.2	5.6	-0.01
	q3oe2.1	2	SSR-6360	64.5	2.384	53.9-79.8	6.7	0.002
	q3oe2.2	2	SSR-6612	53.9	2.065	36.8-64.5	5.8	-0.002
	q3oe8	8	SSR-6990	133.3	2.158	117.8-150.2	6.1	-0.004
	q3oe10	10	SSR-6838	91.9	2.2333	69.0-108.6	6.3	0.0033
3-Octanol	q3ol1	1	SSR-6592	200.5	4.752	178.6-217.2	12.9	-0.001
	q3ol2	2	SSR-6906	287.3	2.155	270.5-301.5	6.0	-0.01
	q3ol4.1	4	SSR-6744-2	143.9	3.491	127.0-159.4	9.6	-0.01
	q3ol4.2	4	SSR-6273-1	67.2	4.125	51.9-84.6	11.3	0.0015
	q3ol10	10	SSR-6838	91.9	2.728	69.0-108.6	7.6	0.0013
1-Octen-3-ol	q1o3ol1	1	SSR-6592	200.5	2.419	178.6-217.2	6.8	-0.04
	q1o30l4	4	SSR-6744-2	143.9	2.641	127.0-159.4	7.4	-0.017
Benzaldehyde	qben2	2	SSR-6666	232.2	2.057	215.5-252.4	5.8	0.296
	qben4	4	SSR-6516	51.9	2.293	34.2-67.2	6.4	-0.163
Benzylalchol	qbzyl1	1	SSR-7117	18.6	2.463	0.0-22.3	6.9	-0.009
	qbzyl2	2	SSR-6314	150.2	2.448	134.4-164.7	6.8	-0.003
	qbzyl3	3	SSR-6577	0.0	2.215	0.0-18.0	6.2	-0.02
	qbzyl4.1	4	SSR-6429	248.2	2.986	232.6-252.3	8.3	-0.004
	qbzyl4.2	4	SSR-6516	51.9	4.657	34.2-67.2	12.6	-0.009
	qbzyl5	5	SSR-7101-2	188.5	2.798	171.6-208.7	7.8	0.005
	qbzyl6	6	SSR-6369	86.2	2.141	72.3-86.2	6.0	-0.016
	qbzyl7	7	SSR-7027-2	87.0	2.004	77.2-97.9	5.6	-0.003
Methyl benzoate	qmben10	10	SSR-6964	236.1	3.226	221.9-236.1	8.9	-0.158
Methyl salicylate	qmsal1	1	SSR-7082	51.6	2.471	34.9-67.1	6.9	-0.001
2-Amino benzaldehyde	q2aben1	1	SSR-6607	159.2	2.683	145.4-172.4	7.5	-0.01
Methyl anthranilate	qmant1	1	SSR-6469	555.6	2.167	537.7-575.1	6.1	-0.06
Acetophenone	qacet1	1	SSR-6469	555.6	3.427	537.7-575.1	9.4	-0.127
	qacet2	2	SSR-6240	535.9	2.935	520.9-536.0	8.1	-0.076
	qacet6	6	SSR-6934	50.6	2.755	33.8-60.8	7.7	-0.064
	qacet7	7	SSR-7000-1	153.9	2.248	143.8-162.7	6.3	0.072
	qacet10	10	SSR-6916-2	171.0	2.097	165.0-186.0	5.9	0.041
Methyl phenylacetate	qmphe1	1	SSR-6469	555.6	2.098	537.7-575.1	5.9	0.01
	qmphe6	6	SSR-6369	86.2	2.391	72.3-86.2	6.7	0.0164
	qmphe8	8	SSR-6324	36.6	2.003	18.7-49.6	5.6	0.01

2-Phenylethanol	q2peth4	4	SSR-6516	51.9	2.028	34.2-67.2	5.7	-0.031
1-Phenylethanol	q1peth1	1	SSR-7045-2	96.9	2.085	84.1-111.5	5.9	-0.004
	q1peth8	8	SSR-6324	36.6	2.336	18.7-49.6	6.5	-0.006
Phenylacetonitrile	qpace7	7	SSR-7000-1	153.9	2.545	143.8-162.7	7.1	-0.01
3-Phenylpropanal	q3ppa2	2	SSR-6515	270.5	2.49	252.4-287.3	7.0	-0.032
	q3ppa4	4	SSR-6516	51.9	3.318	34.2-67.2	9.2	-0.012
3-Phenylpropanol	q3ppo1	1	SSR-7117	18.6	3.105	0.0-22.3	8.6	-0.022
	q3ppo2	2	SSR-6612	53.9	2.315	36.8-64.5	6.5	-0.018
(Z)Cinnamic aldehyde	q(z)cal1	1	SSR-6469	555.6	2.066	537.7-575.1	5.8	0.264
	q(z)cal3	3	SSR-6577	0.0	4.368	0.0-34.3	11.9	-0.839
	q(z)cal4	4	SSR-6516	51.9	4.105	34.2-67.2	11.2	-0.157
	q(z)cal6	6	SSR-6369	86.2	2.211	72.3-86.2	6.2	-0.296
(E)Cinnamic aldehyde	q(e)cal3	3	SSR-6577	0.0	4.864	0.0-18.0	13.1	-2.966
	q(e)cal4	4	SSR-6516	51.9	3.968	34.2-67.2	10.9	-0.756
	q(e)cal6	6	SSR-6369	86.2	2.046	72.3-86.2	5.8	-1.128
	q(e)cal7	7	SSR-7027-4	77.2	2.827	61.3-87.0	7.9	-0.602
	q(e)cal10	10	SSR-6838	91.9	3.44	69.0-108.6	9.5	0.865
Cinnamic alcohol	qca1	1	SSR-6698	145.4	2.098	138.1-159.2	5.9	0.012
	qca5	5	SSR-6733	40.4	2.005	22.2-57.6	5.6	0.0014
Methyl(Z) cinnamate	qm(z)c1	1	SSR-6222	172.4	2.44	159.2-178.6	6.8	0.065
	qm(z)c2	2	61R	598.1	2.044	585.2-600.3	5.7	-0.016
	qm(z)c5	5	SSR-6245	265.6	2.013	251.6-289.5	5.7	0.034
	qm(z)c8	8	SSR-7040-2	75.1	2.003	62.8-88.4	5.6	-0.019
	qm(z)c10	10	SSR-6838	91.9	3.587	69.0-108.6	9.9	0.041
Methyl(E) cinnamate	qm(e)c1	1	SSR-6222	172.4	4.701	159.2-178.6	12.7	0.211
	qm(e)c5	5	SSR-6245	265.6	2.912	251.6-289.5	8.1	0.124
	qm(e)c8	8	SSR-7040-1	88.4	2.023	75.1-103.1	5.7	-0.11
Indole	qind2	2	SSR-6515	270.5	2.372	252.4-287.3	6.6	-0.011
	qind5	5	SSR-7053-2	142.5	2.107	124.9-150.2	5.9	0.003

All together QTLs for volatile compounds putatively involved in cowpea flower scent were found on 9 of the 11 cowpea chromosomes (Table 6, Fig. 15). These are the linkage groups 1, 2, 3, 4, 5, 6, 7, 8 and 10 (Fig.15). A large number of QTLs are located on the LG 1 of this cowpea

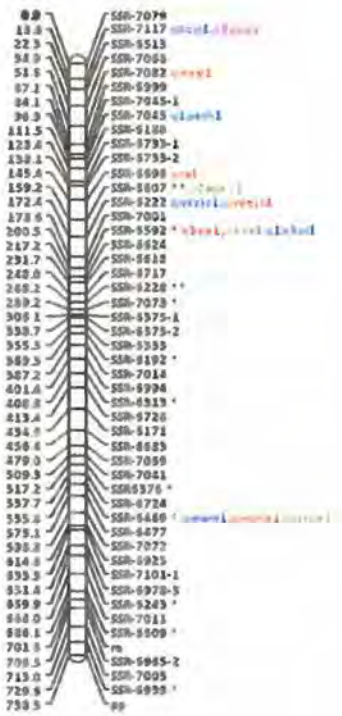
linkage map. Six of the seven alcohols, two of the five aldehydes, five of the six esters and the whole ketones showed QTL effects on this linkage group.

Table 7 Identified compounds analysed in the 159 F_7 recombinant inbred lines and in the parental lines '524B' and '219-01'

Volatile compound		F7 RIL population			524B		219-01	
		Mean	Min.	Max.	Mean	SD	Mean	SD
Alcohols								
A1	3-Octanol	0.0010	0	0.007	0.0010	0.0017	0.0002	0.0013
A2	1-Octen-3-ol	0.0212	0	0.4298	0.0302	0.0628	0.0070	0.0506
A3	Benzylalcohol	0.0082	0	0.0600	0.0018	0.0014	0.0022	0.0015
A4	2-phenylethanol	0.0497	0	0.4597	0.0030	0.0028	0.3240	0.1797
A5	1-phenylethanol	0.0057	0	0.0533	0.0012	0.0012	0.0457	0.0230
A6	3-phenylpropanol	0.0146	0	0.2000	0.0030	0.0055	0.0008	0.0011
A7	Cinnamic alcohol	0.0002	0	0.0039	0.0001	0.00012	0.0000	0.0000
Esters								
E1	Methyl benzoate	0.0230	0	0.7244	0.0075	0.0129	0.0024	0.0068
E2	Methyl salicylate	0.0005	0	0.0068	0.0013	0.0017	0.0003	0.0005
E3	Methyl anthranilate	0.0272	0	0.7623	0.0010	0.0022	0.0066	0.0053
E4	Methyl phenylacetate	0.0002	0	0.0030	0.0	0.0000	0.0011	0.0008
E5	Methyl (Z) cinnamate	0.0294	0	0.2000	0.0002	0.00133	0.0568	0.0718
E6	Methyl (E) cinnamate	0.0925	0	0.7334	0.0010	0.00500	0.2602	0.2499
Ketone								
K1	3-Octanone	0.0020	0	0.032	0.0014	0.003	0.0026	0.0054
K2	Acetophenone	0.1023	0	1.100	0.0246	0.0280	0.7019	0.3985

Aldehyde							
AL1 Benzaldehyde	0.2970	0	3.3000	0.0631	0.087	0.0274	0.0200
AL2 2-Aminobenzaldehyde	0.0103	0	0.2800	0.0002	0.0008	0.0041	0.0046
AL3 3-phenylpropanal	0.0165	0	0.1859	0.0030	0.0054	0.0000	0.0000
AL4 (Z) cinnamic aldehyde	0.2294	0	2.2596	0.0290	0.0370	0.0000	0.0000
AL5 (E) cinnamic aldehyde	0.8226	0	9.1000	0.1230	0.1192	0.0023	0.0060
AL6 Phenyl acetaldehyde	0.0082	0	0.4320	0.0015	0.0017	0.0410	0.0335
Miscellaneous							
M1 Phenylacetonitrile	0.00003	0	0.0014	0.0	0.0	0.0015	0.0017
M2 Indole	0.00590	0	0.0550	0.0001	0.0002	0.0089	0.0040

1



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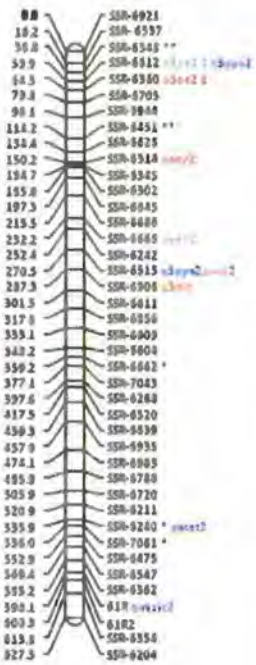

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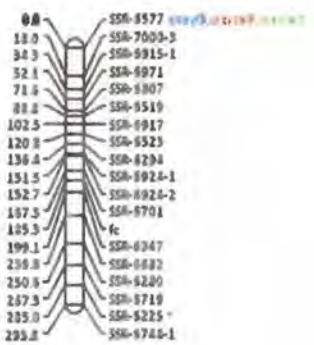


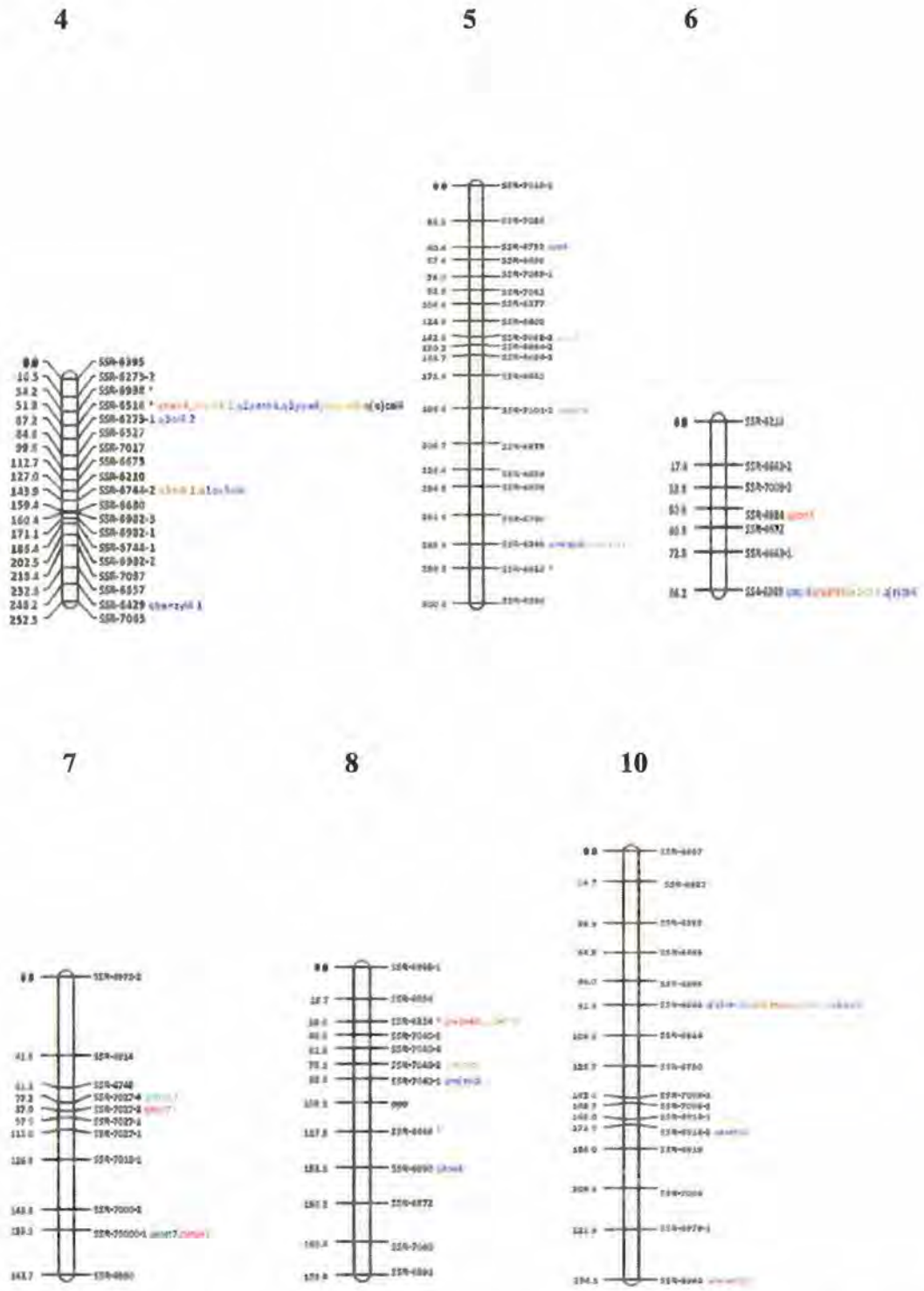
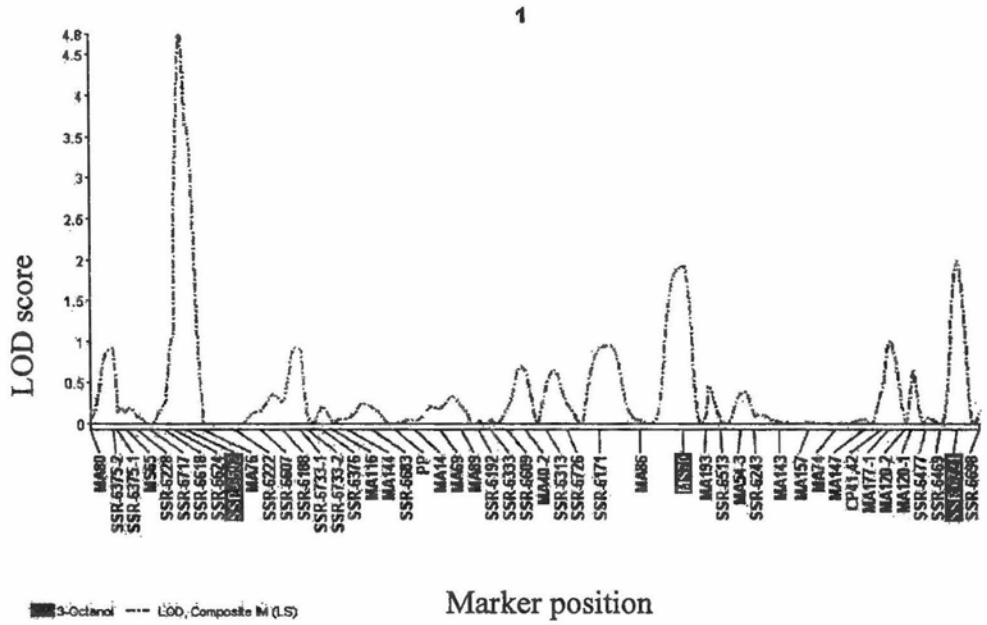
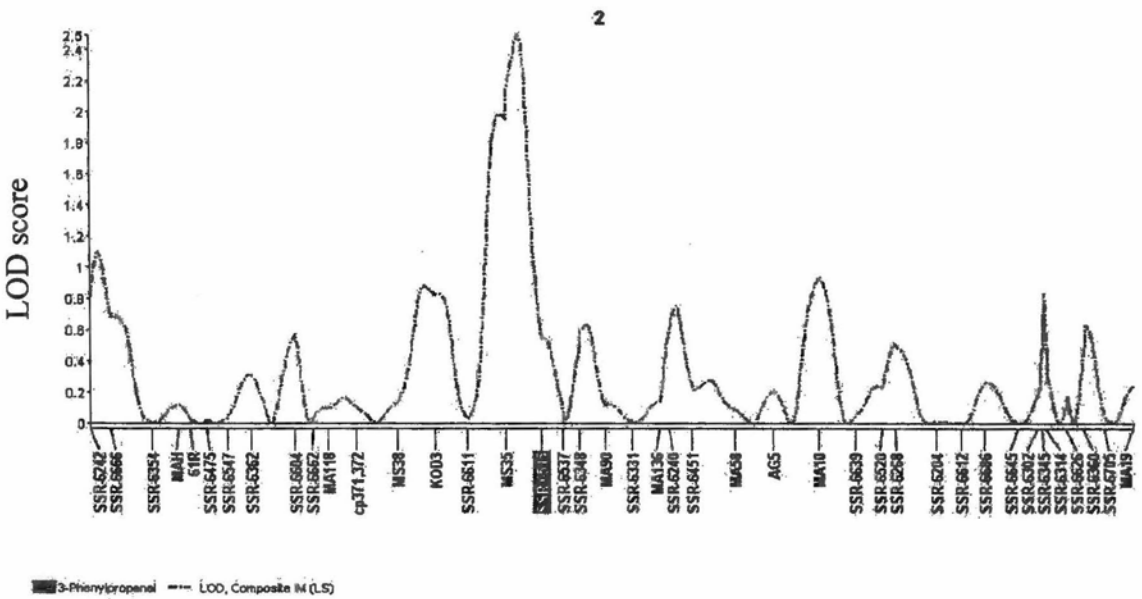


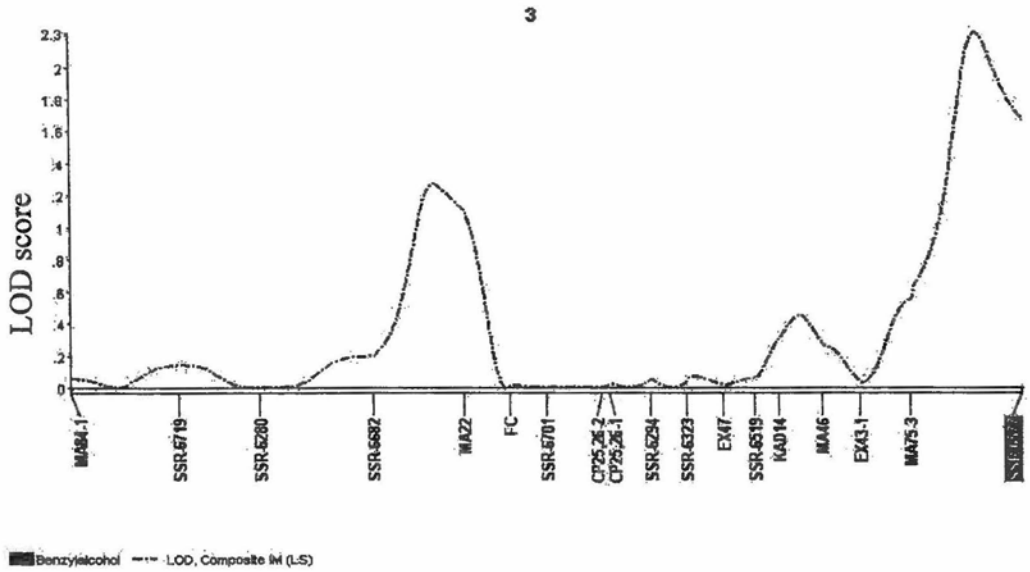
Figure 15 Genetic linkage map of cowpea that comprised the 9 LGs derived from 524B x 219-01 using SSR markers. Distances (in cM) between adjacent markers and the relative marker names are indicated at the left and right side respectively. Detected QTLs are represented by the name of the chemical compound followed by numbers showing the respective LGs. The asterisks show the x^2 p levels of significance (*, 0.05; **, 0.01). Note that only the nine LGs that have the QTLs are represented here.


1
LOD score
4.8
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
MA80
SSR-6375-2
SSR-6375-1
MS25
SSR-6728
SSR-6717
SSR-6618
SSR-6624
MA76
SSR-6222
SSR-6607
SSR-6188
SSR-5733-1
SSR-5733-2
SSR-6376
MA114
MA116
SSR-6683
PP
MA14
MA69
MA89
SSR-6192
SSR-6333
SSR-6689
MA40-2
SSR-5313
SSR-6726
SSR-6171
MA86
MS10
MA193
SSR-6513
MA54-3
SSR-6243
MA143
MA157
MA74
MA147
CP41.42
MA177-1
MA120-2
MA120-1
SSR-6477
SSR-6463
SSR-6698
3-Octanol
LOD, Composite IM (LS)
Marker position

(a)

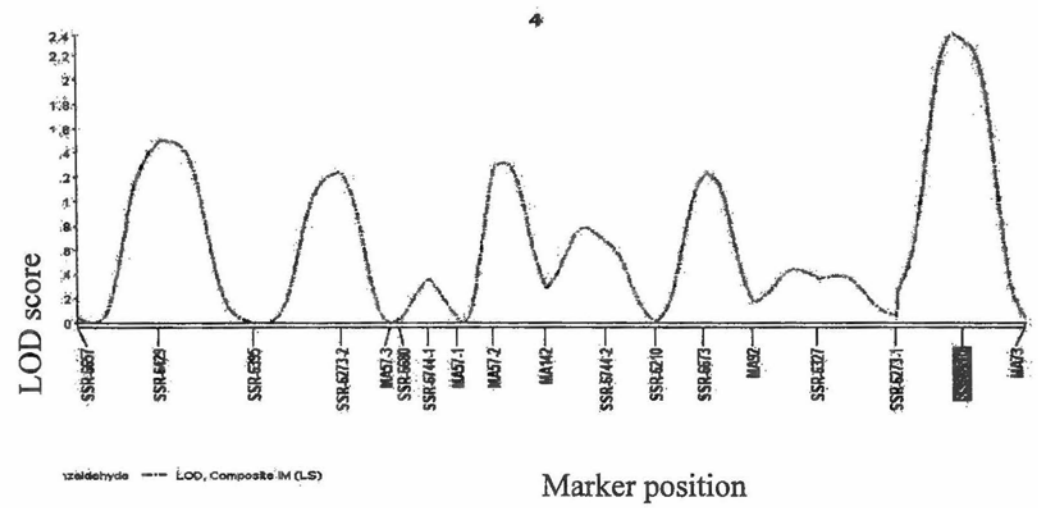

2
LOD score
2.5
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SSR-6242
SSR-6866
SSR-6354
MA31
61R
SSR-6475
SSR-6547
SSR-6362
SSR-6804
SSR-6862
MA118
cp371.372
MS38
K003
SSR-6611
MS35
SSR-6637
SSR-6348
MA90
SSR-6331
MA136
SSR-6240
SSR-6451
MA58
AG5
MA10
SSR-6629
SSR-6520
SSR-6258
SSR-6204
SSR-6612
SSR-6686
SSR-6845
SSR-6302
SSR-6345
SSR-6314
SSR-6626
SSR-6360
SSR-6705
MA19
3-Phenylpropenal
LOD, Composite IM (LS)
Marker position

(b)



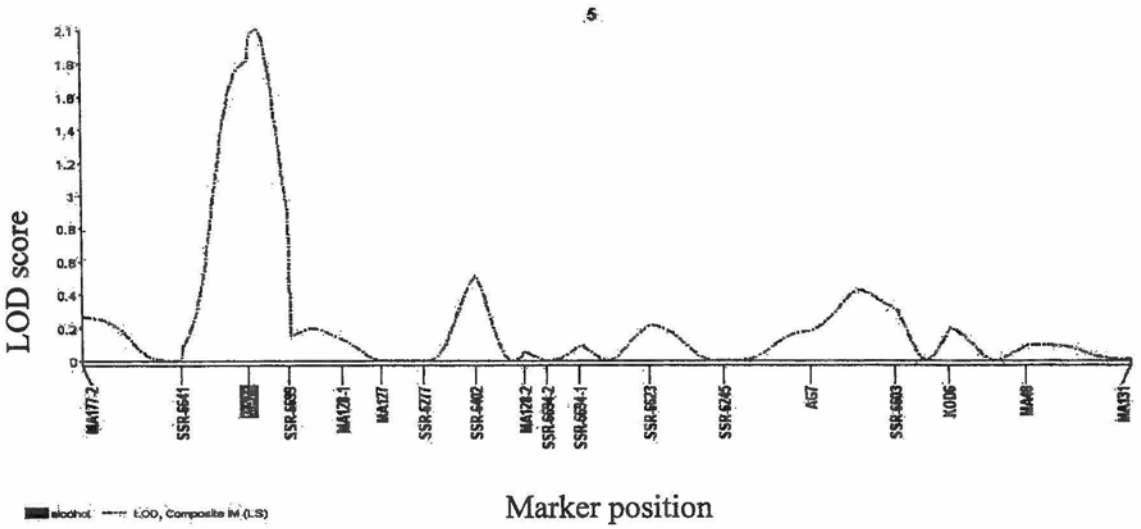
Marker position

(c)

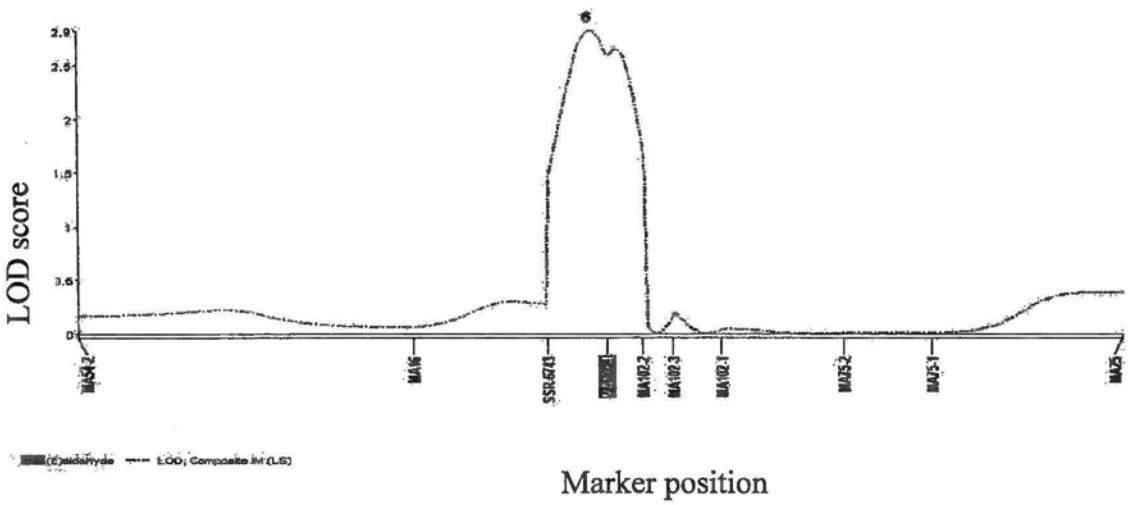


Marker position

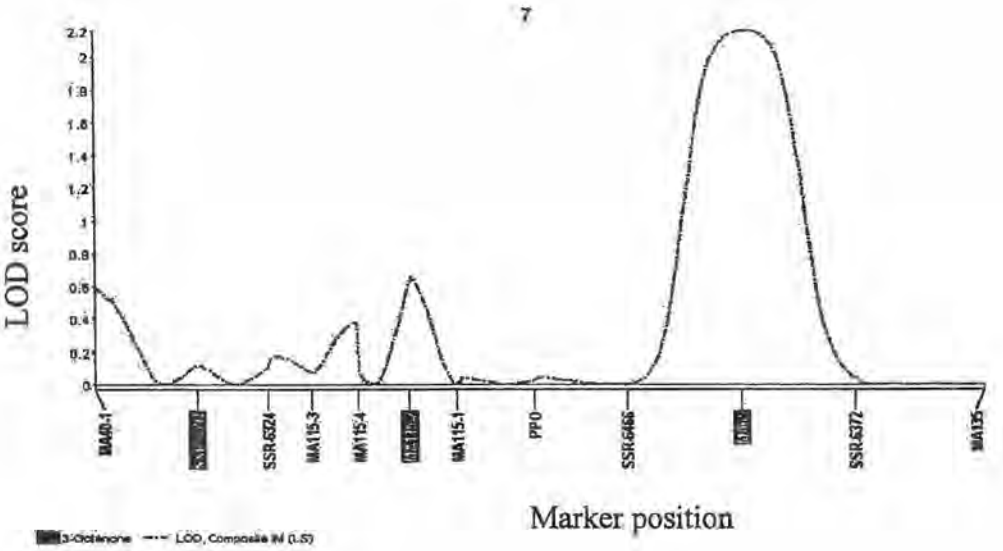
(d)



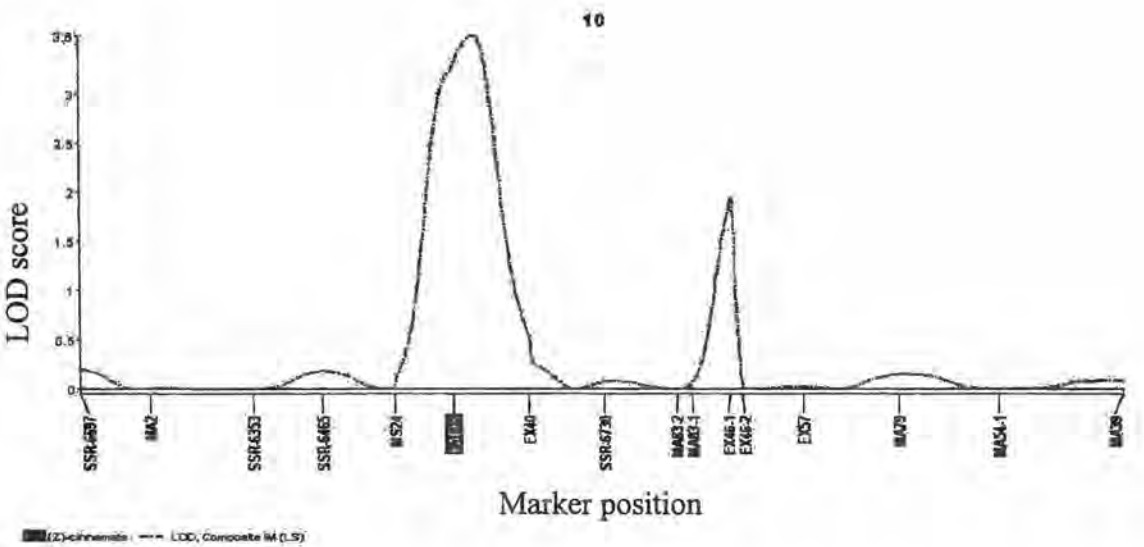
(e)



(f)

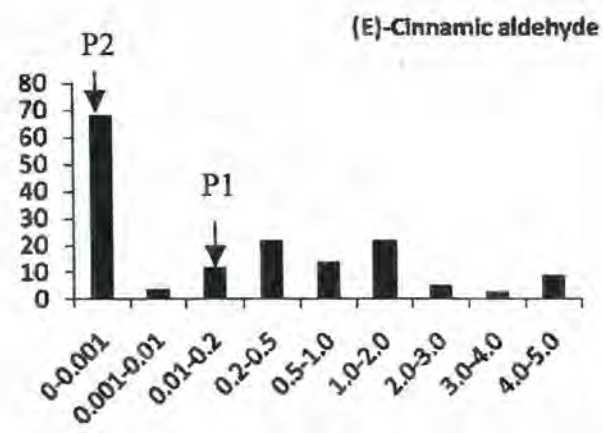


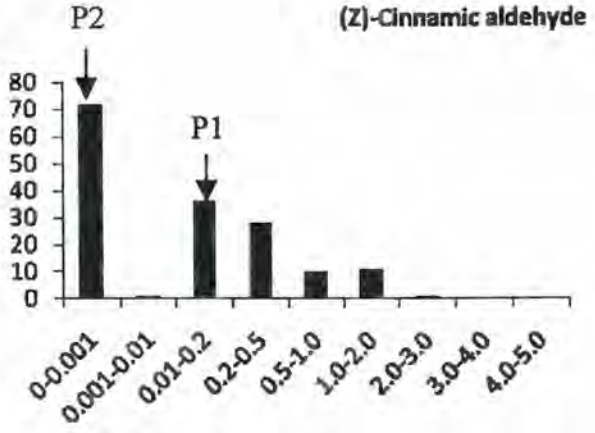
(g)

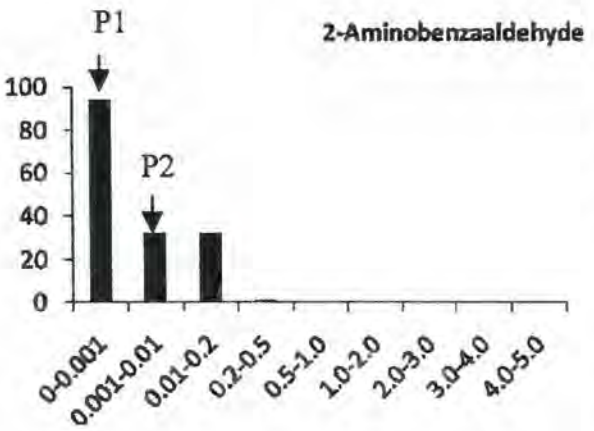


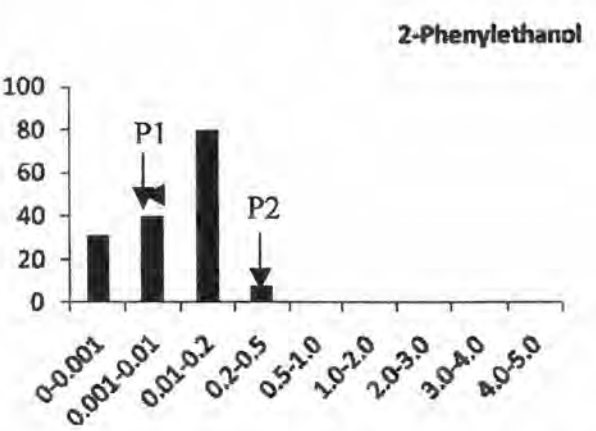
(h)

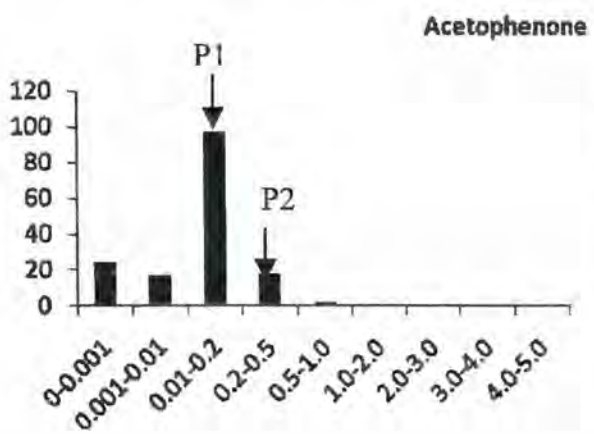
Figure 16 Representative examples that show the relative marker position of QTLs and LOD value that is responsible for different floral scent traits on different linkage groups; a) 3-octanol, b) 3 phenylpropanal, c) benzylalcohol, d) benzaldehyde, e) cinnamic alcohol, f) (E) cinnamic aldehyde, g) 3-octanone, h) Methyl (Z) cinnamate.

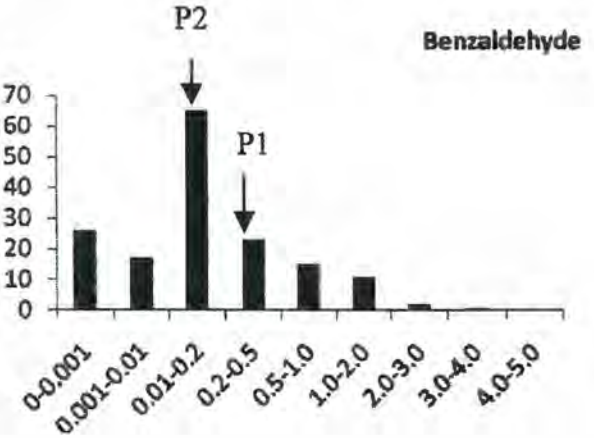

(E)-Cinnamic aldehyde
P2
P1
80
70
60
50
40
30
20
10
0
0-0.001
0.001-0.01
0.01-0.2
0.2-0.5
0.5-1.0
1.0-2.0
2.0-3.0
3.0-4.0
4.0-5.0


(Z)-Cinnamic aldehyde
P2
P1
80
70
60
50
40
30
20
10
0
0-0.001
0.001-0.01
0.01-0.2
0.2-0.5
0.5-1.0
1.0-2.0
2.0-3.0
3.0-4.0
4.0-5.0


2-Aminobenzaaldehyde
P1
P2
100
80
60
40
20
0
0-0.001
0.001-0.01
0.01-0.2
0.2-0.5
0.5-1.0
1.0-2.0
2.0-3.0
3.0-4.0
4.0-5.0


2-Phenylethanol
P1
P2
100
80
60
40
20
0
0-0.001
0.001-0.01
0.01-0.2
0.2-0.5
0.5-1.0
1.0-2.0
2.0-3.0
3.0-4.0
4.0-5.0


Acetophenone
P1
P2
120
100
80
60
40
20
0
0-0.001
0.001-0.01
0.01-0.2
0.2-0.5
0.5-1.0
1.0-2.0
2.0-3.0
3.0-4.0
4.0-5.0


Benzaldehyde
P2
P1
70
60
50
40
30
20
10
0
0-0.001
0.001-0.01
0.01-0.2
0.2-0.5
0.5-1.0
1.0-2.0
2.0-3.0
3.0-4.0
4.0-5.0

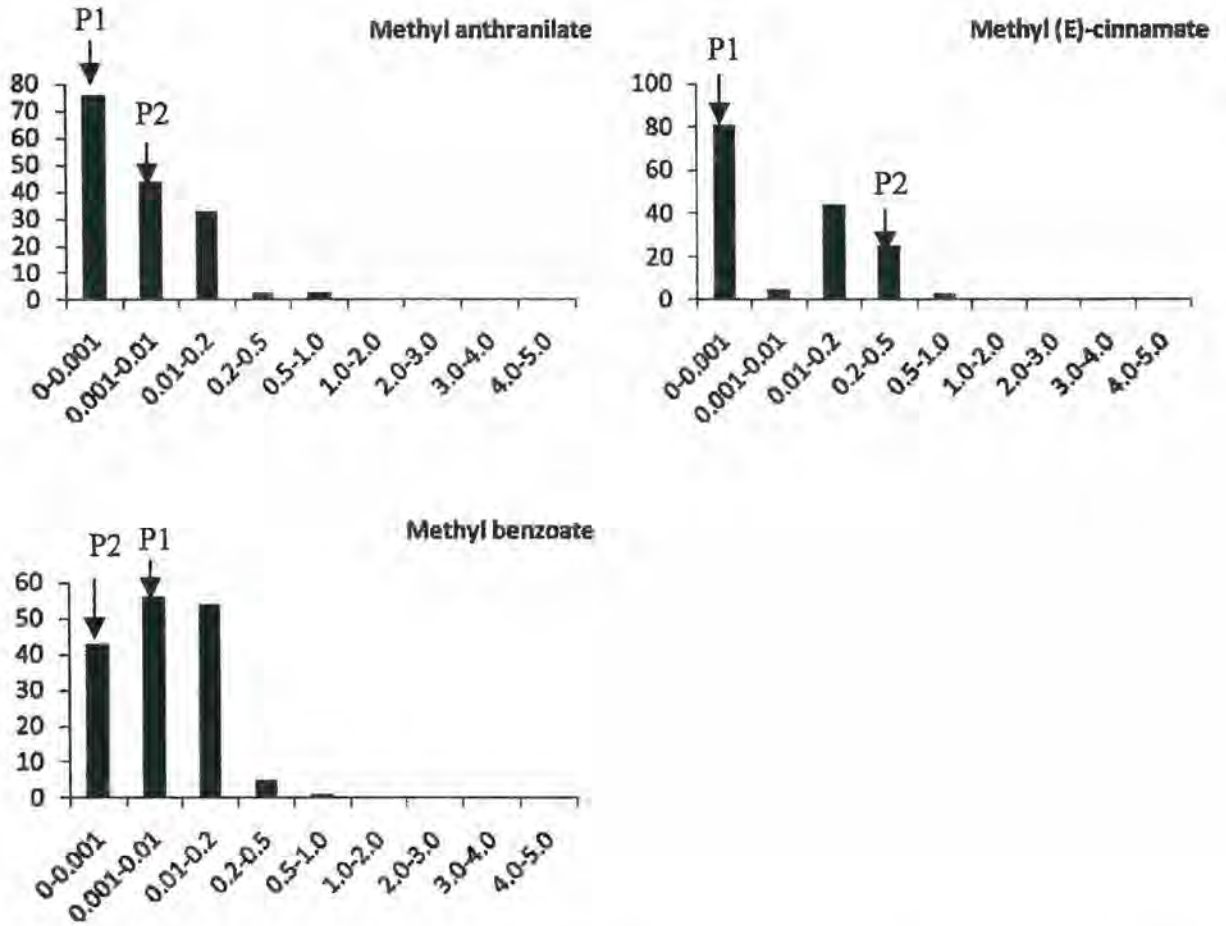


Figure 17 Frequency distributions of key volatile compounds (alcohol A4; aldehydes AL1, AL2, AL4, AL5; ketone K2; and esters E1, E3, E6) measured as absolute peak areas in the '524B' × '219-01' F_7 RILs. The mean values of the parents are indicated by arrows (P1-524B; P2-219-01). X-axis: Relative concentration, y-axis: frequency

CHAPTER FIVE

DISCUSSION, CONCLUSIONS and RECOMMENDATIONS

DISCUSSION

5.1 Genetic linkage map

I developed a linkage map for an F_7 recombinant inbred line population (N= 159) of cowpea using 202 SSR markers and four morphological markers. The map consisted of 11 linkage groups that cover 2991 cM of the genome with an average interval of 14.5cM between markers. The framework map that is presented here has so many diverse advantages due to the number as well as the quality of the markers. Additionally, the strong differences between the parental phenotypes and the segregation of these traits in the progeny, will allow researchers to investigate and map a large number of economically important traits. The map which was constructed by by Ouedraogo *et al.* (2002a) using Amplified Fragment Length Polymorphisms (AFLPs), gave a map that covers 2670 cM in 11 LGs. The size of the map constructed in this study is enhanced from 2670cM which was reported by Ouedraogo *et al.* (2002a) using AFLP to 2991cM. This can be expressed as since SSR markers are more evenly distributed and not clustered like AFLP markers, which provide a better coverage of the genome of cowpea with markers uniformly distributed in each linkage group. The use of a wild parent definitely enlarges the size of the map through access to areas that are not variable in domesticated gene pool. In addition the SSR markers developed in cowpea will show great promise for comparative mapping studies within the leguminosae and across wider species boundaries in the future.

5.1.1 SSR polymorphism

The parents that were used in this study 524B and 219-01 have contrasting characteristics for the traits that were studied. 524B is a California black-eyed type that shows resistance to Fusarium wilt and is developed from a cross between California cultivars CB5 and CB3 while 219-01 is a unique wild perennial outbreeding plant from coastal Kenya still kept after several years of vegetative multiplication. The level of polymorphism was 31.6 %. The low level of polymorphism obtained in the present study is not unexpected since the same levels of polymorphism have been observed in other studies (Varshney *et al.*, 2007) and similar observations were made with these sets of primers between other cultivars (Timko, personal communication).

5.1.2 Segregation of markers

In the present study 22 markers (10.67 %) showed segregation distortion. Almost half of these markers (64 %) fell into two linkage groups LG 1 and LG 2. In LG 1, 11 markers showed segregation distortion, and all the distorted markers were deficient in 219-01 alleles. In LG 2 five out of forty-one markers were distorted and the distortion followed a trend within the linkage group. All the distorted markers in LG 2 were deficient of the 524B alleles. Similar unidirectional distorted segregation has been reported in interspecific crosses of many crops including *Brassica* (Landry *et al.*, 1992), tomato (DeVicente and Tanksley, 1991), *Mimulus* species (Fishman *et al.*, 2001), *Populus* species (Yin *et al.*, 2004), in maize (Doebley *et al.*, 1990; Doebley and Stec, 1993; Xu *et al.*, 1997), rice (Cai and Morisima, 2002), soybean (Keim *et al.*, 1990a; Yamanaka *et al.*, 2001; Watanabe *et al.*, 2004) pearl millet (Poncet *et al.*, 2000, 2002)

intraspecific cross in *Medicago* (Jenczewski, 1997; Thoquet *et al.*, 2002). Segregation distortions can be caused by both pre-zygotic and post-zygotic factors. These include pollen-pistil incompatibility (Diaz and MacNair, 1999), meiotic drive (Lyttle, 1991), inbreeding depression (Remington and O'Malley, 2000), epistatic interaction between gametophytes and selection for specific alleles (Launey and Hedgecock, 2001; Yin *et al.*, 2004).

5.1.3 Mapping of genes controlling morphological traits

Singh and Jindla (1971) showed that erect pod attachment is dominant to drooping pod attachment and is regulated by a single gene. The observation in the parental population was in congruence with that reported by Menendez *et al.* (1997), i.e. while the plants are still young, 219-01 bears drooping pods and 524B shows erect pods; later when the plants mature the 524B pods tend to bend. This locus segregates at a 1:1 ratio in the F_7 generation and the *PPO* locus mapped on the LG8.

The purple and white flower color segregated at a 1:1 ratio in the F_7 generation with a chi-square value of 2.58 and the *fc* locus mapped on LG3 of the current linkage map; this shows that flower color is regulated by a single dominant gene only in this particular population unlike that of two different genes reported by Menendez *et al.* (1997). Jindla and Singh (1970) and Hanchinal and Goud (1978) reported dominant nature of violet color over very light violet color, whereas Uguru (1995) observed partial dominance of purple petal color over white petal color in a cross of white and purple petal colored parents. Harland (1919) and Spillman and Sando (1930) suggested that the R factor is essential for expression of flower color and rr for white color. Based on the above studies, the gene symbols R and rr are assigned for purple and white flower, respectively.

Flower color and pod color being less influenced by environmental variations are used as markers in the identification of species or varieties. Pigmentation is a common feature of cowpeas and its

presence is due to the anthocyanin pigment. Harland (1919) described that the presence of anthocyanin in pods is regulated by a single gene *P* where purple pigmentation of the pods is dominant over green pods. Many *P*-locus alleles have been described and from those described alleles *PP* produces a green pod with a purple tip. The wild parent (219-01) carries the dominant allele with pigmentation. The presence and absence of purple pigmentation at the tips of the pods also segregates at the expected 1:1 ratio in the F_7 generation and the *P* locus mapped on LG1of this map and this result is in congruence with Menendez *et al.* (1997). In addition to this it was possible to map the location of root architecture based on the tap root as well as fibrous root system for the first time and it also segregates in a 1:1 ratio in the F_7 generation and the *ra* locus mapped on the same linkage group 1.

5.1.4 QTL map

In this study, the basic theory of QTL mapping with composite interval mapping was applied to map quantitative trait loci controlling seed weight, domestication traits and flower scent of cowpea in the SSR based genetic linkage map. The method of QTL mapping was proved to be accurate and systematic (Kinzer *et al.*, 1990; Ahn *et al.*, 1993; Backes *et al.*, 1995). In addition, QTL mapping has also been used to identify important genes of human diseases in animal models that may be then studied in human patients and families (Rise *et al.*, 1991; Todd *et al.*, 1991).

5.1.4.1 QTLs for Seed weight

In this study, unlike the previous studies I identified six QTLs in the genetic control of seed weight. As a whole, the QTLs for seed weight were scattered across four linkage groups (LG1, LG2, LG3

and LG 10), and each accounted for around 20% or less of the observed variation. As in our study, previous studies with cowpea and other legumes involving a wild parent showed families with none of the RIL approaching the seed weight of the domesticated parent, and therefore a partial dominance of low seed weight alleles (Drabo *et al.*, 1984; Abbo *et al.*, 1992; Fatokun *et al.*, 1992; Ubi *et al.*, 2000; Liu *et al.*, 2007). Both cowpea studies reported the involvement of 2 main areas (Fatokun *et al.*, 1992) or 4 main areas (Ubi *et al.*, 2000). Here we ended up with 6 QTLs but grouped in four areas in LG1, LG2, LG4, and LG10. Interestingly, the QTL from LG10 is closely linked with the major pod shattering QTL, like in Ubi *et al.* (2000) LG12, and the QTL from LG1 is fairly close to another pod shattering QTL.

Alleles with moderate additive effects were identified for most of the evaluated traits and specifically for seed weight trait. The positive additive effects indicate that the cultivated parent with high SW (524B) contributed the increasing allele, while the negative additive effects indicate that the wild parent with low SW (219-01) contributed the increasing allele. Those alleles, which confer higher seed weight, were derived from both the cultivated and wild parents. Alleles that improve the trait being derived from parents that are agronomically inferior have been identified for several plant species (Xiao *et al.*, 1998; Fulton *et al.*, 2000; Frary *et al.*, 2004; Wang *et al.*, 2004; Yoon *et al.*, 2006).

Given the small number of markers, the small number of progeny, the kind of molecular marker they used, and the unequal number of linkage groups that they had for the cowpea genetic map, the observed QTLs may not represent the number of loci that control seed weight. The importance of population size has been strongly emphasized by many researchers (Tanksley, 1993; Doerge *et al.*, 1997; Ripol *et al.*, 1999 and Collard *et al.*, 2005). A larger population size is critical in order to observe a representative sample of recombinations. Therefore, the larger the

population size, the higher the statistical power and the most likely significant QTLs with minor effects can be detected (Collard *et al.*, 2005). The mapping population used in this study is ideal for identifying the loci that regulates seed weight as well as the other domestication traits when it is compared with the previous studies. In addition, differences in the number and location of QTLs detected herein and those identified in previous studies maybe due to several causes including whether the loci are segregating for contrasting alleles and interaction among loci in the reference population (Holland, 2007).

5.1.4.2 QTLs for domestication traits

QTLs that control the domestication traits were distributed in six of the 11 linkage groups, and individual QTLs accounted for 5.6-26.1 % of the phenotypic variance observed. Previous studies on cowpea pod shattering indicated a one gene (Aliboh *et al*,. 1996) or a two genes (Mohammed *et al.*, 2010) control of the trait. Although we studied the trait using a quantitave measurement (pod fiber layer thickness) instead of a qualitative assessment (dehiscent versus non dehiscent), our results would confirm a two genes control, but with additional QTLs in LG 6. Unlike the QTLs that are responsible for seed weight the alleles which confer a thin testa size and a thick pod size were derived mainly from the cultivated parent (524B). A significant QTL influencing both the domestication traits and yield related trait was detected on linkage groups 1 and 10.

According to Collard *et al.* (2005), QTLs accounting for more than 10% of phenotypic variation (R^2) are major QTLs. The results obtained in this study suggest that the domestication related traits examined are controlled by one or two major QTLs and a number of genotype-dependent minor QTLs. This is in agreement with the genetic basis for domestication related traits (DRTs) reported in many crop species as reviewed by Ross-Ibarra (2005). Another commonly found trend of the genetic

basis of DRTs is a clustering of domestication-related QTLs. To this end , in this study three genomic regions show clustering and held QTLs for different traits, i.e. QTLs for seed weight, pod size and testa size in linkage groups 1 and 10; QTLs for seed weight and testa size in linkage group 2. The low level of clustering of QTLs may be partly due to the absence of developmental constraint towards each other.

By taking into consideration the highly polygenic nature of the traits analyzed and the considerably high number of progenies, it is expected to get QTLs with lower phenotypic variation (R^2 values). Based on QTL mapping studies in other species, it can be generalized that higher phenotypic variation for a given trait in the mapping population and a considerable higher marker density genotyping data are the pre-requisites for identifying the major QTLs that are responsible for explaining a higher phenotypic variation. However, in the present study, the marker density of the linkage map developed is moderate and also the range of variations for the targeted traits was not very high in the RILs. The marker density on this genetic linkage map can be improved after integrating more number of polymorphic markers so that more QTLs can be detected which have a major effect for different traits that are able to explain a higher phenotypic variation.

5.1.4.3 QTLs for flower scent

This study, reports QTLs that are responsible for cowpea flower scent and identified the involvement of 63 QTLs that took part in the genetic control of different scent volatile compounds. The QTLs were scattered across the nine linkage groups (LG1, LG2, LG3, LG4, LG5, LG6, LG7, LG8 and LG10) of the 11 chromosomes of cowpea. This is the first time such QTL have been reported.

From the 23 volatile compounds that were identified by the help of GC-MS technique quantitative trait loci were identified for the 22 volatile compounds in this population. It was only phenyl acetaldehyde (AL 6) that detected no QTL and it could be due to lack of variability for this trait in the parents of the controlled cross progeny. For most of the volatiles more than one QTL was identified and generally at least one QTL was observed for a specific volatile. Similar to the other traits of interest, the positive additive effects indicate that the cultivated parent (524B) very few scent compounds contributed the increasing allele, while the negative additive effects indicate that the wild parent (219-01) rich scent compounds also contributed the increasing allele. Generally the alleles that are mainly responsible for different volatile compounds derived mainly from the wild parent which is very rich in its scent compounds.

QTLs for scent volatiles were clustered mainly on linkage groups LG 1, 2 and 4 suggesting the involvement of these regions in volatile compounds metabolism. The region characterized by the largest cluster of QTLs was the upper portion of LGs 1 and 4. Co-localizations of QTLs for alcohols benzylalcohol, 3-phenylpropanol, 1-phenylethanol, 3-octanol, and 1-octen-3-ol, and the esters methyl salicylate, methyl (Z) - Cinnamate and methyl (E) - Cinnamate at the upper portion of linkage group 1 could be due to biochemical relationships. QTLs for Benzylalcohol, 2-Phenylethanol, 3 – Phenylpropanol and 3- Octanol on LG 4 might also have the same physiological origin as well as the two aldehyde QTLs on the same linkage group. Co-localization of QTLs for aroma volatiles derived from the same metabolic pathway was also shown in tomato (on LG1-fatty acid metabolism derived two volatiles and on LG 9 two phenolic compounds) (Saliba-Colombani *et al.*, 2001). Tiemann *et al.* (2006) identified multiple QTL loci that affected sets of related volatiles. Clustering of QTLs can occur either due to the presence of

a single locus with pleiotropic effects on several volatiles or as a result of tightly linked different loci. Such loci may encode transcription factors that co-ordinately regulate genes, or they may encode enzymes that catalyse limiting steps in single pathways (Tiemann *et al.*, 2006).

Since clustering of QTLs has important implications for plant breeding programmes the finding that I reported here has a positive impact in cowpea breeding because selection of the ideal genotype of one QTL region could simultaneously improve several other traits positively. In general for QTL clusters, where both desirable and undesirable traits map together, fine mapping and analysis of near-isogenic substitution lines is necessary to determine whether there are multiple QTLs or a single QTL with pleiotropic effects.

5.1.5 Variability and scent composition of volatile compounds

Twenty-three volatile compounds were identified by gas chromatography-mass spectroscopy (GC/MS) and they were analyzed for the variability of their concentrations in the parental '524B' and '219-01' as well as in the 159 F_7 recombinant inbred lines. The majority of the volatiles were alcohols, esters and aldehydes but also ketones, phenylacetonitrile and indole were identified and measured in different quantities. In general, based on the result of the floral scent volatile compounds, they are grouped into four main chemical compound classes (i.e. fatty acid derivatives, benzenoids, phenyl propanoids and nitrogen containing compounds). The compounds are ordered in classes reflecting their biosynthetic origin: fatty acid derivatives (products of the malonic acid pathways), benzenoids (products of the shikimic acid pathways), phenyl propanoids (products of an intermediate compound of the shikimate pathways). Generally, fatty acid derivatives were present in low proportions while benzenoids were much

more common and nitrogen – containing compounds were not found except for indole. More compounds are usually detected in strong compared to weaker floral scent samples, because compounds found in minor and trace amounts in strong samples may, even if they are present, be below the analytical detection threshold in weaker samples.

Single scent compounds showed different frequency distributions in the parents as well as in the offsprings indicating a polygenic nature of the traits. This is what to expect because the biosynthesis of the aromatic compounds found in cowpea most likely follow a common route until chorismic acid, where after the nitrogen containing compounds, except phenylacetonitrile, follow the tryptophan pathway and the remaining the phenylalanine pathway (Wakasa & Ishihara, 2009). The diversity and plasticity of the final products derived from phenylpropanoids are catalyzed by oxygenases, ligases, oxidoreductases and various transferases with differing substrate specificities (Boatright *et al.*, 2004; Long *et al.*, 2009; Vogt, 2010). Although scent is a highly appreciated organoleptic attribute, little is known about the inheritance patterns of the scent trait and the main compounds associated with it apart from the information available on the biochemistry of scent volatiles. A few studies have been done in tomatoes (Causse *et al.*, 2001; Saliba-Colombani *et al.*, 2001), strawberries (Carrasco *et al.*, 2005; Olbricht *et al.*, 2008) and the inheritance of Mendelian loci for terpenoid composition in *Mentha* sp. has been reported (Gershenzon *et al*, 2000) along with a study identifying QTLs responsible for terpene oil content in Eucalyptus (Shepherd *et al.*, 1999). In the present study, we found that most of the cowpea scent compounds analyzed showed a distribution in the F_7 recombinant inbred lines, which indicate that they are under genetic control.

Since transgene escape from GM cowpea to its wild or weedy populations through gene flow may pose potential ecological consequences, due to the unique characteristics of transgenes that are genetically modified. QTLs that are specifically associated with floral scent traits are very important in order to block pollen flow from domesticated to wild cowpea, thus preventing the risk that insect-resistance transgene moves into wild cowpea gene pool and potentially turn wild cowpea plants into aggressive weeds. So one of the main concerns linked with the deployment of GM cowpea in Africa would then be overcome.

CONCLUSIONS AND RECOMMENDATIONS

This study showed the construction of molecular marker–based, SSR linkage map for cowpea and its potential exploitation for marker assisted selection, gene localization and cloning studies. The use of microsatellite markers for cowpea mapping was explored and it was shown that they are suitable marker class for these studies. Additionally, the microsatellite markers developed in cowpea will show great promise for comparative mapping studies within the leguminosae and across wider species boundaries.

The moderate marker density and the wide variety of trait loci present on this map promise to make this an invaluable resource for leguminosae researchers. The utility of a genetic map is related to its degree of completeness and as more marker data is obtained from the other leguminosae species under study, it will become possible to create a consensus map for the family, and genome synteny will become established. Furthermore, it will be possible to "shuttle" marker and gene information between the various species, which represents a cost-effective alternative to whole genome sequencing of cowpea or other leguminosae species.

QTL mapping study for domestication related traits and floral scent traits all of which are important traits in cowpea were revealed in this study. Several significant regions or potential QTLs associated with the inheritance and expression of these important traits is reported. These regions were associated with observable SSR markers from the linkage map. However, since this is the first attempt to locate QTLs associated with these traits in cowpea, further analyses are needed to confirm and validate these regions, which will even further enhance the understanding of the inheritance of the complex traits in cowpea.

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APPENDICES

Appendix 1 Segregation, x^2 goodness-of-fit analysis of 206 loci of 159 mapping lines in the F_7 recombinant inbred line population.

S/n	Nr	Locus	a	h	b	c	d	-	X2	Df
1	1	SSR-6375-1	72	0	82	0	0	5	1.30	1
2	2	SSR-6218	94	0	65	0	0	0	3.19	1
3	3	SSR-6323	82	0	77	0	0	0	0.49	1
4	4	SSR-6243*	92	0	67	0	0	0	6.12	1
5	5	SSR-6451**	61	0	98	0	0	0	10.97	1
6	6	SSR-6353	96	0	63	0	0	0	2.86	1
7	7	SSR-6273-1	80	0	79	0	0	0	0.02	1
8	8	SSR-6369	80	0	77	0	0	2	0.02	1
9	9	SSR-6277	77	0	82	0	0	0	0.92	1
10	10	SSR-6268	83	0	76	0	0	0	0.42	1
11	11	SSR-6280	75	0	84	0	0	0	0.44	1
12	12	SSR-6327	77	0	82	0	0	0	0.30	1
13	13	SSR-6294	85	0	74	0	0	0	0.83	1
14	14	SSR-6592*	90	0	69	0	0	0	6.28	1
15	15	SSR-6597	74	0	85	0	0	0	2.16	1
16	16	SSR-6607**	98	2	58	0	0	1	10.26	1
17	17	SSR-6611	87	0	70	0	0	2	1.84	1
18	18	SSR-6639	87	0	72	0	0	0	1.42	1
19	19	SSR-6641	75	0	82	0	0	2	0.86	1
20	20	SSR-6618	80	0	79	0	0	0	0.02	1
21	21	SSR-6645	74	0	85	0	0	0	0.83	1
22	22	SSR-6662*	62	0	90	0	0	7	5.16	1
23	23	SSR-6663-1	88	0	71	0	0	0	0.16	1
24	24	SSR-6666	76	0	83	0	0	0	0.46	1
25	25	SSR-6624	83	0	76	0	0	0	0.62	1
26	26	SSR-6603	69	0	90	0	0	0	2.25	1
27	27	SSR-6891	70	0	89	0	0	0	1.77	1
28	28	SSR-6895	76	0	83	0	0	0	1.10	1
29	29	SSR-6906	71	0	88	0	0	0	0.16	1
30	30	SSR-6856	82	0	77	0	0	0	0.17	1
31	31	SSR-6859	78	0	81	0	0	0	0.07	1
32	32	SSR-6367	84	0	75	0	0	0	0.29	1
33	33	SSR-6362	89	0	70	0	0	0	2.05	1
34	34	SSR-6360	78	0	81	0	0	0	0.15	1
35	35	SSR-6348**	67	0	92	0	0	0	8.53	1
36	36	SSR-6920	85	0	74	0	0	0	0.83	1
37	37	SSR6376*	93	0	63	0	0	3	5.77	1
38	38	SSR-6345	82	0	77	0	0	0	0.12	1

39	39	SSR-6372	78	0	81	0	0	0	0.15	1
40	40	SSR-6466*	90	0	69	0	0	0	6.85	1
41	41	SSR-6354	75	0	84	0	0	0	1.72	1
42	42	SSR-6331	87	0	72	0	0	0	1.37	1
43	43	SSR-6314	79	0	80	0	0	0	0.02	1
44	44	SSR-6302	86	0	73	0	0	0	1.49	1
45	45	SSR-6324*	71	0	88	0	0	0	6.43	1
46	46	SSR-6429	79	0	80	0	0	0	0.02	1
47	47	SSR-6313*	90	0	69	0	0	0	6.85	1
48	48	SSR-6921	86	0	73	0	0	0	1. 37	1
49	49	SSR-6922	84	0	75	0	0	0	1.72	1
50	50	SSR-6477	76	0	83	0	0	0	1.10	1
51	51	SSR-6516*	70	0	89	0	0	0	6.83	1
52	52	SSR-6513	98	0	61	0	0	0	2.90	1
53	53	SSR-6515	80	0	79	0	0	0	0.02	1
54	54	SSR-6519	79	0	79	0	0	1	0.83	1
55	55	SSR-6240*	69	0	89	0	0	1	6.80	1
56	56	SSR-6242	75	0	84	0	0	0	1.10	1
57	57	SSR-6228**	92	0	67	0	0	0	10.78	1
58	58	SSR-6225*	69	0	90	0	0	0	7.05	1
59	59	SSR-6222	76	0	83	0	0	0	0.42	1
60	60	SSR-6210	81	0	78	0	0	0	0.15	1
61	61	SSR-6211	61	0	96	0	0	2	2.90	1
62	62	SSR-6188	89	0	70	0	0	0	2.05	1
63	63	SSR-6192*	88	0	69	0	0	2	6.36	1
64	64	SSR-6395	79	0	80	0	0	0	0.02	1
65	65	SSR-6469*	88	0	69	0	0	2	6.36	1
66	66	SSR-6245	72	0	87	0	0	0	1.84	1
67	67	SSR-6520	86	0	73	0	0	0	1.64	1
68	68	SSR-6475	85	0	74	0	0	0	1.72	1
69	69	SSR-6465	81	0	78	0	0	0	0.28	1
70	70	SSR-6402	71	0	88	0	0	0	1.82	1
71	71	SSR-6604	95	0	64	0	0	0	3.38	1
72	72	SSR-6612	74	0	82	0	0	3	1.10	1
73	73	SSR-6623*	69	0	90	0	0	0	6.85	1
74	74	SSR-6626	83	0	75	0	0	1	1.72	1
75	75	SSR-6673	80	0	79	0	0	0	0.02	1
76	76	SSR-6909	81	0	78	0	0	0	0.16	1
77	77	SSR-6537	76	0	83	0	0	0	0.42	1
78	78	SSR-7078*	88	0	70	0	0	1	5.30	1
79	79	SSR-7079	78	0	81	0	0	0	0.83	1
80	80	SSR-6547	72	0	87	0	0	0	1.82	1
81	81	SSR-6609*	88	0	71	0	0	0	5.29	1
82	82	SSR-6657	92	0	67	0	0	0	2.36	1
83	83	SSR-6577	65	0	93	0	0	1	2.77	1
84	84	SSR-6807	78	0	81	0	0	0	0.15	1
85	85	SSR-6810	80	0	79	0	0	0	0.02	1
86	86	SSR-6838	80	0	78	0	0	1	0.16	1
87	87	SSR-6934	84	0	75	0	0	0	0.72	1
88	88	SSR-6927	74	0	85	0	0	0	1.10	1
89	89	SSR-6935	89	0	70	0	0	0	2.27	1

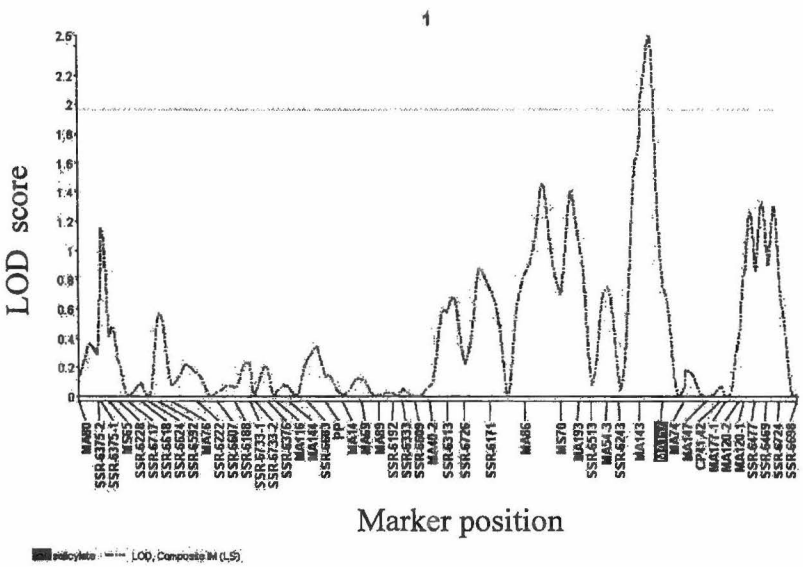
90	90	SSR-6939*	89	0	70	0	0	0	5.69	1
91	91	SSR-6944	86	0	73	0	0	0	1.03	1
92	92	SSR-6950	74	0	85	0	0	0	0.76	1
93	93	SSR-6962	75	0	83	0	0	1	1.70	1
94	94	SSR-6964	69	0	90	0	0	0	2.77	1
95	95	SSR-6941	82	0	77	0	0	0	0.57	1
96	96	SSR-6971	94	0	65	0	0	0	2.74	1
97	97	SSR-6973	84	0	72	0	0	3	1.10	1
98	98	SSR-6979-1	86	0	73	0	0	0	1.63	1
99	99	SSR-7014	85	0	74	0	0	0	2.58	1
100	100	SSR-7025	84	0	74	0	0	1	1.57	1
101	101	SSR-6996	73	0	86	0	0	0	1.49	1
102	102	SSR-7005	74	0	85	0	0	0	0.76	1
103	103	SSR-6998*	69	0	90	0	0	0	5.16	1
104	104	SSR-6994	74	0	83	0	0	2	0.74	1
105	105	SSR-7001	83	0	76	0	0	0	0.69	1
106	106	SSR-7015	87	0	70	0	0	2	1.92	1
107	107	SSR-7009-1	77	0	82	0	0	0	0.57	1
108	108	SSR-6990	80	0	79	0	0	0	0.02	1
109	109	SSR-6983	80	0	79	0	0	0	0.02	1
110	110	SSR-7017	85	0	74	0	0	0	1.10	1
111	111	SSR-6999	77	0	82	0	0	0	0.94	1
112	112	SSR-7004	83	0	75	0	0	1	0.43	1
113	113	SSR-6790	89	0	67	0	0	3	2.37	1
114	114	SSR-6788	76	0	83	0	0	0	0.67	1
115	115	SSR-6682	81	0	78	0	0	0	0.28	1
116	116	SSR-6683	83	0	76	0	0	0	0.52	1
117	117	SSR-6697	75	0	84	0	0	0	0.49	1
118	118	SSR-6699	86	0	71	0	0	2	1.33	1
119	119	SSR-6914	86	0	71	0	0	2	1.33	1
120	120	SSR-6719	96	0	63	0	0	0	3.89	1
121	121	SSR-6720	72	0	87	0	0	0	1.89	1
122	122	SSR-6717	80	0	79	0	0	0	0.02	1
123	123	SSR-6701	82	0	77	0	0	0	0.57	1
124	124	SSR-7052	75	0	84	0	0	0	1.27	1
125	125	SSR-7053-1	72	0	84	0	0	3	0.76	1
126	126	SSR-7061*	71	0	83	0	0	5	4.19	1
127	127	SSR-7068	74	0	84	0	0	1	0.85	1
128	128	SSR-7072	80	0	79	0	0	0	0.02	1
129	129	SSR-7063	70	0	89	0	0	0	2.27	1
130	130	SSR-7045-1	61	0	98	0	0	0	3.89	1
131	131	61R	77	0	82	0	0	0	0.57	1
132	132	61R2	73	0	86	0	0	0	1.74	1
133	133	SSR-7041	83	0	76	0	0	0	0.67	1
134	134	SSR-6915-1	86	0	73	0	0	0	1.42	1
135	135	bok	72	0	84	0	0	3	1.89	1
136	136	SSR-6680	79	0	80	0	0	0	0.02	1
137	137	SSR-6686	81	0	78	0	0	0	0.06	1
138	138	SSR-6694-1	75	0	84	0	0	0	1.70	1
139	139	SSR-6694-2	79	0	80	0	0	0	0.02	1
140	140	SSR-6698	80	0	79	0	0	0	0.02	1

141	141	SSR-6705	89	0	70	0	0	0	2.33	1
142	142	SSR-6916-1	76	0	82	0	0	1	1.60	1
143	143	SSR-6916-2	85	0	74	0	0	0	1.10	1
144	144	SSR-6917	77	0	82	0	0	0	0.57	1
145	145	SSR-6918	86	0	73	0	0	0	1.42	1
146	146	SSR-6919	71	0	86	0	0	2	1.29	1
147	147	SSR-6724	76	0	83	0	0	0	0.67	1
148	148	SSR-6726	96	0	63	0	0	0	3.93	1
149	149	SSR-6730	75	0	84	0	0	0	0.85	1
150	150	SSR-6743	78	0	81	0	0	0	0.06	1
151	151	SSR-6744-1	82	0	77	0	0	0	0.16	1
152	152	SSR-6744-2	79	0	80	0	0	0	0.02	1
153	153	SSR-6947	73	0	86	0	0	0	1.06	1
154	154	SSR-6965-1	82	0	77	0	0	0	0.57	1
155	155	SSR-6965-2	82	0	77	0	0	0	0.57	1
156	156	SSR-7027-1	72	0	84	0	0	3	1.85	1
157	157	SSR-7027-2	87	0	72	0	0	0	1.42	1
158	158	SSR-7027-3	81	0	78	0	0	0	0.06	1
159	159	SSR-7027-4	78	0	81	0	0	0	0.06	1
160	160	SSR-7028	94	0	65	0	0	0	2.77	1
161	161	SSR-7040-1	79	0	80	0	0	0	0.02	1
162	162	SSR-7040-2	83	0	76	0	0	0	1.94	1
163	163	SSR-7040-3	77	0	82	0	0	0	0.57	1
164	164	SSR-7040-4	67	0	92	0	0	0	2.74	1
165	165	SSR-7043	70	0	89	0	0	0	2.27	1
166	166	SSR-7056	81	0	78	0	0	0	0.06	1
167	167	SSR-7060	89	0	70	0	0	0	2.27	1
168	168	SSR-7067	86	0	73	0	0	0	1.40	1
169	169	SSR-7069	77	0	82	0	0	0	0.57	1
170	170	SSR-6982-1	83	0	76	0	0	0	0.31	1
171	171	SSR-6982-2	65	0	94	0	0	0	2.77	1
172	172	SSR-6982-3	81	0	78	0	0	0	0.06	1
173	173	SSR-7000-1	77	0	82	0	0	0	0.57	1
174	174	SSR-7000-2	86	0	73	0	0	0	1.70	1
175	175	SSR-7000-3	94	0	65	0	0	0	2.77	1
176	176	SSR-7009-2	82	0	77	0	0	0	0.16	1
177	177	SSR-7011	77	0	82	0	0	0	0.16	1
178	178	SSR-7013-1	86	0	73	0	0	0	1.06	1
179	179	SSR-7013-2	79	0	80	0	0	0	0.02	1
180	180	SSR-7082	82	0	77	0	0	0	0.16	1
181	181	SSR-7101-1	76	0	83	0	0	0	0.31	1
182	182	SSR-7101-2	88	0	71	0	0	0	1.82	1
183	183	SSR-7117	71	0	86	0	0	2	1.29	1
184	184	SSR-6375-2	73	0	86	0	0	0	1.06	1
185	185	SSR-6273-2	82	0	77	0	0	0	0.16	1
186	186	SSR-7053-2	65	0	94	0	0	0	2.77	1
187	187	SSR-7045-2	79	0	80	0	0	0	0.02	1
188	188	SSR-6663-2	84	0	75	0	0	0	0.52	1
189	189	SSR-6204	77	0	82	0	0	0	0.16	1
190	190	SSR-6733-1	71	0	88	0	0	0	1.82	1
191	191	SSR-6733-2	82	0	77	0	0	0	0.16	1

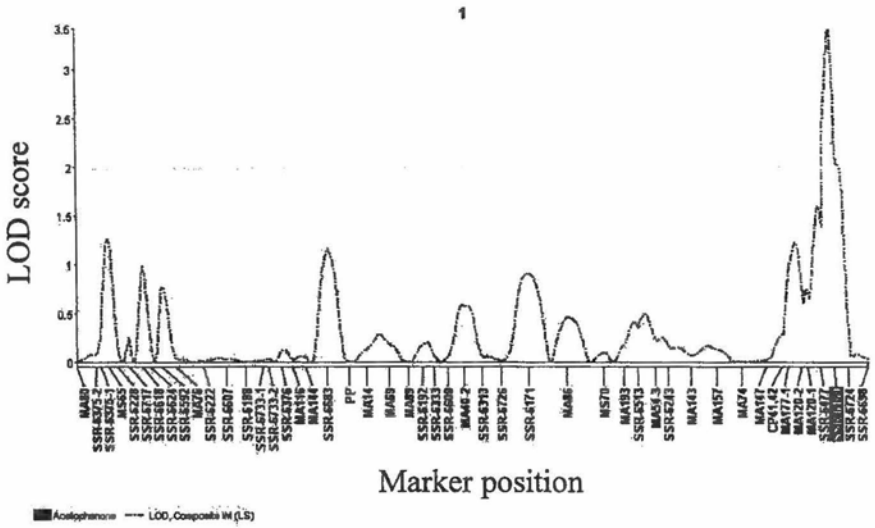
192	192	SSR-6923	76	0	83	0	0	0	0.31	1
193	193	SSR-6171	84	0	75	0	0	0	0.52	1
194	194	SSR-6924-1	89	0	70	0	0	0	2.27	1
195	195	SSR-6924-2	91	0	68	0	0	0	3.33	1
196	196	SSR-6312	75	0	84	0	0	0	0.52	1
197	197	SSR-6594	77	0	82	0	0	0	0.16	1
198	198	SSR-6333	77	0	82	0	0	0	0.16	1
199	199	SSR-7008-1	84	0	75	0	0	0	0.52	1
200	200	SSR-7008-2	79	0	80	0	0	0	0.02	1
201	201	SSR-6978-2	80	0	79	0	0	0	0.02	1
202	202	SSR-6978-3	73	0	86	0	0	0	1.06	1
203	203	fc	70	0	89	0	0	0	2.58	1
204	204	pp	73	0	86	0	0	0	1.06	1
205	205	ppo	86	0	73	0	0	0	1.06	1
206	206	ra	80	0	79	0	0	0	0.02	1

* and ** are levels of significance at $p \leq 0.05$ and $p \leq 0.01$, respectively.

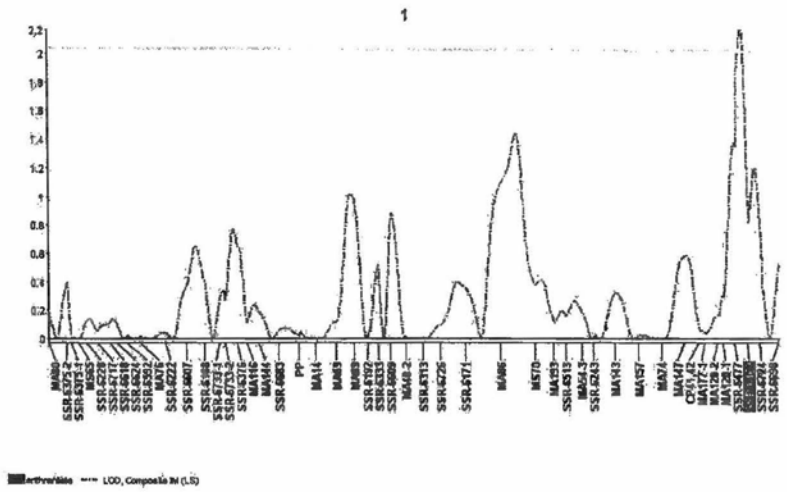
Appendix 2 Relative marker position of QTLs and LOD value for different scent traits at different linkage groups of the map. On LG1 (a) Methyl salicylate, (b) Acetophenone (c) Methylanthranilate, (d) Methyl phenylacetate (e) (Z) Cinnamic aldehyde; on LG2 (f) Acetophenone, (g) Indole, (h) 3 Phenylpropanol; on LG3 (i) (Z) cinnamic Aldehyde; on LG4 (j) (Z) cinnamic aldehyde; on LG5 (k) cinnamic alcohol, (l) methyl (E) cinnamate; on LG6 (m) Phenylacetonitrile; on LG8 (n) (Z) cinnamic aldehyde; on LG10 (o) Methyl benzoate.



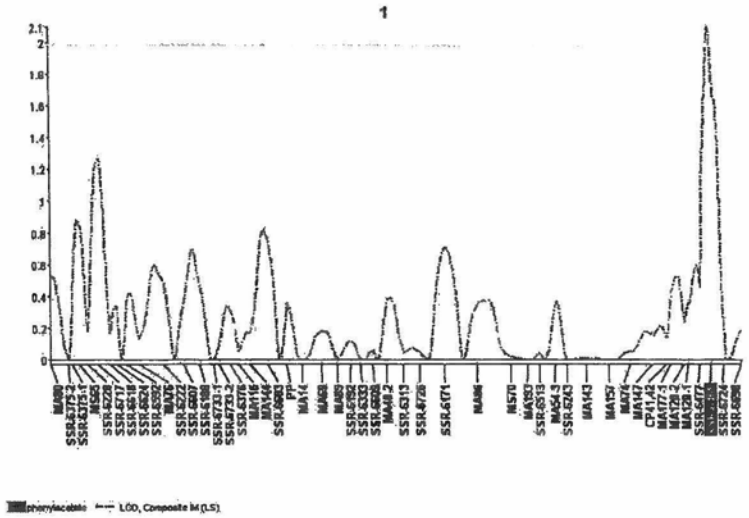
(a)


1
LOD score
Marker position
Acetophenone
LOD, Composite IM (LS)

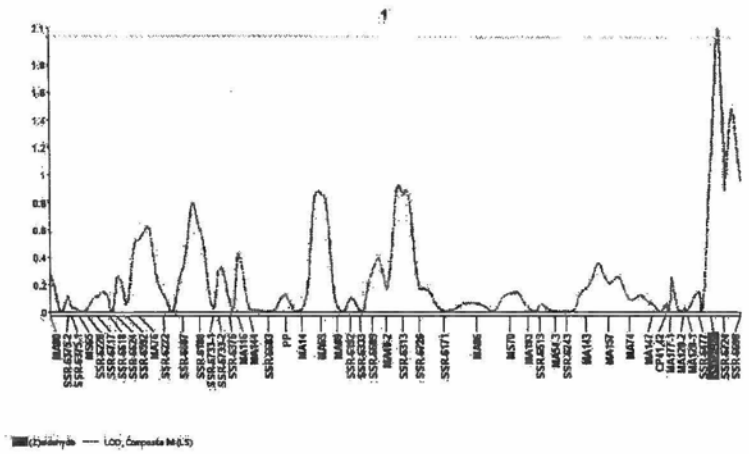
(b)


1
LOD, Composite IM (LS)

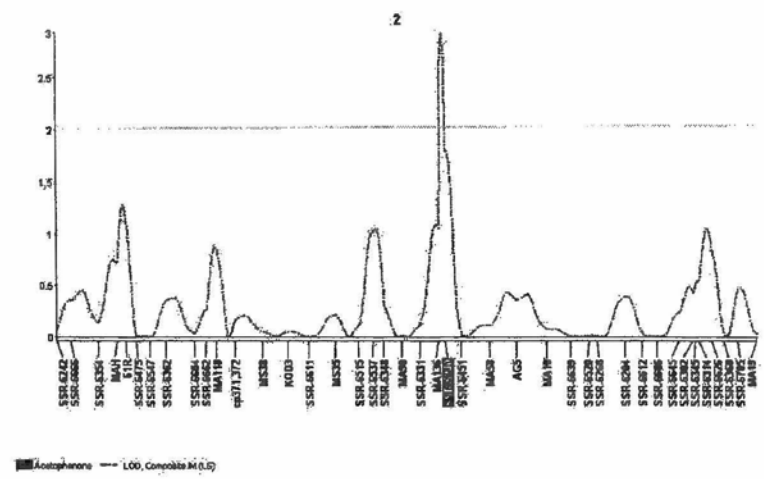
(c)


1
LOD, Composite Int (LS)

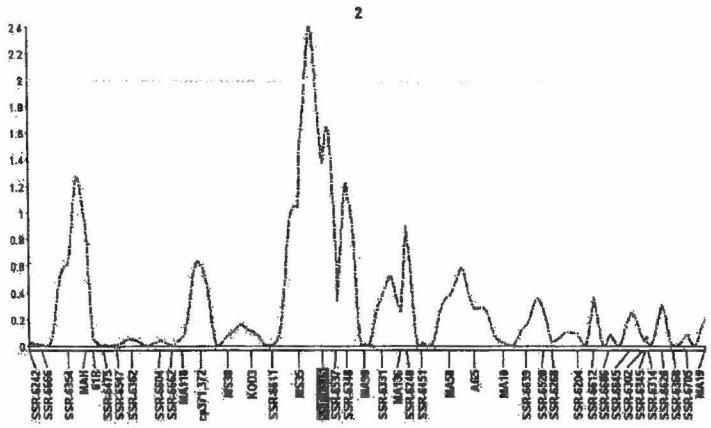
(d)


1
LOD, Composite Int (LS)

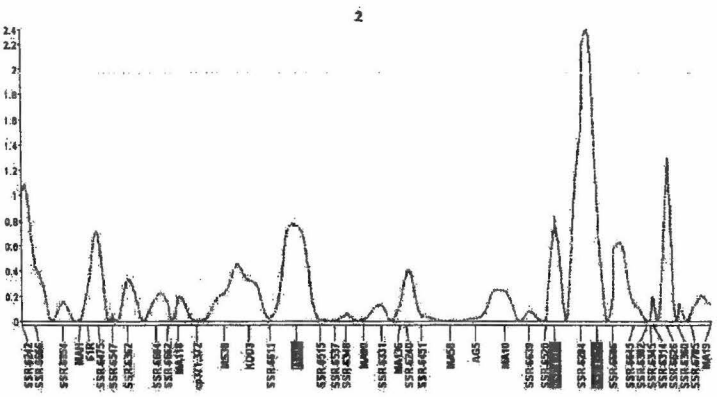
(e)


2
LOD, Composite Int (LS)

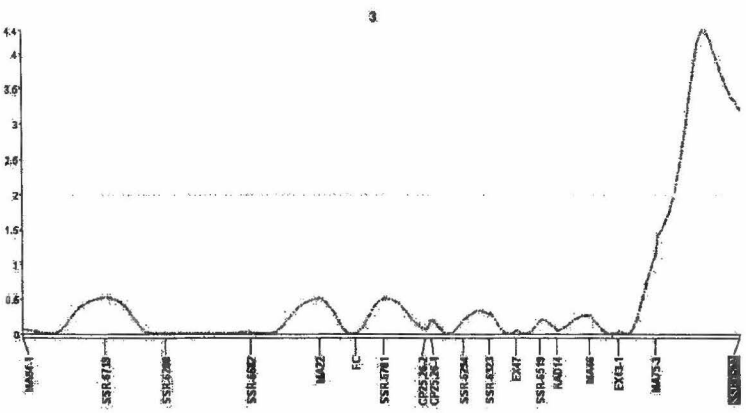
(f)



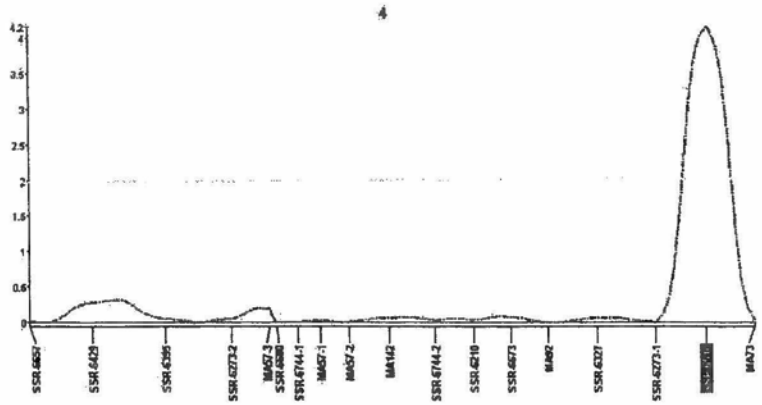
(g) Indole --- LOD, Composite IM (LS)



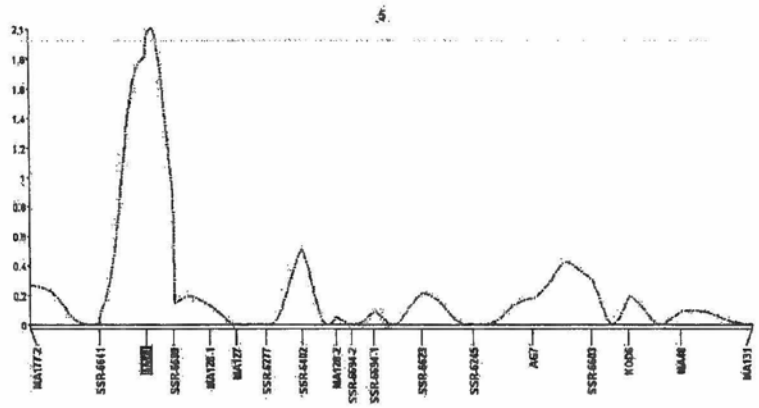
(h) 3-Phenylpropanol --- LOD, Composite IM (LS)



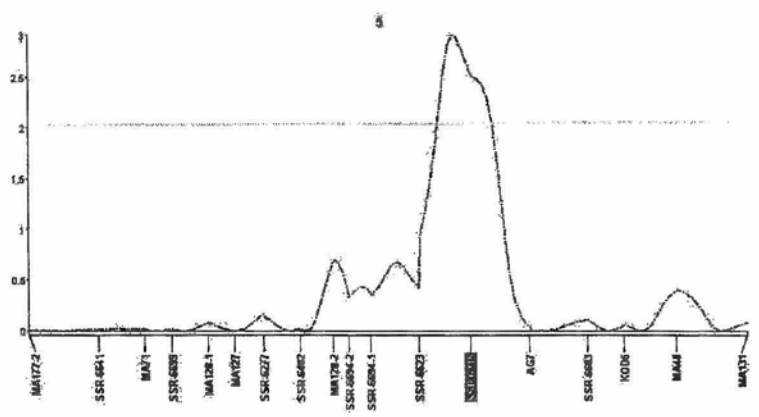
(i) (Z)-Methyl --- LOD, Composite IM (LS)



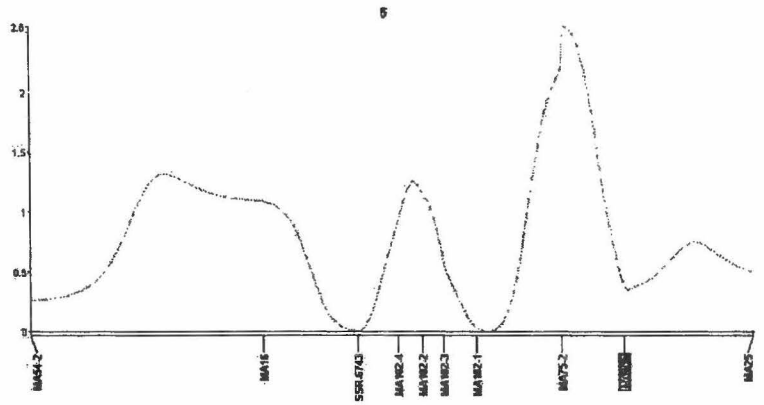
(j)



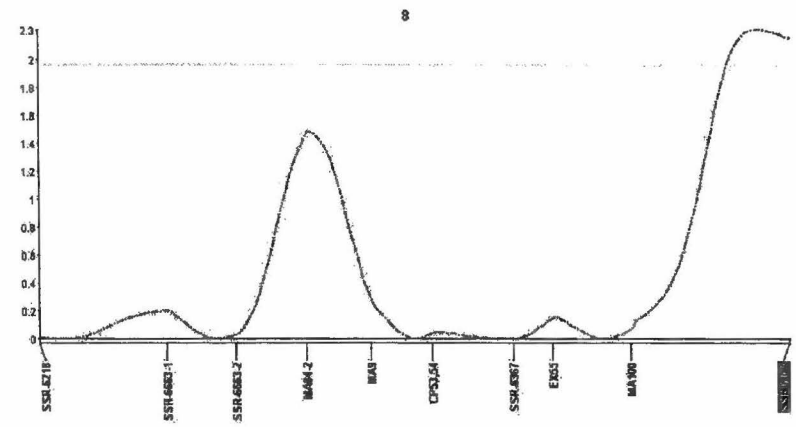
(k)



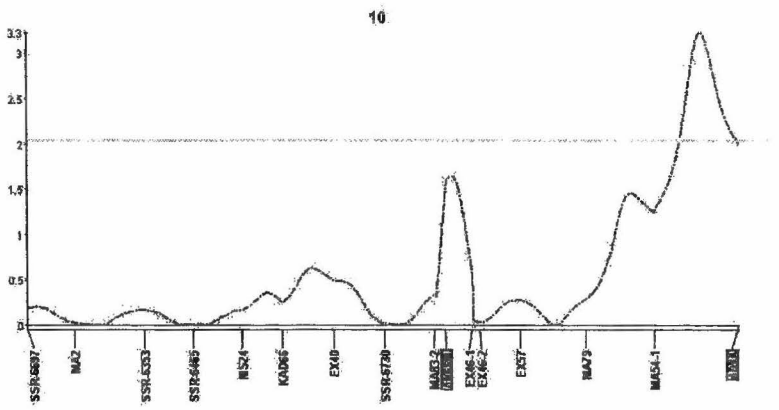
(l)



(m) [illegible]phenylacetonitrile — LOD, Composite IM (LS)



(n) [illegible](Z)-dodec-9-enal — LOD, Composite IM (LS)



(o) [illegible]benzoate — LOD, Composite IM (LS)

Appendix 3 Publications

3.1 Abstracts:

Mebeaselassie, A., Pasquet, S.R., Gowda, S.B., Muluvi, M.G., and Timko, P.M. (2010) Mapping of Quantitative Trait Loci (QTLs) associated with domestication characteristics using a recombinant inbred population derived from a cross between wild and cultivated cowpea (*V. unguiculata* (L.) Walp.) Presented at the Vth International Congress on Legume Genetics and Genomics, 2 – 8 July, Asilomar, California, USA.

Mebeaselassie, A., Pasquet, S.R., Knudsen, J., Muluvi, M.G., and Timko, P.M. (2010) QTL mapping of flower scent compounds analyzed by headspace adsorption gas chromatography in cowpea (*V. unguiculata* (L.) Walp.) Presented at the 2nd International Symposium on Genomics of Plant Genetic Resources, 24 – 27 April, Bologna, Italy.

3.2 Proceeding:

Mebeaselassie, A., Emana, G., and Bekele, J. 2006. Studies on the pest status of bean bruchids & management of major bruchid species in central rift valley of Ethiopia. Proceedings of the XVIth Conference of the Biological Society of Ethiopia, 10-11 February 2006, Pp. 41-53, Addis Ababa,
Ethiopia.

3.3 Thesis:

Mebeaselassie, A. (2004) Bioefficacy of plant derivative against the bean bruchid, *Zabrotes subfasciatus* (Bruchidae: Coleoptera) in stored beans. M.sc research thesis submitted and defended at the Department of Biology, Addis Ababa University, Ethiopia, in partial fulfillment of the award of Masters Degree in Entomology.

3.4 Articles published:

Mebeaselassie, A., Pasquet, S.R., Gowda, S.B., Muluvi, M.G., and Timko, P.M. 2010. Mapping of Quantitative Trait Loci (QTLs) associated with domestication characteristics using a recombinant inbred population derived from a cross between wild and cultivated cowpea (*V. unguiculata* (L.) Walp.) Theoretical Applied Genetics (in press).

Mebeaselassie, A., Pasquet, S.R., Gowda, S.B., Muluvi, M.G., and Timko, P.M. 2010. The first SSR-based genetic linkage map of cowpea (*V. unguiculata*) from the interspecific cross between inbred and wild lines. Genome (in press).

Mebeaselassie, A., Emana, G., and Bekele, J. 2007. Bioefficacy of products derived from *Milletia ferruginea* (Hochst) baker against the bean bruchid, *Zabrotes subfasciatus* (Bruchidae: Coleoptera) in stored beans. Insect Science and Its Application. 27(1): 33-45.

3.5 Articles in preparation:

Mebeaselassie, A., Pasquet, S.R., Knudsen, J., Muluvi, M.G., and Timko, P.M. 2010. QTL mapping of flower scent compounds analyzed by headspace adsorption gas chromatography in cowpea (*V. unguiculata* (L.) Walp.) (in progress).

Mebeaselassie, A., Pasquet, S.R., Muluvi, M.G., and Timko, P.M. 2010. Quantitative trait loci analysis of flowering-time-related traits identified in recombinant inbred lines of cowpea (*V. unguiculata* (L.) Walp.) (in progress).