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GENETIC ANALYSIS OF

PLANT MORPHOLOGY IN BAMBARA GROUNDNUT

[Vigna subterranea (L.) Verdc.]

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Abstract

Bambara groundnut (Vigna subterranea (L.) Verdc.) is an important underutilised legume crop, grown mainly by female subsistence farmers in Africa under traditional low input agricultural systems. Bambara groundnut is known as being of high nutritional value, as an atmospheric nitrogen fixer and to possess high levels of drought, pest and disease tolerance. Bambara groundnut is a predominantly self-pollinated crop and is grown as locally adapted landraces. These are expected to exist as non-identical inbred lines and are generally low yielding. Strategies involving genetic analysis of this species could provide important data for breeding programmes that could enhance food security in Africa.

A set of 124 SSR primers designed from different library sources were tested to screen a 'narrow' genetic cross (F_3) and a 'wide' genetic cross (F_2) . The former is a cross between domesticated landraces (DipC and Tiga necaru) while the latter is a cross between a domesticated landrace and a wild ancestor (DipC and VSSP11).

Residual heterozygosity in the F_3 'narrow' cross was confirmed to be around 25% based on 33 polymorphic SSR primers, consistent with an F_3 population. A 'narrow' cross linkage map was constructed for the first time in bambara groundnut using 269 polymorphic markers (236 DArT and 33 SSR). The map consisted of 238 markers in 21 linkage groups of two or more linked markers, totalling 608.1cM and covering a predicted 54% of the bambara groundnut genome, although the high marker-marker linkage (at 89%) suggests a more comprehensive coverage. QTL analysis was carried out for 73 bulked lines of an F_3 population and plants were evaluated for traits in a controlled glasshouse suite and a field trial in Indonesia. Data from single plant analysis of the F_2 generation of this cross grown in a controlled environment

glasshouse was also used. Most of the QTLs detected were clustered on linkage groups 1, 4 and 12. Major QTLs for internode length and biomass dry weight were detected on LG4 and LG1, respectively, for the FutureCrop glasshouse and field datasets. The highest LOD score of 9.7 was detected for peduncle length and was located within the confidence interval for a QTL for internode length locus. Marker locus bgPabg-596774 was detected to be associated with QTL for six traits; node no./plant, pod no/plant, pod weight, seed no./plant, seed yield and biomass dry weight, on LG1 within one LOD score of confidential interval, potentially suggesting pleiotropic effects of a more limited number (or even one) gene(s).

One hundred and fifty-nine additional markers (136 DArT and 23 SSR) were used to improve the existing partial 'wide' map (141 AFLP, 1 SSR) constructed in an F_2 population of 98 plants. A total of 194 markers were assigned to 20 linkage groups spanning a total of 901 cM. The linkage map derived from the 'wide' cross (DipC x VSSP11) had an expected genome coverage of 79.6%. An attempt to combine both maps through 32 common markers allowed a common QTL for days to emergence to be detected in both populations in close association with the common DArT markers 601384 and 601748.

The main segregating traits were found to be plant spread, internode length, growth habit, peduncle length, pod weight, seed yield and biomass dry weight. Detecting the same QTL positions for a number of traits, suggested that common underlying genes might be responsible. The QTL-DNA marker associations developed in this study could be used practically for MAS in a future breeding program of this crop.

Publication

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List of abbreviations

μL   Microlitre (0.000001 Litres)

μM   Micromole, equal to 10^{-6} mole

ABI   Applied bio system

AFLP   Amplified Fragment Length Polymorphism

BC   Back cross

BDW   Biomass dry weight

bp   Base pair

CAPS   Cleaved amplified polymorphic sequences

CFFRC   Crops for the Future Research Centre

CID   Carbon isotope discrimination

CIM   Composite interval mapping

CISR   Conserved intron spanning region

cM   CentiMorgan

COS   Conserved orthologous sequences

CP   Cross Pollinator

CRD   Complete randomised design

DArT   Diversity Arrays Technology

DE   Days to emergence

DF   Days to flowering

DNA   Deoxyribonucleic Acid

dNTP   Deoxynucleotide Triphosphates (usually mix of dATP/dTTP/dCTP/dGTP)

DPL   Pod length of double seeded

DPN   double seeded pods/plant

DPW   Pod width of double seeded

EDTA   Ethylenediaminetetraacetic acid

E_i Expected frequency

EP   Eye pattern around hilum

EST-SSR   Expressed Sequence Tag-SRR

FN   Flower no./plant

FTh   Flower bud thrip

GFU   Global Facilitation unit for Underutilized species

GH   Growth habit

GMM   Genic molecular marker

HSW   100-seed weight

ICUC   International Centre for Underutilized Crops

IITA   International Institute of Tropical Agriculture

IL   Internode length

IM   Interval mapping

ISSR   Inter-simple sequence repeat

KW   Kruskal-Wallis analysis

LA   Leaf area

LG   Linkage group

LOD   Logarithm of odds

LPA   Linear polyacrylamide

MAS   Marker-assisted selection

MIM   Multiple interval mapping

MISA   Microsatellite identification tool

mM   Millimole, equal to 10^{-3} mole

ng   Nanogram (One billionth (10^{-9}) of a gram)

NN   Node no./stem

O_i Observed frequency

PCR   Polymerase chain reaction

PEL   Peduncle length

PLE   Pod length

pmol   Picomole, equal to 10^{-12} moles

PN   Pod no./plant

PS   Plant spread

PT   Permutation test

PVE   Phenotypic variation explained

PWD   Pod width

PWE   Pod weight

QTL   Quantitative trait loci

RAPD   Random Amplification of Polymorphic DNA

RFLP   Restriction fragment length polymorphism

RIL   Recombinant inbreed line

SDW   Sterile distilled water

SEL   Seed length

SEN   Seed no./plant

SEW   Seed width

SH%   Shelling percentage

SLA   Specific leaf area

SLS   Sample loading solutions

SNP   Single Nucleotide Polymorphism

SMA   Single marker analysis

SS   Size standard

SSR   Simple sequence repeat

STN   Stem no./plant

STR   Short tandem repeat

SWT   Seed weight

TBE   Tris/Borate/EDTA

TCRU Tropical Crops Research Unit

TLL   Terminal leaflet length

TLW   Terminal leaflet width

tRNA Transfer ribonucleic acid

UV   Ultraviolet

Chapter 1. INTRODUCTION

1.1 Legumes: an overview

The legume family is one of the largest families of the angiosperms. Plants in the leguminosae family (alternative name, Fabaceae) form three identified sub-families; Papilionoideae, Caesalpinioideae and Mimosoideae. The first sub-family, with approximately 70% of the Leguminosae species, is the largest subfamily and includes the important edible legume crops and major model legume species (Cannon et al., 2009; Doyle and Luckow, 2003). The Fabaceae family is the third largest amongst flowering plants, with 18,000 species and 650 genera (Polhill et al., 1981).

The wide distribution of leguminosae throughout the world and their high protein content gives them great potential as food sources. Their further cultivation and increased utilization would positively contribute toward improving food security. In Europe consumption of legume seeds is 2.5 kg/capita per annum while in other parts of the world higher consumption rates have been recorded (5.4 – 14.4 kg/capita) (Schuster-Gajzágó, 2009). Focusing on accessible plant protein sources like legumes is advisable in developing countries to improve the nutritional status of the low-income groups and restrict malnutrition (Iqbal et al., 2006). Although, legume crops rank second in importance to cereals, legumes are up to 2-3 times richer in protein than cereal grains. Thus they are an important component of a balanced diet and are a cheap source of protein in many African countries where animal protein is costly and beyond the purchasing power of the low income groups in these areas (National Academy of Sciences, 1979).

The low nutrient and degraded status of many soils is a particular problem for small landholders, as many cannot afford to use artificial fertilizers. Legume yields are less dependent upon an external supply of nitrogen fertilizer (although most do respond to additional nitrogen) and the ability of most of them to grow in poor soils has favored their cultivation (Sandal et al., 2002). They are an important part of subsistence agriculture as in addition to providing a protein-rich food they also improve the soil structure (Sato et al., 2010). Faba beans are among the best legume crops to increase and restore organic matter in the soil, in developing countries and arid zones (Solh and Saxena, 2011).

Proteins are major components of legume seeds as seeds can contain 200-250g protein/kg. Legume seeds are rich in lysine which cereals are deficient in. While they contain a lower percentage of methionine and cysteine compared to the cereals, this combination can be balanced by integrating grain legumes with cereals in the diet (Schuster-Gajzágó, 2009). In an investigation of nutritional value of some alternative crops in organic agriculture, grain legumes were reported as a source rich in protein, carbohydrates and minerals (Table 1-1). The grain seed of faba bean, chickpea and lentil contains around 21g protein/100g, which is higher than the content of pseudo cereals. The highest energy value was registered for faba bean, with 396.58 kcal/100g seed (Toader et al., 2011). These pseudo cereals themselves were found to be in higher contents for carbohydrate, protein and oil compared to wheat (Souci et al., 2000).

More than 30 species of grain legumes are grown across the tropics, in sub-Saharan Africa and South Asia and they are an important component of sustainable agriculture (Abate et al., 2012).

Table 1-1: Nutritional values of alternative crops (Moara Domneasca Experimental Field, Bucharest; (Toader et al., 2011)

Alternative crops	Species	Carbohydrate (g/100g)	Proteins (g/100g)	Lipids (g/100g)	Minerals (g/100g)	Energy value (kcal/100g)
Pseudo cereals	Amaranthus spp.	66.87	16.47	4.91	2.61	389.97
	Quinoa	64.32	16.71	5.80	2.89	389.06
	Buckwheat	65.50	16.03	3.53	2.31	351.05
Grain legumes	Faba bean	63.90	21.50	4.40	5.85	396.58
	Chickpea	56.20	21.23	4.31	3.41	360.95
	Lentils	33.29	22.18	3.03	4.00	259.60
Oil crops	Safflower	26.41	12.60	28.38	3.60	426.73
	Camelina	36.27	20.43	31.75	4.28	532.02
	Oil flax	27.73	22.56	34.10	5.25	528.56

The recent millennium report of ecosystem assessment indicates that desertification threatens over 41% of the world's land area, mostly in the dry areas of Middle East, North Africa and sub-Saharan Africa. Water scarcity and available water quality are potentially serious threats to food security and health in dry areas countries of developing world which characterized by marginal production with scarce resources and very high population growth rates of 2.5% (Erskine, 2003; Solh and Saxena, 2011).

1.2 Bambara groundnut

Bambara groundnut (Vigna subterranea (L.) Verdc.) (Figure 1-1) is an indigenous, underutilized African legume crop from semi-arid Africa, grown mainly by subsistence farmers for food. It has potential to assist with providing food security in the dry areas of Africa and also in other parts of the world. Bambara groundnut gives reasonable yields with little rainfall and is a favored food crop for many local people, being nutritionally comparable to other legumes, such as soybean, in the essential amino acids of lysine, methionine and cysteine (Brough and Azam-Ali, 1992). It is also grown more broadly, having shown the ability to adapt to a broad range of

ecological conditions and can be found in South East Asia. In Indonesia it is a minor

crop, while the average rainfall can exceed 2300 mm per year across Java (Aldrian

and Djamil, 2008).

Figure 1-1: Bambara groundnut [Vigna subterranea (L.) Verdc.]. (a) a botanical sketch, (b) flower, (c) fruits, (d) seed, (Maesen and Somaatmadja, 1989), (e) freshly harvested plant.

A phylogenetic tree is presented here (Figure 1-2) showing that bambara groundnut

belongs to the Phaseoloid-Millettioid clade, which diverged some 45–50 million years

ago from the Hologalegina clade. The Phaseoloid-Millettioid clade contains most

legume crops, such as pea, alfalfa (Medicago spp.), chickpea, soybean and common

bean (Choi et al., 2004b).
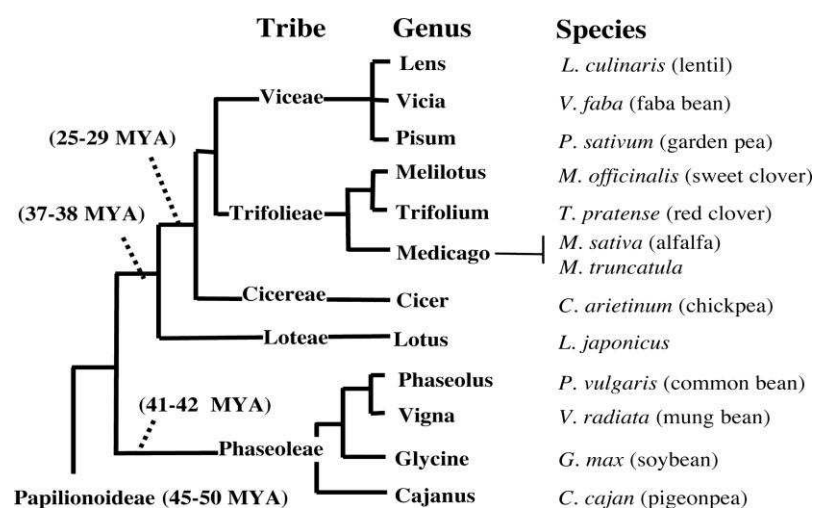
Figure 1-2: Taxonomic relationships between crop legumes. Penalised Likelihood analysis (Doyle and Luckow, 2003) was used to estimate the species divergence. Most crop legumes occur either within the Galegoid clade, including tribes Viceae, Trifolieae, Cicereae and Loteae, or within the phaseoloid clade (tribe Phaseoleae). MYA, million years ago (Choi et al., 2004b).

1.3 The importance of Bambara groundnut

Bambara groundnut ranks as the third most important food legume crop in semi-arid Africa in terms of production and consumption after groundnut (Arachis hypogaea L.) and cowpea (Vigna unguiculata L. Walp) (Aremu et al., 2006; Doku and Karikari, 1970; Howell et al., 1994; Linnemann, 1992; Sellschop, 1962) because of the shortage of sufficient rainfall for major legume cultivation. Drought tolerance is the most important trait that this crop possessed as it can be grown and gives acceptable yields in marginal areas where the cultivation of other legumes is not practicable (Vietmeyer, 1979). In addition it has undergone a prolonged period of exposure to pest and disease pressure under low input agriculture (Azam-Ali et al., 2001). It is a rich source of protein and along with other local sources of protein could help to alleviate nutritional problems in areas where staple foods are predominantly carbohydrate sources (Massawe et al., 2005; Okpuzor et al., 2010). It has been concluded that bambara groundnut seed is a useful ingredient for different food

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products of beverages, infants, children milk food and different drinks (Eltayeb et al.,
2011). Bambara groundnut seed makes a 'complete food', as on average the seed
contains sufficient protein (19%), carbohydrate (63%) and fat (6.5%) for a
nutritionally balanced diet (Amarteifio and Moholo, 1998; Brough and Azam-Ali,
1992; Ijarotimi and Esho, 2009). Mineral content was also estimated for 100g seed,
giving; iron 59 mg, potassium 1240 mg, phosphorus 296 mg, sodium 3.7mg and
calcium 78 mg (Amarteifio and Moholo, 1998). In addition it has high protein quality
with a good balance of essential amino acids, compared to most of other grain
legumes, with relatively high lysine (6.8%) and methionine (1.3%) (Brough et al.,
1993; Ellah and Singh, 2008; Okpuzor et al., 2010) which are often only available at
low levels in legumes. In a cream testa bambara groundnut a methionine content of
2.84% (of total crude protein) was reported (Olaleke et al., 2006). Also the seed pods
and other by-products have been recommended for feeding all kinds of livestock.
Some anecdotal medicinal uses of bambara groundnut seed and leaves mixed with
other crops in North Eastern Nigeria have also been reported (Atiku, 2000;
Directorate plant production, 2011). Symbiosis of bambara groundnut with Rhizobium
bacteria to fix atmospheric N_2 enhances the value of this crop for crop rotation and
intercropping, as it contributes to the supply of soil nitrogen for other crops (Karikari
et al., 1999). Additionally, naturally-occurring NO_3^- ion tolerant symbioses in
bambara groundnut have been identified. These compare well to tolerance of artificial
nitrate in other legumes, where there is a strong inhibitory effect on symbiosis. This
potentially allows Nitrogen fertilization in intercropping systems without inhibiting
N_2 fixation in the associated legumes (Dakora, 1998).

The annual world production of bambara groundnut is estimated to be around 330,000
tons, with West Africa (Nigeria, Niger, Burkina Faso, Chad, Cote d'Ivoire) producing

nearly half of this amount, and the demand for bambara currently is reported to be greater than the quantities produced (Brink and Belay, 2006; Directorate plant production, 2011).

Despite its importance as a part of the diet of much of sub-Saharan Africa, bambara groundnut has not as yet received significant research interest. There are no established varieties of bambara groundnut and the crop is still cultivated mainly from local landraces, rather than as genotype-specific varieties (Massawe et al., 2005). Bambara groundnut is characterized by higher genetic diversity in the wild ancestor (Pasquet et al., 1999) compared to domesticated landraces. Many landraces also consist of multiple genotypes, the mixes of which potentially increase the tolerance of the landrace to biotic and abiotic stresses and make them better able to survive and adapt to marginal areas under harsh conditions (Massawe et al., 2005). Both wild material and genotypes within landraces could represent an important potential source of beneficial genes for bambara groundnut breeding programs. Landraces could have the advantage of yield stability through genetic and phenotypic heterogeneity, but this is also likely to be one reason for limited yield compared to pure line crops. To improve this crop through breeding and enable the distribution of this crop to currently non-producing areas, detailed genotypic information and modern processing methods for domesticated and wild landraces are required. The ability of the breeder to exploit molecular technology to generate information at the gene level using different kinds of molecular markers (based on mapping and marker-trait analysis to locate important qualitative and quantitative trait loci (QTLs)) offers the opportunity to gain a better understanding of bambara groundnut genetics. This could potentially contribute to increased farmer income, better food security and also improve the quality of local diets.

1.4 Literature review

1.4.1 Underutilized crops

Underutilized crop species are often highly adapted to marginal environments and are inherited as part of the local culture and production systems. Many such crops are considered to be adapted to uncertain environments and niches which cannot be filled by other major crop species (Bhag, 2007). They have often been selected both directly and indirectly to resist harsh conditions under low agricultural input, both in terms of nutrients and prophylactic chemicals.   Plant scientists have emphasized the availability of these species because of their significant adaptability to less favored areas and harsh environments (IPGRI, 2002; Padulosi and Hoeschle-Zeledon, 2004; Padulosi et al., 2002). Underutilized plant species are those grown primarily in their centre of origin or centers of diversity that have been maintained by cultural preferences and traditional practices. They play an important role in food security, nutrition, and income generation for local subsistence farmers, as well as often having important cultural functions (International Atomic Energy Agency, 2004). Some underutilized crops were once widely grown but are today neglected by research and conservation for a variety of agronomic, genetic, economic and cultural reasons and the understanding of their utilization is restricted often to only indigenous knowledge (Magbagbeola et al., 2010; Padulosi et al., 2002). Using nutritionally-rich underutilized species which are adapted to low input agriculture systems could have immediate consequences for food security and the income of local people (IPGRI, 2002; Naylor et al., 2004; Oniang'o et al., 2006). These facts suggest that the conservation of, and more research on, these underutilized species is required by current and subsequent generations (Dansi et al., 2012).

1.4.2 Important considerations for underutilized crops

Humans have vigorously practiced plant and animal breeding for more than 12,000 years by selection within the crops they grow and animals they rear. The development of crop agriculture, in particular, has encouraged the development of permanent settlements and has been key in the development of civilization (Moschini, 2008). They have selected and developed those crop and breeds with traits of interest appropriate to the regions where they were first domesticated and have subsequently altered these species by artificial selection as their range has changed geographically and climatically, through the process of selective adaptation. Current climate change, urban expansion, deforestation, and other human activities have accelerated genetic erosion and are increasing the rates at which species are becoming threatened and vanishing. While the development of high yielding varieties in the Green Revolution allowed the rapidly increasing populations to be fed, but they have arguably also increased the rate that local crops are displaced from many farmland areas, especially in developing countries (Thies, 2000). At present, human beings rely on a limited number of food and non-food crops to meet the requirements of their staple diets and associated needs. Although, in the past more than 7,000 species of plants have been cultivated for human consumption, 95% of human food energy currently comes from only 30 crops, four of which (rice, wheat, maize and potato) provide more than 60% of our energy intake (FAO, 2011b). So a small number of species occupy the majority of the agricultural land area for cultivation.

Although some underutilized species are globally distributed, they remain inadequately characterized and neglected by research and conservation (Eyzaguirre et al., 1999). They are barely represented in ex situ gene banks and have no supply system for their germplasm. The plant genetic resources conserved today for food and

agriculture exceeds 6 million accessions. However, underutilized crops have poor representation at around 20% of the total collection of crop germplasm with on average only eight accessions per species (Padulosi et al., 2002) (Figure 1-3)
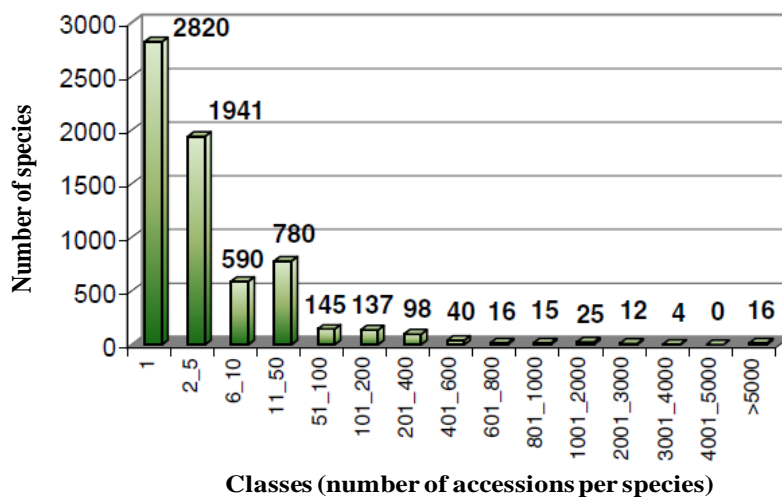
Figure 1-3: Minor crop representation in ex situ gene banks (Padulosi et al., 2002).

Much of the underutilized species germplasm is preserved by farmers in their field through their use of them, which makes loss of germplasm more of a risk than for species where there are extensive genebanks (IPGRI, 1998).

Food production to sustain a steadily increasing population is becoming one of the most important issues facing Humankind, with world population expected to exceed 9 billion by 2050 (Conforti, 2011). Global agriculture production has to grow substantially, by between 70% and by 100% in developing countries over the next four decades to feed this population (FAO, 2011a). Developing more sustainable practices to produce the food required for this number of people is a big challenge, especially with the potential impact of climate change and the increased demand for water resources for human use on agricultural production. It has been reported that climate change has decreased agricultural production in low-latitude regions, where

most developing countries are located (Easterling et al., 2007). In addition, population growth rates are highest in these areas, especially in the regions of the greatest poverty and food shortages, making an existing problem potentially far worse (Davies, 2003).

Research has focused primarily on the current major crops in recent decades, as they have dominated agriculture and human consumption. Among the commercialized major crops for human consumption three crops (maize, wheat, and rice) supply half of the world's daily protein and calories (Bharucha and Pretty, 2010). The displacement of indigenous and underutilized crops by introduced major staples has also lead to a serious erosion of germplasm for many local landraces (Brookfield, 2002). Thus, relying only on a small number of crops is potentially dangerous given the circumstances of increasing population pressure and climate change (Taylor et al., 2009). Any attempt to improve global food security has to include staple crops, as our reliance on them is so great at the moment. However, we also have no choice but to focus on increased sustainability of crop production in the future and one means of realizing this may be through growing crops which require fewer inputs or are more resistance to stress. (Mayes et al., 2011). Growing indigenous crops could help resource poor farmers obtain a satisfactory (and more stable) income. In many cases, women in these areas are often responsible for growing additional crops, while the main staple crops are grown by men. Although, germplasm has been selected by local farmers in marginal areas, food shortages have often not been alleviated, because their farming practice is limited by the resources available and often associated depleted, degraded or simply poor soils. From this argument it is obvious that a focus on breeding for neglected crop germplasm, especially in more hostile environments, by focusing on minor crops which are resistant to drought, pests and disease and which

are of high nutritional value is required to help to tackle the increasing demands for food due to population pressure (Holmens, 1998).

It has been proposed that improving the genetics of existing underutilized crop varieties, exploring post-harvest technology and better management, particularly of soils, in the regions of Africa and Latin America could bring underutilized species back into cultivation to fill some of the yield gaps and enhance food security. As they are generally more adapted to the extreme soil and climatic conditions predominantly found in Africa than the major crops and they are extremely important source for food production in low income, food deficit countries (Foley et al., 2011; Mohan and Suprasanna, 2011).

1.4.3 Strategic approaches for underutilized species

Many successful projects for underutilized crops have focused on a specific and important single end-use, however many underutilized crops are multipurpose and their end-uses vary from place to place. For example, the legume Lathyrus is used largely for fodder in Turkey, and as a food legume in South Asia, while it is used as animal feed and as a green manure in Australia (Williams and Haq, 2002). Identifying a specific and important single end-use of a product which could substitute for a major crop product from minor crops such as Triticale, Amaranthus, buckwheat and sesame has proven successful in development of strategic approaches to increase uptake of these crops. This process was applied through decades of research and the development of substantial germplasm collections, followed by selection, breeding and multilocational trials and commercialization (Ashri, 1994; Williams and Brenner, 1995).

Some organizations are engaged in strategic frameworks for research and development activities to enhance the use of underutilized species, aiming to improve the living condition of people worldwide. The International Centre for Underutilized Crops (ICUC) was established in 1992 as a global research, development and training organization. It provided expertise and acted as a knowledge center for tropical, sub-tropical and temperate plant development. ICUC has conducted research programmes in partnership with international and local research groups in over 30 countries to address increasing the use of underutilized crops for food, medicinal and industrial products, and for environmental conservation (ICUC, 2006).

The Global Facilitation Unit for Underutilized Species (GFU) was established in 2002 as a global partnership program focusing on networking and knowledge-sharing globally and to encourage incorporation of underutilized plant species into international and national research. It focused on the analysis of existing policies and legal frameworks that affect the conservation and use of underutilized species rather than being directly involved in the implementation of projects on particular plant species.

In 2008 a new international organization was formed under the name of 'Crops for the Future.' This organization has evolved from a union of the International Centre for Underutilized Crops (ICUC) and the Global Facilitation Unit for Underutilized Species (GFU). It supports, collects, synthesizes and promotes knowledge on neglected and underutilized species for the benefit of the poor and the environment (Jaenicke et al., 2009). An associated research centre (CFFRC) was established recently as a unique public-private partnership between a national government and an international research-led university (http://www.nottingham.edu.my/CFFRC/index.aspx). It will operate as the research

arm of the global Crops for the Future (CFF) body hosted in Malaysia by Bioversity International and University of Nottingham Malaysia Campus (UNMC) and is a new approach of a limited company without shareholders, but guaranteed by the Government of Malaysia and the University of Nottingham in Malaysia. The Government of Malaysia has provided money for facilities and for running costs for seven years (end-2017), by which time CFFRC is expected to be self-financing, through competitive grant funding and direct interaction with industry. CFFRC has a remit to implement research and development of underutilized plants for food and non-food purposes worldwide (http://www.nottingham.ac.uk/news/pressreleases/2011/june/newcropsforthefutureres earchcentre.aspx). The strategy of CFFRC is to focus on different minor and underutilized plant species, as an attempt to promote diversification of agriculture. This will hopefully improve nutritional food security, but also provides some resilience if climate change or other factors such as disease and pests caused major crops to fail long-term in parts of the world (Mayes et al., 2011). Underutilized crops could also help to provide purchasing power for poor and subsistence farmers as the recent spikes in food commodity prices show that even if food is physically available, the poor often do not have the purchasing power to obtain it (Padulosi et al., 2011).

1.4.4   Origin and distribution of bambara groundnut

'Bambara groundnut' (botanically known as Vigna subterranea (L) Verdc. ;2n=2x=22; (Heller et al., 1995) belongs to the family Leguminosae, subfamily Papilionoideae. It is related to cowpea (Vigna unguiculata L.) and is believed to have originated from Central Africa, before the introduction of groundnut (Arachis hypogaea L.) from South America (Goli, 1997). It comprises two botanical forms; the

wild sub-species (V. subterranea var. spontanea) found in Cameroon, Sudan and some parts of Nigeria, and the cultivated type (V. subterranea var. subterranea) which is found widely distributed in Sub-Saharan Africa (Basu et al., 2007b). Recently, a study of genetic diversity provided evidence for Cameroon/Nigeria as the putative centre of origin for bambara groundnut, using both phenotypic descriptors and DArT markers (Olukolu et al., 2012). In an investigation by Doku and Karikari (1971) it was proposed that cultivated bambara groundnut had developed gradually from var. spontanea. The suggested developments involved a shift in growth habit from open to bunch type, a change from outbreeding to inbreeding and a reduction in pod shell thickness. The germination of cultivated forms is rapid and uniform while in the wild forms it is erratic and germination takes longer (15-30 days) (Basu et al., 2007b). Other differences were also observed between the two types; domesticated landraces were found to have larger seeds and do not wrinkle upon drying, compared to the wild type (Basu et al., 2007b; Pasquet, 2003). It has been demonstrated that wild bambara groundnut (spontanea) is likely to be the true progenitor of domesticated bambara groundnut (subterranea) using both morphological and isozyme data (Pasquet et al., 1999).

It is believed that bambara groundnut was brought first to East Africa and Madagascar, then later it was introduced by slaves at the time of the Slave Trade and distributed to South and South East Asia (Hanelt, 2001). Somta et al., (2011b) using molecular markers proposed that Thai bambara groundnut landraces originate from both West Africa (Nigeria) and East Africa, implying that it was introduced to Thailand more than once. Bambara groundnut is widely cultivated throughout tropical Africa, India, Sri Lanka, Indonesia and Malaysia (Goli, 1997). It is also reported to be

cultivated in other parts of the world, including South and Central America and parts of northern Australia (Linnemann and Azam-Ali, 1993; Suwanprasert et al., 2006).

Evaluation and collection of bambara groundnut germplasm was carried out initially in the 19th century (Anonymous, 1947). The International Institute of Tropical Agriculture (IITA) in Nigeria has carried out numerous collection expeditions in 26 countries around the world and now there are 2030 germplasm accessions in their collections (Stadler, 2009).

1.4.5 Morphological and physiological characteristics of bambara groundnut

1.4.5.1 Morphological characteristic

Bambara groundnut is a herbaceous, intermediate, annual plant and believed to be mainly self-pollinating (Heller et al., 1997). The morphological structure of the crop largely matches that of the groundnut (Arachis hypogaea), in that the pale yellow flower stalk bends downwards after fertilization bearing its pods below the ground (Uguru and Ezeh, 1997).

It has two main contrasting growth habits; the branched form and the bunched habit, with a reproductive cycle of usually 90 to 150 days, depending on environment and landraces (Berchie et al., 2010; Goli, 1997). The tap root is well developed with many profuse geotropic lateral roots of around 20 cm long on the lower part (Akpalu, 2010). Nodules formed on the roots fix atmospheric nitrogen through symbiosis with Rhizobium bacteria, which makes them useful for crop rotation and intercropping (Karikari et al., 1999; Linnemann and Azam-Ali, 1993).

Bambara groundnut is believed to be autogamous and floral reproduction starts 30 to 35 days after sowing and may continue until the end of the plant's life (Directorate

Plant Production, 2009; Swanevelder, 1998). Flowers are normally carried in pairs on short peduncles by a pedicle which arises from the axis formed by the petioles and the stem (Doku, 1968). Flowers produced on the same peduncle do not open synchronously, although they will open within a 24 hours interval. Delayed flower opening may be caused by low temperatures and cloudy skies (Massawe et al., 2003). It has been reported that fertilization in bambara groundnut takes place on the same day as anthesis (Linnemann and Craufurd, 1994). After fertilization, the flower stem elongates. During this time, the peduncle elongates to bring the ovaries to the soil level and the pedicels penetrate the soil surface after fertilization to form the pods (Heller et al., 1995). The sepal enlarges and the fruit develops above or just below the soil surface. Pod development lasts up to 30 days after fertilization and the seed develops over a further 10 days (Swanevelder, 1998).

The pod is small, round or slightly oval shaped and wrinkled. Generally a single seed is produced in the pod, although two seeds per pod have been reported (Pasquet and Fotso, 1997). Seeds are mature when the parenchymatous layers surrounding the embryo have disappeared and the pods become a light brown (Toungos et al., 2009). The seeds are round, smooth and very hard when dried, with highly variable testa colors, including cream, brown, red and blotched (Stephens, 2003) (Figure 1-4).
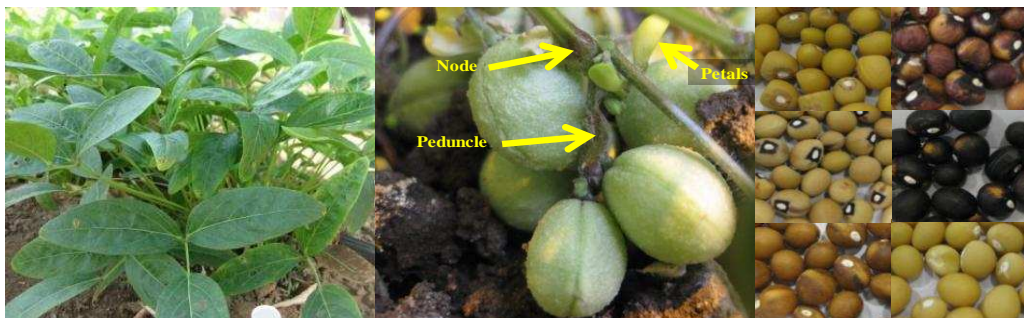
Figure 1-4: Bambara groundnut; plant, flowers, pods and seeds with different testa colors.

1.4.5.2 Physiological characteristic

The growth and development of bambara groundnut is affected by the major abiotic factors of moisture, temperature and photoperiod. Bambara groundnut requires warm temperatures with an average day temperature of 20 to 28°C being ideal for the crop. Germination in bambara groundnut usually takes seven to 15 days under favorable temperature conditions (28.5°C-32.5°C) for bambara groundnut; while under lower temperatures, the germination and seedling emergence can be delayed up to 31 days with some seeds remaining dormant indefinitely (Linnemann and Azam-Ali, 1993; Swanevelder, 1998). Development in bambara groundnut appears to be indeterminate with flowers often small and yellow color arising alternatively with leaves at each node (Dimakatso, 2006).

A number of physiological studies have been undertaken in bambara groundnut. They confirmed that different landraces have different responses to photoperiod, sowing date (Sesay et al., 2008), moisture deficit (Collinson et al., 1997; Mwale et al., 2007), growth rate (Massawe et al., 2003) and drought tolerance. These recorded variations in the response of bambara groundnut provides a good background to improve the performance and develop varieties for bambara groundnut in future breeding programs.

Photoperiod

The life cycle of the bambara groundnut crop varies from 3-6 months, depending on the landrace and climatic conditions. Photoperiod affects the days to maturity and it has been found that the podding is retarded by long photoperiods in some landraces (Harris and Azam-Ali, 1993) and different landraces have different photoperiod requirements for pod production. In the photothermal response of the onset of flowering and the onset of podding for three Nigerian landraces it was found that the

flowering of Ankpa 2 and Yola was controlled by temperature only, while Ankpa 4 was sensitive to temperature and photoperiod. Whereas the onset of podding was affected by temperature and photoperiod in all three landraces and the most photoperiod-sensitive was Ankpa 4, followed by Yola and Ankpa 2. (Linnemann and Craufurd, 1994). In another study it was also determined that photoperiod influences the flowering and podding in Ankpa 4, with no pods being produced under 14 and 16 hour photoperiods, while in Tiga necaru only the onset of podding is affected by photoperiod (Linnemann et al., 1995). A greenhouse experiment was conducted under 16-17 hours photoperiod and 20°C - 25°C temperature to identify photoperiod neutral lines in bambara groundnut. Three landraces (TZA-1498, TZA-1505 and TZA-2114) collected from Tanzania close to equator have been reported as photoperiod-neutral lines having clear pod setting whereas all other landraces remained in the vegetative stage (Jorgensen et al., 2009). It has been generally reported that floral initiation is not photoperiod dependent but this is now being questioned, although the current data suggests that flowering is more likely affected by temperature, while the reproductive development and yield generation in bambara groundnut is affected by photoperiod after flowering (P. Kendabie, personal communication).

Drought tolerance

With the potential risk of drought associated with climate change, drought tolerance is likely to become even more important in African agriculture. Bambara groundnut as a drought tolerant plant has the potential as a crop to provide improved food security in the dry areas of Africa (Berchie et al., 2012). The plant is most suited for hot dry areas, is highly adaptable and tolerates harsh conditions (Karunaratne et al., 2011). The strong root system with a compact tap root has been suggested to enhance the resistance of this plant to drought (Begemann, 1986). Bambara groundnut is

apparently able to maintain turgor through a combination of osmotic adjustment, reduction in leaf area index and effective stomatal regulation of water loss (Collinson et al., 1997). Although the mechanisms that allow bambara groundnut to still produce some yield during severe droughts are poorly understood, it has been stated that this mechanism may be linked with its relatively high root/shoot biomass ratio and small leaf area, which restricts transpirational loss of water (Collinson et al., 1996).

Drought response was identified to be different among bambara groundnut landraces. Uniswa Red from Swaziland defined as a drought avoiding landrace, a 'water-saver' according to the three adaptation terms defined by Turner (1979). It had the most 'optimistic' growth with the largest leaf area reduction and the earliest stomata closure which is important for survival during intermittent drought. While S19-3 from the low rainfall area of Namibia is defined as a 'water-spender' with a late closure of stomata, with a more 'conservative' growth pattern, with compressed phenology combined with reduction in leaf area and stomata closure. The drought responses of S19-3 are fit well with the short cropping season of Namibia with annual terminal drought problems (Jorgensen et al., 2010).

Berchie et al. (2012) evaluated the performance of five bambara groundnut landraces (Black eye, Burkina, NAV 4, NAV Red and Tom) to drought and heat stress, at Tono-Navrongo, upper east region of Ghana. They stated the existence of variation among landraces with respect to drought tolerance as Burkina proved the most drought and heat tolerant, exhibiting the greatest root dry weight and leaf area at 120 DAS, and the longest leaf area duration. The authors believe that this tolerance to drought may be a result of adaptation to environmental conditions in the material.

1.4.5.3 Management and agronomy

Different plant spacings are used in bambara groundnut cultivation and the recommended spacing is broadly 10 to 15cm in rows of 45 to 90cm apart (for rows cultivation) and a spacing of 20×20cm (250,000 plants ha^{-1}) in flat seedbeds, with semi-bunch types giving the highest yields in Côte d'Ivoire (Directorate plant production, 2011; Kouassi and Zoro, 2010).

Bambara groundnut crops can grown in poor soils low in nutrients and requires soil of pH 5-6.5, preferring well-drained sandy loam and the average rainfall requirement of this crop ranges between 600-700mm during the growing season (Swanevelder, 1998). It can be cultivated up to 1600m above sea level (Chittaranjan, 2007).

Yields of bambara groundnut vary widely. A yield of 400-1400 kg ha^{-1} unshelled pods was reported in Zimbabwe (Heller et al., 1995). In Swaziland yields of 2600 kg ha^{-1} was recorded in the field (Sesay et al., 2008), and over 3000 kg ha^{-1} have been obtained in South Africa (Swanevelder, 1998). While in Côte d'Ivoire seed yield was recorded as high as 4000 kg ha^{-1} (Kouassi and Zoro, 2010) with similar yields in Ghana (J. Berchie, personal communication).

1.4.6 Genetic diversity of bambara groundnut

The evaluation of available genetic diversity is a pre-requisite for genetic improvement in crop plants, especially in underutilized crops such as bambara groundnut (Olukolu et al., 2012). Investigation of genetic diversity in both wild and domesticated species is equally important. Wild populations are known to be a potential source of useful genes and traits which could be introduced into the domesticated gene pool; in particular, genes responsible for adaptation to stressful environments such as those providing a particular resistance to a pathogen or to arid

conditions (Cattan-Toupance et al., 1998). Wild populations in centers of diversity or domestication constitute the initial gene pool of crops species. Crop failures and dispersal of germplasm within the centre of origin or limited introduction or isolated locations ('Founder Effects') could lead to reduced genetic diversity in particular breeding populations, which could have long-term negative consequences for production (Trethowan and Mujeeb-Kazi, 2008). By focusing on commercial and elite germplasm the breeder may further reduce the genetic diversity of the domesticated gene pools (Rauf et al., 2010; Yi et al., 2008). Studies of genetic diversity can help to guide the exploitation of wild relatives in a breeding program to retrace or enhance gene flow between wild and domesticated populations which may increase the genetic diversity in domesticated gene pools (Gepts and Papa, 2002).

Estimating the genetic diversity of crop species can be achieved using different marker methods, including; morphological, trait/agronomic, biochemical and molecular. The latter has several advantages over conventional phenotypic markers, as they can be used efficiently regardless of the developmental stage of the plant under investigation (Mondini et al., 2009).

Genetic diversity was analyzed within 100 single plant accessions of bambara groundnut from a wide range of locations in Tanzania. They were assessed with 49 polymorphic bands of 11 informative AFLP primers. Cluster analysis revealed that bambara groundnut has two major groups in line with their putative geographic origins (Ntundu et al., 2004). The results of this study agreed with a previous study that used RAPD markers on 25 African accessions from the collection in IITA (Ibadan and Nigeria) showing two main groups of accessions corresponding to their geographic distribution (Amadou et al., 2001).

High genetic identity between wild and domesticated accessions was detected in an isozyme diversity study of bambara groundnut, Ht= 0.087 with 14 polymorphic loci and Ht= 0.052 with only 7 loci for the wild and domesticated, respectively. The study suggests that wild bambara groundnut is likely to be the true progenitor of domesticated bambara groundnut. Beside the high value of intra-population diversity in both wild and domesticated accessions, the study also suggests that self pollination is the major mode of sexual reproduction for both accession types (Pasquet et al., 1999). Two hundred and forty single plant accessions of bambara groundnut were assessed using 22 SSR markers. Higher gene and allelic diversity were obtained in the West African and Cameroon/Nigeria regions than others (east African, central African, and Southeast Asian) with 6.68 and 6.18 alleles per locus, and 0.601 and 0.571, respectively (Somta et al., 2011b).

An extensive and diverse range of germplasm was investigated to study genetic diversity of bambara groundnut in the study by Olukolu et al. (2012). Morphological and quantitative descriptors, alongside DArT markers that represent wide genome coverage, were used and a high genetic diversity was observed for the Cameroon/Nigeria region relative to other regions. This supports the hypothesis that this region is the centre of diversity for bambara groundnut (Olukolu et al., 2012).

The available literature reveals a number of studies of genetic diversity in bambara groundnut in the wild and domestication material. They offer a reasonable start to understanding the genetic basis of the domestication event(s) in this crop, potentially enabling parents with a wide genetic base to be identified for developing mapping populations and subsequent QTL analysis.

1.5 Molecular markers

Evaluation of genetic variation within and between populations and the interaction of genes with the environment has been studied using different types of markers over the last century. The first phenotypic markers were mapped in fruit fly in 1913 (Sturtevant, 1913). In plants, in 1923 a limited number of morphological markers were tested and identified an association between seed size and seed-coat pigmentation patterns in the common bean, Phaseolus vulgaris (Sax, 1923). However, such phenotypic markers can often not be used as reliable indicators of the traits of interest due to an often complex interaction of the genotype and the environment. Moreover, in some species these markers are expressed at a very low frequency which makes their effective utilization in plant breeding difficult (Farooq and Azam, 2002). Marker development moved forward with the discovery and development of protein and isozyme markers which resolved some of these problems (Markert and Moller, 1959). Protein and isozyme markers can be affected by the environment and their use is constrained by the tissue and developmental stage of the species under study (Avise, 1993). In addition, they often have limited polymorphism to assess genetic diversity within a species. A different method is required to detect different isozyme systems, so a range of application techniques are required to generate data for a number of isozyme markers (Fairbanks and Andersen, 1995). In a study based on DNA markers to evaluate genetic polymorphism within cultivated tomatoes (L. esculentum and L. pennellii), none of the isozyme markers revealed polymorphism compared to 63% that detected polymorphism using RAPD markers between these varieties (Foolad et al., 1993).

Breeding a new variety with conventional methods takes many years especially when there are effects of trait pleiotropism and or when there is a multifactorial basis to

morphological traits. Hence breeders are interested to try new techniques to make this process more efficient. Developments in molecular marker technology offers such a possibility by adopting a wide range of novel approaches which have altered the way plant breeding is being undertaken, allowing the breeder to use them potentially in estimating the genetic diversity and the level of heterozygosity among plants and animals (Dani et al., 2008; Kumar et al., 2008) as a first step to determining the best parents and best strategies for breeding.

DNA marker systems were introduced to genetic analysis in the 1980's, primarily based on the development of the Southern blot technique to assess polymorphism in complex genomes. DNA-based molecular markers have acted as versatile tools in various fields, such as taxonomy, physiology, embryology, genetic engineering (Joshi et al., 1999). DNA molecular markers are used to identify a particular DNA sequence. DNA markers have become a popular means for identification of plant and animal species; they are generally stable and detectable in any part of the genome. Additionally, they are not usually affected by the age, physiological condition of the cell or environmental factors and are generally held to have no pleiotropic or epistatic effects (Mondini et al., 2009).

Currently, extensive use of molecular markers derived from different technical approaches allows the segregation patterns of different alleles to be scored easily and construction of genetic maps from them. Construction of linkage maps is one of the main uses of DNA markers in research on crop species (Collard et al., 2005). Such genetic maps serve several purposes, including detecting association between the genes and traits studied in QTL analysis, with the aim to use the markers to tag those traits, allowing the application of marker assisted selection of these target traits in subsequent breeding programs (Semagn et al., 2006b).

DNA-based markers have been established in many agricultural crops and the availability of reliable molecular markers is of great importance for plant breeding as molecular markers linked to desirable traits have been used to accelerate plant breeding programs (Ribaut and Hoisington, 1998). The ideal molecular marker technique should generate hundreds of molecular markers that cover the entire genome in a single, simple and reliable experiment (Luikart et al., 2003). DNA markers are divided based on the method of their detection into three classes, hybridization-based; polymerase chain reaction (PCR) based and DNA sequence-based (Gupta et al., 1999; Jones et al., 1997; Joshi et al., 1999; Winter and Kahl, 1995).

DNA markers that discriminate between genotypes are termed polymorphic. Co-dominant markers reveal the allelic state of both alleles for a marker in a diploid, whereas dominant markers do not provide full information on the alleles present. Co-dominant markers may have many different alleles, whereas dominant markers only have two alleles i.e. presence or absence (Collard et al., 2005; Mondini et al., 2009). The polymorphic site of a genetic marker may be a short or even single base DNA sequence difference, such as in a single base-pair change (single nucleotide polymorphism, SNP) (Cotton, 1997) or could be due to greater variation, as with repeat units for minisatellites.

Various types of molecular markers have been used. Some of the more commonly used systems are; RFLPs (Restriction Fragment Length Polymorphism), AFLPs (Amplified Fragment Length Polymorphism) RAPDs (Random Amplification of Polymorphic DNA), VNTRs (Variable Number Tandem Repeat), Microsatellites (or Simple Sequence Repeat; SSR), SNPs (Single Nucleotide Polymorphism), STRs (Short Tandem Repeat), SFP (Single Feature Polymorphism), and DArT (Diversity

Arrays Technology). No single technique fulfills all research needs and it is difficult

to predict the emergence of new standard techniques (Semagn et al., 2006b). Different

aspects of cost-effectiveness, accuracy, sensitivity and reproducibility in addition to

the availability of markers specific to an organism and their limitations should be

taken into account to determine the best suitable technology for a specific genotyping

purpose and approach. The most important features, details on the nature of these

markers, their use, advantages and disadvantages of different applications as adapted

from Zalapa et al. (2012) can be found in Table 1-2.

Table 1-2: Main characteristics of major types of molecular markers.

Characteristic	RFLP	SSR	AFLP	RAPD	SNP
Locus number detected	Single locus	Single locus	Multi-loci	Multi-loci	Single locus
Allelism	Co-dominant	Co-dominant	Dominant	Dominant	Co-dominant
Level of polymorphism	Good	Excellent	Good	Good	Excellent
Polymorphism at the locus	2 to 5 alleles	Multiple alleles	Presence/absence	Presence/absence	Up to 4 alleles
Quantity of DNA needed	Large	Small	Small	Small	Small
Quality of DNA needed	V. Good	reasonable	Good	reasonable	reasonable
Reproducibility	Good	Good	Good	Low	Good
Time	Long	Fast, once markers are developed	Fast	Fast	Fast, once markers are developed
Cost	Expensive	Average	Cheap	Cheap	Expensive[a]
Technical difficulty	High	Low	Medium	Medium	High[a]

a: Both cost and technical difficulty are highly dependent on the chosen method of visualization and, hence, on
the expected throughput level

1.5.1   Restriction Fragment Length Polymorphisms

Restriction Fragment Length Polymorphism or RFLP was once the most widely used

molecular marker type. The technique reveals differences in restriction sites between

individual genotypes which are contained in or flank the region of the hybridization

probe. The differences are caused by evolutionary processes, spontaneous mutations or unequal crossing over (Gonzalez-Chavira et al., 2006). In RFLP analysis the DNA sample is digested using restriction endonucleases which recognize specific DNA sequence motifs, generally 4 or 6 base-pair palindromic sequences. Digested DNA fragments are size fractionated according to their length by gel electrophoresis, then they are transferred to a nylon filter and fixed into place before undergoing the hybridization of a cloned sequence of DNA which has been labeled. The relative positions of the hybridizing fragments are visualized through exposure of the hybridized and washed filter to a light or X-ray sensitive surface. This gives a banding pattern which reflects the position of probe hybridization which can differ between individual genotypes due to fragment length differences due to the restriction cut sites present (Gonzalez-Chavira et al., 2006). High reproducibility, co-dominant inheritance and good transferability between laboratories are considered the major strengths of RFLP markers. However their use requires high quality and quantities of DNA, and it is expensive, time consuming, often involving radioactive/toxic reagents (Mondini et al., 2009). Although now largely obsolete, RFLP analysis was the first DNA profiling technique cheap enough to see widespread application. In addition to genetic fingerprinting, RFLP was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing. RFLP markers were used for the first time in 1975 to identify polymorphisms in DNA sequence for genetic mapping of adeno-virus serotypes (Grodzicker et al., 1975), then they were used in human genome mapping (Botstein et al., 1980), and later applied in plant genomes (Helentjaris et al., 1986; Weber and Helentjaris, 1989).

1.5.2   Random Amplification of Polymorphic DNA

RAPD (pronounced "rapid") is a PCR-based technology in which random short segments of DNA are amplified (Kumar et al., 2009). This technique was developed due to the limitations in the routine use of RFLP (Garcia et al., 2004). The assay was developed first by Williams et al. (1990). RAPDs are DNA fragments amplified by PCR using short single synthetic oligonucleotide primers (8-12 bp) of random sequence. These primers serve as both forward and reverse primer at two different sites on complementary strands, to amplify fragments from multiple genomic sites where the product size is within the range of PCR, simultaneously. The resultant PCR fragments are visualized on agarose gels stained with ethidium bromide, and there is no need for hybridization with labeled probes as in RFLP (Kumar et al., 2009). The main advantages of RAPDs are that they are quick and easy to assay, only relatively low quantities of template DNA are required, and no prior sequence information from the organism under study is required. However, they have the drawback of sensitivity to reaction conditions which can lead to their profile varying between laboratories, even for identical reagents. Additionally, as RAPD markers are not locus-specific and several loci are amplified by each primer, heterozygous and homozygous individuals cannot be distinguished (Kumar et al., 2009; Paraguison et al., 2012). Although RAPDs are less popular due to their limitations, they have been applied in genetic variability analysis and individual-specific genotyping. RAPD has been used to characterize, and trace, the phylogeny of various plant and animal species (Vos et al., 1995; Zabeau and Vos, 1993). RAPDs have been applied in gene mapping studies to fill gaps not covered by other markers (Williams et al., 1990). In chickpea RAPDs have been used for identification and mapping QTLs conferring resistance to Aschochyta blight (Santra et al., 2000).

Using RAPDs in bambara groundnut gave high levels of polymorphism in comparison to isozyme markers among landraces investigated by Pasquet et al. (1999). They also have been applied in bambara groundnut to study the genetic diversity in some landraces (Amadou et al., 2001; Massawe et al., 2003).

1.5.3 Amplified fragment length polymorphism (AFLP)

Amplified Fragment Length Polymorphism PCR (AFLP-PCR) or just AFLP is a PCR-based tool used in DNA fingerprinting. This method is based on the combination of the sequence specificity of digestion of DNA through restriction endonuclease enzymes and the PCR technique. It was developed to overcome the limitation of reproducibility associated with RAPD (Mondini et al., 2009). AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. The primer used is extended (by addition of a random base) into the restricted fragments to achieve selective amplification of a subset of those fragments, followed by acrylamide gel analysis of amplified fragments (Kumar et al., 2009; Vos et al., 1995). The amplified fragments are visualized on denaturing polyacrylamide gels in combination with radioactivity (Matthes et al., 1998) or on fluorescent imaging gels systems, such a LiCor or Capillary Electrophoresis systems, such as the Beckmann CEQ 8000. The AFLP technique is highly reliable and reproducible, does not require prior sequence information from the species of interest, gives high levels of polymorphism and allows multiple locus detection. The disadvantages are; it has a complicated methodology, requires both restriction endonuclease and adapter ligation reaction, so the template DNA used is required to be pure and free from inhibitor compounds which interferes with the restriction enzyme or ligation reactions (Gonzalez-Chavira et al., 2006; Kumar et al., 2009). The

technique is also patented, which makes using AFLPs in programme which may lead to commercial products more complex.

AFLP markers have been used to generate a genetic map in maize. 246 markers were mapped to cover 2057 cM (Ajmone Marsan et al., 1999). It was realized that the generation of this marker type was faster than RFLP and markers mapped on the same population could be compared, showing that the AFLP markers show good distribution over the genome and almost all markers in this case (98.1%) followed Mendelian segregation patterns.

Application of AFLP markers was also used in the genetic characterization and mapping of some legume crops. They have been recognized as useful markers to characterize the genetic relationships between wild common bean and lima bean and to deduce the predominant direction of gene flow and the spatial differentiation between domesticated and wild bean (Papa and Gepts, 2003). In chickpea, 89.6% of AFLP bands were found to be polymorphic in 95 accessions that represented the 17 species of Cicer (Nguyen et al., 2004). The introgression of the rhizomatous trait from Trifolium ambiguum to the important pasture species of white clover (T. repens) has been studied and bulked segregant AFLP analysis was used to identify markers linked to the rhizomatous habit (Abberton et al., 2003) to enable this work.

AFLP markers were employed in bambara groundnut to assess genetic diversity and to investigate the genetic relationships among 100 selected landraces from a diverse geographic area of Tanzania, using 49 polymorphic amplification fragments scored bands from 11 markers (Ntundu et al., 2004). In another investigation of genetic diversity in bambara groundnut, AFLP analysis provided sufficient polymorphism to

determine the genetic diversity and to establish genetic relationships among 16 landraces (Massawe et al., 2002).

The AFLP marker technique has also been used to construct an initial genetic map of bambara groundnut. A total of 115 polymorphic AFLP bands were generated through selective amplification using EcoRI/MseI primers with a total of 6 base-pairs of additional selection. These primers along with 1 SSR marker were scored in the F_2 population and used to construct the first genetic linkage map in this crop derived from the cross between the domesticated and wild landraces (DipC x VSSP11) (Basu, 2005).

1.5.4   Simple sequence repeats

Simple sequence repeats (SSR) or short tandem repeats (STR) are comprised of basic short motifs generally between 2 and 6 base-pairs long (di- to hexa- nucleotide repeat units), they can be found in both coding- and non-coding regions for prokaryotes and eukaryotes (Chambers and MacAvoy, 2000; Ellegren, 2004; Kelkar et al., 2008). They are reliable and popular markers as they possess; co-dominant inheritance, are relatively abundant, are multi-allelic, have extensive genome coverage, are highly reproducible and can often be simply detected (Mondini et al., 2009). SSR markers are tandem repeats interspersed throughout the genome and can be amplified using a primer that flanks these regions (Grist et al., 1993). These markers are often present at high levels of inter- and intra-specific polymorphism, particularly when the tandem repeat number is ten or greater (Bowen and Wheals, 2006). The number of SSR motifs at a locus is variable and it is believed that the basis for generation of high levels of polymorphism is unequal crossing over and replication slippage, where the

transient dissociation of the replicating DNA strand is followed by misaligned re-association (Levinson and Gutman, 1987; Richards and Sutherland, 1992).

Microsatellites are not only present in the nuclear genome of bi-parental inheritance; there are also chloroplast and mitochondrion genome microsatellites which are usually inherited in a uniparental pattern (Soranzo et al., 1999).

In plants, the presence of SSRs was first demonstrated by hybridization of oligonucleotide probes on phage libraries derived from tropical tree genomes (Condit and Hubbell, 1991). SSRs have become one of the most widely used molecular marker systems in plant genetics and breeding as the ultra-variability of SSRs among related organisms and a high degree of transferability between species make them an excellent markers for genotype identification, analysis of genetic population and diversity, phenotype and genetic mapping and marker assisted selection. It also enables the investigation of comparative genomic analysis (Powell et al., 1996; Tautz, 1989; Varshney et al., 2005a). SSRs are the only molecular markers to provide clues about which alleles are more closely related to each other (Caporale, 2003) with mutation often leading to repeat unit changes in fragment size.

1.5.4.1 Inter-Simple Sequence Repeat

ISSR (inter-simple sequence repeat) is a general term for a marker system based on amplification of the genomic region between microsatellite loci (Kebour et al., 2012). The technique is reported by Zietkiewicz et al. (1994) and is similar in many ways to RAPDs. DNA fragments of about 100–3000 bp located between adjacent, oppositely-oriented microsatellite regions (Kumar et al., 2009) are amplified. ISSRs are mostly dominant markers, although a few are codominant (Gonzalez-Chavira et al., 2006). They are not proprietary and can be synthesized easily and no sequence information is

required (Joshi et al., 2000). Although the ISSR technique has a reproducibility problem, it is considered by some authors as simple, fast to detect polymorphism, cost-effective and highly discriminative (Shafie et al., 2011) and reasonably reliable. ISSRs are considered useful in gene mapping studies (Zietkiewicz et al., 1994).

1.5.4.2 Expressed Sequence Tag-SRR

EST-SSR was identified as a class of microsatellite by Adams et al. (1991). Such markers can be obtained by partial sequencing of random cDNA clones (Adams et al., 1991). The expressed genes can be detected within EST libraries (Bouck and Vision, 2007). EST-SSRs have received reasonable attention with increasing numbers of ESTs being deposited in databases for various plants, especially model species (Ner-Gaon et al., 2007). Between 1% and 5% of ESTs from different plant species have been found to contain SSRs suitable for marker development. (Kantety et al., 2002; Scott et al., 2000).

The generation of EST or genic SSR markers is relatively easy and inexpensive, especially in plant species with characterized genes and full-length cDNA clones, such as rice (Kikuchi et al., 2003), because they are a by-product of the sequence data from genes that are publicly-available. Additionally, the sequence data for EST-SSRs are often available in GenBank and so can be easily downloaded to screen them for the presence of microsatellite repeats.

EST-SSR markers have been developed for a number of plant species, including cultivated peanut (Liang et al., 2009) grape (Cordeiro et al., 2001), rice (Temnykh et al., 2001), durum wheat (Eujayl et al., 2002), rye (Hackauf and Wehling, 2002),

barley (Thiel et al., 2003), barrel medic (Eujayl et al., 2004), ryegrass (Faville et al., 2004), wheat (Peng and Lapitan, 2005), and cotton (Han et al., 2006).

EST databases can be processed rapidly to develop EST-SSR markers at relatively low cost (Liang et al., 2009). As EST-SSR markers are derived from expressed genes, they are more conserved and have a higher level of transferability to related species than genomic DNA markers (Saha et al., 2004). In addition these SSRs appear to be more closely related to important agronomical traits (Qi et al., 2010). They are, therefore, useful as anchor markers for comparative mapping across species, comparative genomics, and evolutionary studies (Cordeiro et al., 2001; Eujayl et al., 2004; Kantety et al., 2002; Saha et al., 2004; Scott et al., 2000; Thiel et al., 2003), however the conserved nature of EST-SSRs in transcribed regions may also limit their degree of polymorphism as lower levels of polymorphism are often reported compared with genomic SSRs in crop plants (Rungis et al., 2004; Scott et al., 2000). The transferability of EST-SSR loci across species within a genus has in several studies been above 50% (Dirlewanger et al., 2002; Eujayl et al., 2004; Gaitán-Solís et al., 2002; Peakall et al., 1998; Thiel et al., 2003) whereas the transferability of genomic-derived SSR loci across genera was poor (Peakall et al., 1998; Roa et al., 2000; Thiel et al., 2003; White and Powell, 1997). Recently, the application of next-generation sequencing (NGS) technology (Illumina and 454 sequencing) for genome sequencing leads to the discovery of a large number of genome-wide and genebased microsatellites in plant much more efficiently (Jun et al., 2011; Wang et al., 2012; Zalapa et al., 2012).

1.5.4.3 Application of microsatellites in different plant species

Microsatellites as a DNA marker have been intensively used in a wide variety of organisms including humans (Beckmann and Soller, 1990), animals (Moore et al., 1991), plants (Condit and Hubbell, 1991) and bacteria (E. coli; (Gur-Arie et al., 2000).

However single nucleotide polymorphisms (SNPs) are replacing SSRs for some applications as more species are sequenced, their application in related minor species is limited due to the restriction of their transferability within species (Bakker et al., 2006; Vezzulli et al., 2008). In contrast, SSR markers will potentially continue to play an important role in different genetic studies of many minor plant and insect species in the future due to their efficiency, transferability to related species, multiple alleles and cost-effectiveness and ease of use for assessing small numbers of loci (Wang et al., 2009).

Microsatellite markers can facilitate comparative mapping among different plant species to discover genome syntenies in order to develop DNA markers to specific chromosomal regions for marker-assisted selection. Anchoring the physical map of model plant species of Arabidopsis and soybean required SSRs in specific chromosome regions (Shoemaker et al., 2008; Shultz et al., 2007; Wang et al., 1997) to assemble genome DNA sequences and joins together large pieces of overlapped DNA fragments.

SSR markers can be transferred to related species and to minor crops, which can facilitate the construction of genetic maps in minor species and enrichment of regions with low marker saturation. Placing barley SSR markers on rye and wheat genetic maps is an example of one such case (Varshney et al., 2005b).

SSRs have become the marker of choice for evolutionary studies of natural plant populations as they highly polymorphic, genome-wide and multiallelic. Data collected from Chloroplast SSR molecular assays has been used to survey the cytoplasmic diversity among wild and cultivated barley populations. (Provan et al., 1999).

There are many different applications of microsatellite in plant species. Over 80 genetic linkage maps have been constructed which include SSR markers with the first one instance being reported in tropical trees (Condit and Hubbell, 1991), followed by major and minor crops, fruits and vegetables (Akkaya et al., 1992; Wu and Tanksley, 1993; Zhao and Kochert, 1993). In soybean, iron deficiency chlorosis associated with two common SSR markers was co-located in two separate populations (Wang et al., 2008).

Germplasm classification for choice of parental selection has been calculated in different plant species through genetic distance and diversity assessment using the SSR marker system. Genetic diversity and phylogenetic relationships from germplasm collections for cultivated and wild peanut collection have been evaluated by SSR markers (Barkley et al., 2007; Cuc et al., 2008).

Microsatellite markers have also been used in bambara groundnut. Microsatellite primers developed from related legume species including cowpea, mungbean, common bean and soybean, were screened for identification of polymorphisms in the two parents 'DipC' and 'VSSP11' of a segregating F_2 mapping population in bambara groundnut. Out of 15 tested nine primers were amplified in both parents and only one (from soybean) was detected as a polymorphic marker and this was used with the polymorphic AFLP markers in constructing wide genetic map in this crop (Basu, 2005). In another investigation a set of 22 polymorphic SSR markers (from bambara

groundnut, azuki bean, cowpea and mungbean) were used to study genetic diversity in a collection of 240 accessions of bambara groundnut of Africa and Asia. A total of 166 alleles were detected, with a mean of 7.59 alleles per locus (Somta et al., 2011b). Later a set of 75 microsatellites within species were characterized and used to investigate the genetic diversity of 24 bambara groundnut landraces of Africa (Molosiwa, 2012)

The level of inter- and intra-landrace polymorphism was assessed within a small collection of 10 Ghanaian landraces, using 10 reproducible SSR markers. A high level of polymorphism was detected among the landraces investigated (Siise and Massawe, 2012).

1.5.5   Single Nucleotide Polymorphism

SNPs are a marker system that can differentiate individuals based on variation detected at the level of a single nucleotide base (or more) in the genome. Such variation represents all sequence differences between individuals (Kumar et al., 2009). Although SNPs can be used as a powerful and high throughput automated marker system in different applications of linkage disequilibrium and QTL analysis of plant species, they are only amenable in major crops which have already sequenced (Park et al., 2009). Although SNP can now be developed in coding sequence through Next Generation Sequencing approaches at reasonable cost, they are more common in non-coding regions of the genome as coding sequences are often under selective constraints (Mondini et al., 2009). On average, one SNP every 170 bp was identified comparing the sequences from two different rice cultivars, which makes this marker system an attractive tool in plant genomes in constructing linkage maps, QTL analysis and marker assistant selection (Gupta et al., 2001; Rafalski, 2002). Generally, the

frequency of SNPs in plant species is estimated to range from 1 in 30 bp to 1 in 500 bp (Park et al., 2009).

A set of 359 SNP markers derived from genic and intronic regions of the wheat genome were tested against 20 wheat cultivars. Reasonable levels of diversity were observed among wheat cultivars for SNPs located in two of the genomes; A and B. Markers in the D genome show approximately half the level of polymorphism of those for the A and B genomes. It is anticipated that SNP markers will play an increasingly important role in the genetics and breeding of wheat (Chao et al., 2009).

1.5.6   Diversity array technology marker (DArT)

Diversity Arrays Technology (DArT) is a hybridization-based microarray platform, generic and cost effective as a genotyping technology and recognized as a suitable technique for genome-wide discovery and description of genetic variation. This technique was developed to overcome some of the limitations of other molecular marker technologies such as RFLP, AFLP and SSR (Akbari et al., 2006).

This technology can be summarized as follows: complexity reduction using a chosen combination of restriction endonucleases, followed by adaptor ligation to amplify PstI-PstI fragments to obtain a genomic representation of the available genetic diversity, library creation so that each E coli colony contains one of the fragments, micro-arraying the library onto glass slides, treatment of individual genotypes with the same complexity reduction technique and hybridization of fluoro-labeled DNA onto the slides, scanning of the slides for hybridization signal and finally data extraction and analysis using DArT software (Mondini et al., 2009).

DArT allows the simultaneous scoring of thousands of restriction site based polymorphisms between genotypes and does not require DNA sequence information or site-specific oligonucleotides (Alexander et al., 2005). Screening a library of several thousand fragments from a sample of DNA fragments representing the whole genomic diversity in the species is key to the efficient discovery of new DArT markers, with all markers on a particular array being scored simultaneously (Jaccoud et al., 2001). For these reasons, it is considered an important tool for scientists studying and managing genetic diversity with high throughput and low-cost data. (Semagn et al., 2006b). DArT markers are applicable to model and non-model species/crops in addition to complex polyploid genomes, whereby the presence of individual fragment versus absence in genomic representative can be detected (Jaccoud et al., 2001).

The state of DArT development in plants

DArT technology has been used successfully in genotyping of many species. Initially it was developed for rice, a diploid species with a small genome of 430 Mbp (Jaccoud et al., 2001) and afterward it has been applied to many other species including Arabidopsis (Wittenberg et al., 2005), cassava (Xia et al., 2005), and for species with more complex genomes, e.g. barley (Wenzl et al., 2006) and the allopolyploid wheat (Triticum aestivum L.) (Akbari et al., 2006). This technology has been used recently to study the genetic diversity of bambara groundnut (Molosiwa, 2012; Olukolu et al., 2012).

The first version of a high density functional DArT genotyping microarray has been reported in 64 different Eucalyptus species of flowering trees and shrubs, it has been identified that 56% of the total number of cloned fragments were found to be

polymorphic among 284 individuals. This array will provide a high-throughput platform for population genetics and phylogenetics in Eucalyptus where many of the hybrids used commercially are inter-specific (Sansaloni et al., 2010).

Diversity Array Technology has been applied to oat (Avena sativa L.) allowing the construction of a high density genetic map, solving most of the problems of lower-throughput obtained using other technologies. Three discovery arrays were run and 2000 polymorphic markers were identified and 1010 of them were used to saturate and improve the 'Kanota' × 'Ogle' genetic map (Tinker et al., 2009).

DArT markers have been integrated with other markers types in different crop species. A high-density genetic map for a doubled haploid population from a wide barley cross was constructed using 442 DArT markers along with 536 SSRs. High levels of polymorphism, co-linearity and telomeric coverage of the genome were obtained, compared to a previously constructed map (Hearnden et al., 2007).

The DArT array has proved to be a useful marker system in Sorghum bicolor. Over 500 markers have been detected in this staple food and fodder crop, to be used in the analysis of genetic diversity and in construction of a medium-density genetic linkage map. This large number of DArT markers offers a more precise estimation of genetic relationships and provides valuable molecular breeding and genomic applications for this crop (Mace et al., 2008).

1.5.7   Next generation sequencing

The original Sanger sequencing method developed in 1977 (Sanger et al., 1977) and its modifications (Madabhushi, 1998; Smith et al., 1986) after discovery of the polymerase chain reaction (PCR) in 1983 (Inoue and Orgel, 1983) have prevailed in

the DNA sequencing field for nearly three decades. Although in the past decade the length of Sanger sequence reads has increased dramatically from 450 bases to more than 1kb, the primary restrictions are cost per sequence and the need to prepare each sequencing template separately (Smailus et al., 2005; Varshney et al., 2009a). So to try to overcome these limitations alternative next generation sequencing technologies have been developed, which dramatically increase throughput (although are currently still limited in sequence read length) (Angeloni et al., 2012).

Next generation sequencing can be defined as a high-throughput sequencing method that parallelizes the sequencing process, producing thousands or millions of sequences at once (Stapley et al., 2010). Genome analysis has taken off with the advent of NGS systems with the production of vast quantities of sequence at relatively low cost and in a very short time compared to an automated Sanger sequencing (Schloss, 2008). However NGS technologies still produce shorter reads and have greater error rates than Sanger sequencing, sacrificing assembled sequence quality for speed and greatly reduced costs (Berkman et al., 2012).

Next generation sequencing technologies, including Roche 454, Illumina GA, and ABI SOLiD, have been developed to generate more sequencing with lower cost than Sanger methods on ABI 3730xL platform (Bonetta, 2006; Schuster, 2008). The types and volumes of data produced through these technologies have increased greatly over the last few years, promising further accelerated progress (Pareek et al., 2011). Sequencing more than 20 million base pairs in a 4 hours period and considered as the first available system for NGS was developed by 454 and commercialized by Roche as the GS20 (Margulies et al., 2005). The greatest data volume of any NGS platform is generated with HiSeq2000 from Illumina with been up to 600 Gbp of usable data per run (http://www.illumina.com) (Barski et al., 2007; Johnson et al., 2007;

Varshney et al., 2009a). The SOLiD System from Life Technologies based on sequential ligation with dye-labeled oligonucleotides, can generate 20 – 30 Gbp of data per day, with read lengths up to 75 bp for the latest 5500xl system (http://www.appliedbiosystems.com) (Berkman et al., 2012).

The efficient use of two major NGS technologies of 454 pyrosequencing and Illumina has been demonstrated recently to generate large numbers of genome or transcriptome sequences. The sequence data generated from both techniques can be used efficiently to discover SSR loci and identifying the high quality loci for marker development, using the latest computational tools and high-throughput methods, with reduced cost and effort compared to the traditional Sanger approach (Zalapa et al., 2012).

Next-generation transcriptome sequencing (either by de novo assembly of the transcriptome sequence data or by aligning reads to a genome sequence) can be applied normally to analyse gene expression and sequence variation of genomic loci (Morozova and Marra, 2008). With next generation sequencing, the whole genome of many related organisms can be achieved, allowing comparative and evolutionary studies in different organisms regardless of the state of previous genetic information (Metzker, 2010). With modern NGS technologies it is possible to identify and track genetic variation more efficiently and precisely, and hundreds or even thousands of candidate genes could be tracked within a large genebank (Kilian and Graner, 2012).

Application of NGS in crop species

Traditional approaches of genome sequencing are progressively being substituted by assemblies of NGS technologies and they are currently being used to explore de novo genome sequencing in several crop species such as wheat, pigeonpea and common

bean (Varshney et al., 2009a). Genome sequencing and /or resequencing with the ongoing revolution of steady cost reduction and increased read length, could be extended beyond the major crops and model plant species to involve underutilized species and even the progeny lines of mapping populations. This will allow the breeder to follow the segregation of fragments easily from parents to their progeny. These fragments can be placed on the genetic and physical map with more certainty, which helps to confirm the introgression lines for the target traits and introduction of specific fragment from one species to another (Varshney et al., 2009a). An overview of NGS applications relating to crop genetics and breeding is shown in Figure 1-5.
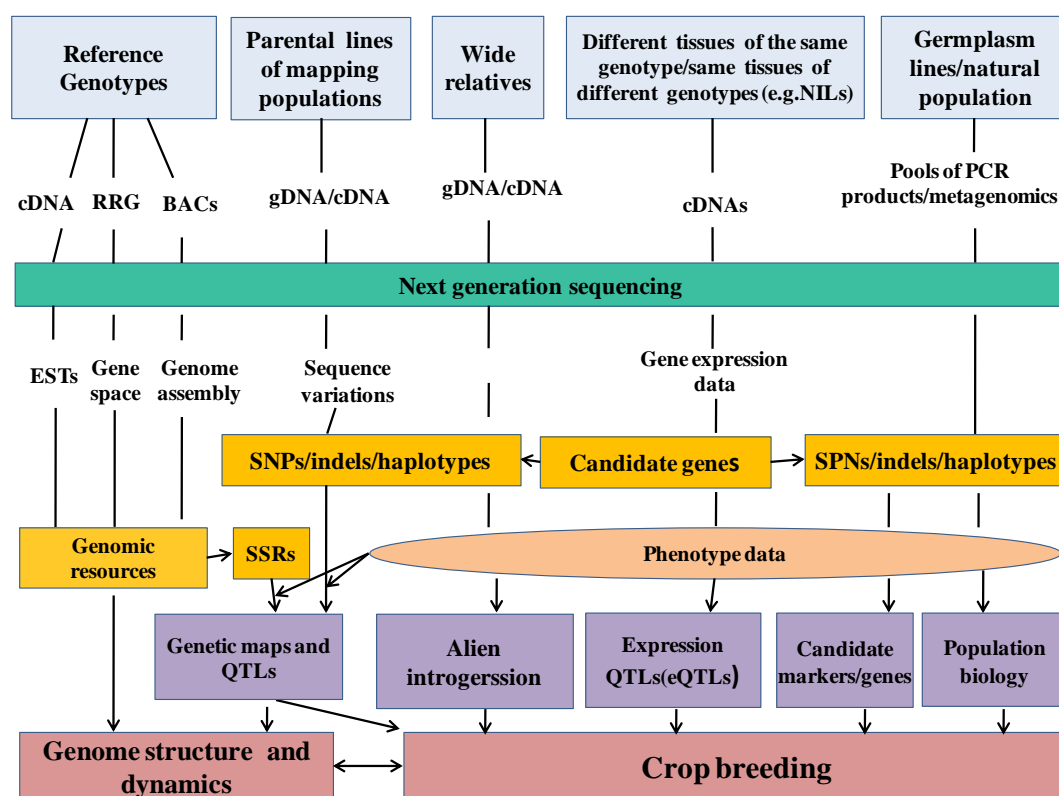
Figure 1-5: An overview of potential NGS applications in crop genetics and breeding. The progress including the generation of genomic resources, marker development and QTL mapping, wide crosses and alien gene introgression, expression analysis, association genetic and population biology (Varshney et al., 2009a).

Using such innovate technologies as NGS will accelerate the possibility of importing genetic knowledge into underutilized species from major or model species and gives a new vision of working with the genomes of clusters of related species instead of single species. The significant sequence data generated through these techniques also allows DNA markers to be produced at reasonable cost in an unknown genetic background which can be exploited for quality control, genetic diversity analysis and practical breeding approaches (Mayes et al., 2011).

RNA-seq is one of the most important applications of NGS technologies that is speedily replacing other methods of studying gene expression in non-model species without a sequenced genome (Morozova et al., 2009). With this method all RNA transcripts can be sequenced. It is practical in underutilized crop species because a reference genome is not strictly required (Strickler et al., 2012). Legumes Pisum sativum and Cicer arietinum were among the first transcriptome characterized species (Garg et al., 2011; Iorizzo et al., 2011).

1.6 Construction of genetic maps

The study of genetic linkage mapping dates back to the beginning of last century when scientists began to understand the recombinational nature and cellular behaviour of chromosomes (Wu et al., 2008). The first genetic linkage map of chromosome X of Drosophila melanogaster was constructed by Sturtevant (1913). With the advent of different molecular markers derived from different techniques it is now possible to follow the segregation of valuable alleles in the populations and map them (Cholin, 2009).

By the end of last century the introduction of DNA based markers caused genetic maps to become much more densely populated, generally into the range of several hundred to more than a thousand markers per genome (Wu et al., 2008).

Linkage maps indicate the position and relative genetic distances between markers on the chromosomes, allowing the identification of regions that contain genes and QTL related to interested traits (Paterson, 1996; Vinod, 2009). Genes which are tightly linked will be inherited together in the progeny much more frequently than those that are located further apart or even on different chromosomes (Semagn et al., 2006a). The recombination frequency of genetic markers allows the genetic distance between the markers to be determined. There are three main steps to constructing a linkage map: production of a mapping population, identification of polymorphic markers for mapping and linkage analysis of the markers which includes statistical procedures and the use of appropriate software packages (Collard et al. 2005).

1.6.1   Mapping populations

A segregation population is required to construct the genetic linkage map. Differences should be present at least in one trait between the parents of the cross selected for the mapping population. If the marker-trait association is to be attempted the individual offspring of the population must be phenotypically evaluated (Collard et al., 2005). Segregating populations such as F_2, F_3 or backcross populations are frequently used to construct a genetic linkage map, however the 'immortal' populations of recombinant inbred (RIL) and doubled haploid are preferable because they allow replicated and multi-location experiments (Collard and Mackill, 2008). It has been reported that the basic population size for genetic mapping ranges from 50-250 individuals; however

larger populations are often required for high resolution mapping (Ferreira et al., 2006; Mohan et al., 1997).

Types of population used to investigate the genetic control of traits

Different kinds of genetic population are often developed in autogamous species, such as: F_2, recombinant inbred lines (RILs), near isogenic lines (NILs), backcross populations and doubled haploid populations (DH) (Semagn et al., 2010). These populations are developed by crossing two (often inbred) parents with clear contrasting differences in the phenotypic trait(s) of interest, to follow the segregation pattern of the desirable traits and subsequent genetic analysis (e.g. Quantitative Trait Locus analysis) or for direct breeding selection.

The steps to generate these populations involve selecting the parents, crossing them with each other through controlled transfer of pollen from one parent onto the receptive stigma of the other parent, then advancing the progeny in an appropriate manner to obtain a set of individual plants or lines with variation for the traits of interest. Besides differing for phenotypic traits, the parents of a mapping population must also be sufficiently different at the genotypic level for the chromosome recombination events to be tracked using molecular markers (Collard et al., 2005; Young, 1994). Sometimes relatively little genetic polymorphism exists between the parents of crosses and this is often overcome through choosing genotypes which are highly genetically distinct, sometimes crossing to wild species to increase the level of polymorphism present (Kennard et al., 1994; Muños et al., 2011).

F_2 and F_3 populations

These are quickly and easily developed by self-pollinating the F_1 hybrid. In inbreeding species, this is relatively simple, with a single controlled cross being the rate limiting step to develop the F_1 hybrid from inbred parental lines (Semagn et al., 2006a). The primary advantage of both F_2 and F_3 populations is their ability to measure the effect of additive and dominant gene action at specific loci because they contain heterozygote individuals; however such populations are not fixable due to the heterozygous genetic constitution present in individual lines, which reduces with each generation of inbreeding, such that the proportion of heterozygous loci halves each generation, as the individual lines head towards being fully inbred. This lack of immortality of the lines restricts their use in analysis of QTL (Rakshit et al., 2012). In an F_2 population a single plant represents each genotype, so replication over time or space cannot be carried out (Semagn et al., 2010) and phenotypic evaluation on single plants are usually not considered reliable for quantitative traits like yield. This limitation of F_2 populations can be overcome by cloning F_2 plants in species that are easily cloned (e.g. by taking cuttings) to increase the replication of the traits. Another possibility is to produce F_3 plants from F_2 self-pollinated plants, each F_3 family consisting of multiple plants to be used for phenotypic evaluation. The assessment of single plants is replaced by an emphasis on the performance of F_3 families.

Recombinant inbred lines (RILs) population

Recombinant inbred lines are homozygous (fixed) lines and can be developed from F_2 individuals using single seed descent or continuous inbreeding (Keurentjes et al., 2011). One seed is taken from each F_2 individual and grown for a number of generations (until around F_8 to F_{10}) with single seed descent at each generation until

the lines are virtually homozygous at all loci. The result is a set of homogeneous, homozygous lines for almost all loci (Darvasi and Soller, 1995). These lines are true breeding and large amounts of seed can be produced for replicated trials, making such populations potentially immortal, for fully inbreeding species (Huang et al., 2011). RILs (or doubled haploids, where available) are considered powerful to analyze quantitative traits effectively, with marker data only needing to be generated once (Collard et al., 2005; Tanksley, 1993). Fully inbred lines can be readily circulated and new data could be continuously added to the previously constructed map. Genotype in this kind of population is represented by an inbred line, rather than by an individual, so genetic components of variance could be assessed more accurately when studying quantitative traits (Bailey, 1981). The main limitations in using this population type include the time and high cost for construction. Such populations will detect only additive effects without dominance effects. For species where the final product (such as the cultivar) is fully inbred, additive effects are likely to be sufficient. Where the final product is a hybrid between two inbreds, then dominance effects become important and these population cannot intrinsically reveal the effects of particular combinations of alleles within the hybrid (Haley and Anderson, 1997).

Backcross populations

This type of population is easy to develop by crossing an F_1 plant to one of its parents (usually the 'better parent' for a trait). Similar to the F_2 population, each primary backcross genotype is represented by a single plant. This kind of population is less informative for linkage mapping compared to an F_2 population as recombination among markers occurs in only one set of gametes (either male or female), although interpretation is simpler (Lander et al., 1987). BC1 populations can provide abundant

genetic information to analyze both additive and dominant effects. It can also be used to analyze QTL, however it is a temporary population and the population genetic structure will change with further self-pollination or backcrossing (Wang and Chee, 2010).

Doubled haploid populations

Heterozygous F_1 individuals are used to produce doubled haploid lines (DH lines) using microspore culture or wide pollination techniques. They can also be produced by female gametophyte (usually unfertilized egg cells) culture (Devaux and Pickering, 2005; Zhang et al., 2011). Production of haploids and chromosome doubling are two key steps in DH production. Haploid plantlets produced from embryos can undergo spontaneous doubling or this can often be induced through the use of Colchicine to produce doubled haploid lines (Forster et al., 2007). DH lines have the advantage of producing homozygous or fixed lines that are no longer segregating in a single generation (Collard et al., 2005; Semagn et al., 2010). This kind of populations can be used efficiently in map construction and QTL analysis, particularly when the effect of the QTL is small (Devaux and Pickering, 2005). They have been used for QTL mapping in many species e.g. (Behn et al., 2005; Semagn et al., 2007; Semagn et al., 2006c; Xu and Crouch, 2008).

1.6.2   Polymorphism identification

Identification of the polymorphic markers is the second step in genetic map construction. Enough polymorphic markers are required to be present between the parents of cross to construct the map (Young, 1994). The identified markers must then be screened against DNAs of the individuals of population and they have to reveal

differences between individuals to be called as polymorphic. Depends on their discrimination of homozygotes and heterozygotes they are classified in to dominant and codominant markers. Codominant markers can differentiate between homozygotes and heterozygotes, with the differences in the size 'alleles' apparent, while dominant markers are either present or absent within individuals (Collard et al., 2005; Semagn et al., 2006a).

1.6.3   Linkage analysis of the markers

Analysis of a linkage map composed of molecular markers makes it possible to detect the gene or QTLs controlling traits based on the genetic recombination during meiosis (Tanksley, 1993). Mapping programs are used to analyse the segregation patterns of DNA markers throughout coding the data for each marker in each individual of a population and conducting linkage analysis (Collard et al., 2005). Logarithm of odds (LOD) value or LOD score is used to express the strength of linkage between markers (Risch, 1992). The Distance between these markers along a linkage map is measured in terms of the frequency of recombination between them, however the genetic distance on a linkage map does not depend on the genome size of the plant species (the physical distance of DNA between genetic markers) but on the frequency of recombination between them (Paterson, 1996). Since recombination frequency is not linear, mapping functions are required to convert recombination fractions into additive centiMorgans (cM) distances. Two commonly used mapping functions are Kosambi and Haldane, assuming the presence of interference and assuming the absence of interference (i.e. no effect of crossing over in the neighbor regions, respectively (Hartl and Jones, 2001; Kearsey and Pooni, 1996)). Commonly used software programs and freely available online include Mapmaker/ EXP (Lander et al.,

1987; Lincoln et al., 1993) and MapManager QTX (Manly et al., 2001). The JoinMap software is another commonly-used program in constructing linkage maps, although it is not free (Stam, 1993; Van Ooijen, 2006).

Construction of a genetic linkage map is one of various applications of molecular markers in plant breeding. It allows detailed genetic analysis of qualitative and quantitative traits that enable the localization of genes or quantitative trait loci (QTL) (Doerge, 2002; Semagn et al., 2006a; Yim et al., 2002).

1.7 Quantitative Trait Loci

Genetic variation in nature often takes the form of a quantitative phenotypic range, with an approximately normal distribution, rather than of qualitative phenotypes that fall into discrete categories (Paran and Zamir, 2003). A QTL is a genome region that appears to contribute to a quantitative trait which can be localize by marker linkage map as first demonstrated by Paterson et al. (1988). It can be a single gene or it might be a cluster of linked genes that affect the trait. Mapping of QTL allows a statistical description of the effects of each genome region on quantitative traits (Vinod, 2009). QTL analysis studies the genetic variation in a controlled cross to locate the genes responsible and to explore their effects and interactions (Kearsey, 1998).

QTL mapping has facilitated investigations into the molecular basis of several traits in plant species such as Arabidopsis (El-Assal et al., 2001), rice (Kojima et al., 2002), maize (Thornsberry et al., 2001), tomato (Frary et al., 2000) and in soybean (Panthee et al., 2007).

QTLs mapping analyses the association between the QTLs and genetic markers for linkage disequilibrium, requiring the information on both markers and phenotypic values of each individual in the population used (Huynh, 2009). When genes

controlling quantitative traits interact with each other or there is a genotype and environment interaction QTL mapping has to involve more sophisticated analysis (Mackay, 2001). With increasing complexity levels of QTLs, different techniques have been developed in plants to associate QTL with molecular markers using mapping population (Nagabhushana et al., 2006). The commonly used techniques in QTL mapping are:

Single Marker Analysis (SMA): Considered as the simplest method, does not require a complete linkage map. This analysis can be performed with basic statistical software programs to detect QTLs associated with single markers by testing the association between trait values and the genotype at each marker locus. Linear regression is the most commonly used statistical method for this analysis (Collard et al., 2005). The programs used to perform this analysis include QGene (Nelson, 1997) and MapManager QTX (Manly et al., 2001). The sample size is the first consideration as more robust associations can be obtained with larger samples of individuals and also provides greater sensitivity to detect QTL (Doerge, 2002). The main disadvantage of this method is that apparent QTL effects at a marker will be smaller than the actual QTL effect as a result of recombination between the marker and the QTL. Also when the markers are widely spaced (more than 15 cM) the QTL may be quite far from all markers causing the power of QTL detection to be decreased (Tanksley, 1993).

Simple Interval Mapping (SIM or IM): Considered a more powerful method statistically compared to single marker analysis. It involves a test of association between trait values and positions within marker intervals along the linkage map to check for the presence of a QTL at many positions between two marker loci (Lander

and Botstein, 1989). The interval mapping (IM) approach uses information from informative markers simultaneously to estimate the QTL location and its effect (Knott et al., 1996). The disadvantage of this sort of mapping is that the detection of QTLs could be misleading by giving positive or negative results due to possible interference of other markers (Rodriguez-Zas et al., 2002). MapMaker/QTL (Lincoln et al., 1993) and MapQTL (Van Ooijen, 2009) have been developed to carry out this analysis.

Composite Interval Mapping (CIM): This method has been developed to address problems with the previous two methods of QTL analysis. It combines interval mapping with linear regression and the possibility to allow additional genetic markers in the model in addition to an adjacent pair of linked markers for interval mapping (Jansen and Stam, 1994; Zeng, 1993; Zeng, 1994). In CIM the variance from other QTL is accounted for by including partial regression coefficients from markers in other regions of the genome (Basten et al., 2001) and the effects of other QTL are not present as residual variance (Chakraborty and Zeng, 2011). Morever the bias that would normally be caused by QTL that are linked to the position being tested could be eliminated by performing this analysis (Nagabhushana et al., 2006). To perform this analysis a number of programmes have been developed such as: Window QTL Cartographer (Basten et al., 2001; Wang et al., 2007), MapManager QTX (Manly et al., 2001) and PLABQTL (Utz and Melchinger, 1996).

Multiple Interval Mapping (MIM): QTL mapping by MIM model was first proposed by Kao et al., (1999). This model could fit multiple putative QTLs simultaneously to reveal their individual effects in addition to analyse epistatic QTL and heritability of quantitative traits. Thus, the components of genetic variance contributed by individual

QTL can be calculated and markers assigned to represent them (Chakraborty and Zeng, 2011). The software R/qtl can be used to estimate multiple QTL mapping for quantitative trait (Arends et al., 2010).

However, none of these programs incorporates all the functions of QTL methodological analysis into one QTL mapping system, the QTLNetwork software package has been introduced to enable the detection of multiple QTLs which can simultaneously map quantitative trait loci (QTL) with individual effects, epistasis and QTL–environment interaction (Yang et al., 2008).

1.8 Marker-Assisted Selection

In plant breeding, as quantitative traits are potentially controlled by several QTLs, and due to the cost of utilizing several QTLs only a few DNA markers that are tightly linked to agronomical important traits (no more than 3 QTLs) have be used as molecular tools for marker assistant selection in practice (Collard et al., 2005; Ribaut and Betran, 1999). Up to 5 QTLs has been reported as being introgressed into tomato via MAS (Lecomte et al., 2004). Markers detected to be associated with QTLs from preliminary mapping studies were not directly useful in MAS because of the possibility of inaccurate position of QTL due to sampling bias (Melchinger et al., 1998). Confirmation and validation steps for these QTLs are preferable before using them without subsequent phenotyping in MAS (Langridge et al., 2001), although there are some examples of highly accurate preliminary QTL mapping data determined by subsequent QTL mapping research (Price, 2006).

All QTLs selected for MAS should ideally be stable across environments and they should account for the majority of phenotypic variance for the trait (Hittalmani et al.,

2002; Ribaut and Betran, 1999; Tanksley, 1993). The pipeline of marker

developments in QTL analysis and MAS can be described in Figure 1-6.

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┌─────────────────────────────────────┐
│      Population development          │
│  parental selection and hybridization│
└─────────────────────────────────────┘
                  │
                  ▼
┌─────────────────────────────────────┐
│           QTL mapping               │
│      linkage map construction       │
│   phenotypic evaluation for trait(s)│
│           QTL analysis              │
└─────────────────────────────────────┘
                  │
                  ▼
┌─────────────────────────────────────┐
│          QTL validation             │
│ confirmation of position and effect of QTLs│
│    verification of QTLs in independent│
│  populations and testing in different genetic│
│            backgrounds              │
│            fine mapping             │
└─────────────────────────────────────┘
                  │
                  ▼
┌─────────────────────────────────────┐
│          Marker validation          │
│ testing of markers in important breeding│
│               material              │
│ identification of 'toolbox' of polymorphic│
│               markers               │
└─────────────────────────────────────┘
                  │
                  ▼
┌─────────────────────────────────────┐
│       marker-assisted selection      │
└─────────────────────────────────────┘
```

Figure 1-6: Marker and QTL validation pipeline for MAS (Collard and Mackill, 2008).

Other considerations for the markers used in marker assisted selection are they should

be tightly linked to target loci at less than 5 cM genetic distance is preferable.

Flanking markers or intragenic markers increase the reliability of the markers to

predict phenotype. Good quantity and quality of DNA and simplicity of technical

procedure for marker assay are also required. In addition they have to be highly

polymorphic in breeding material and be cost-effective (Mackill and Ni, 2000; Mohler and Singrun, 2004).

Marker-assisted selection (MAS) is an approach whereby a phenotype is selected based on the genotype of a marker. This approach has been developed to solve the problems of selection criteria in conventional plant breeding through shifting from phenotypic selection toward gene selection either directly or indirectly, which may greatly increase the efficiency and effectiveness in plant breeding compared to conventional breeding methods (Collard et al., 2005). Once markers that are tightly linked to genes or QTLs of interest have been identified, prior to field evaluation of large numbers of plants, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs (Michelmore, 1995; Ribaut et al., 1997).

Applying MAS in the early generations has a great advantage allowing the elimination of plants with undesirable gene combinations. Thus greater attention can be concentrated on fewer high-priority lines in subsequent generations. When the linkage between the marker and the selected QTL is not very tight, the greatest efficiency of MAS is in early generations due to the increasing probability of recombination between the marker and QTL in later generations (Collard and Mackill, 2008).

The trend of utilization of molecular markers arrays and genetic maps make MAS possible both for traits controlled by major genes and for quantitative trait loci (Francia et al., 2005). Although the availability of public databases for markers and QTLs will lead to the greater adoption of MAS in major crops, the incorporation of MAS will still be very slow in plant breeding programmes for orphan crop species and in developing countries (Naylor et al., 2004). Focusing on comparative genome

analysis for closely related crops especially between model crops and orphan species might be one of the solutions to facilitate the construction of high-density molecular linkage maps for gene localization and QTL mapping of important agronomic traits in these minor crops.

1.9 Aim and objectives of this study

The aim of this project is to construct and develop a genetic linkage map of an F_3 segregating population derived from the intra-subspecific cross (DipC x Tiga necaru). The cross has been developed from domesticated landraces which contrast for a number of agronomically important traits, including; extent of bunching, internode length, canopy spread, peduncle length, biomass and seed yield. A partial genetic map for an inter-subspecific cross between a domesticated landrace and a wild ancestor (Basu et al., 2007c) will also be improved, traits analysed and the genetic maps compared. The mapping will develop, characterize and utilize microsatellites and Diversity Arrays Technology (DArT) markers to carry out a QTL analysis of economically important traits (Jing et al., 2009; Semagn et al., 2006b; Xue et al., 2010; Zhang et al., 2004). This should help to identify marker-trait linkages useful for the introgression of desirable genes or QTLs for marker-assisted selection in breeding programs of bambara groundnut. The analysis should also give a fundamental insight into the genetics underlying important agriculturally relevant traits (as well as the process of domestication, through a more detailed analysis of the 'wide' cross) which could help inform conventional breeding selection.

The objectives of this study were:

- Validation of the population under study using microsatellite markers to reveal the residual heterozygosity in the population.

- Development of polymorphic SSR and DArT markers to construct the genetic linkage map.

- Construction of a genetic map in the segregating population derived from an intra-subspecific narrow cross.

- Comparison of the 'narrow' intra-subspecific genetic map with the 'wide' inter-subspecific genetic map.

- Evaluation of important phenotypic and agronomic traits through selection in two different environments (Field and controlled environment glasshouses in Indonesia and the UK) for QTL trait analysis.

- QTL analysis of the important agronomic traits.

Thesis outline

Chapter1: Presents the introduction and the aims of the project. It also provides a review of the existing literature on underutilized crops, focusing on bambara groundnut. Different types of molecular marker and their application in different plant species, construction of genetic linkage maps, QTL analysis and MAS are also reviewed in this chapter.

Chapter 2: Explains the methodology of molecular marker analysis using SSR and Diversity Arrays Technology (DArT) markers, phenotypic evaluation in glasshouse and field experiments, data preparation, construction of the genetic maps and subsequent QTL analysis.

Chapter 3: Focuses on the results of screening the SSR primer pairs (derived from different microsatellite-enriched libraries) for polymorphism in the segregating populations, DipC X Tiga necaru and DipC x VSSP11. It also describes the development of DArT markers and the validation of the populations under study, through evaluating the presence of residual heterozygosity and evaluation of the populations for contaminants/out-crosses.

Chapter 4: Describes the distribution, segregation of agronomic traits and their associations for the F_3 population derived from the DipC and Tiga necaru landraces, in controlled environment glasshouses (Sutton bonington campus, UK) and in the Field (Bungah field, Gresik, Indonesia). Data collected previously from an F_2 population of the same cross in the Tropical Crops Research Unit was also involved alongside the F_3 data analysis.

Chapter 5: Presents the results of genetic linkage map construction for both narrow (DipC X Tiga necaru) and wide (DipC x VSSP11) crosses, with a shared maternal landrace (DipC) parent, using AFLP, SSR and DArT markers. This chapter also describes the initial attempts to combine the wide and narrow genetic linkage maps.

Chapter 6: Describes the QTL analysis of agronomically important traits and their inter-correlations.

Chapter 7: Overviewed the results and discussion of different chapters and also concluded some future works.

Chapter 2. MATERIALS AND METHODS

This chapter is divided into four sections. It starts with the molecular biology experiments in the laboratory (DNA and marker techniques). A segregating F_3 population derived from a narrow intra-subspecific cross and an F_2 segregating population from a wider inter-subspecific cross were used. The second section describes characterization of agronomic traits and their segregation patterns in a F_3 population derived from a controlled cross between the DipC and Tiga necaru landraces, assessed both in the field and the glasshouse. The third and fourth sections explain the methodology of constructing a genetic linkage map and QTL analysis, respectively, in both segregating populations from the narrow and wide crosses of bambara groundnut. Details of the materials and methods used for each aspect of the investigation have been described in their appropriate sections.

2.1 Development of polymorphic SSR and DArT markers and validation of the F_3 population

2.1.1 Plant materials

A segregating F_3 population derived from an intra-subspecific cross between the DipC (female) and Tiga necaru (male) landraces which were obtained originally from IITA was used as the plant material for molecular analysis using SSR and DArT markers. Although there was limited seed material available for the F_3 segregating population under study, it was possible to investigate a total of 73 lines in the glasshouse and in a field trial. Another segregating F_2 population, previously studied in the construction of an initial genetic linkage map for the cross of DipC x VSSP11, was also analysed.

2.1.2 DNA Extraction

For each line, approximately 5g of fresh leaf from the Glasshouse (25 leaflets in total) was picked from the bulk of plants within lines - from 4-10 plants per line were sampled, depending on the seed availability for that line - and flash frozen in liquid nitrogen. All 73 lines were transferred to the laboratory. The leaves were ground with liquid nitrogen using a pre-chilled mortar and pestle, after which the powder was transferred to a pre-chilled 50 ml falcon tube (Sarstedt) to begin DNA extraction. Genomic DNA was extracted from all the lines of the segregating population using the Dellaporta protocol (Dellaporta et al., 1983) with some modifications. DNA extracted was re-suspended in 500ul of 1X TE buffer. Samples were kept in 1.5 ml Eppendorf tubes and stored at -20^{o}C. All work with DNA stocks was carried out on ice. For the individuals of the F_2 population GenElute Plant Genomic DNA kit (Sigma Aldrich) was used to extract DNA from silica dried leaves for all individuals and their parents.

2.1.3 DNA quantitation

Gel electrophoresis was used to quantify and test the quality of genomic DNA. A known volume of each sample was loaded onto a 1% Agarose Molecular Grade (Bioline) gel in 0.5 x TBE (Tris borate EDTA) buffer alongside a range of uncut lambda DNA standards containing 500 to 25 ng DNA. The gel contained 1µL of 10mg/mL ethidium bromide stock per 100ml of gel (Promega Corporation). This was added once the dissolved gel had cooled enough to be handled safely and the gel was swirled before pouring. The gel was allowed to fully set before submerging in the same buffer in a Biorad Mini Sub-Cell GT kit and the comb removed. In the first lane, 5µL of 2-log DNA ladder (New England Biolabs) was loaded, followed by DNA

samples of bambara groundnut premixed with 6x loading buffer (2µL DNA + 5µL 6x loading buffer) for each sample. Uncut lambda DNA (50ng/µL) was loaded in the following amounts; 10µL, 5µL and 2.5µL to act as a fluorescence comparison with the unknown samples, allowing an estimate of sample concentration to be made. The gel was run at 90 Volt for 2 hours. The concentration of each DNA sample was determined approximately by comparing the fluorescence of the unknown sample under UV light with lambda DNA bands for the same gel. The quality of DNA can also be determined by visualization, comparing the size of extracted fragments with the 2-log ladder and lambda samples. After quantitation DNA samples were diluted to 10ng/µL to be used in PCR reactions.

2.1.4 Molecular markers

Two molecular marker techniques, microsatellite (SSR) and Diversity Array Technology (DArT) were investigated in this study to analyze the differences at molecular level in the segregating F_3 population of the 'narrow' cross. Residual heterozygosity was also revealed with polymorphic SSR markers, allowing the validation of the identity and generation of the population under study. Microsatellite markers were also applied to the F_2 population derived from the 'wide' cross (DipC x VSSP11) and the population was also analysed by DArT, allowing additional markers to be added to the initial linkage map (primarily based on AFLP markers).

2.1.4.1 Microsatellite markers developed and used in this study

A total of 124 SSR markers were tested for segregation in the wide and narrow crosses of bambara groundnut. These markers were derived from a number of sources,

including a 454-sequenced microsatellite-enriched library and a bambara groundnut leaf transcriptome. One SSR marker from soybean was also used in this investigation.

Roche 454 pyrosequencing technology was used to facilitate microsatellite discovery and sequencing. The libraries were developed using the method of Edwards et al. (1996). The technique involved digestion of genomic DNA with a restriction enzyme. Digested fragments were ligated to an adapter, then they were amplified with PCR, before hybridization to immobilized synthetic SSR repeat motif oligonucleotides. After stringent washing, the repeat-enriched amplicons were recovered from the hybridization filters and amplified. The pool of PCR fragments enriched for simple sequence repeats was 454 Pyrosequenced (Roche) using a 1/16th plate run with Titanium reagents. This was expected to give an average read length 400 –450bp, as defined by the manufacturer.

Sequences of microsatellite repeat motifs were used to screen for repeats within individual sequence amplicons using the MISA.pl (Perl) script (pgrc.ipk-gatersleben.de/misa/). The M13 5'-Tag approach was used to allow generic labeling with a third (M13 sequence) primer which was directly labeled with one of the following dyes; D2-D4 (Black, Green, Blue; WellRED, Sigma Aldrich) (Schuelke, 2000).

The primers used in this investigation were classified to three primer sets according to their development sources:

The first set of 57 primers was previously developed. Primer pairs amplified PCR fragments of the expected size were screened for polymorphism in the segregating populations. These were developed using Sanger sequencing of individual clones. Labeling used a M13 5'-Tag approach. One microsatellite marker from the related

legume, soybean (Glycine max L.) (Peakall et al., 1998) was also tested for polymorphism within the individuals of F_3 progeny (Appendix 1).

The second set of 37 SSR primer pairs were also designed from the bambara groundnut microsatellite-enrich library (Mayes, unpublished data), this time using a $1/16^{th}$ 454 Pyrosequencing run with Titanium reagents, allowing longer sequences (expected length 300-350bp) to be generated. A total of 37 primer pairs were designed by the current author with 32 fragment sequences being screened for polymorphism in the populations under study (Appendix 2). The first work on this library started with identifying the unique fragments containing microsatellite repeats from sequences contained SSRs. SSR parameters, including motif, size, repeat start point and end point were used to identify unique fragments from fasta dataset.

The third set of 29 primers was developed from bambara groundnut RNA deep sequencing transcriptome library. RNA-based sequence data was generated previously using a 1 plate 454 Pyrosequencing from the S19-3 genotype of bambara groundnut as part of an investigation into temperature effect on gene expression in bambara groundnut leaf by Sean Mayes' group. The sequences were screened in silico for microsatellite repeat motifs. Of 68 primer pairs synthesized 29 gave polymorphic SSR primers against a standard set of 24 genotypes representing Bambara groundnut germplasm. These were tested as the third set of microsatellites on both wide and narrow cross populations (Appendix 3).

Detailed microsatellite methods

Primer Design

Web-based programs Primer3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/) by Rozen and Skaletsky (2000) was used to design the primers flanking the identified SSR. In the program the unique sequences were pasted into the input field. The target SSR was identified using a square bracket "[ ]". Triangular brackets "< >" were used to exclude parts of the sequence such as adapter or obvious repetitive or poor sequence regions. Some of the default parameters were adjusted manually in the software package to screen for the required primer pair; for instance the product size range was fixed to start from 100 bp instead of the default of 150bp, primer GC% minimum not to be less than 40 and an effort was made for Max 3' Self Complementarity not to be more than 3 (Figure 2-1).

Figure 2-1: Default parameters condition to pick the primer using Primer3 (v. 0.4.)

The result of designed primer by web-based program (Primer3 v.0.4.) was picked and
saved as a text file (Figure 2-2).

```
F4GBQGD03F5L0A_LEN=295_QL=36_QR=262, (GA)14, primer name BN 145


OLIGO              start   len        tm      gc%    any     3' seq
LEFT PRIMER         126    19     59.86    52.63    4.00   0.00 GGCACTGGTAGCAACGAAA
RIGHT PRIMER        248    22     61.33    50.00    4.00   0.00 CGTGGACGTAACAACACAACAC
SEQUENCE SIZE: 295
INCLUDED REGION SIZE: 295

PRODUCT SIZE: 123, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00
EXCLUDED REGIONS (start, len)*: 1,35

    1 tcagcgacgtgactCTCTTGCTTACGCGTGGACTACATACCCATTGTGCCAGAGGCATCG
      XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

   61 CTTCCACCGTAGAAAGTAGCGTGGGCACCCTGCCATGCACCGCCGGTGTAGACACCGGGA


  121 ATTCTGGCACTGGTAGCAACGAAAAGGGTAACCAACGATGCAATGCAGAGCGAAACCGTT
           >>>>>>>>>>>>>>>>>>>

  181 CCAGCCATGGCGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGTAGTTGTGTTGTGTTGTTA
                                                   <<<<<<<<<<<<<<

  241 CGTCCACGACGTAAGACAAGAGctgagactgccaaggcacacgagggganaggnn
      <<<<<<<<
```

**Figure 2-2: Primer pairs designed with the Primer3 software. The SSR motif repeat has been
highlighted, the forward and reverse primers were denoted by ">>>" and "<<<", respectively.**

The modified M13 (-21) tail sequence (CAC GAC GTT GTA AAA CGA C) was
added to the 5'-end of the forward primers (Schuelke, 2000), to give Forward-Tag
primers. Fluorescently labeled M13 universal primer, labeled with blue dye (D2-D4),
was ordered from Sigma Aldrich/Proligo at the 10 OD scale. Reverse and F-Tag
primers were all ordered from MWG Eurofins. The three primer labeling approach
(Schuelke, 2000) with modified M13 sequence was used to label the SSRs prior to
running the PCR products on a Beckmann CEQ^{TM} 8000.

Preparation of main stocks of primer (200pmol/µL)

To prepare a 200pmol/µL (1000x main stock) from the ordered primers, Sigma Molecular biology grade water was added into the lyophilized primer containing vials to give a final concentration of 200pmol/L, based on the synthesis report. The tubes were vortexed, spun down and placed on ice for 30 min. After that a 5µL volume was taken from the main stock of both Forward & Reverse primers, separately, and mixed with 495 µL SDW, to produce a 10x primer stock 10X (20 µM/µL) for each primer. These were kept in a freezer at -20°C.

Three primer design

The system of three primer design was reported by Schuelke (2000). This approach allows a large number of potential microsatellites to be screened at reduced cost. Detecting the length of PCR product by capillary sequencer requires one of the primers to carry a fluorescent label. In this system, a specific sequence is added to the 5' end of the microsatellite-specific forward primer. This F-tag primer is used along with the previously used sequence–specific Reverse primer and 'universal' fluorescently–labeled M13 (-21) in the PCR reaction.

The forward primer used in the reaction was $1/10^{th}$ of the standard amount. The other $9/10^{th}$ of the forward primer are made up with the Universal fluorescent-labeled M13 primer. The reaction conditions were chosen such that during the early cycles, the forward primer with its M13 (-21) Tag (CAC GAC GTT GTA AAA CGA C) is incorporated into the accumulating PCR products. Thus, the universal M13 (-21) 'takes over' as a Forward primer and incorporates the fluorescent dye into the PCR product in later cycle.

Primer optimization

All the primers were optimized to identify the optimal and working annealing temperature at the gradient range of 50-65°C using a Thermo Hybaid Express PCR machine (Electron Corporation, Milford, MA, USA). Equal amounts of genomic DNA from six landraces at 10ng/µL concentration were mixed and used as a template in the primer optimization. The PCR reaction mixture is described as below:

2µL PCR Buffer (New England BioLabs; w/MgCl$_2$, final 1.5mM, 0.2µL Forward and 2µL Reverse primers (20 µM/µL), 1.8 µL of M13 Tag (20 µM/µL), 0.2 µL of dNTPs (0.25 mM each final concentration (Promega corporation), 0.1 µL of 5 unit Taq DNA polymerase (New England BioLabs), 2 µL of template DNA (10ng/µL) and 11.7 µL of sterilized distilled water. The master mix for each primer set was dispensed into 12 wells of 96-well plate (1-12) per primer pair. Eight primer pairs were screened per 96-well plate (Thermo Scientific). The plates were sealed with a Thermowell$^{®}$ Sealing mat (Fisher Scientific) and spun briefly in an Eppendorf refrigerated centrifuge (5180). Amplification was carried out across a gradient of 12 different temperatures with the following cycling regime: 94°C for 3 minutes, 35 cycles of 94°C for 1 minute, 12 temperatures from wells 1 to 12, ranging between 50-65°C for 1 minute, 72 ^{o}C for 2 minute; with a final extension at 72°C for 10 minutes.

Gel electrophoresis of PCR products

A 500ml agarose 2% gel was prepared in 0.5 X TBE. 2µL of ethidium bromide stock (10mg/ml) was added to the gel solution which was then poured into a taped Scot lab gel tray, with 26-well combs (Scotlab Maxi gel; model). 12 rows of combs (each row contained 26 wells) were fitted into the gel tray immediately after pouring. This allowed 3 x 96 well-plates to be analyzed at the same time. 5µL of 6x loading buffer

was added into each well of PCR products after amplification. The plate was spun briefly and 10μL from each well was loaded into the submerged gel, after removing the combs. Each primer pair reaction was loaded into one half of a row of the gel (each primer pair had 12 reactions across a 15°C annealing gradient). 5μL of 2-log DNA ladder (New England Biolabs) was loaded in the first lane of each primer pair. The gel was run for 45 minutes at 120 Volt. It was visualized by illumination with UV light and images taken using a Biorad (Gel DOC 2000), then printed on a thermal printer (Mitsubishi P91). Based on the intensity and size of bands, the optimal annealing temperatures were determined for the primers.

PCR reactions for the individual lines of segregating populations

The PCR reactions were carried out in a total volume of 20μL as above. The only difference was in the DNA template, with each well a single individual from the cross being analysed (plus parents and controls). The amplification was carried out in a ABI PCR 9700 Thermocycler machine (ABI, Carlsbad, CA, USA) with the following profile: Initial denaturation at 94°C for 3 minutes was followed by 35 cycles of 94°C for 1 minute, 50-65°C for 1 minute (at the optimal annealing temperature derived from a gradient annealing temperature PCR reaction) and 72°C for 2 minutes, followed by a final extension at 72°C for 10 minutes.

Gel electrophoresis for PCR products of the individual lines

Visualizing the PCR products of individual lines was conducted in the same way as described for primer optimization. Products were visualized to ensure that all samples had amplified before loading samples onto the Beckmann CEQ^{TM} 8000.

Capillary electrophoresis

The fluorescently labeled PCR products were loaded onto the Beckmann CEQ^{TM} 8000 (Beckman Coulter Inc, Fullerton, USA). The Beckman Coulter CEQ^{TM} 8000 Genetic Analyzer is a fully automated system for genetic analysis. It automatically fills the capillary array with a patented linear polyacrylamide (LPA) gel, denatures and loads the sample, applies the voltage program, and analyzes the data. All PCR products (SSR fragments) were sized on with a 400 bp size standard. The sample loading solution (SLS; Beckman coulter Inc, Fullerton, USA) was mixed with the size standard (SS; Beckman Coulter Inc, Fullerton, USA) in the ratio of 1:100 (v/v) and 25μL of the mixture was loaded into the individual wells of a new PCR plate. 2 - 4μl of the single or pooled PCR products (depending on the intensity of bands visualized on agarose gel and size range of SSRs under analysis) from each genotype was added into the mixture of SLS and SS solutions. Each well was overlaid immediately with a drop of mineral oil (Beckman Coulter, Inc Fullerton, USA).

Capillary result analysis

The CEQ^{TM} 8000 Fragments Analysis Software Version 8 (Beckman Coulter Inc., Fullerton, USA) was used to measure and analyze the fragment sizes of PCR products. The results were transferred to a Microsoft Office Word Document file. For each microsatellite marker the product sizes were scored manually in all individuals. The largest peak size (rounded to the nearest whole number) from the electrophoretograms was recorded as the allele size for each individual. Scoring was based on overall microsatellite allele patterns, rather than a specific peak, as the relative peak height can change between alleles and it is the overall pattern which is important to maintain a consistent scoring pattern.

2.1.4.2 DArT marker

Diversity Array Technology marker assays were developed and performed by DArT

Pty. Ltd (Yarralumla, Australia; www.diversityarrays.com) as previously described

(Akbari et al., 2006; Semagn et al., 2006c; Stadler, 2009; Wenzl et al., 2004).

Genomic representations were prepared from 73 DNA samples from the individual F_3

plants of the cross (DipC x Tiga necaru), using both restriction enzymes

representations (bgPabg and bgPt) as described in Stadler (2009). These

representatives were hybridized to the existing 7700 clone array and scored by DArT

Pty Ltd.

2.1.5 Application of the microsatellite markers in the F_2 population

A segregating F_2 population derived from the wide cross of DipC and VSSP11 were

prepared from dried leaves that had been stored for 10 years. Genomic DNA was

extracted from 98 individual lines of the F_2 population along with their parents and F_1

hybrid, using the GenElute Plant Genomic DNA kit (Sigma Aldrich). All

microsatellite markers developed from different sources were tested for

polymorphism in this population. The parents and F_1 hybrid were used first to detect

potentially polymorphic markers and those showing different allele sizes on the

Beckmann CEQ^{TM} 8000 were screened against all individuals. Genomic DNA of

individuals from this cross was amplified with a slightly increased cycle number

(37-38) due to the relatively poor quality of the genomic DNA from this cross.

2.1.6 Isolating DNA from Bambara groundnut seeds

2.1.6.1 DNA Extraction

An experiment was carried out to extract the DNA directly from the endosperm of bambara groundnut seed, without affecting seed viability. The seeds were drilled on the opposite side to the embryo with a Dremel craft drill (Dremel 300 series, model 300 F013030046, mexico). The GenElute Plant Genomic DNA kit (Sigma Aldrich) was used (with some modifications) to extract the DNA from the endosperm powder. Three different incubation periods (10, 30 and 60 minutes at 65^{0}C) were tested after adding both parts of lysis buffer A and B, to optimize the incubation period to obtain the highest yield of DNA from dried seed.

2.1.6.2 Germination test

The drilled seeds were tested for viability in a germination test, to check the influence of drilling the sample. Two drill diameters (3/64" and 1/16"), two different seed sizes (1 and 0.5 cm diameter) and untreated seeds, were used in a (complete randomized design) CRD experiment with three replicates of 5 seeds each. The seeds were treated with 10% Parazone bleach for 15 minutes, prior to the experiment, to surface sterilize them. Then they were rinsed three times with distilled water before use, to remove the effects of the Parazone. Germination testing was conducted in the germination room at 29°C on Whatman filter paper in plastic petridishes, with 5 seed per dish. Water was applied once a day to ensure that the paper remained wet and the irrigation water was treated with File-X fungicidal (0.0625 ml/2.5L water) to protect the seeds from fungal disease.

2.2 Characterization of the segregating population

The segregating F_3 population lines derived from an intra-subspecific cross of DipC x Tiga necaru landraces were evaluated in a controlled environment and in a field experiment, to determine the segregation patterns and inheritance of morphological and agronomic traits identified in the two parents of the cross. F_3 seed derived from self-fertilization of 73 F_2 plants were utilized for both evaluations. Previous phenotypic data recorded for the second filial generation (F_2) of the same cross grown in the Tropical Crops Research Unit (TCRU) at Nottingham University were also used in this study for trait and QTL analysis.

Phenotypic evaluation was performed for the F_3 segregating lines and their parents in a fully controlled glasshouse (FutureCrop glasshouses) at the School of Biosciences, Sutton Bonington campus, University of Nottingham. The experiment was set in complete randomized design (CRD) with 4 replicates. The individual plants represent each replicate. The seeds were planted on 8th Aug 2011 after preparation of the soil bed and seed were planted at a 2-3 cm depth under the soil. The glasshouse experiment was run for 5 months and plants harvested on the 8th Jan 2012. They were grown in rows with a 25cm x 25cm spacing between individual plants. Photoperiod was set at 12 hours using an automatic blackout system with a $28^{o}C$ day time temperature and $23^{o}C$ during the night, set for a 12/12 split. Trickle tape irrigation was used to irrigate the plant for 20 minutes, twice per day. Irrigation was adjusted as needed to maintain well watered conditions (Figure 2-3). A field trial was also carried out for all F_3 progeny lines in Bungah field, Gresik, Indonesia on 30th May 2010. Seasonal constraints at that time of the year in Indonesia constrained the life cycle of this crop to around four months and it was harvested on 26th Sep 2010. The available seeds for each line (5-15 seeds) were planted directly into the soil with 40 cm between

rows and 40 cm between the plants within rows. The morphological and important agronomic traits here were also characterized according to descriptors in the book 'Bambara groundnut (Vigna subterranea) (IPGRI, 2000).

Figure 2-3: Trickle tape irrigation for F₃ population of bambara groundnut grown in the FutureCrop glasshouses after 5 weeks from sowing.

2.2.1 Characterization of the traits

Glasshouse and field traits were characterized according to descriptors in the book 'Bambara groundnut (Vigna subterranea) (IPGRI, 2000) with some modifications. Data was recorded for the individual plants at different growth stages and during harvesting as follows:

2.2.1.1 Vegetative characters

Days to emergence: Number of days from sowing to the appearance of first true leaf on the soil surface.

Flower no./plant: Counted each 2-3 days from the first day of flowering for the duration of study.

Leaf no./plant: Recorded at the 4th month after planting as total leaf no/plant in the field experiment. In the glasshouses it was recorded weekly from 5 weeks after emergence for a further three months.

Days to flowering: Recorded from seedling emergence to the appearance of the first flower(s).

Plant height (cm): Recorded from 10 weeks after sowing and measured from the base of the plant at ground level to the highest point of the terminal leaflet.

Petiole length (cm): Recorded as the average length of five leaves of each plant at 10th week after sowing.

Terminal leaflet length and width (cm): Maximum length and width of central leaflet was recorded for 5 leaves per plant at 10 weeks from sowing.

Leaf area (cm^2): Estimated based on the central leaflet length and width using the method of Cornelissen et al. (2002) in the following equation:

$$A_{plant} = 0.86 * \text{Leaf number} \left[0.91 * 3 \left(0.95 * \text{Length} * \text{Width} * \pi /4 \right) \right]$$

Where leaf number = leaf number/plant; length and width being mean length and width of the terminal leaflet of five leaves/plant, and $\pi = 3.1416$.

Plant spread (cm): Greatest distance between two opposite points at the base and top of plant, measured at harvest.

Stem no./plant: Recorded at harvest.

Branch no./stem: Average number of branches on three stems/plant, counted at harvest.

Node no./stem: Average node number on three stems/plant, recorded at harvest.

Internode length (mm): Average length of fourth internodes measured for five longest stems per plant at harvest.

Growth habit: Recorded 10 weeks after sowing for all individual plants, based on the 4th petiole (P)/4th internode (I) length ratio (P/I) as measured in descriptors; Bunch type (P/I = >9), Semi-bunch type (P/I = 7 − 9) and Spreading type (open) (P/I = <7). In our investigation to follow the segregation pattern of growth habit in the segregating F_3 we used the following classification: Bunch types were recorded for the DipC parent class (P/I = >13.53), semi-bunch (intermediate) type (8.567< [P/I] <13.53) and spreading type for Tiga necaru parental class (P/I = <8.567). This modification of the standard classification was introduced to allow segregation in comparison to parental types to be scored.

Peduncle length (mm): Average length of five peduncles per plant, measured at harvest.

Days to podding onset: Number of days from seedling emergence to the discovery of the first pod(s) (at least 0.5 cm long).

2.2.1.2 Yield evaluation traits

Pod no./plant: Counted at harvest. Number of pods with more than one seed was also determined.

Pod weight (g/plant): Weight of dried pods (at 12% moisture content) was recorded after maintaining the harvest pods for three weeks at 37°C.

Pod length and width (mm): Digital Vernier Caliper (model no. OD-15GP, serial no. 211810, Mitutoyo UK Ltd.) was used to measure the greatest length and width of five dried pods containing one seed and five pods containing more than one seed, when available.

Seed length and width (mm): Digital Vernier Caliper was used to measure the greatest length and width of four dried seeds (at 12% moisture content).

Seed no./plant: counted after removing the shells of all pods.

Seed weight (g/plant): Weight of dried seed (at 12% moisture content).

Biomass dry weight (g/plant): The dry weight of all organic materials produced by the plant was measured.

Shelling percentage (%): measured as an average of all pods/plant, based on the weight of matured dried seeds compared to the weight of dried pods.

100-seed weight (g): Recorded after harvest at 12% moisture content.

Eye pattern around hilum: taken as a presence verses absence of eye pattern around hilum for the seeds of individual lines in the population under study.

2.2.2 Statistical analysis:

Anderson-Darling Normality tests (Stephens, 1974) were used to screen the distribution of the trait data. The inheritance and segregation of contrasting morphological traits were studied in the segregating population derived from the DipC and Tiga necaru landraces.

Chi-Square (Goodness-of-fit test) (Snedecor and Cochran, 1989) was used as an alternative to Anderson-Darling test for trait data that was non-normally distributed.

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

Where:

O_i = observed frequency.

E_i = an expected (theoretical) frequency, based on the null hypothesis.

The Chi-Square value was calculated depending on the expected model for complete and incomplete dominance of the trait distribution which depend on filial generation.

For instance the expected ratio for the phenotype of Mendelian traits is 3:1 and 1:2:1 for complete and incomplete dominance in the second filial generation, respectively. The result was compared to the chi-squared distribution probability to determine the goodness-of-fit at k-1 degrees of freedom.

Box-Cox transformations were applied for the traits showing non-normal distribution and not following Mendelian patterns of segregation, before retesting to determine whether the transformed trait was normally distributed.

Statistical software GenStat 14th Edition and MINITAB (Release 16) were used to analyze the variance, construct residual plots and detect significant association between the traits ($p < 0.05$).

2.3 Construction of the genetic linkage maps

JoinMap4 software (Van Ooijen, 2006) was used to construct linkage maps for the two segregating populations of the narrow and wide crosses. All 73 individuals of segregating F_3 population derived from the cross of DipC x Tiga necaru were used as the first mapping population. The population was screened with SSR and DArT markers and polymorphic markers were scored for all individuals to be used in constructing the map. The segregating F_2 population of 98 individuals derived from the cross of DipC and VSSP11 was also screened with both marker types; SSR and DArT. Polymorphic markers for this population were used to add to the earlier version of the linkage map based largely on AFLP markers.

2.3.1 Preparation of the data files:

Plain text files 'Locus genotype file; .loc' were prepared for categorized segregating marker loci. These were prepared with the text editor program Wordpad and loaded

into JoinMap4 software to be analyzed. The locus genotype file (loc-file) contains the marker segregation patterns within the population. It has a sequential structure, containing four line header instructions. The data body contains the actual genotype information for all individuals grouped according to locus as defined in the manual. The four instructions defined the name of the population, the type of population, the number of loci, and the number of individuals. The syntax of the four instructions is:

name = NAME

popt = POPT

nloc = NLOC

nind = NIND

where NAME was the name given to the population, NLOC and NIND were the numbers of loci and individuals, respectively, and POPT was the code for the population type, which must be one of the codes given in 'Population type codes' in the manual for JoinMap 4.0.

In the segregating F_3 population derived from the 'narrow' cross (cultivated landraces) the parental data was not available by the time of running SSR and DArT markers. Phase determination for DArT markers was carried out by analyzing the population initially as a Cross Pollinator (CP) to determine linkage phase for these markers, using the segregation type code of <hkxhk> (Figure 2-4).

Linkage analysis was carried out (as described below for the F_3 dataset) and all markers showing linkage were collected into a new linkage file, for conversion (using the determined phase data) into the new F_3 loc file.

Figure 2-4: The locus genotype file (.txt) used to detect the phases of DArT markers in segregating F$_3$ population derived from 'narrow' cross in bambara groundnut.

Phase in the linkage groups was used to convert the DArT marker loci to different classifications in an 'RIL3' population type dataset ({0,0} giving a or c, while {1,1} gave b,d). The newly arranged marker locus loc file for the F$_3$ population was prepared again in plain text file to assign linkage groups.

In the wide cross population the F$_2$ population code was selected for use in mapping, as shown in Figure 2-5.

Figure 2-5: The locus genotype file (.loc) used the phases of DArT markers in segregating F$_2$ population derived from 'wide' cross in bambara groundnut.

2.3.2 Genotyping codes for population types

It is very important that the order of the individuals is identical over all loci in the file.

The genotype code for population types RIL3 and F$_2$ were as follows:

Code	Description
a	homozygote as the one parent
b	homozygote as the other parent
h	Heterozygote
c	not genotype a (dominant b-allele)
d	not genotype b (dominant a-allele)
-	genotype unknown

Classification type codes at each locus were summarized for all individuals automatically by the software into different genotypic classes with the expected ratio adjusted according to the expected segregation ratios (Table 2-1).

Table 2-1: Classification codes of F_2 and RIL3; the ratios of expected Mendelian segregation

Code	Ratio		Classification into genotype classes
	F_2 population type	RIL3 population type	
(a,c)	1:3	3:5	a and c; h and b will be included in class c
(b,d)	1:3	3:5	b and d; a and h will be included in class d
(a,h,b)	1:2:1	3:2:3	a, h, and b

2.3.3 Linkage analysis of the markers

The linkage map was assembled for both populations, using the JoinMap4 software. The default calculation options were applied, started with calculating the LOD scores and pairwise recombination frequencies between markers. The loci for all individuals in both population types were grouped according to 'independence LOD'. They were manually selected using thresholds from LOD 3-5 and Regression mapping was carried out. The Haldane mapping function was used to convert recombination frequencies into map distances (centimorgan; cM). Segregation distortion was determined through a chi-square test for goodness of fit by comparing the observed ratios to those expected, at the threshold of $p = 0.05$. The third mapping round forces markers which have been grouped, but rejected in the first and second rounds of mapping into the genetic map. The markers those forced to be grouped after first round but showed negative map distances and large jumps (threshold value >5) in mean chi-square values were removed from the map.

2.4 QTL mapping

Phenotypic data for all agronomical traits for each individual across different environments were combined separately with genotypic data and the linkage map in

order to identify QTLs associated with these traits using MapQTL v.6 software, for each environment (Van Ooijen, 2009).

In the narrow cross, QTL analysis was conducted for the means of agronomic data for the segregating F_3 population in both glasshouse and field trials. Phenotypic data recorded previously for the segregating F_2 population of the same cross (TCRU, 2004) were also used for QTL analysis. In the wide cross QTL analysis was performed for the phenotypic data recorded for the F_2 population, analysed using the improved genetic map derived from the wide cross of DipC x VSSP11.

2.4.1 Data files for QTL analysis

Three types of data files were prepared for QTL analysis. They were prepared according to the instructions given in the software manual (Van Ooijen, 2009). All three data files were imported into the program and placed in the same directory to avoid confusion. These data files are:

1. Locus genotype file: It also called loc-file, being the same file as was used for JoinMap 4 software to construct the map. This file contains the genotype codes for the loci of segregating lines.

2. Map file: containing the mapped positions of all loci, resulting from grouping and mapping the markers to yield the constructed map. This file will be used by the software to dictate the positions of the markers relative to each other for applications such as Interval Mapping. In non parametric mapping the loci are analysed one by one and the positions of map are only used to sort loci, while in the interval mapping they are used to calculate recombination frequencies, necessary for likelihood calculation.

Map file has no header and starts with the instruction of group number. The loci with their map positions must be given on subsequent lines in ascending order (Figure 2-6).

Figure 2-6: Part of a map file (.map) used for QTL mapping of narrow cross population.

3. Quantitative data file: (also called qua-file) has the data for all quantitative traits for all individuals. The header of the file contains three instructions, followed by the names of the traits. The data body contains the trait information for all individuals. The three instructions define the numbers of traits (ntrt = NTRT), individuals (nind = NIND) and the text that indicated a missing value (miss = MISS), here denoted by '*' (Figure 2-7).

```
Fielddata.qua - Notepad
File  Edit  Format  View  Help
ntrt=29
nind=73
miss=*
Emergence
leafno
plantheig
plantspr
leafletlen
leafletwid
Leafarea
petiolelen
pedunclele
Podweight
Podno.
Podno.2seed
podwidth
podlength
flowering
stemno
Branchno
Nodeno
seedsweight
seedno
seedlenght
seedwidth
shelling%
100-seedwei
biomass
internode
growthhabit
T-podlenght
T-Podno.
9       32.00    21.9    26.8    5.1     2.37    713.2150446        *        6
*        *        *       *       *       *       0.74       *     4.472135955
6       20.00    15.6    15.5    3.67    1.7     230.0895933     9.33     3
8       7.6      6.4     71.05263158     33.75   8.77    0.3     31.11111111
7       39.00    21.98   34.32   5.92    3.06    1302.745961     13.31    4.54
10.2    8.3      6.17    93.33333333     37.05882353     19.12   1.515714286
6       44.33    23.32   41.18   6.05    2.65    1310.640639     15.37    4.53
8.5     9.01     6.67    57.64499121     48.23529412     19.23   1.621809524
6       27.63    19.7    22.97   5.32    2.13    577.225512      10.46    5.13
5.75    7.94     6.23    45.71917808     38.69565217     14.50   0.652    16.0403
6       38.17    19.93   30.03   5.51    2.14    829.8538236     12.53    4.99
7       7.94     6.31    92.40506329     41.71428571     17.28   1.272    9.84888
6       39.92    18.69   30.45   5.35    2.06    811.3290066     10.61    3.62
8.33    7.11     5.67    86.4594498      32.26666667     14.66   1.425777778
6       28.27    17      20.77   4.32    2.04    459.4450886     9.05     3.09
7.8     8.63     6.54    67.81725888     42.82051282     10.29   0.546666667
```

Figure 2-7: The trait file (.qua) of the field data used for QTL mapping in the narrow cross population.

Transformation was used to try to normalize some of the traits which had not been distributed normally, before performing a QTL analysis. The Box-Cox transformation (Box and Cox, 1964) was performed with QI macros SPC Software for Excel.

2.4.2 The analysis of QTL

Nonparametric mapping (Kruskal-Wallis analysis; KW)

This analysis was done for all normal and non-normally distributed trait data. Interval mapping has an assumption of normally distributed residuals, but KW analysis does not rely on this assumption, so is suitable for both normally and non-normally distributed traits. The Kruskal-Wallis test is regarded as the nonparametric equivalent of the one-way analysis of variance. The test ranks all individuals according to the quantitative trait, while it classifies them according to their marker genotype. A segregating QTL (with big effect) linked closely to the tested marker will result in large differences in average rank of the marker genotype classes. A test statistic based on the ranks in the genotype classes is calculated. For individuals in ties, i.e. several individuals have equal values of the quantitative trait, the average rank (midrank) is used, while for the test the statistic adjusted for ties is used (indicated by K*) (Lehmann, 1975).

The Kruskal-Wallis statistic K* is a Chi-square like distribution, including the degrees of freedom (n-1 df for F_2 and F_3 populations) and the significance level in asterisks. [Significance level: *:0. 1, **: 0.05, ***: 0.01, ****: 0.005, *****: 0.001, ******: 0.0005, *******: 0.0001]

Interval mapping

In interval mapping a so-called QTL likelihood map is calculated. In each position in the genome (every centiMorgan) the likelihood for the presence of a segregating QTL is determined [the likelihood under the alternative hypothesis (H1)]. At the same time the genetic effects of the QTL and the residual variances are calculated. Coefficient determination is performed from the model of analysis to estimate the proportion of

phenotypic variance attributed to a particular QTL and the additive effects. This likelihood under H1 is compared to the likelihood for the situation when a locus with zero genetic effects (no segregation) [the likelihood under the null-hypothesis (H0)]. This comparison is done with a likelihood ratio statistic called the LOD (or LOD score), which is the 10-base logarithm of the quotient of the two respective likelihoods. The maximal LOD position was determined as a most likely position of QTL and a one LOD decrease on either side of LOD peak was considered as a confidence interval for the effect. The appropriate threshold was estimated, based on the actual data under study, to detect the significance threshold for interval mapping. This can be determined by performing a Permutation Test using 10,000 reiterations to develop Genome Wide (GW) threshold equivalent to $p \leq 0.05$ (Churchill and Doerge, 1994).

Chapter 3: THE DEVELOPMENT OF POLYMORPHIC SSR AND DArT MARKERS FOR MAP CONSTRUCTION AND VALIDATION OF THE F$_3$ POPULATION

3.1 Introduction

Molecular markers act effectively as chromosomal landmarks which possess multiple alleles. These sequence variants are distributed in different individuals, so the pattern of inheritance of alleles from parental genotypes can be traced after the construction of a controlled cross between them. This allows the testing of the patterns of inheritance of these loci and the surrounding chromosomal region that has been co-inherited. Generally, such markers are considered to be phenotypically neutral (Chauhan and Varma, 2009). They are passed on by the standard laws of inheritance through the generations, potentially allowing target allele association with trait inheritance (Semagn et al., 2006b). They have several advantages over traditional phenotypic markers, particularly in terms of access to very large numbers of phenotypically neutral markers which can be used to objectively assess associations between marker and phenotypes. This creates opportunities to improve crop varieties in a shorter time-scale, through the association of marker alleles and trait genes (Farooq and Azam, 2002). There are several types of molecular markers which differ in principle, methodology, and application. Careful consideration is required to choose one or more classes of such markers (Semagn et al., 2006b). In the current study detection and exploitation of DNA polymorphism used both microsatellite and DArT markers.

This chapter includes a discussion of the methodologies of SSR and DArT markers, their application, analysis and interpretation in the 'narrow' and 'wide' cross populations. As a starting point for mapping, validation of the F_3 population under study was carried out, both in terms of legitimacy and also to confirm the generation of inbreeding of the lines. As an early application relevant to breeding programmes, the isolation of DNA from dried bambara groundnut seed were also investigated with a view to rapid fingerprinting of putative hybrids in the dried seed state.

3.1.1 Microsatellite markers

A microsatellite contains many copies of a short tandem repeat sequence. These sites are usually in the size range of 16 to 60 bases in total, composed of individual repeat units of up to six base pair repeats in length. The sequence repeat unit and overall repeat distribution of different SSR motifs has been investigated in many organisms (Trivedi, 2004). In plants, the average distance between SSRs was found to be 6-7 kb which is similar to reports in mammals (Cardle et al., 2000). Microsatellites are often very polymorphic, scattered throughout genomes, usually located in the same place on the genome across individuals within a species, but often with a difference in the number of repeat sequences (Kalia et al., 2011; Trivedi, 2004).

Amplification of SSR

Microsatellites can be identified by amplification with the polymerase chain reaction, using primers designed to unique sequences flanking the repeat region. As these markers are PCR-based, only small amounts of DNA are needed for amplification and polymorphism can be detected in a number of ways to reveal size differences between alleles (Hammock and Young, 2005).

90

Limitations of SSR markers

Microsatellites have proven to be adaptable molecular markers, particularly for population analysis, but have some limitations. Microsatellites developed for a particular species can often be applied to closely related species, but the percentage of amplified loci may decrease when the genetic distance between design and tested species increases (Jarne and Lagoda, 1996). PCR failure may result when a particular allele at a locus fails to amplify, whereas others alleles may amplify more efficiently and may appear homozygous on a gel assay, when they are actually heterozygous in the genome (Gholizadeh and Mianji, 2007). Such 'null' alleles are found in most taxa and complicate the interpretation of microsatellite allele frequencies. These are considered a technical limitation which might lead to high frequencies of false homozygotes and cause problems for diversity or parentage analysis (Dakin and Avise, 2004; Reece et al., 2004). Large allele dropout and slip-strand mispairing for microsatellites during PCR are considered another problem that can cause reduced signal strength or increased stutter (Van Oosterhout et al., 2004). Stutter bands are artifacts having less signal intensity and usually they are shorter than the actual SSRs. They are thought to arise due to the inherent instability of the repeat sequence in the PCR reaction, reflecting their instability in vivo which leads to their high mutation rates. These bands have to be taken into consideration during microsatellite scoring, as improper scoring of the bands will lead to incorrect results during data analysis (Wang et al., 2009).

Alleles containing more repeat sequence bases are more likely to be mis-replicated as the long alleles have more chance of recombination instability during meiosis (Hancock and Simon, 2005).

3.1.2 Diversity array technology markers (DArT)

Due to a number of limitations for other markers types, such as; time, cost and resources required to discover a large number of markers for a species, the DArT system has been developed to overcome some of these limitations. DArTs have the advantage of being highly locus specific, due to their detection by hybridization (Jaccoud et al., 2001) but unlike SSRs they are considered dominant, therefore unable to differentiate between homozygous and heterozygous loci. Although, SSR markers have an advantage over DArT markers as they are co-dominant, highly polymorphic and widely distributed in the genome (Yang et al., 2006), the requirement of substantial sequence information to generate SSRs makes DArT more applicable, especially in underutilized crops where no sequence information exists, as is the case for bambara groundnut.

Diversity Arrays Technology offers a low-cost per marker high-throughput, robust system with minimal DNA requirements, capable of providing comprehensive genome coverage even in organisms with limited or no DNA sequence information. The "genotyping array" is hybridized with an individual DNA sample using slide microarray technology; the resulting hybridization signal is measured and converted into a genotype score for 'presence' or 'absence'. Such arrays have been developed by Dr. Kilian and his team for about seventy species, includes; sorghum, rice, barley, wheat, chickpea, pigeon pea and many others including animal species (www.diversityarrays.com; as at July 2012).

DArT uses an array of individual clones from a genomic representation prepared by amplification of fragments between 'rare' cutting restriction fragments (often PstI) using adaptors to the cut sites. A second frequent cutting enzyme reduces the complexity of the pool and the number of amplifying PstI-PstI fragments. Labeled

genomic representations of the individuals to be genotyped prepared in exactly the same way are then hybridized onto individual arrays. The polymorphisms scored are the presence versus absence of hybridization to individual array elements. The platform allows high-throughput screening of tens of thousands of potential molecular markers in parallel, and it especially suited for the generation of genome-wide markers for genetic linkage mapping.

3.2 Results

A total of 37 primer pairs were designed in this study and used as additional microsatellite markers to those developed previously; here referred to as 'the second set'(as described in Chapter 2). The pool of PCR fragments enriched for simple sequence repeats was generated from a bambara groundnut microsatellite-enrich library using a $1/16^{th}$ 454 Pyrosequencing run with Titanium reagents. A total of 5443 sequences were produced giving a total length of 1,697,965bp. As a result, 261 sequences were found to be unique and had enough sequence flanking the SSR repeat motif to design the primer pair. These unique sequences include the SSRs from 2-5 unit size, having allelic sizes from 10-60 bp for repeated unit and 19 of them were with compound format of SSR. The results of the microsatellite motifs search, using the MISA.pl script, and the distribution of repeated type for library fragments are shown in Table 3-1.

Table 3-1: Result of microsatellite-enriched library and the distribution of repeated type.

Results of microsatellite search						
Total number of sequences examined					5443	
Total size of examined sequences (bp)					1697965	
Total number of identified SSRs					1559	
Number of SSR containing sequences					1290	
Number of sequences containing more than 1 SSR					145	
Number of SSRs present in compound formation					227	
Distribution of different repeat type classes						
Unit size	1	2	3	4	5	6
Number of SSRs	68	740	669	58	21	3

Thirty seven primer pairs were designed in this study from a total of 32 fragments out of 261 unique sequences. These primer sets were used along with the other two sets of primers designed previously to give a total number of 124 primer pairs. These were test for segregation in the both 'wide' and 'narrow' crosses of bambara groundnut.

3.2.1 Microsatellite markers applied to the F$_3$ population of the 'narrow' cross

3.2.1.1 Primer optimization

The amplification was performed with a Thermo Hybaid Express PCR machine to optimize annealing gradient temperatures for all the primers. The best annealing temperature was identified for each primer based on the intensity of the amplified bands from 12 different temperatures along the gradient. To detect polymorphic alleles all individuals were amplified with the primers at the best annealing temperature using the M13-Tag Forward primer in a three primer reaction to allow fluorescent labelling.

In the first primer set all markers amplified as expected except Primer81, while bam2coL58 gave a weak band with fragment size larger than expected (more than 500bp). Generally, most of the primers amplified with a wide range of gradient temperature (Figure 3-1).

In the validation of the second primer set with bambara groundnut genomic DNA, the product sizes in general were less than 300 bp, however the fragments amplified with some of the primers were not in agreement with their predicted sizes. Based on original sequences the product sizes of primers BN11 and BN21 were expected to be 152 and 114 bp, while the product sizes for both showed fragments more than 400 and

500bp on agarose gel, respectively (Figure 3-2) and they eliminated from further test. In total the optimal annealing temperature was identified for 22 out of 37 primers as the rest were not amplified well or they had unexpected product sizes.
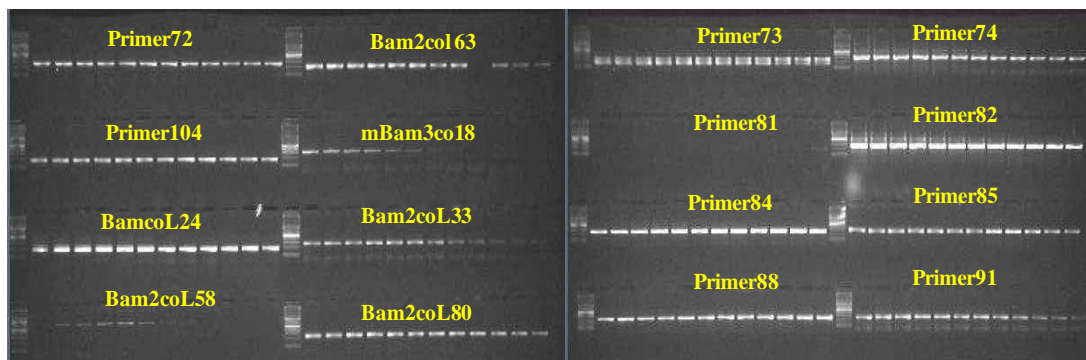
Figure 3-1: Annealing gradient evaluation of a range of primers from the bambara groundnut microsatellite enriched-library, showing amplification across a wide range of temperatures (50-65°C) with different expected fragment sizes (168bp-294) and unexpected (incorrect) fragment size (>500bp) for primer bam2coL58.
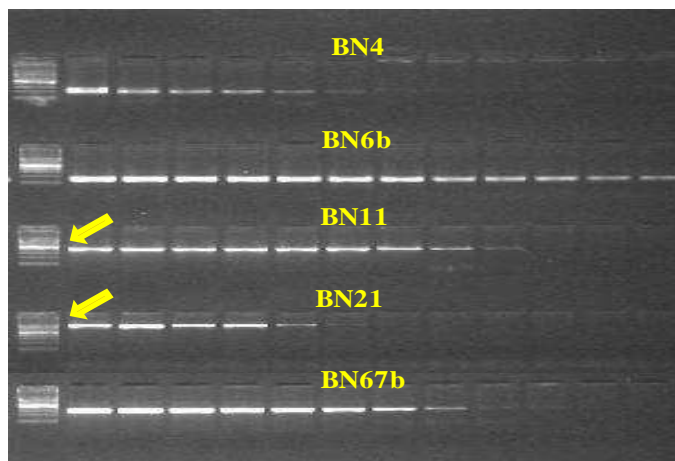
Figure 3-2: Annealing gradient evaluation for a number of the second primer set of bambara groundnut genomic enrich-library designed using Primer3 from sequence generated with Titanium reagents. The temperature gradient ranges from 50°C to 65°C and uses mixed genomic DNAs representative of bambara groundnut germplasm. An expected fragment sizes of around 400bp and 500bp were amplified for both primers BN11 and BN21, respectively, which they were too big to be use in capillary analysis.

All 29 primers developed from bambara groundnut transcriptome amplified products of the expected size in an annealing gradient test with bambara groundnut genomic DNA. They were then used to amplify individual lines at the best optimized temperature to screen for allelic size differences.

3.2.1.2 Detection of polymorphism

All primer sets with identified optimal annealing temperatures were used to screen for polymorphism within the F_3 segregating population. Each primer pair was run against 8 lines of the cross, initially. When polymorphism was detected for primers, the remaining lines were included to generate a full dataset for the mapping. The correct size of different alleles were determined with the CEQ^{TM} 8000 Fragment Analysis Software Version 8 (Figure 3-3) and data scored manually for segregation.
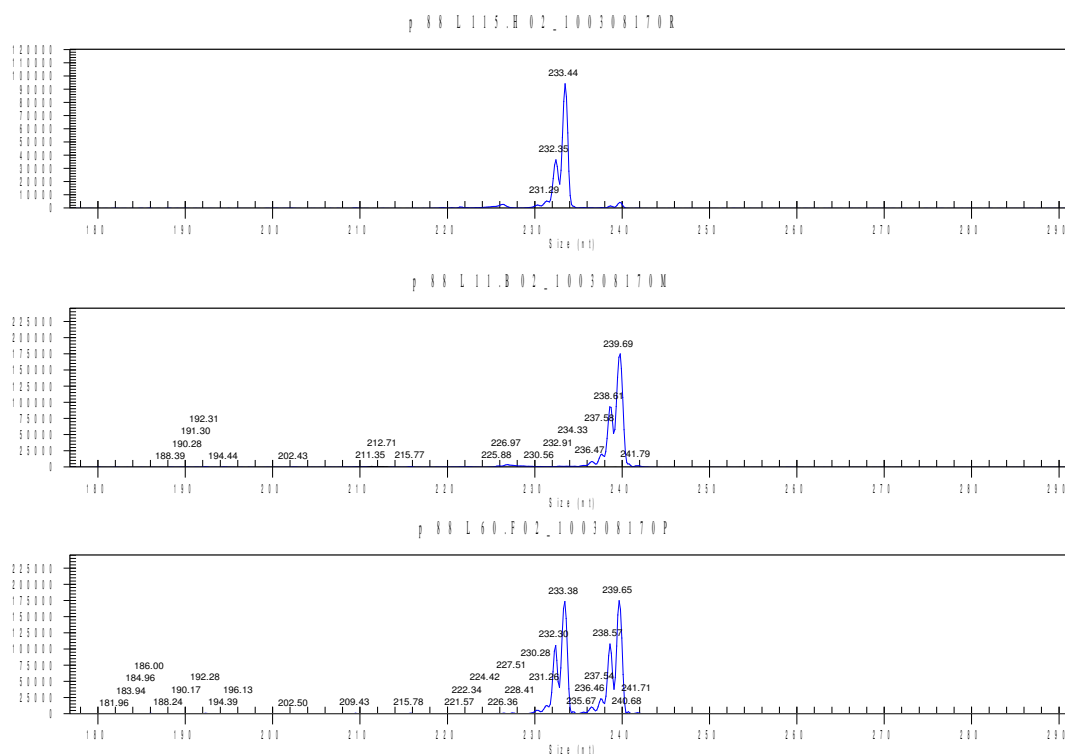
Figure 3-3: Capillary result for the polymorphic Primer 88 showing different fragment lengths of parental alleles (DipC and Tiga necaru) within selected lines of cross. Line 11 and 115 contained one parental allele each, while line 60 has both parental alleles.

The parents of F$_3$ population were confirmed by genotyping of available seed pools by the time of developing primers from the RNA deep sequencing leaf transcriptome for bambara groundnut. All 29 primers were run directly against the parents of cross (DipC and Tiga necaru). The PCR products were run on capillary electrophoresis and different fragment lengths for parental alleles were identified for 15 primer pairs (Table 3-2).

Table 3-2: Polymorphic microsatellite primers derived from the RNA sequence data in the F$_3$ population of 'narrow' cross

No.	Primer name	Optimal annealing temperature °C	'narrow' cross	
			DipC	Tiga necaru
1	D.5953	50	315	297
2	D.25551	60	200	198
3	D.42026	60	238	229
4	D.8148	65	244	229
5	D.8999	55	203	205
6	D.37053	55	181	178
7	D.12522	60	328	324
8	D.14265	55	182	176
9	D.16501	50	255	267
10	D.24269	60	246	238
11	D.35497	55	168	202
12	D.51646	60	185	189
13	D.7215	62	208	202
14	D.125	60	277	273
15	D.2094	60	224	227

Out of these 15 polymorphic primers, one of them (D.35497) was screened by agarose gel electrophoresis. A 2.5% agarose gel in 0.5 X TBE (500ml tray size) was used (adding 3µL of ethidium bromide stock; 10mg/mL) and the gel was run for 3 hours on 90Volts (Figure 3-4).
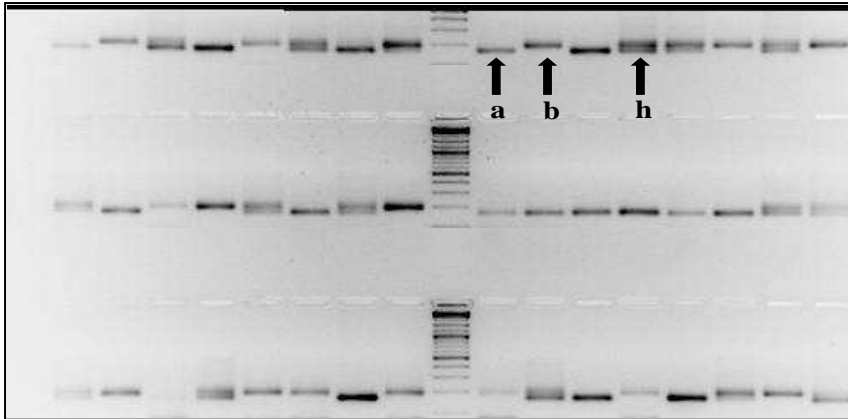
Figure 3-4: Reverse gel plate image of PCR products amplified by primer D.35497 (derived from the leaf transcriptome library) with a selection of the individual F$_3$ lines of the 'narrow' cross. (a) allele from DipC parent, (b) allele from Tiga necaru and (h) for a heterozygote line within the cross. Fragments are visualised on a 2.5% agarose gel run on 90 Volt for 3 hours.

3.2.1.3 Validation of the F$_3$ population

All 73 individual lines of the F$_3$ population derived from the cross of DipC x Tiga necaru were screened with the 33 polymorphic SSR markers derived from these three sources of SSRs. Heterozygote alleles were scored across all 73 individual lines by each marker and the average level of residual heterozygosity in this population was identified to be 24.86% (Table 3-3). This result shows that this population is effectively an F$_3$ population, as expected from planting history. In addition, no lines which were not consistent with the expected origin were identified.

3.2.2 DArT markers applied to the F$_3$ population 'narrow' cross

The slide microarray was developed as previously described (Mayes et al., 2009; Olukolu et al., 2012; Stadler, 2009) . Of the 7680 fragments detected in DArT array, 236 (3.1%) were assigned with at least one different score within the population and so identified as putative polymorphic markers in the 'narrow' cross population derived from the cross of DipC x Tiga necaru landraces. Later the DArT marker data

Table 3-3: Primers used in the initial screening of the segregating F₃ population, showing optimal annealing temperature, heterozygosity and parental alleles.

Name	Forward sequence (5′-3′)	Length	Reverse sequence (3′-5′)	Length	Optimal annealing temperature (°C)	Hyterozygosity %	Fragment length (bp) Parent 1	Parent 2
PRIMER2	CGTGGATACCCATACCGTCT	20	TAAGTCCATTTTGTCCGATTGA	22	51	34.25	171	173
PRIMER7	GTAGGCCCAACACCACAGTT	20	GGAGGTTGATCGATGGAAAA	20	54	32.88	210	212
PRIMER10	TCAGTGCTTCAACCATCAGC	20	GACCAAACCATTGCCAAACT	20	54	27.40	260	234
PRIMER15	AGGAGCAGAAGCTGAAGCAG	20	CCAATGCTTTTGAACCAACA	20	58	30.14	238	212
PRIMER16	CCGGAACAGAAAACAACAAC	20	CGTCGATGACAAAGAGCTTG	20	55	16.9	189	187
PRIMER19	AGGCAAAAACGTTTCAGTTC	20	TTCATGAAGGTTGAGTTTGTCA	22	57	28.77	273	235
PRIMER26	CGCTCATTTTAACCAGACCTC	21	CAAACAAACCAACGGAATGA	20	55	20.83	183	185
PRIMER32	TTCACCTGAACCCCTTAACC	20	AGGCTTCACTCACGGGTATG	20	55	19.18	247	251
PRIMER37	CCGATGGACGGGTAGATATG	20	GCAACCCTCTTTTTCTGCAC	20	60	35.62	258	260
PRIMER38	TCACACTTGCAATGGTGCTT	20	TCGTTGTTTCTCTTTTCATTGC	22	57	31.51	194	191
PRIMER43	CTTGATGCTACCGAGAGAGAG	21	AGGCTCCAACAATGCGATAG	20	55	20.55	199	205
PRIMER45	CGTGGATACCCATACCGTCT	20	AAGTCCATTTTGTCCGATTGA	21	52	36.99	171	173
PRIMER48	TACCTGCATTCGGGACAGTT	20	TTCACTCTTTCTTGATCACATGC	23	60	20.55	238	230
PRIMER65	GGACGTGAATCGATGGAGAT	20	TCCTTCCCCCTTCTCTGATT	20	55	35.62	172	176
PRIMER66	CGTTAGATCTGAGACGCCATT	21	CATCCATCACCTGTCACCAG	20	60	27.40	225	213
PRIMER85	TTTCCAGATTGGATCGTTGA	20	TGTCTTCACACCGGAATTTG	20	58	22.54	248	252
PRIMER88	TGTGGTTGTGCTCCTTCTCA	20	GGGAAGAAGAGTGAAGTTGGAA	22	62	28.77	233	239
PRIMER95	AAGTCCATTTTGTCCGATTGA	21	CGTGGATACCCATACCGTCT	20	58	31.51	168	170
PRIMER98	TTTTGTCACTGTTTGCCACAA	21	AGATTTATATCTGGATGAGAGAGAGAG	27	57	15.07	264	294
PRIMER103	AAATTCAAAGGCCTGGAAAAA	21	TTTTTGAGTTCTGCGAGCAA	20	57	26.76	210	220
GH-19-B2-D9	ATCAAAATCAAGCAAATGAGA	21	ACCTTTTACGCTCATTTTAACCAG	24	50	26.03	236	238
BamcoL17	AACCTGAGAGAAGCGCGTAGAGAA	24	GGCTCCCTTCTAAGCAGCAGAACT	24	58	28.77	162	166
Bam2coL33	ATGTTCCTTCGTCCTTTTCTCAGC	24	AAAACAATCTCTGCCCCAAAAAGA	24	54	20.55	253	255
Bam2coL63	AAAATCTCACTCGGATGGCATGTG	24	TGGAATCACCTGATAGTAGTGTATTGG	27	55	23.29	293	295
Bam2coL80	GAGTCCAATAACTGCTCCCGTTTG	24	ACGGCAAGCCCTAACTCTTCATTT	24	58	13.70	220	224
mBam3co7	GGGTTAGTGATAATAAATGGGTGTG	25	GTCATAGGAAAGGACCAGTTTCTC	24	59	28.77	267	275
mBam3co33	TGTGTCTGTTTGTGGGGATATGTA	24	TTATCCCGGTCCTAATTCATCTTA	24	58	21.92	295	319
AG81	ATTTTCCAACTCGAATTGACC	21	TCATCAATCTCGACAAAGAATG	22	52	10.96	202	190
BN 6b	CACTACCCTGTTCTTCATCCGT	22	CATTGCACGTCATAGAATTTGG	22	53	26.03	146	150
BN 145	GGCACTGGTAGCAACGAAA	19	CGTGGACGTAACAACACAACAC	22	50	13.70	150	154
BN 259	CGATTGCACGTCATAGAATTTG	22	GTTCCAGACACTACCCTCGTTC	22	50	21.92	159	163
D.24269	AGGTTCATGATCGTAGATGTGGAT	24	ACGATaTCATACTGACaTgtTTCATAC	27	60	17.81	246	238
D.35497	ACTTTTAGCTCTTGTCAGGAAACG	24	TCTTTCTACTTTTCTCTGGCTGGT	24	55	23.61	168	202

was combined with the SSR marker data to construct the genetic linkage map. As the parental seed sources were not confirmed by the time of the preparation of DNA representations from the individual lines, the data for the DArT markers had only two genotypic classes, present and absent, without knowing which parent was transmitting the absence of the band (the fully informative state). For this reason, the coding for unknown phase, two genotypic classes, was used (k-, hh) (Figure 3-5).

DArT marker	Individual lines														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
bgPabg-593965	hh	k-	k-	k-	hh	hh	hh	hh	hh	k-	k-	k-	hh	hh	k-
bgPabg-596335	k-	hh	k-	k-	k-	hh	k-	k-	k-	k-	hh	hh	hh	hh	k-
bgPabg-596774	hh	k-	hh	k-	hh	hh	hh	hh	--	k-	k-	k-	hh	k-	k-
bgPabg-597624	hh	hh	k-	k-	k-	hh	k-	k-	k-	k-	hh	k-	k-	k-	hh
bgPabg-597858	hh	hh	k-	k-	k-	k-	k-	hh	hh	hh	k-	hh	k-	k-	k-
bgPabg-594335	k-	k-	hh	hh	k-	hh	k-	k-	k-	k-	hh	k-	hh	k-	k-
bgPabg-594261	k-	k-	--	k-	k-	hh	k-	k-	hh	k-	k-	hh	k-	k-	--
bgPabg-595641	hh	hh	k-	hh	hh	--	hh	hh	hh	hh	k-	k-	hh	k-	k-
bgPabg-595315	k-	k-	hh	hh	hh	k-	k-	hh	k-	hh	hh	k-	k-	hh	k-
bgPabg-595273	k-	k-	hh	hh	hh	k-	k-	hh	k-	hh	hh	k-	k-	hh	k-
bgPabg-594877	k-	k-	k-	k-	k-	k-	k-	k-	hh	hh	k-	hh	k-	k-	k-
bgPabg-597436	k-	hh	k-	hh	k-	hh	k-	k-	k-	k-	hh	--	hh	hh	k-
bgPabg-593983	hh	k-	hh	hh	hh	hh	hh	k-	k-	k-	hh	k-	hh	hh	hh
bgPabg-594142	k-	--	k-	hh	k-	hh	k-	k-	k-	k-	hh	k-	hh	hh	k-

Figure 3-5: Allelic differences at loci for different DArT markers among in a selection of the individual F$_3$ plants derived from the narrow cross of DipC x Tiga necaru. Alleles were scored depending on the presence and the absence of the allele, with the absence being fully informative and presence unable to distinguish between a homozygous or heterozygous band, due to the dominant nature of the hybridization-based DArT array marker system.

3.2.3 Microsatellite markers scored in the F$_2$ population of the 'wide' cross

The population lines and their parents were extracted from dried leaves. The gel picture of extracted DNA showed a smear, suggesting relatively low DNA quality for

the parents and the individual F_2 plants (Figure 3-6), as might be expected from old silica gel dried leaf material. These DNA templates could be amplified with different primers in PCR using greater numbers of cycles (37-38 cycles). Different strengths of amplification were produced depending on the primer pair, but they all had enough PCR products to allow visualization on agarose gel and size calling on the Beckman CEQ^{TM} 8000 Fragments Analyzer (Figure 3-7)
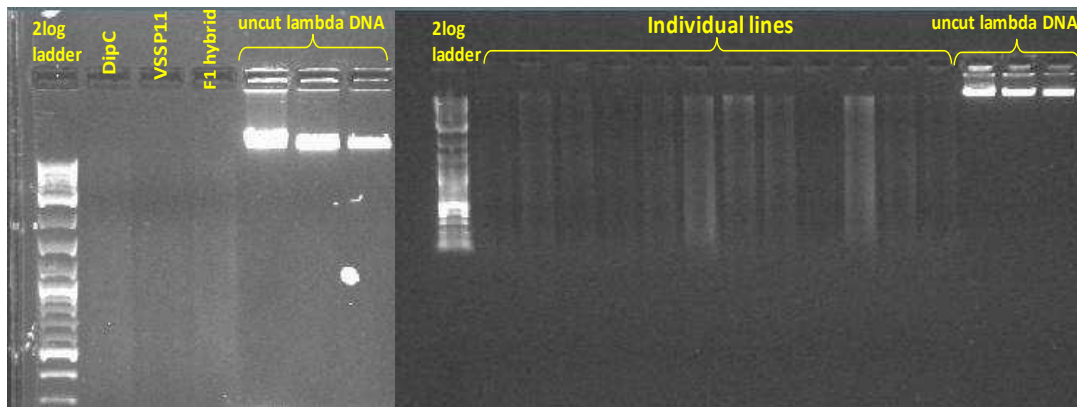
Figure 3-6: Gel picture of extracted DNA from silica dried leaf tissue of parents (DipC and VSSP11), their F_1 hybrid and selected individuals of the F_2 population for the 'wide' cross, showing poor DNA quality and quantity.
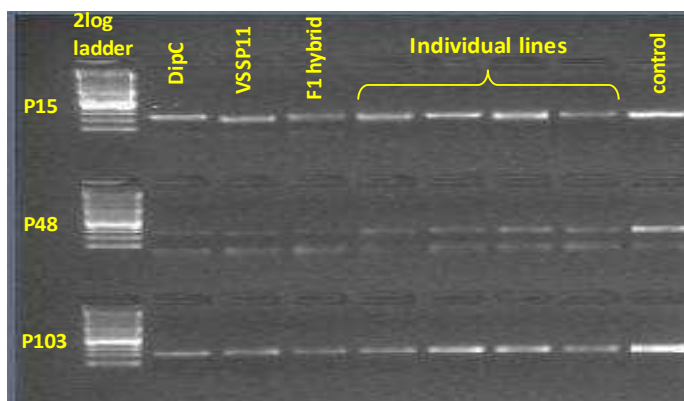
Figure 3-7: Amplification of parents and selected individuals of the wide cross population for a number of different primer pairs (Primer15, primer48 and primer103) compared with amplified DNAs extracted from DNA derived from a single fresh leaf sample (control; narrow cross individual).

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This population was also screened with the same primer sets used for the 'narrow' cross population to identify polymorphic markers. The aim of this application was to further develop the initial map of the 'wide' cross population with more markers (in addition to the current AFLP markers) and then compare it with the 'narrow' cross population through a number of common markers. Out of 95 SSR markers established from the first and second sets genomic library of bambara groundnut and soybean , 22 markers were polymorphic when amplified in the parents of the cross (DipC and VSSP11) (Table 3-4). Among these polymorphic primers four of them did not show polymorphism when applied to 'narrow' cross population. All of these polymorphic primers except Primer81 and Primer96 were screened with the individual lines of F_2 population.

Table 3-4: Polymorphic primers for the wide cross population recording the different allelic size for the parents of the cross.

No.	Primer name	Optimal annealing temperature ^{o}C	'wide' cross	
			DipC	VSSP11
1	PRIMER10	53	268	237
2	PRIMER15	59	240	212
3	PRIMER16	55	189	185
4	PRIMER19	59	270	244
5	PRIMER26	55	183	189
6	PRIMER32	50	220	218
7	PRIMER38	56	194	191
8	PRIMER48	55	244	228
9	PRIMER65	55	172	176
10	PRIMER66	55	219	225
11	PRIMER73[a]	56	236	234
12	**PRIMER81**[a]	53	189	185
13	PRIMER82[a]	53	270	242
14	PRIMER85	55	248	240
15	**PRIMER96**[a]	53	182	184
16	PRIMER98	59	274	276
17	GH-19-B2-D9	50	236	242
18	Bam2coL80	55	220	218
19	Bm2coL33	50	239	249
20	mBam3co7	55	267	271
21	AG81[b]	52	202	190
22	BN145	50	143	147

a: polymorphic only in the wide cross population

b: primer from soybean

Amplified fragments of the individual F_2 plants by primers Primer10 and Primer82 could be scored for allelic sized differences using agarose gel electrophoresis. A 2.5% agarose gel in 0.5 X TBE (500ml tray size) was made adding 3µL of ethidium bromide stock (10mg/mL) and the gel was run for 3 hours on 90 Volts (Figure 3-8).
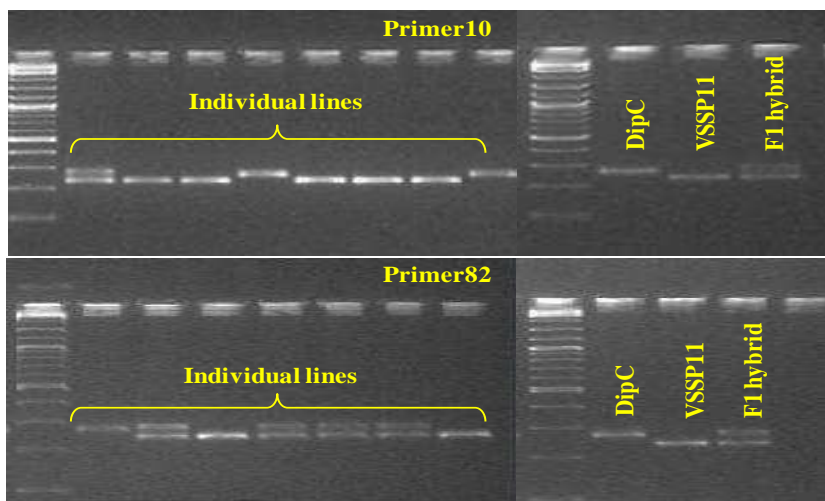
Figure 3-8: Scoring the SSR markers in lines of wide cross population (F_2) for parental alleles using agarose gel 2.5% for primer10 and primer82. Two different fragment sizes for both parents in the F_1 hybrid and a selection of lines from the "wide" cross population were distinguishable on this system.

Scoring the F_2 population with the other identified polymorphic primers was conducted through running the PCR products on the Beckman CEQ^{TM} 8000 capillary electrophoresis machine. The electrophoretograms of the results were analyzed with the CEQ system software and the product sizes called manually from the traces (Figure 3-9).
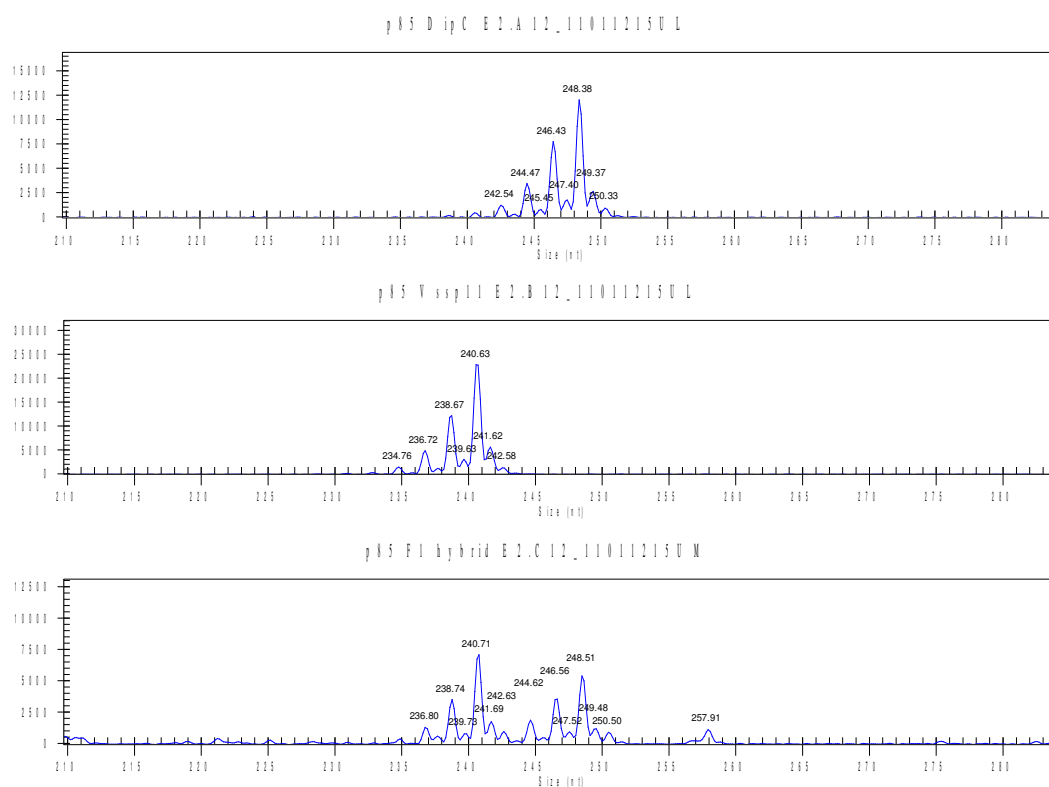
Figure 3-9: Electropherogram for polymorphic Primer85 using the Beckman CEQ^{TM} 8000 capillary electrophoresis machine. Different alleles can be seen at 248bp for DipC, at 240bp for VSSP11 and the presence of both parental alleles in the F$_1$.

Primers developed from the bambara groundnut transcriptome were run directly against the parents (DipC and VSSP11) of the F$_2$ segregation population. Out of 29 primers 19 of them showed polymorphism when amplified from the parents and F$_1$ hybrid. Only 3 primers (D.32936, D.48339 and D.51646) were used to screen the whole population of individual lines of 'wide' cross population (Table 3-5) due to cost constraints in this part of the work. The population was screened with the second primer (D.48339) using agarose gel electrophoresis. Additional primers could be added when funding allows to increase the density of SSR markers in the wide cross.

Table 3-5: Polymorphic microsatellite primers derived from the RNA sequence data for the parents of 'wide' cross.

No.	Primer name	Optimal annealing temperature °C	'wide' cross	
			DipC	VSSP11
1	D.15508	62	307	311
2	D.36186	62	172	163
3	D.42026	60	238	229
4	D.8148	65	244	229
5	D.8999	55	203	195
6	D.37053	55	181	190
7	D.12522	60	328	326
8	D.15619	55	221	217
9	D. 21310	50	178	186
10	D.24269	60	246	250
11	**D.32937***	60	291	305
12	D.35497	55	168	166
13	**D.48339***	60	197	161
14	**D.51646***	60	185	195
15	D.1006	60	161	158
16	D.7215	62	208	202
17	D.8387	55	187	190
18	D.11860	55	271	283
19	D.2094	60	224	227

* : primer used to screen the whole population individuals

3.2.4 Genotyping errors in SSR analysis

During the course of the present study a number of issues were encountered with the results of Beckman Coulter CEQ^{TM} 8000. These problems were:

- Stutter bands: One of the potential genotyping errors in analyzing the electropherogram data of microsatellites is the production of stutter bands. In primer48, heterozygosity was difficult to score in line 106 for the F_3 derived from the cross DipC x Tiga necaru (Figure 3-10), but it became clear when the peaks of both alleles were compared. The results of parental alleles enabled a clear scoring of line 106 as heterozygous.
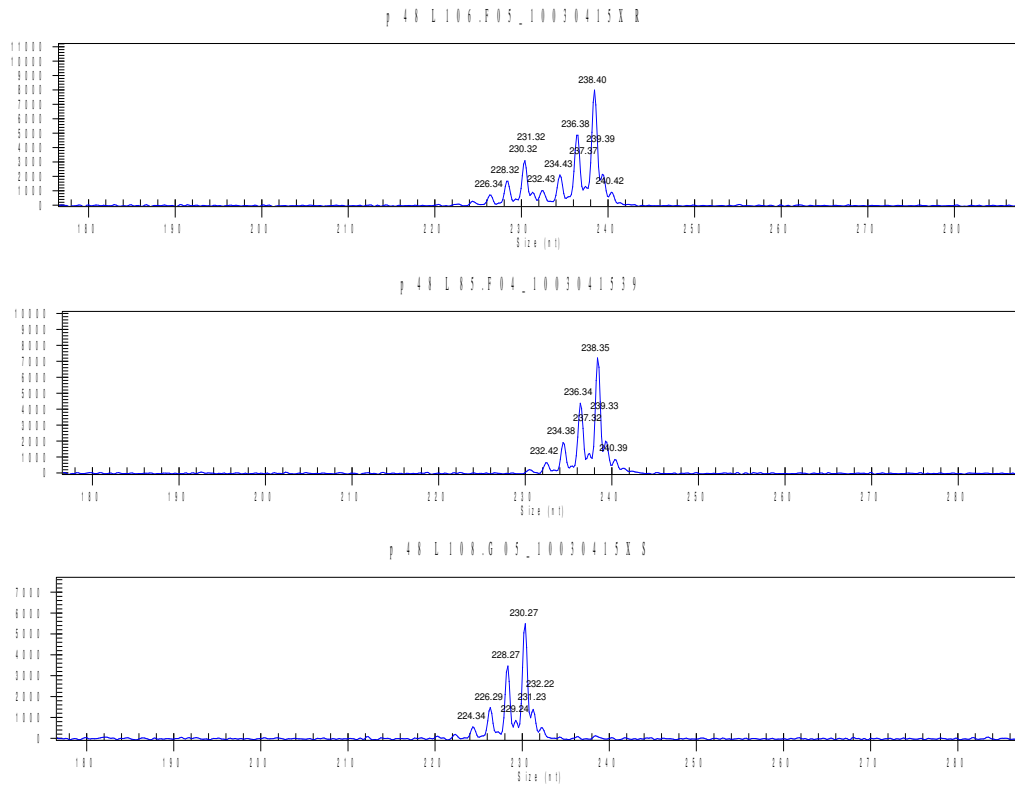
Figure 3-10: Capillary electrophoresis showing a potential scoring error to detect the both parental alleles in the heterozygote sample of Primer48 L 106.F05 due to the effects of stutter bands and overlap. While both alleles showed normal band separately in different homozygote lines of L85 and L108.

-   Allele size calling: Allele sizes should be a whole number of bases, but as the mass of bases is not identical, there is some degree of genuine mass difference between sequences which may be the same length, but which have different base compositions. In addition, the capillary electrophoresis also can experience technical issues relating to operating temperatures between runs, quality of reagents and age of gel matrix which can cause minor shifts between runs or even within runs, if the calibration ladder is poor. These non-integer values can lead to mistyping errors due to rounding off of alleles during binning. As there were only 2 different sizes of parental alleles within the individuals for a particular SSR product, sample peak sizes was recorded

to the nearest whole number and it is not believed that this will be a major
source of mistyping, for this application.

- Noisy peak shape: some samples had a high background noise which made
scoring more difficult, especially when allele size differences between parents
are small. Sample (a) of Figure 3-11is line 56 in the 'narrow' cross population
amplified using Primer 37 and shows an irregular peak shape, making it
difficult to score, as there is only a 2bp difference between the parental alleles.
A new PCR product gave a clear peak in sample (b), suggesting that there is a
technical basis in PCR for this effect.
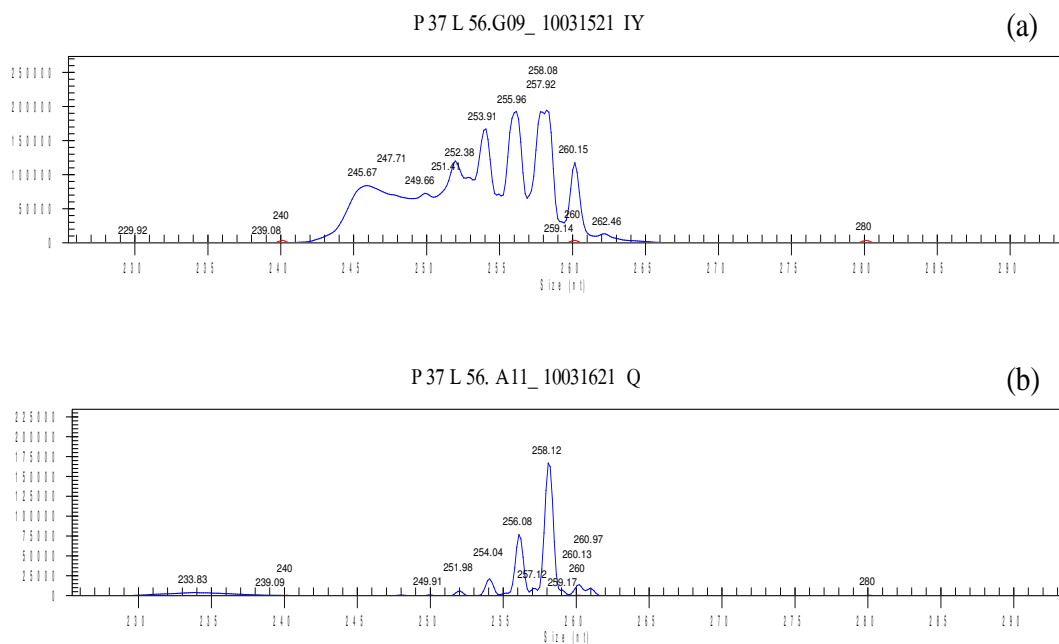
**Figure 3-11: Capillary electrophoresis showing a noisy background that made the scoring of
allele size more difficult for Primer37 in the 'narrow' cross population. Sample (a) shows
irregular peak shape, while in sample (b) the reamplified PCR product has a regular peak shape.**

3.2.5 DNA Isolation from endosperm

3.2.5.1 DNA Extraction and quantitation

Endosperm powder was made from bambara groundnut seeds with a Dremel craft drill. Genomic DNA was extracted using the GenElute Plant Genomic DNA kit (Sigma Aldrich). Isolated DNA with three incubation periods was quantified using gel electrophoresis (1% agarose). The result of different incubation periods revealed that the 30 minutes incubation had the best result for extraction of genomic DNA from dried endosperm, as shown on the agarose gel (Figure 3-12).
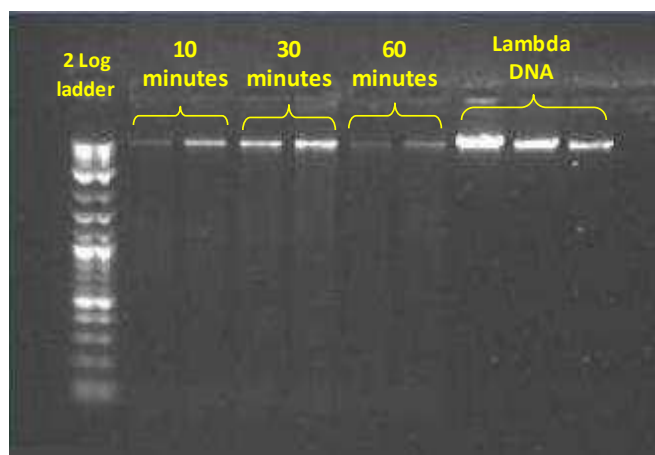
Figure 3-12: Quantified DNA extracted from the endosperm of bambara groundnut seed with three incubation periods for extraction (10, 30 and 60 minutes), using the GenElute Plant Genomic DNA Extraction kit. Thirty minutes incubation produced the best results for quantity of DNA extraction.

3.2.5.2 Germination tests for treated seeds

Analysis of variance was conducted using the Minitab software (release 16). Three levels of drilling treatment (not drilled and two drill diameters (3/64" and 1/16") were applied against two seed sizes of bamabra groundnut, big and small seed sizes of 1cm and 0.5 cm in diameter. According to the results, the germination percentage was 48.9% and an analysis of variance shows that there was no significant difference for germination between the levels of drilling treatment [F (2, 12) = 2.8, $\underline{p}$ < .01], seed size [F (1, 12) = 0.4, $\underline{p}$ < .01] and also for their interaction [F (2, 12) = 0.4, $\underline{p}$ < .01] (Table 3-6).

Table 3-6: Analysis of variance for germination test of drilled seeds.

Source of Variation	df	SS	MS	F value	probability
Drilling	2	1244	622	2.8	0.1
Seed size	1	89	89	0.4	0.539
Drilling*seed size	2	178	89	0.4	0.679
Error	12	2667	222		
Total	17	4178			

3.3 Discussion

3.3.1 Microsatellite markers

Microsatellites essentially consist of short tandem repeated units and can be found in both coding and non-coding regions in all prokaryotic and eukaryotic genomes (Kalia et al., 2011; Trivedi, 2004). It is considered to be a marker of choice to study molecular genetics in plant species (Somta et al., 2011b). Microsatellite markers used in this study were developed from a genomic-enriched repeat library sequenced with non-titanium and titanium reagents (Roche 454 Pyrosequencing) and from a bambara groundnut leaf transcriptomic library (Roche 454 Pyrosequencing). Only one primer derived from another legume genome (soybean) was also mapped in the current study.

The RNA sequence data was generated as a part of an experiment to look at the effect of temperature on gene expression in leaf. Primer pairs were designed for microsatellite repeat motifs after their screening in silico. The development of a comprehensive set of genic-SSR markers has been reported in pigeon pea using deep transcriptome sequencing. They are considered as an important genetic resource in their application for understanding the genetic relationship among different accessions and regional origins in this crop (Dutta et al., 2011).

The soybean microsatellite marker (AG81) was able to identify polymorphism in the parents of both populations. High sequence similarity at the amino acid level was identified between the AG81 sequence and a seryltRNA synthetase gene (Arabidopsis thaliana, Z70313), suggesting that the conservation of this locus could be due to functional constraints on its evolution (Peakall et al., 1998).

However the cross-genus amplification rate of SSRs among Medicago and other forage legume (32%) was low in comparison to some other non-legumes (Dirlewanger et al., 2002), the transferability of SSR markers in genetic studies of legume crops potentially opens a new approach to utilize them for the applications of comparative mapping, functional analysis and for assessing genetic diversity (Chandra, 2011).

The transferability of SSR markers across legume families has been investigated. SSRs from existing databases of different legumes (including Medicago, soybean, cowpea, and peanut) were used to screening 24 different legume accessions. About one-third of DNA markers from Medicago and soybean were reproducible and amplification was obtained in peanut, clover, cowpea, guar, and other legumes, suggesting that transferring SSRs across species and/or across genera could be an efficient approach to develop DNA markers, especially for minor crops (Wang et al., 2004a).

Although a few SSR markers (10 markers) have been reported for bambara groundnut (Basu et al., 2007a), markers from counterpart species such as azuki bean (Wang et al., 2004b), cowpea (Xu et al., 2010), and mungbean (Somta et al., 2008) show a high rate of amplification when applied in bambara groundnut and their utilization was considered useful for genomics study in this crop (Somta et al., 2011a).

DNA extracted from the individuals of the F_3 'narrow' cross and the F_2 population of the wide cross using both the protocols of Dellaporta and the GenElute Plant Genomic DNA kit (Sigma Aldrich), respectively, were used to generate DNA stocks. DNA stocks of the 'wide' cross (F_2 population) were used directly in the PCR reaction without dilution because of the poor quality of the DNA. However, with additional cycles of amplification (+2/3 cycles) strong amplification was generally achieved,

making it unlikely that lack of template could be leading to issues with null alleles (allelic dropout and false alleles) caused by quality alone (Pompanon et al., 2005)

The correct size of microsatellite amplification is considered to be critical for the confirmation of identity and correct locus amplification of SSR markers. In PCR amplification for a limited number of SSR (three) the product sizes of target sequences was greater than expected (bam2coL58, BN11 and BN21). Insertion/deletion of additional DNA between the designed primers could lead to differences in size for particular genotypes, compared to the design sequences. (Hackauf and Wehling, 2002). Incorrect priming or the abolition/mutation of the original priming site might also lead to an additional product being amplified and with transcriptome derived SSRs, the presence of an intervening intron when amplifying from genomic sequences is always a possibility (although primer pairs were tested against genomic DNA during the characterization phase). Genomic SSR fragments of unexpected size are usually monomorphic (Roder et al., 1998) and these primers have been eliminated from further analysis and scoring to avoid genotyping errors. Furthermore running such fragments with capillary electrophoresis was not possible as all PCR products were sized with a 400 bp size standard and the software cannot provide a size estimate beyond this range (as the calibration curve would not extend this far).

Detection of SSR alleles

Although agarose gel electrophoresis has relatively low resolution (Wang et al., 2009), it was possible to score allelic size differences for some of the markers. Parental alleles were scored within the individuals of F_3 population derived from the 'narrow' cross on agarose gel using primer D.35497, as well as in the F_2 population of

'wide' cross for Primer10, Primer82 and D.48339. Parental allele differences within the individuals using these primers were in the order of 28-36bp, allowing their detection easily with 2.5% agarose gels and 1-3 hour run times on 80-90 Volt. The agarose gel system using a 3% concentration gel for 3.5 hours has been applied by other researchers to screen for polymorphism of SSR markers (Beyene et al., 2005). The CEQ^{TM} 8000 (Genetic Analysis System, Beckman Coulter, USA) was used to estimate different allele sizes for other primers in both 'narrow' and 'wide' cross populations. Although the CEQ^{TM} 8000 contains an automated allele binning wizard to determine the sizes of alleles of markers, product sizes were scored manually from the electrophoretograms. Visual inspection is recommended to score sized fragments with the CEQ system to avoid mis-reporting of automated sizing caused by scoring stutters as a real peak in automated scoring. Combining automated allele size calling and human inspection of electropherogram data has been suggested to provide efficient and accurate detection of novel alleles (DeWoody et al., 2006), but only peak size estimation can be relied upon. The automated calling system is unable to cope with changes in relative peak heights and it is essentially that the entire microsatellite profile is taken into consideration. Changes in this more realistically reflect genuinely different alleles. The heterozygosity of the individuals may have to be confirmed by direct comparison of their peaks to the parental allele traces.

All markers were screened individually before multiplexing, using the blue M13 WellRed Dye to provide information on peak patterns and size ranges of the expected alleles. This helped to avoid unidentified large-allele dropout in multiplexing, when one allele of a heterozygote randomly fails to amplify and leads to mistyping the heterozygote loci as homozygotes (Johnson and Haydon, 2007). Large allele dropout has been reported a common error in microsatellite scoring (Bonin et al., 2004). The

amount of PCR products added to a post-PCR multiplex depended on the strength of the amplification products before pooling, as assessed on agarose gel. All primers were fluorescently labeled with the D4 (blue) dye to avoid the problem of false peaks due to colour bleed through and the PCR products were multiplexed for 2-3 primers which showed a wide allelic size difference between microsatellites in their pre-screening. The problem of false signal was reported in multiplexing different PCR products with different labeled dyes of D3 green and D4 blue (Molosiwa, 2012) where product allele sizes were similar.

SSR polymorphism

Polymorphism of the microsatellite markers was estimated to be around 36.3% in the segregation population derived from the cross of DipC x Tiga necaru, while it was about 33.1% in the 'wide' cross population from the cross of DipC and VSSP11. A similar percentage of polymorphism (40%) was identified for bambara groundnut SSR markers in the eight tested Bambara groundnut single genotype accessions from all major growing regions of the world, while a lower level of polymorphism (17.4%) was identified for these accession using SSR primers from the relatives of bambara groundnut, azuki bean, cowpea and mungbean (Somta et al., 2011b). A high polymorphism level for SSR markers (70.3%) was identified between the two chickpea cultivars (BG 256 and WR 315). These were used as the parents of mapping populations in linkage and quantitative trait loci (OTL) analysis to determine the position of a Fusarium wilt resistance gene (Qadir et al., 2007). A higher polymorphism rate (81.3%) was presented in an investigation of pigeon pea. Sixteen newly developed SSR markers were screened on 40 genotypes representing different Cajanus species including eight wild pigeon pea genotypes (Saxena et al., 2010).

The higher polymorphism observed in the 'narrow' cross compared to the 'wide' cross population could be due to the fact that parents of the 'narrow' cross came from different origins and domestication backgrounds. The results of a genetic similarity analysis of 87 bambara groundnut genotypes based on the similarity matrix of 296 unique polymorphic DArT markers revealed considerable structural differences between DipC and Tiga necaru populations (Stadler, 2009), while the wild accession of VSSP11, used as a male parent in constructing the 'wide' cross population, came from a similar background (despite being a wild ancestor/undomesticated individual) as DipC . This would argue that the 'wide' cross segregates mainly for domestication characters, but otherwise is genetically relatively uniform, while the 'narrow' cross does not segregate for domestication loci, but is genetically quite diverse.

Thirty SSR markers developed from the genomic microsatellite-enriched library of bambara groundnut and a single SSR from soybean were polymorphic in the 'narrow' cross population, while 22 markers were polymorphic in the 'wide' cross. While 15 markers derived from the transcriptome data were polymorphic in the narrow cross and 19 were polymorphic in the 'wide' cross, giving overall figures of 45 SSRs polymorphic in the narrow cross and a similar number, 41 polymorphic in the wide cross, which suggests levels of overall polymorphism in these crosses are similar, despite the wide cross being from the domesticated subspecies to the ancestor subspecies, spontenea

The DipC landrace was used as the female parent in both 'wide' and 'narrow' cross populations when crossed with landraces of VSSP11 (V. subterranea spontenea) and Tiga necaru (V. subterranea subterranea), respectively. The legitimacy of the DipC landrace as the source of the maternal parent was confirmed through production of the same allelic size in both populations with all the polymorphic transcriptome primers

and around half of the genomic microsatellite-enriched library primers applied. The
fact that the maternal alleles are not identical in both crosses is likely to be a
consequence of different individuals of the DipC landrace being used for the maternal
parent (Massawe et al., 2005).

3.4.2 Validation of the segregating F$_3$ population

The F$_3$ population derived from the cross between DipC and Tiga necaru landraces
was checked for the residual levels of heterozygosity before using it to construct a
linkage map and to carry out the subsequent QTL analysis. Segregating patterns of
parental loci were followed and screened for all individuals using 33 polymorphic
SSR markers. Residual heterozygosity was confirmed with these markers to match
with the predicted Mendelian ratios expected from the population history.

3.4.3 DArT Assay

DArT is a genetic technology which has the ability to type thousands of loci in
parallel, with reduced costs per data point compared to other genetic marker systems
(Kilian et al., 2005). Array development and genotyping was carried out in
collaboration with Diversity Arrays Technology Pty Ltd (Yarralumla, Australia). In
this study DArT arrays were developed and a total of 3.1% polymorphism per array
element was obtained. Approximately 2% polymorphism of DArT array generated
previously for the inter-subspecific cross of bambara groundnut was also used in this
study. DArT as a high-throughput approach and has proven to be most cost- and time-
efficient in studying genetic diversity in bambara groundnut and it is considered as a
useful approach for other under-utilized crops (Mayes et al., 2009; Olukolu et al.,
2012; Stadler, 2009). A total of 236 polymorphic markers were detected for the

individuals of the F_3 population derived from the cross of DipC x Tiga necaru. Due to the high locus specificity of DArT markers, these can be easily placed into genetic linkage maps with reasonable confidence that the same array element will usually detect the equivalent chromosomal location in all individuals (Wenzl et al., 2006). Thus these developed markers along with SSR markers will be used for constructing a genetic linkage map of bambara groundnut in the segregating F_3 population. This was also used to improve the existing initial map from the cross of DipC and wild accession (VSSP11).

3.4.4 DNA isolation from the endosperm

Many attempts were made to develop a successful hybridization technique for bambara groundnut (Basu, 2005; Massawe et al., 2004; Suwanprasert et al., 2006). Hybrid seed production in bambara groundnut is by hand emasculation and pollination, the number of successful F_1 hybrids developed by artificial cross pollination can be reduced by self-pollination and it is not possible to distinguish genuine F_1 hybrids from self-pollination in the F_1 seed, as the testa is genetically maternal. It is only with the development of F_2 seed that non-maternal characters could be expressed and only with F_3 seed that segregation for these can be observed (Basu et al., 2007c) although if there are substantial differences in morphology between the parents, these factors may be informative in the F_1 plant. PCR-based molecular marker techniques have been used to rapidly verify F_1 hybrids in plant crops (Sundaram et al., 2008). The standard method has been by growing all the presumed hybrids and extracting genomic DNA from all of them to check their status as F_1 hybrids using molecular markers. In order to accelerate this process we have investigated the possibility of extracting DNA directly from the seed endosperm

before planting, in a way that does not reduce their viability. Genomic DNA was extracted from the endosperm of the seeds after optimizing the period of incubation in the extraction process. The DNA extracted with this process could be amplified in the polymerase chain reaction using molecular marker to identify genuine F_1 hybrids. The seeds tested showed no effect on their germination frequency compared to controls, suggesting that there was no reduction in viability (although viability was comparatively low in this experiment and the experiment needs to be confirmed with a large-scale analysis on recently harvested seed). This approach seems feasible to allow a rapid determination of hybrid status before planting of F_1 seed under optimal conditions to ensure good seed production for F_2 populations for research and breeding. Alternatively, confirmed F_1 seed could be sent for germination in countries with in-field programmes of bambara groundnut research.

Chapter 4: SEGREGATING POPULATIONS AND TRAIT INHERITANCE

4.1 Introduction

The genetic improvement of crops is one of the most promising approaches for addressing future resource constraints (particularly land pressure and environmental concerns) and to enhance food production to meet the pressure of expected population growth in the near future.

Genetic improvement of crop species and genetic resource conservation and evaluation using both conventional and biotechnological approaches is an on-going activity, focused on improving tolerance to biotic and abiotic stresses while maintaining high levels of productivity, profitability and quality. Estimating the heritability of morphological and agronomical traits is an important part of effective selection for breeding traits (Firouzian, 2003). The development of molecular markers to such traits can assist the integration of desirable alleles into the crop genotype to accelerate progress in agriculture and the delivery of new cultivars and technologies.

4.1.1 Inheritance of agronomic traits in crop plants

Traits inheritance falls into two main classes; continuous variation and discrete classes. In crop plants most traits of agronomic important are quantitative and are likely to be controlled by many genes, often with an approximately normal distribution, in contrast to qualitative phenotypes which usually form discrete classes. The multiple genes often involved in many quantitative traits makes their control

genetically more complex as each gene may have a minor phenotypic effect and can be very sensitive to the environment (Choudhary et al., 2008). The combination of many genes and their environmental interaction makes the study of quantitative traits more complicated (Chenu et al., 2011; Ikram and Chardon, 2010; Koornneef et al., 2004; Paran and Zamir, 2003).

 Breeders often have limited information on the gene-to-phenotype architecture for the traits under study (Cooper et al., 2005). Predictions of gene properties in populations of genotypes can be obtained through understanding the genetic basis of variation for simple and quantitative traits.

Although, very few morphological and developmental studies have been carried out in bambara groundnut, studies conducted on genome conservation and information gained from other legume crops could shed light on the inheritance patterns of important traits that are also present in bambara groundnut.

4.1.2 Inheritance patterns of phenotypic traits in selected legume crops

4.1.2.1 Qualitative trait samples

Thirty three domestication-related traits in azuki bean (Vigna angularis) were studied by Isemura et al. (2007). The traits were evaluated for the segregating populations (BC_1 and F_2) which were derived from the cross between the wild species Vigna nepalensis (female) and the cultivated azuki bean (male and recurrent parent for backcross). Three traits of epicotyl colour, seed coat colour and black mottling of seed coat were identified as qualitative traits. The segregation ratios for the three qualitative traits matched the ratios expected for control by a single gene in both

populations, except that seed coat colour in the F_2 population which showed distorted segregation significant at the 5% level, perhaps suggesting that the single gene model did not adequately explain the genetic control of this trait.

The expression of morphological traits was studied by Damayanti et al. (2010) in hybrids of the cross of domesticated var. macrosperma x wild Australian of tropical tuberous legume (Vigna vexillata (L.) A. Rich). Several traits relating to domestication were evaluated, including; broad leaflet size, ovate leaflet shape, non-pigmented stems, non-dehiscent pods, uniform seed testa colour and green seed testa. The standard chi-square goodness of fit test suggested that the segregation ratios for these traits except seed testa colour were consistent with control by single dominant genes, with the domesticated version acting recessively compared to the wild type traits. Seed testa colour was consistent with being controlled by two genes in dominance-epistatic relationship (Damayanti et al., 2010).

In common vetch (Vicia sativa L.) a full diallel cross was conducted by Chowdhury et al. (2004) using five parents of different cotyledon and testa colour . The segregation patterns of cotyledon colour was studied in F_2, BC_1 and BC_2 populations and found to be governed by a single gene of two alleles with incomplete dominance. Testa colour was also found to be a qualitative trait and to be regulated by a single gene with complete dominance. Similar results of monogenic inheritance for seed coat color were reported by Milczak (1971) based on the analysis of F_1 population data.

Hasan et al. (2006) analysed a half diallel cross in French bean (Phaseolus vulgaris L.) for yield related traits. The result indicated that recessive alleles govern days to 50% flowering while dominant alleles are more important in controlling days to maturity.

In a diallel experiment using dry bean (Phaseolus vulgaris L.) the importance of both additive and dominance genetic components was emphasized in the control of different yield related traits, including days to first flowering, days to podding and days to maturity, however the high value of heritability in the narrow sense (explaining more than 50% of the trait variation) for days to maturity indicating the largely additive control of this trait and possibility of selection for this trait to improve this crop (Islam and Newaz, 2000).

4.1.2.2 Quantitative trait examples

In a genetic study of agronomic traits of 82 genotypes of mungbean (Vigna radiata (L.) Wilczek.), major additive gene effects were reported for 100-seed weight, seed yield/plant, and plant height (Rohman et al., 2003). Another investigation by Khattak et al. (2002) reported the importance of additive gene action in specifying seed yield and its components in mungbean. Similarly, inheritance analysis of agronomic traits of seven long bean varieties (Vigna sesquipedalis) found higher additive genetic variation compared to dominance variation in the control of seed weight and pod length. However, dominance was more important for pod no./plant and seed no./pod (Mak and Yap, 1980).

Phenotypic parameters were estimated in cowpea by Lopes et al. (2003) to understand the genetic control of seed size. The F_1, F_2, BC_1 and BC_2 populations from the cross TVx5058-09C X Manteiguinha along with their parents were analysed. The additive-dominance model fitted the data for 100-seed weight and the additive effects were the more important. Five genes were predicted to control the expression of 100-seed weight and the authors suggested that selection could be made in the early segregating generations for seed size in cowpea.

An inheritance study in French bean (Phaseolus vulgaris L.) by Hasan et al. (2006) estimated narrow sense heritability to be greater than 50% for plant height and 100-seed weight, indicating that the majority of the phenotypic variation was due to additive gene action. It was concluded by the authors that selection would be effective for the improvement of these characters in French bean. Seed yield/plant was identified as a trait largely controlled by non-additive effects.

In dry bean (Phaseolus vulgaris L.) a diallel experiment was carried out using an F_2 population and evaluated in two different environments by Islam and Newaz (2000). They emphasized the importance of both additive and dominance genetic components in controlling the different yield related traits of plant height, pods/plant, pod length, seeds/pod, seeds/plant and 20-seed weight. Heritability, in the narrow sense, showed high values (above 50%) for pod length in both environments, and 20-seed weight in one environment, indicating that the major portion of the phenotypic variation of these two traits was additive in nature and selection of these traits could be an effective means to improve yield in dry bean.

Roy et al. (2006) studied ten traits in bush bean (Phaseolus vulgaris L.) to identify suitable traits for yield improvement. High heritability estimates in the broad sense were observed for pods/plant (90.76), 100-seed weight (91.13), seed yield/plant (90.54) and protein percentage (99.78) in the genotypes, which indicated the presence of additive gene effects in addition to dominance effects. The authors stated that this crop could be improved through selection of these traits. An inheritance study on days to flower and seed size in pigeon pea (Cajanus cajan (L.) Millsp.) was conducted by Gupta et al. (1981). The authors used F_1, F_2 and backcrosses populations of a cross between two parents with large-seed (ICP-8504) and small seed (Prabhat). Additive gene effects were found to be most important in the expression of both earliness and

seed size. The result showed that selection could be used to improve the seed size in early maturing cultivars of pigeon pea.

In an inheritance study of seed yield and related traits in 15 lentil genotypes (Lens culinaris Medik) significant genetic variation was observed by Rasheed et al. (2008) for all traits studied. High heritability values and expected percent of genetic advance (respectively) were observed for seed yield (98.30%, 128.20%), harvest index (97.10%, 79.40%), biological yield (94.30%, 56.10%) and 100-seed weight (88.30%, 50.80%) indicating the role of additive genes in controlling these traits. It was concluded that the traits of 100-seed weight, harvest index and biological yield could be exploited for the improvement of seed yield in this crop.

The studies made in legume crops suggest the differences in number of genes controlling different traits. The effect of single or a few genes has been identified for some of the phenology traits such as days to emergence, days to flowering and maturity, seed and coat colour, while the other traits (yield related) were found to be under multiple gene effect. Identify the genetic control of these traits will delight the process to study their inheritance with the suitable breeding program whether to be through selection of hybridization. The inheritance studies of these legume related crops could be exploited for the improvement of these traits in bambara groundnut.

4.1.3 Trait correlation studies in bambara groundnut

A number of studies have been made in bambara groundnut on cultivars development and the relationship between yield and its components. However, it is important to note that bambara groundnut generally exists as landraces. As a strongly inbreeding species, the landraces are likely to be mixtures of inbred lines. How genetically diverse landraces are will depend upon the cultivation history of the landrace.

Ofori (1996) estimated the effects of yield components on the seed yield in bambara groundnut germplasm from Ghana. Both pod no./plant and 100-seed weight were identified to have a positive effects on seed yield, as might be expected. For the bunch morphology type, leaf no./plant was more important in determining seed yield, while leaf size was more important in the spreading morphology types. Association was detected between the traits of seed size and leaf size. Thus selection of genotypes with bigger leaf size could contribute to improve the productivity of bambara groundnut.

Another correlation coefficient analysis for pod yield components of bambara groundnut was made by Makanda et al. (2009). Twenty bambara groundnut landraces were evaluated at four planting dates in Zimbabwe outside the traditional growing season of bambara groundnut. A significant correlation was indicated for most of the traits studied in different planting dates.

Another experiment was conducted under screen house conditions at the Sokoine University of Agriculture, Tanzania. Leaflet length was found to be the only vegetative variable which had a significant and positive correlation with seed yield. This positive relationship suggests plant architecture for bambara groundnut that favors longer leaves, results in more pods and higher seed yield. Plant height and leaflet length were both positively correlated with number of pods produced. The authors also reported that pod no./plant was highly correlated with seed yield. Whereas days to flowering was negatively correlated with seed yield and petiole length was negatively correlated with 100-seed weight; this was largely due to its negative effect on seed size (Misangu et al., 2007).

Chijioke et al. (2010) performed a comparative study in bambara groundnut to evaluate the contribution of agronomical traits to yield in this crop. They evaluated 30 local bambara groundnut cultivars over two cropping seasons in Nsukka, Nigeria. The

correlation coefficients suggested that seed weight was significantly associated with leaves per plant, plant height, flowers per plant and pods per plant, considering these traits as major determinant of yield in bambara groundnut.

Inheritance patterns in bambara groundnut were investigated for a number of traits using a segregating F_2 population and 14 small F_3 families derived from the inter-subspecific cross of a domesticated DipC and wild VSSP11 accession. The additive gene effects and several genes were suggested to control the traits of leaf area, specific leaf area, carbon isotope discrimination (CID) and 100-seed weight. In contrast, the variation for traits such as internode length, stems/plant, days to emergence and seed eye pattern around the hilum was likely to be under largely monogenic control (Basu et al., 2007c).

The aim of the present study was to identify the inheritance patterns of domestication and agronomically important traits in an F_3 progeny segregating from the cross of a DipC (female) landrace with a Tiga necaru (male) landrace grown under both glasshouse and field conditions. The population itself was used to construct a genetic linkage map using DArT and SSR molecular markers. The combination of this linkage map with the trait data from current and previous studies was used to conduct a QTL analysis in this cross. An analysis of the traits and their inheritance patterns and distribution is presented here, before subsequent chapter go on to describe the mapping and QTL analyses.

4.2 Results

The results presented in this chapter represent the analysis of the distribution and associations of morphological traits for the segregating F_2 and F_3 populations derived from the intra-(sub-specific) cross between the DipC and Tiga necaru landraces.

The F_2 population data of the "narrow" cross was collected previously in the Tropical Crops Research Unit from the individual plants for each line. Data measured in the FutureCrop glasshouse were recorded for four replicates in a CRD design. A single plant represented each replicate and the QTL analysis was based on the means of replicates however the variance between the lines for all 4 replications is also presented for the glasshouse data. Data recorded for the Indonesian field trial of the same cross (F_3) came from the mean of (5-15) individual plants per line (depending on the availability of seed).

4.2.1 Data distribution of morphological traits for the F_3 population

Traits measured are (in concordance with the IPGRI descriptors for Bambara groundnut), describing below a number of them evaluated in both FutureCrop glasshouse and the field.

Days to emergence: Data recorded for the number of days from sowing to attaining the first true leaf for the segregation F_3 population lines of FutureCrop glasshouse showed non-normal distribution (Table 4-1). As the P-value (P = 0.000) for the Anderson-Darling normality test was less than an alpha of 0.01, significant departure from normality is indicated. The parental data in the glasshouse revealed that the Tiga necaru parent emerged within 7 days (sd= 0.94) and DipC in 10.7 days (sd= 0.82),

classifying this trait into distinct classes. The field data also indicated a non-normal

distribution of this trait (Table 4-2).

Leaf area (cm^2): Leaf area data using the method of Cornelissen et al. (2002),

showed a non-normal distribution in the glasshouse, while the leaf area calculated in

the field was revealed a normal distribution (Appendix 4). A Box-Cox transformation

was applied to normalize the leaf area data of glasshouse (Figure 4-1).

Table 4-1: Statistical analysis and the distribution of trait data in the F$_3$ population and the parents in the glasshouse.

Trait	Mean value	Variance[a]	Variance[b]	Standard deviation	Skewness	Kurtosis	P value	Individual no.	normality	parents DipC	parents Tiga necaru
Days to emergence	7.9	1.3	1.5	2.80	3.0	13.5	0.00	73	non-normal	10.7	7.0
Days to flowering	36.4	9.1	11.1	12.28	2.3	8.2	0.00	64	non-normal	39.3	33.7
Flower no./plant	105.2	1187.2	1285.3	47.13	0.4	-0.2	0.47	64	normal	116.7	82.0
Plant height	25.5	4.8	6.6	8.63	-0.3	-0.0	0.07	71	normal	27.3	21
Petiole length	19.1	2.9	4.5	6.47	-0.8	0.4	0.01	71	normal *	20.6	14.1
Terminal leaflet length	8.2	0.7	1.0	2.81	-0.5	0.0	0.22	64	normal	7.8	7.2
Terminal leaflet width	3.5	0.2	0.4	1.24	-0.1	-0.7	0.71	64	normal	3.6	2.4
Leaf area	3461.2	1925714	2153621	1719.54	1.8	5.6	0.00	70	non-normal	2678	2312.1
Plant spread	33.7	40.4	51.8	12.54	0.8	0.5	0.02	64	normal*	26.3	31
Stem no./plant	10.3	3.0	4.6	3.76	0.6	0.7	0.01	64	normal*	11.0	9.7
Node no./stem	8.7	4.7	6.6	3.49	0.6	1.1	0.08	64	normal	6.2	12.0
Internode length	2.0	0.9	1.1	1.12	0.6	0.1	0.17	64	normal	1.1	1.6
Growth habit	12.0	43.0	51.7	7.26	1.3	1.3	0.00	64	non-normal	13.5	8.6
Pod no./plant	47.6	256.0	319.2	21.57	0.6	0.2	0.08	64	normal	48.3	19.5
Double seeded pods/plant	5.4	16.9	24.3	4.22	1.3	1.6	0.00	64	non-normal	6.0	2.3
Peduncle length	2.9	1.1	1.5	1.35	0.2	-0.7	0.24	64	normal	2.2	3.8
Pod weight/plant	36.8	216.4	287.6	18.26	0.7	0.1	0.03	64	normal*	40.0	35.0
Pod length/ plant	16.1	1.3	1.6	5.41	-0.1	-0.6	0.89	64	normal	17.4	14.8
Pod width/plant	13.0	0.7	1.0	4.33	-0.2	-0.4	0.59	64	normal	13.4	12.1
Double seeded pods length	24.8	6.7	8.9	9.19	-0.5	1.3	0.04	62	normal	27.1	20.9
Double seeded pods width	13.3	0.6	0.8	4.82	0.1	-0.5	0.58	62	normal	13.0	12.1
Seed length	11.1	0.7	0.9	3.73	0.1	-0.4	0.42	64	normal	11.5	10.4
Seed width	9.2	0.3	0.4	3.08	-0.8	1.7	0.08	64	normal	9.7	9.0
Seed no./plant	51.5	317.9	423.2	23.68	0.5	-0.5	0.01	64	normal*	52.3	35.0
Seed weight	28.9	144.9	173.3	14.68	0.7	-0.1	0.00	64	non-normal	33.4	14.7
Biomass dry weight	61.3	524.3	663.4	29.31	0.8	0.0	0.00	64	non-normal	60.8	49.8
Shelling%	79.1	40.8	48.3	26.68	-0.2	0.1	0.37	64	normal	83.4	43.8
100-seed weight	55.6	75.6	97.8	19.98	0.2	-0.3	0.65	64	normal	63.6	43.8
Eye pattern around hilum	1.45	0.20	0.3	0.65	0.25	-1.76	0.00	64	non-normal	1	2

* Normal distributed data at 99% of critical value

a: variance for the means of lines

b: variance of lines for all four replicates

Internode length (mm): Wide variation was recorded for internode length counted as the average of the five longest stems per plant at the 4th internode, with the trait ranging from 0.33-4.72 cm in the glasshouse and 0.28-2.24cm in the field. An Anderson-Darling normality test indicated a normal distribution of this quantitative trait in both locations (Appendix 5 and Appendix 6).

Table 4-2: Statistical analysis and distribution of the trait data in the F_3 population in the field

Trait	Mean value	Variance	Standard deviation	Skewness	Kurtosis	Individual No	P value	A-Squared	Distribution
Days to emergence	6.5	0.4	1.41	1.3	2.7	70	0.00	9.57	non-normal
Days to flowering	42.9	4.7	17.83	1.2	1.1	65	0.00	4.78	non-normal
Plant height	18.5	11.1	6.57	-0.5	1.4	65	0.08	0.67	normal
Petiole length	10.7	5.7	4.15	-0.8	0.8	64	0.04	0.77	normal
Terminal leaflet length	4.8	0.7	1.70	-1.3	1.8	65	0.00	1.96	non-normal
Terminal leaflet width	2.1	0.1	0.76	-0.7	1.1	65	0.02	0.94	normal*
Leaf area	672.2	108700	377.69	0.1	-0.7	64	0.51	0.33	normal
Plant spread	27.1	76.6	11.77	-0.7	0.6	65	0.13	0.57	normal
Stem no./plant	4.0	0.5	1.54	0.8	3.9	63	0.02	0.90	normal*
Node no./stem	14.6	15.4	6.18	0.9	4.3	63	0.09	0.64	normal
Internode length	1.0	0.2	0.53	0.7	0.3	62	0.05	0.76	normal
Growth habit	12.8	40.1	7.51	1.6	3.2	60	0.00	2.18	non-normal
Pod no./plant	7.7	21.5	4.99	0.9	0.8	64	0.007	1.08	non-normal
Double seeded pods/plant	1.4	1.3	1.16	1.0	1.6	61	0.00	1.89	non-normal
Peduncle length	4.5	2.9	2.13	1.2	0.6	65	0.00	3.81	non-normal
Pod weight	4.0	6.4	2.69	0.7	0.0	64	0.02	0.89	normal*
Pod length	13.6	3.1	4.96	0.7	3.7	63	0.01	1.07	normal*
Pod width	9.5	0.9	3.39	-0.6	0.9	63	0.19	0.51	normal
Seed length	8.1	1.3	3.44	-1.8	6.6	58	0.00	1.23	non-normal
Seed width	6.1	0.6	2.54	-1.9	5.2	58	0.00	2.53	non-normal
Seed no./plant	6.9	17.5	4.61	0.7	0.5	59	0.07	0.68	normal
Seed weight	2.7	3.4	1.97	0.7	0.2	59	0.06	0.72	normal
Biomass dry weight	8.5	13.8	5.06	0.4	-0.4	63	0.40	0.38	normal
Shelling%	63.3	379.7	30.38	-0.6	0.2	59	0.31	0.42	normal
100-seed weight	38.1	162.0	18.80	-0.1	2.1	59	0.01	1.00	normal*

* Normal distributed data at 99% of critical value
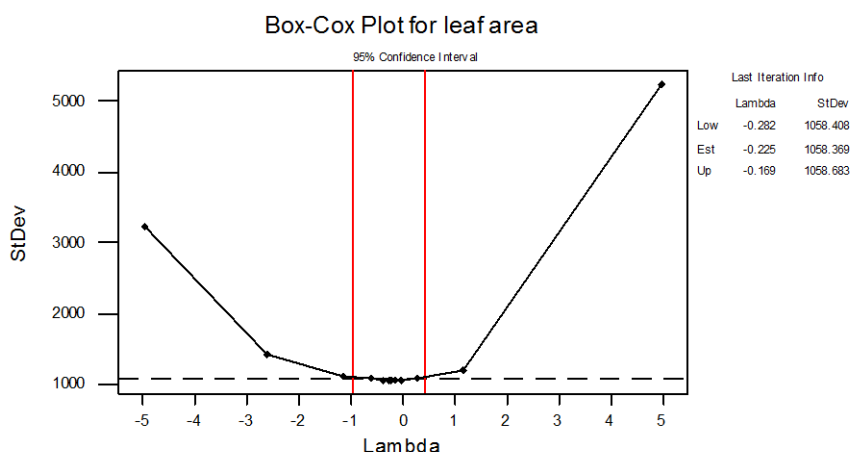
Figure 4-1: Box-Cox plot for leaf area for the F₃ population grown in glasshouse.

Growth habit: Non-normal distribution of both glasshouse and field data was revealed (Appendix 7 and Appendix 8). Trait distribution of growth habit in the population under study showed a highly significant non-normal distribution (P-value= 0.000). Based on the parental values obtained an attempt was made to classify offspring into parental classes, bunched habit of 13.53 or more for DipC parent in contrast to Tiga necaru parent of semi bunched types of 8.567 or less (spreading types). A Chi-square test was carried out by grouping these trait data into three probable classes; beyond the parental classes and intermediate class. The results were consistent with segregation in a ratio of 3:2:3, with 21 plants representing the DipC parental class, 25 belonging to the Tiga necaru class and 19 classified as the intermediate type (21:19:25), out of 65 segregated F₃ lines. Chi-square tests gave a result of 0.95 at 2 df <5.99 of probability 0.05. The result of this hypothesis was consistent with co- dominance of growth habit in the present cross.

Pod no./plant: In order to estimate the yield of bambara groundnut the number of pods/plant was recorded. A normal distribution was observed in the F₃ population

with a P-value of 0.08 (Appendix 9). While this trait was not normally distributed having a P-value of 0.007) in the field. Box-Cox transformation was used to normalize this trait to give a 0.25 P-value in an Anderson-Darling test (Appendix 10). Double seeded pods/plant was recorded and found to be non-normally distributed in both glasshouse and field experiments. The data distribution of double seeded pods/plant in the glasshouse was normalized using a Box-Cox transformation (Appendix 11).

Seed weight (g/plant): A wide range of trait segregation was observed for seed weight/plant. Despite non–normal distribution of the data for this trait in the glasshouse, normal distribution was revealed for the data recorded in the field (p = 0.056). Box-Cox transformation was applied for glasshouse data to maximize the closeness to normality, the transformed data was revealed to be normally distributed (p = 0.19) (Appendix 12).

Biomass dry weight (g/plant): Biomass dry weight reflects the amount of energy stored in the plant through capturing carbon and sunlight. The data recorded for biomass in the glasshouse, unlike the field data collected for this trait, did not show a normal distribution pattern in the F_3 population lines. A Box-Cox transformation test was used to normalize the data for the glasshouse a P-value of 0.06 (Figure 4-2)
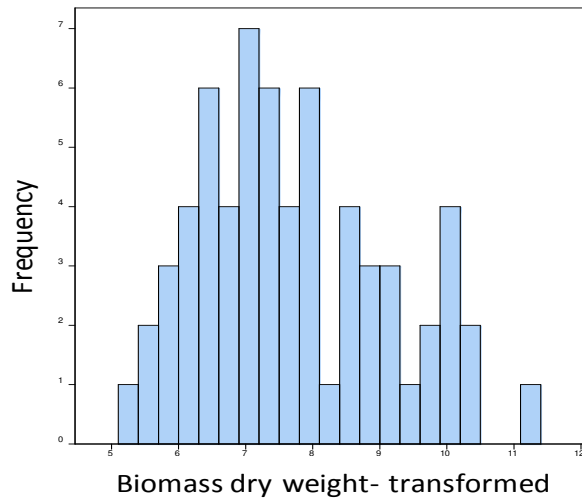
Figure 4-2: Histogram of normal distribution for transformed biomass dry weight in the glasshouse for the F_3 population.

Eye pattern around hilum: Since both parents have a creamy testa color and the eye pattern is present only in DipC maternal parent, the eye pattern segregation was studied in the glasshouse. Discontinuous distribution was revealed with the eye pattern trait (Figure 4-3). Chi-square tests were applied to the ratio of 40 presence: 24 absence. A hypothesis of complete dominance of eye pattern was proposed. 41 plants belonged to the maternal type (DipC) and 23 plants had no eye pattern around hilum, belonging to the Tiga necaru paternal type. The X^2 value at 1df was 0.06< 3.84 at 0.05 probability, indicating that this trait could be under the control of a single dominant gene for eye pattern presence.

Figure 4-3: Presence versus absence of eye pattern around hilum for the seed in both parents of DipC and Tiga necaru landraces.

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4.2.2 Distribution of the F_2 population data

Previous data collected from the segregating F_2 population derived from the same intra-subspecific cross of DipC x Tiga necaru landraces was also used to study the inheritance of phenotypic traits of this crop before QTL analysis. The distribution of all these data except seed weight matched with data recorded for the traits of F_3 progenies of the glasshouse, although some of them were transformed to follow the continuous distribution (Table 4-3).

Trait data of the segregating F_2 population:

As in the segregating F_3 data grown in glasshouse and field, days to emergence for the F_2 segregating population of the same cross grown in TCRU showed non-normal distribution pattern. It was not possible to partition the data into different categories depends on their parents' data as both DipC and Tiga necaru had no difference in the time spending to germinate.

Table 4-3: Statistical analysis and the distribution of traits data of F_2 population and the parents of narrow cross (DipC x Tiga necaru).

Trait	Mean value	Variance	Standard deviation	Skewness	Kurtosis	Individual no.	P value	Normality	Parents	
									DipC	Tiga necaru
Days to emergence	12.0	6.4	2.52	1.8	3.4	73	0.00	non-normal	10	10
Days to flowering	6.7	0.1	4.14	0.7	0.3	73	0.00	non-normal	43	40
Plant height	23.2	32.5	5.66	-0.2	-0.6	73	0.04	normal*	21	10
Petiole length	18.3	22.5	5.32	0.3	0.2	73	0.04	normal*	20.64	11.7
Terminal leaflet length	9.6	2.3	1.51	0.5	1.4	73	0.11	normal	7	7
Terminal leaflet width	4.1	0.7	0.82	0.0	0.2	73	0.16	normal	2.8	1.7
Plant spread	56.1	160.7	12.59	0.0	-0.6	73	0.40	normal	53	29
Pod no./plant	120.9	6358.6	79.19	1.1	1.8	73	0.02	normal*	102	27
Double seeded pods	8.3	71.0	8.36	1.2	0.4	73	0.00	non-normal	3	1
Seed no./plant	115.4	6049.2	77.24	1.0	1.0	73	0.00	non-normal	70	22
Seed weight	39.6	784.0	27.81	0.4	-0.8	73	0.00	non-normal	23.49	5.94
Biomass dry weight	99.2	4482.2	66.49	0.6	0.0	73	0.00	non-normal	66.12	14.56
Shelling%	73.1	46.0	6.73	-1.2	2.2	73	0.01	normal*	77.77	74.32
100-seed weight	32.8	133.4	11.47	0.0	-0.6	73	0.30	normal	33.57	27

* Normal distributed data at 99% of critical value

Other traits including terminal leaflet length, terminal leaflet width, plant spread, pod no./plant, shelling% and 100-seed weight (Figure 4-4 and Appendix 13) also tested as normally distributed with Anderson-Darling normality tests. While the traits of double seeded pods/plant, seed no./plant and biomass dry weight were transformed with a Box-Cox transformation to correct to a normal distribution (Appendix 14). Seed dry weight was not distributed normally even after transformation analysis.
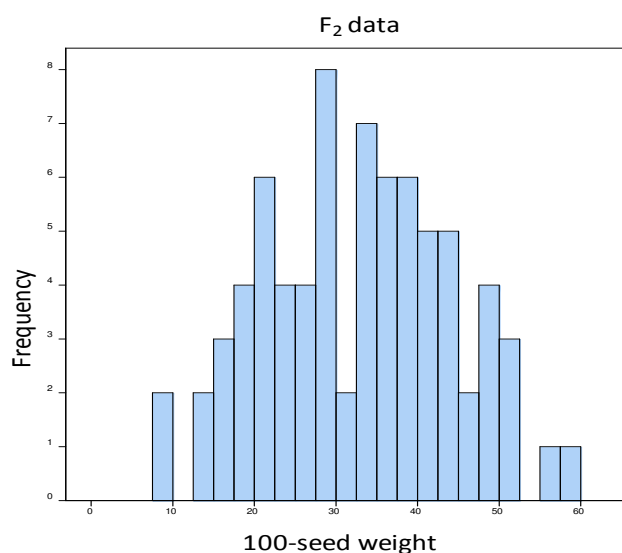

Figure 4-4: Histogram of normal distribution of 100-seed weight for F$_2$ progeny data

4.2.3 Association of the traits

Phenotypic correlations were conducted between the evaluated vegetative and yield-related traits based on the means of F$_3$ segregating population derived from the cross between DipC x Tiga necaru using Pearson's Correlation Coefficient analysis. MINITAB (release 16) software was used to construct the regression plots and to detect the significant association between the traits data for all F$_2$ and F$_3$ populations evaluated under different environments.

4.2.3.1 Association between traits for the F₃ generation in the glasshouse

The correlation and adjusted regression values for the glasshouse trait data are presented in Table 4-4.

Flower no./plant: was significantly associated with all vegetative growth traits except the traits days to emergence, days to podding and growth habit. This trait could be account for as much as 32% of the variation observed in plant spread. Flower no./plant also had significant negative correlation with days to flowering (Pearson's correlation coefficient, -0.48; p=0.000). Whereas it was found to be associated significantly with seed weight and a number of yield component traits such as pod weight, seed no./plant, biomass dry weight and 100-seed weight. It was accounted for 44% of the variation associated with biomass dry weight (Appendix 15).

Plant spread: A negative and significant correlation of this trait was found with days to flowering and growth habit (Pearson's correlation coefficient, -0.382; p= 0.002 and -0.615; p=0.000, respectively). Plant spread explained 56% of the variation in biomass dry weight of this population. Excluding the traits of emergence, days to podding, stem no./plant, seed length and Shelling%, plant spread showed a positive and significant associations with all the others. A regression analysis suggested plant spread accountable for 37% of the variation observed in growth habit (Appendix 16).

Internode length: Among the vegetative growth traits the highest positive correlation was observed between internode length and peduncle length at +0.799 (p= 0.000) followed by plant spread, whereas for yield related trait a higher Pearson's Correlation Coefficient value (r= +0.408; p= 0.001) was observed between internode length and double seeded pods/plant. Regression analysis suggested that 15% of the variation in double seeded pods could be accounted for by internode length (Figure 4-5). Plant spread had a significantly negative correlation with growth habit; -0.793 at

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p= 0.000. The regression analysis suggested that internode length accounted for 62.4% of the variation in growth habit in the population under study.
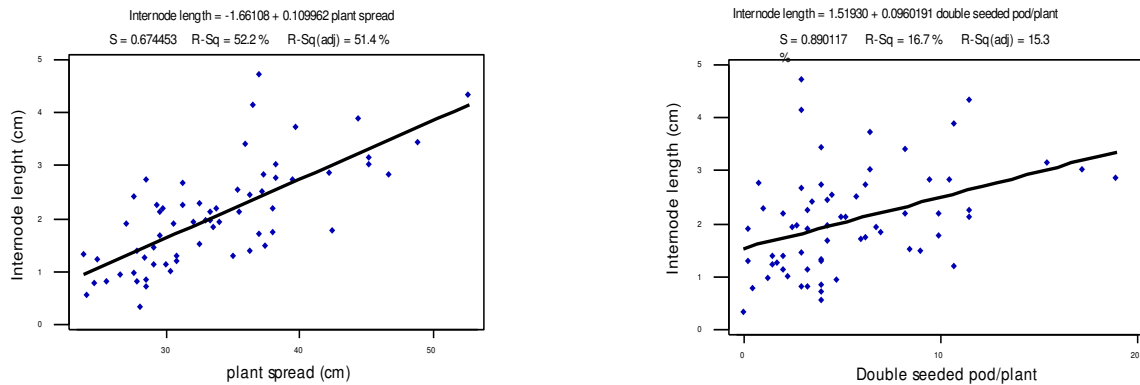

Figure 4-5: A fitted line regression plot of internode length with both plant spread and double seeded pods/plant in F$_3$ population of glasshouse.

Pod no./plant: As a yield component trait, it had a significant positive association with all the vegetative growth traits, while it was in negative association with days to flowering (r= -385; p= 0.002). Regression analysis revealed that pod no./plant accounted for 51% of the variation in node no./plant of the population under study. Pod no./plant, beside its strong association with biomass dry weight, was found to have positive and significant correlation with seed no., seed weight and pod weight/plant (r=+0.969, +0.881 and +0.875 at p= 0.000, respectively) but has no significant relationship with the dimensions of the pod and seed. Seed yield accounted for as much as 77% of the variation in pod no./plant in this population (Appendix 17). Similar pattern of the relationship for double seeded pods/plant with the other traits was scored with different significant level.

Table 4-4: Relationship between traits in the F$_3$ population of the cross DipC x Tiga necaru for glasshouse data based on the Pearson's correlation analysis showing correlation and P values.

Legend:
- r > 0.7 (yellow)
- r = 0.5-0.69 (green)
- r = 0.30 – 0.49 (blue)

Each cell shows correlation (top) / P value (bottom).

Trait	#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Days to emergence	1																														
Flower no./plant	2	-0.196 / 0.121																													
Leaf no./plant	3	-0.158 / 0.212	0.553 / 0.000																												
Days to flowering	4	0.442 / 0.000	-0.478 / 0.000	-0.391 / 0.001																											
Days to podding	5	0.418 / 0.001	0.054 / 0.672	0.105 / 0.409	0.546 / 0.000																										
Plant height	6	-0.230 / 0.068	0.435 / 0.000	0.410 / 0.001	-0.426 / 0.000	-0.195 / 0.122																									
Petiole length	7	-0.390 / 0.001	0.442 / 0.000	0.299 / 0.017	-0.477 / 0.000	-0.336 / 0.007	0.859 / 0.000																								
Leaf area	8	-0.101 / 0.426	0.624 / 0.000	0.873 / 0.000	-0.454 / 0.000	0.029 / 0.818	0.456 / 0.000	0.372 / 0.002																							
Terminal leaflet length	9	-0.128 / 0.313	0.445 / 0.000	0.133 / 0.293	-0.489 / 0.000	-0.288 / 0.021	0.341 / 0.006	0.379 / 0.002	0.485 / 0.000																						
Terminal leaflet width	10	-0.045 / 0.721	0.344 / 0.005	0.291 / 0.020	-0.308 / 0.013	-0.155 / 0.222	0.333 / 0.007	0.335 / 0.007	0.645 / 0.000	0.563 / 0.000																					
Plant spread	11	-0.112 / 0.379	0.576 / 0.000	0.385 / 0.002	-0.382 / 0.002	-0.121 / 0.339	0.254 / 0.043	0.330 / 0.008	0.525 / 0.000	0.449 / 0.000	0.447 / 0.000																				
Stem no./plant	12	-0.349 / 0.005	0.352 / 0.004	0.443 / 0.000	-0.381 / 0.002	-0.263 / 0.036	0.583 / 0.000	0.474 / 0.000	0.416 / 0.001	0.212 / 0.093	0.270 / 0.031	0.041 / 0.750																			
Branch no./plant	13	0.240 / 0.056	0.441 / 0.000	0.496 / 0.000	-0.041 / 0.748	0.253 / 0.044	0.174 / 0.168	0.056 / 0.662	0.466 / 0.000	0.147 / 0.246	0.093 / 0.464	0.380 / 0.002	-0.046 / 0.719																		
Node no./stem	14	0.103 / 0.420	0.568 / 0.000	0.620 / 0.000	-0.297 / 0.017	0.126 / 0.321	0.142 / 0.264	0.108 / 0.394	0.600 / 0.000	0.257 / 0.041	0.188 / 0.138	0.612 / 0.000	-0.117 / 0.358	0.772 / 0.000																	
Internode length	15	-0.007 / 0.958	0.395 / 0.001	-0.047 / 0.713	-0.321 / 0.010	-0.128 / 0.312	0.051 / 0.691	0.231 / 0.066	0.139 / 0.273	0.401 / 0.001	0.290 / 0.020	0.722 / 0.000	-0.132 / 0.297	0.080 / 0.528	0.343 / 0.006																
Pod no./plant	16	-0.121 / 0.342	0.684 / 0.000	0.763 / 0.000	-0.385 / 0.002	-0.006 / 0.964	0.448 / 0.000	0.382 / 0.002	0.772 / 0.000	0.331 / 0.008	0.411 / 0.001	0.660 / 0.000	0.262 / 0.036	0.539 / 0.000	0.720 / 0.000	0.254 / 0.043															
Double seeded pods/plant	17	-0.219 / 0.082	0.185 / 0.143	0.346 / 0.005	-0.325 / 0.009	-0.202 / 0.110	0.347 / 0.005	0.379 / 0.002	0.410 / 0.001	0.238 / 0.058	0.309 / 0.013	0.641 / 0.000	-0.057 / 0.653	0.182 / 0.149	0.433 / 0.000	0.408 / 0.001	0.554 / 0.000														
Peduncle length	18	-0.049 / 0.703	0.370 / 0.003	0.065 / 0.609	-0.315 / 0.011	-0.068 / 0.592	0.035 / 0.784	0.160 / 0.208	0.211 / 0.095	0.394 / 0.001	0.243 / 0.053	0.750 / 0.000	-0.172 / 0.173	0.224 / 0.076	0.472 / 0.000	0.799 / 0.000	0.268 / 0.032	0.533 / 0.000													
Pod weight/plant	19	-0.160 / 0.206	0.604 / 0.000	0.619 / 0.000	-0.418 / 0.001	-0.138 / 0.278	0.514 / 0.000	0.456 / 0.000	0.728 / 0.000	0.431 / 0.000	0.535 / 0.000	0.701 / 0.000	0.180 / 0.156	0.489 / 0.000	0.657 / 0.000	0.287 / 0.021	0.875 / 0.000	0.691 / 0.000	0.414 / 0.001												
Pod length	20	-0.173 / 0.171	0.233 / 0.064	0.134 / 0.292	-0.189 / 0.134	-0.260 / 0.038	0.463 / 0.000	0.468 / 0.000	0.303 / 0.015	0.493 / 0.000	0.416 / 0.001	0.405 / 0.001	0.291 / 0.009	0.032 / 0.800	0.086 / 0.500	0.158 / 0.213	0.171 / 0.176	0.294 / 0.018	0.342 / 0.006	0.438 / 0.000											
Pod width	21	-0.176 / 0.164	0.215 / 0.089	0.121 / 0.342	-0.258 / 0.040	-0.286 / 0.022	0.465 / 0.000	0.411 / 0.001	0.301 / 0.016	0.466 / 0.000	0.442 / 0.000	0.350 / 0.005	0.327 / 0.008	-0.025 / 0.846	0.005 / 0.968	0.134 / 0.290	0.106 / 0.405	0.219 / 0.015	0.303 / 0.015	0.387 / 0.002	0.903 / 0.000										
Pod length of double seeded	22	-0.178 / 0.166	0.139 / 0.281	0.071 / 0.583	-0.051 / 0.694	-0.116 / 0.369	0.361 / 0.004	0.366 / 0.003	0.214 / 0.096	0.356 / 0.005	0.298 / 0.018	0.424 / 0.001	0.104 / 0.423	0.010 / 0.940	0.155 / 0.229	0.261 / 0.040	0.217 / 0.090	0.481 / 0.000	0.449 / 0.000	0.453 / 0.000	0.782 / 0.000	0.621 / 0.000									
Pod width of double seeded	23	-0.032 / 0.802	0.102 / 0.428	0.136 / 0.290	0.033 / 0.800	-0.224 / 0.081	0.319 / 0.011	0.296 / 0.019	0.325 / 0.010	0.355 / 0.005	0.449 / 0.000	0.320 / 0.011	0.194 / 0.131	0.092 / 0.479	0.040 / 0.758	0.033 / 0.801	0.147 / 0.253	0.278 / 0.029	0.264 / 0.038	0.423 / 0.001	0.840 / 0.000	0.839 / 0.000	0.661 / 0.000								
Seed length	24	-0.189 / 0.135	0.223 / 0.076	0.056 / 0.660	-0.237 / 0.060	-0.278 / 0.026	0.486 / 0.000	0.485 / 0.000	0.261 / 0.037	0.475 / 0.000	0.441 / 0.000	0.237 / 0.059	0.318 / 0.011	-0.052 / 0.861	0.082 / 0.681	0.086 / 0.520	0.222 / 0.498	0.236 / 0.078	0.399 / 0.061	0.803 / 0.001	0.737 / 0.000	0.644 / 0.000	0.723 / 0.000	0.723 / 0.000							
Seed width	25	-0.389 / 0.001	0.294 / 0.018	0.051 / 0.689	-0.366 / 0.003	-0.437 / 0.000	0.432 / 0.000	0.513 / 0.000	0.269 / 0.031	0.536 / 0.000	0.499 / 0.000	0.312 / 0.000	0.326 / 0.000	-0.003 / 0.979	0.017 / 0.894	0.193 / 0.126	0.114 / 0.371	0.241 / 0.055	0.325 / 0.009	0.423 / 0.000	0.775 / 0.000	0.750 / 0.000	0.601 / 0.000	0.734 / 0.000	0.884 / 0.000						
Seed no./plant	26	-0.145 / 0.252	0.623 / 0.000	0.703 / 0.000	-0.419 / 0.000	-0.077 / 0.544	0.437 / 0.000	0.405 / 0.001	0.725 / 0.000	0.343 / 0.005	0.416 / 0.001	0.718 / 0.000	0.182 / 0.000	0.519 / 0.000	0.725 / 0.000	0.332 / 0.007	0.969 / 0.000	0.698 / 0.003	0.362 / 0.000	0.910 / 0.000	0.187 / 0.139	0.109 / 0.391	0.264 / 0.038	0.153 / 0.234	0.098 / 0.441	0.146 / 0.250					
Seed weight/plant	27	-0.136 / 0.285	0.645 / 0.000	0.642 / 0.000	-0.446 / 0.000	-0.115 / 0.367	0.485 / 0.000	0.438 / 0.000	0.745 / 0.000	0.448 / 0.000	0.523 / 0.000	0.728 / 0.000	0.149 / 0.149	0.538 / 0.000	0.713 / 0.000	0.342 / 0.006	0.881 / 0.000	0.680 / 0.000	0.449 / 0.000	0.981 / 0.000	0.382 / 0.002	0.326 / 0.008	0.389 / 0.002	0.342 / 0.007	0.374 / 0.002	0.400 / 0.001	0.920 / 0.000				
Biomass dry weight	28	-0.099 / 0.436	0.671 / 0.000	0.667 / 0.000	-0.414 / 0.000	-0.010 / 0.939	0.445 / 0.000	0.394 / 0.000	0.774 / 0.000	0.441 / 0.000	0.520 / 0.000	0.754 / 0.000	0.137 / 0.240	0.565 / 0.000	0.777 / 0.000	0.371 / 0.003	0.886 / 0.000	0.644 / 0.000	0.486 / 0.000	0.948 / 0.000	0.363 / 0.002	0.310 / 0.013	0.382 / 0.002	0.308 / 0.015	0.298 / 0.017	0.323 / 0.009	0.903 / 0.000	0.956 / 0.000			
Shelling %	29	0.052 / 0.683	0.303 / 0.015	0.232 / 0.065	-0.134 / 0.291	0.115 / 0.364	-0.112 / 0.380	-0.030 / 0.812	0.200 / 0.114	0.064 / 0.614	-0.029 / 0.821	0.184 / 0.146	-0.104 / 0.412	0.244 / 0.052	0.333 / 0.007	0.279 / 0.025	0.174 / 0.169	0.049 / 0.699	0.129 / 0.309	0.066 / 0.604	-0.298 / 0.017	-0.334 / 0.007	-0.282 / 0.026	-0.392 / 0.002	-0.104 / 0.412	-0.125 / 0.324	0.190 / 0.133	0.234 / 0.063	0.167 / 0.187		
Growth habit	30	0.083 / 0.512	-0.245 / 0.051	0.051 / 0.689	0.364 / 0.018	0.295 / 0.018	0.003 / 0.978	-0.140 / 0.270	-0.115 / 0.366	-0.427 / 0.000	-0.246 / 0.050	-0.615 / 0.000	0.049 / 0.702	0.067 / 0.017	-0.299 / 0.017	-0.793 / 0.000	-0.165 / 0.191	-0.349 / 0.005	-0.726 / 0.000	-0.216 / 0.086	-0.314 / 0.011	-0.245 / 0.051	-0.405 / 0.001	-0.155 / 0.229	-0.158 / 0.214	-0.253 / 0.044	-0.233 / 0.064	-0.243 / 0.053	-0.294 / 0.018	-0.100 / 0.430	
100-seed weight	31	-0.060 / 0.636	0.349 / 0.005	0.129 / 0.311	-0.256 / 0.041	-0.148 / 0.243	0.349 / 0.005	0.317 / 0.011	0.351 / 0.004	0.444 / 0.000	0.457 / 0.000	0.287 / 0.022	0.109 / 0.390	0.188 / 0.136	0.200 / 0.113	0.175 / 0.166	0.189 / 0.135	0.224 / 0.076	0.305 / 0.014	0.524 / 0.000	0.549 / 0.000	0.564 / 0.000	0.385 / 0.002	0.503 / 0.000	0.800 / 0.000	0.726 / 0.000	0.206 / 0.102	0.550 / 0.000	0.474 / 0.000	0.242 / 0.054	-0.113 / 0.375

Biomass dry weight: was found to be associated significantly with almost all other traits except the traits of days to emergence and podding, stem no./plant, shelling % and growth habit. Among the vegetative and growth stage traits node no./stem was correlated most significantly positively with biomass (r= 0.77; p= 0.000). A regression analysis suggested that 59.7% of the variation in biomass dry weight could be explained on the basis of node no./stem (Figure 4-6). Both leaf area and plant spread were followed node no./stem in their highest association with biomass dry weight, valued + 0.774 and +0.754, respectively (p= 0.000).

Pearson's correlation coefficient presented that the association of biomass dry weight with both seed weight and pod weight had recorded the highest positive value (r= +0.956 and 0.948; p= 0.000), respectively, among the yield component traits and explained 91.3% and 89.7% of the trait variation (Figure 4-6).
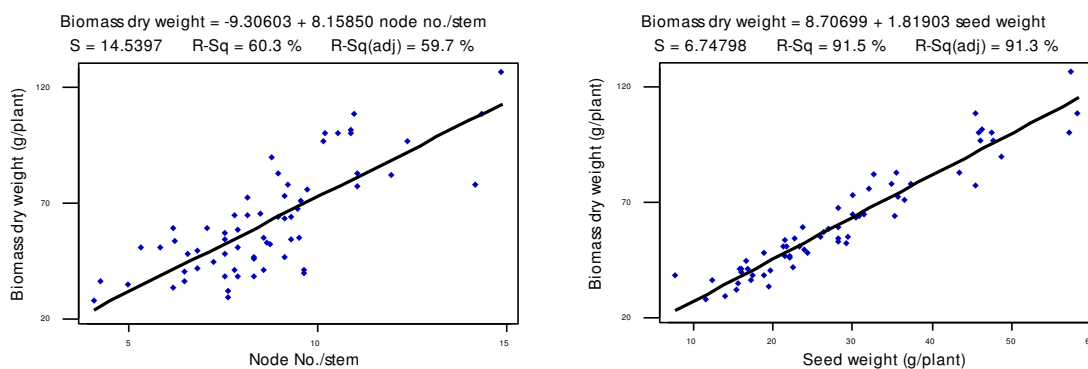
Figure 4-6: A fitted line regression plot for biomass dry weight with both of node no./stem and seed weight in the F_3 population of glasshouse.

4.2.3.2 Association of the traits for F_3 generation in field

Phenotypic relationship among the characters was studied and the coefficients are given in Table 4-5.

Table 4-5: Relationship between traits in the F₃ population of the cross DipC x Tiga necaru for field data based on the Pearson's correlation analysis showing correlation and P values.

Legend:
- r > 0.7 (yellow)
- r = 0.5–0.69 (green)
- r = 0.30–0.49 (blue)

Traits	#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Days to emergence	1																										
Leaf no./plant	2	-0.123 / 0.327																									
Days to flowering	3	0.284 / 0.034	0.025 / 0.855																								
Plant height	4	-0.065 / 0.609	0.577 / 0.000	-0.082 / 0.544																							
Petiole length	5	-0.136 / 0.288	0.734 / 0.000	-0.028 / 0.837	0.818 / 0.000																						
Leaf area	6	-0.038 / 0.767	0.909 / 0.000	0.006 / 0.964	0.724 / 0.000	0.858 / 0.000																					
Terminal leaflet length	7	-0.073 / 0.568	0.617 / 0.000	-0.102 / 0.452	0.763 / 0.000	0.847 / 0.000	0.779 / 0.000																				
Terminal leaflet width	8	-0.020 / 0.873	0.565 / 0.000	0.046 / 0.734	0.681 / 0.000	0.806 / 0.000	0.778 / 0.000	0.806 / 0.000																			
Plant spread	9	0.016 / 0.898	0.808 / 0.000	-0.020 / 0.885	0.691 / 0.000	0.766 / 0.000	0.879 / 0.000	0.770 / 0.000	0.678 / 0.000																		
Stem no./plant	10	0.005 / 0.967	0.019 / 0.880	-0.406 / 0.002	0.072 / 0.580	0.100 / 0.445	0.015 / 0.909	0.144 / 0.265	-0.040 / 0.755	0.032 / 0.807																	
Branch no./stem	11	-0.020 / 0.879	0.098 / 0.443	-0.222 / 0.101	0.207 / 0.107	0.180 / 0.164	0.188 / 0.148	0.243 / 0.057	0.189 / 0.141	0.233 / 0.068	0.117 / 0.363																
Node no./plant	12	0.179 / 0.165	0.146 / 0.253	-0.249 / 0.064	0.277 / 0.029	0.221 / 0.086	0.214 / 0.098	0.267 / 0.036	0.262 / 0.040	0.148 / 0.250	0.318 / 0.011	0.421 / 0.001															
Internode length	13	0.068 / 0.604	0.079 / 0.541	-0.308 / 0.022	0.099 / 0.446	0.107 / 0.416	0.085 / 0.516	0.265 / 0.039	0.161 / 0.215	0.171 / 0.188	0.228 / 0.074	0.154 / 0.232	0.519 / 0.000														
Growth habit	14	-0.083 / 0.533	0.259 / 0.045	0.350 / 0.010	0.161 / 0.218	0.241 / 0.064	0.212 / 0.107	0.010 / 0.937	0.040 / 0.763	0.133 / 0.312	-0.155 / 0.236	-0.293 / 0.023	-0.416 / 0.001	-0.797 / 0.000													
Peduncle length	15	0.245 / 0.051	-0.051 / 0.687	0.173 / 0.197	-0.129 / 0.305	-0.234 / 0.063	-0.058 / 0.650	-0.153 / 0.225	-0.142 / 0.259	-0.120 / 0.342	-0.160 / 0.216	0.200 / 0.119	0.049 / 0.704	-0.046 / 0.724	-0.080 / 0.541												
Pod no./plant	16	-0.254 / 0.044	0.059 / 0.645	-0.339 / 0.010	0.040 / 0.755	0.113 / 0.382	0.103 / 0.424	0.099 / 0.438	0.208 / 0.101	0.099 / 0.440	-0.049 / 0.702	0.244 / 0.054	0.061 / 0.633	0.331 / 0.009	-0.336 / 0.009	-0.116 / 0.365											
Double seeded pods/plant	17	0.017 / 0.899	-0.106 / 0.415	-0.253 / 0.063	-0.051 / 0.697	-0.130 / 0.328	-0.042 / 0.751	-0.129 / 0.326	-0.154 / 0.241	-0.025 / 0.849	0.067 / 0.608	0.276 / 0.031	0.105 / 0.421	0.066 / 0.617	-0.268 / 0.042	0.111 / 0.400	0.315 / 0.013										
Pod weight/plant	18	-0.184 / 0.149	0.030 / 0.814	-0.292 / 0.028	0.097 / 0.448	0.169 / 0.188	0.153 / 0.234	0.162 / 0.204	0.278 / 0.027	0.174 / 0.173	-0.067 / 0.601	0.246 / 0.052	0.061 / 0.632	0.268 / 0.035	-0.299 / 0.020	-0.104 / 0.419	0.913 / 0.000	0.384 / 0.002									
Pod length	19	0.407 / 0.001	0.022 / 0.864	0.102 / 0.454	0.328 / 0.009	0.427 / 0.001	0.284 / 0.025	0.243 / 0.058	0.346 / 0.006	0.237 / 0.064	-0.047 / 0.718	0.072 / 0.577	0.218 / 0.089	-0.046 / 0.726	-0.124 / 0.350	-0.088 / 0.495	0.166 / 0.194	0.333 / 0.009	0.376 / 0.002								
Pod width	20	0.147 / 0.255	0.086 / 0.503	-0.105 / 0.439	0.301 / 0.017	0.339 / 0.008	0.219 / 0.087	0.229 / 0.073	0.369 / 0.003	0.262 / 0.040	-0.042 / 0.746	0.039 / 0.765	0.216 / 0.092	0.281 / 0.028	-0.243 / 0.064	-0.152 / 0.238	0.464 / 0.000	0.083 / 0.531	0.580 / 0.000	0.676 / 0.000							
Seed length	21	-0.102 / 0.451	0.268 / 0.042	0.214 / 0.123	0.320 / 0.014	0.401 / 0.002	0.348 / 0.008	0.173 / 0.193	0.384 / 0.003	0.362 / 0.005	-0.093 / 0.491	0.110 / 0.416	-0.042 / 0.759	-0.132 / 0.332	0.192 / 0.156	-0.092 / 0.491	0.284 / 0.030	0.089 / 0.520	0.477 / 0.000	0.539 / 0.000	0.553 / 0.000						
Seed width	22	-0.238 / 0.075	0.131 / 0.328	0.105 / 0.455	0.192 / 0.148	0.275 / 0.037	0.199 / 0.138	0.109 / 0.416	0.288 / 0.028	0.218 / 0.101	-0.275 / 0.038	0.044 / 0.747	-0.123 / 0.364	0.014 / 0.917	0.062 / 0.648	-0.128 / 0.340	0.587 / 0.001	0.090 / 0.515	0.587 / 0.000	0.396 / 0.002	0.601 / 0.000	0.879 / 0.000					
Seed no./plant	23	-0.077 / 0.564	0.065 / 0.625	-0.367 / 0.007	-0.006 / 0.967	0.075 / 0.577	0.059 / 0.665	0.076 / 0.569	0.204 / 0.124	0.116 / 0.385	-0.079 / 0.558	0.177 / 0.183	0.112 / 0.402	0.418 / 0.001	-0.376 / 0.004	-0.064 / 0.633	0.858 / 0.000	0.312 / 0.019	0.831 / 0.000	0.264 / 0.046	0.537 / 0.000	0.304 / 0.022	0.474 / 0.000				
Seed weight	24	-0.081 / 0.544	0.084 / 0.526	-0.331 / 0.016	0.051 / 0.706	0.154 / 0.249	0.126 / 0.349	0.171 / 0.200	0.282 / 0.032	0.187 / 0.160	-0.083 / 0.535	0.186 / 0.163	0.116 / 0.387	0.392 / 0.003	-0.339 / 0.011	-0.069 / 0.605	0.830 / 0.000	0.318 / 0.017	0.894 / 0.000	0.411 / 0.001	0.635 / 0.000	0.460 / 0.000	0.601 / 0.000	0.947 / 0.000			
Biomass dry weight	25	0.051 / 0.680	0.392 / 0.001	-0.041 / 0.763	0.758 / 0.000	0.817 / 0.000	0.769 / 0.000	0.947 / 0.000	0.761 / 0.000	0.746 / 0.000	0.096 / 0.460	0.249 / 0.051	0.166 / 0.196	0.138 / 0.289	0.045 / 0.732	-0.124 / 0.327	0.041 / 0.751	-0.021 / 0.876	0.148 / 0.246	0.358 / 0.004	0.207 / 0.107	0.202 / 0.129	0.127 / 0.341	-0.030 / 0.824	0.077 / 0.560		
Shelling%	26	0.052 / 0.696	0.233 / 0.075	-0.192 / 0.169	-0.098 / 0.463	0.025 / 0.851	0.089 / 0.512	-0.017 / 0.899	0.161 / 0.229	0.190 / 0.154	-0.169 / 0.203	-0.014 / 0.917	-0.133 / 0.320	0.167 / 0.216	-0.073 / 0.592	0.075 / 0.576	0.153 / 0.247	0.057 / 0.674	0.164 / 0.216	0.023 / 0.862	0.280 / 0.033	0.385 / 0.003	0.464 / 0.000	0.504 / 0.000	0.515 / 0.000	-0.131 / 0.322	
100-seed weight	27	-0.008 / 0.952	0.090 / 0.497	-0.008 / 0.952	0.227 / 0.087	0.351 / 0.007	0.350 / 0.008	0.237 / 0.074	0.410 / 0.001	0.381 / 0.003	-0.101 / 0.452	0.065 / 0.627	-0.077 / 0.567	-0.096 / 0.476	0.048 / 0.723	-0.096 / 0.471	0.249 / 0.057	0.236 / 0.080	0.487 / 0.000	0.696 / 0.000	0.644 / 0.000	0.782 / 0.000	0.736 / 0.000	0.195 / 0.139	0.410 / 0.001	0.339 / 0.009	0.266 / 0.042

Plant height and plant spread: A significant positive correlation existed between them (r= +0.691; p=0.000) and both showed significant positive correlation with each of petiole length, leaf area, terminal leaflet length and width, leaf no./plant pod width and seed length. Plant height itself presented a very high positive correlation with biomass dry weight (Appendix 18). Most of these associations reflected results from data collected from the glasshouse.

Internode length: like the data from the glasshouse it had positive and significant relationship with node no./plant, pod no./plant, pod weight, seed no and seed weight. It also had significant and negative correlation with growth habit valued -0.797 at p= 0.000, while internode length explained 63% of the variation in the growth habit among the individuals of this population (Appendix 19). In addition, this trait was in a negative association with days to flowering (-0.308; p<0.05).

Seed weight: was positively and significantly associated with most of the other yield component traits and with internode length and leaflet width (Appendix 20). It also showed a significant negative correlation with growth habit (r= -339; p<0.05).

Biomass dry weight: As with the glasshouse experiment the coefficient analysis showed that biomass had a strong and positive correlation with some of vegetative growth traits included leaf no./plant, plant height, plant spread, leaflet length, leaflet width, leaf area and petiole length, among them a higher value of +0.947 was recorded in the association of terminal leaflet length with biomass at p= 0.000 level of significant. Regression analysis made for biomass dry weight accountable for 89.6% and 55% of the variations observed in leaflet length and plant spread, respectively (Figure 4-7). Among the yield-related traits biomass was only showed a significant

correlation with both of pod length and 100-seed weight (r= +0.358 and +0.339; p= 0.004 and 0.009, respectively).
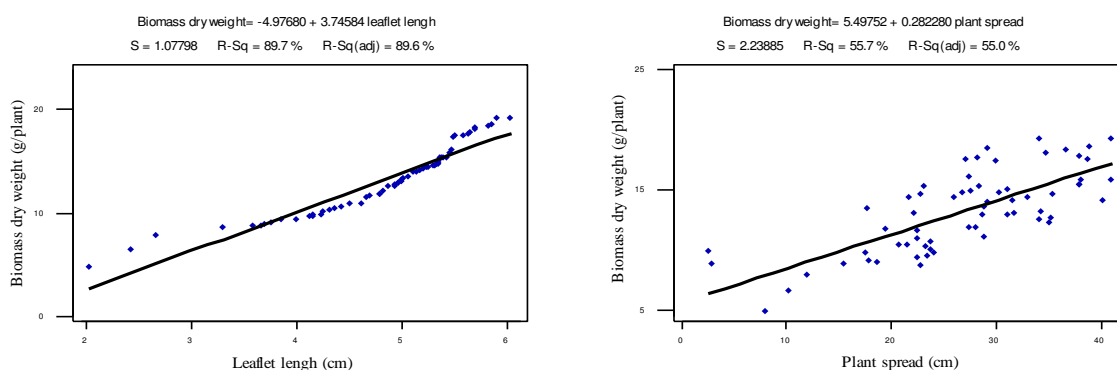
Figure 4-7: A fitted line regression plot for biomass dry weight with both of leaflet length and plant spread in the F₃ population of the field.

The correlation between the same traits of different trials:

There was a positive significant correlation between the data measured for the same traits of both glasshouse and the field experiments of the F_3 population derived from the narrow cross of DipC and Tiga necaru landraces (Table 4-6).

Table 4-6: Correlation coefficient values for the same traits values of both glasshouse and the field trial of the F₃ population derived from the cross of DipC x Tiga necaru

No	Trait	Correlation value	P value
1	Days to emergence	0.410	0.001
2	Leaf area	0.283	0.030
3	Terminal leaflet length	0.349	0.006
4	Terminal leaflet width	0.328	0.011
5	Plant spread	0.285	0.030
6	Internode length	0.611	0.000
7	Double seeded pods	0.291	0.021
8	Biomass dry weight	0.331	0.009
9	Seed weight	0.320	0.016
10	100-seed weight	0.304	0.024

4.2.3.3 Association of the traits for F_2 generation in TCRU

The result of associations between the traits of this generation can be found in Table 4-7.

Table 4-7: Relationship between traits in the F_2 population of the cross DipC x Tiga necaru for TCRU data based on the Pearson's correlation analysis showing correlation and P values.

Traits		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Days to emergence	1														
Leaf no./plant	2	-0.107 0.366													
Days to flowering	3	0.609 0.000	-0.199 0.091												
Plant height	4	0.275 0.018	0.575 0.000	0.217 0.065											
Petiole length	5	0.125 0.301	0.673 0.000	0.200 .097	0.749 0.000										
Terminal leaflet length	6	-0.029 0.810	0.183 0.129	-0.192 0.104	0.213 0.071	0.312 0.009									
Terminal leaflet width	7	-0.064 0.593	0.495 0.000	-0.219 0.063	0.350 0.002	0.447 0.000	0.752 0.000								
Plant spread	8	0.055 0.641	0.635 0.000	0.049 0.681	0.615 0.000	0.832 0.000	0.547 0.000	0.590 0.000							
Pod no./plant	9	0.079 0.507	0.530 0.000	0.133 0.263	0.545 0.000	0.768 0.000	0.388 0.001	0.456 0.000	0.728 0.000						
Double seeded pods/plant	10	0.032 0.790	0.407 0.000	0.099 0.412	0.329 0.005	0.508 0.000	0.328 0.005	0.332 0.005	0.653 0.000	0.615 0.000					
Pod weight/plant	11	0.126 0.288	0.632 0.000	0.198 0.094	0.655 0.000	0.836 0.000	0.276 0.018	0.410 0.000	0.800 0.000	0.909 0.000	0.702 0.000				
Seed weight	12	0.166 0.159	0.648 0.000	0.228 0.053	0.677 0.000	0.840 0.000	0.264 0.024	0.396 0.001	0.788 0.000	0.888 0.000	0.690 0.000	0.990 0.000			
Biomass dry weight	13	0.114 0.336	0.636 0.000	0.203 0.083	0.665 0.019	0.854 0.000	0.273 0.019	0.411 0.000	0.807 0.000	0.918 0.000	0.666 0.000	0.987 0.000	0.973 0.000		
Shelling%	14	0.304 0.009	-.001 0.994	0.294 0.011	0.117 0.323	0.107 0.379	-0.147 0.216	-0.165 0.162	-0.062 0.604	-0.081 0.497	-0.074 0.542	0.064 0.588	0.156 0.186	0.025 0.832	
100-seed weight	15	0.306 0.009	0.445 0.000	0.296 0.011	0.481 0.000	0.456 0.000	-0.004 0.975	0.176 0.137	0.370 0.001	0.156 0.188	0.234 0.050	0.447 0.000	0.507 0.000	0.418 0.000	0.628 0.000

Legend: r > 0.7 (yellow); r = 0.5–0.69 (green); r = 0.30 – 0.49 (blue).

Days to emergence: It was behaved as like as F_3 generation data grown in the glasshouse being in a positive and significant association with shelling percent and 100-seed weight, however the significant associations with other traits was not recorded significantly.

Plant spread: Found to be associated significantly with other traits of terminal leaflet length and width, and with some yield related traits such as pod no./plant, growth habit, biomass dry weight and 100-seed weight. The highest association of this trait

was recorded with biomass dry weight (r= +0.81 at p= 0.000) and the regression analysis between these two traits revealed an R-Sq (adj) value of 64.6% (Appendix 21). This result supported those for the F_3 populations in glasshouse and field trial.

Seed weight: Was found to be correlated with leaf no./plant, plant height, plant spread, pod no./plant, pod weight and biomass dry weight (Appendix 22).

4.3 Discussion

The inheritance and the segregation patterns of morphological traits were investigated in the segregating F_3 population lines derived from an intra-subspecific cross between two domesticated landraces. DipC from Botswana was crossed with the wild accession (VSSP11). This investigation into the genetic relationships between wild and domesticated landraces is likely to reflect the genetic changes which occurred during domestication. The same landrace (DipC) was chosen as the female parent and crossed with the Tiga necaru landrace to construct a segregating population from a fully domesticated background. Tiga necaru from Mali was found to be from a different localized cluster using DArT markers in a genetic diversity analysis of bambara groundnut. This analysis suggested that the major molecular variation division was between West African and South/East African accessions (Bamlink annual report, 2008). The wide (although intra-subspecific) genetic differences between the parents of cross was also supported by another investigation studying the diversity in bambara groundnut using 201 DArT and 65 SSR markers (Molosiwa, 2012).

The current study with a series of statistical investigations has described the nature of trait variation observed for a wide range of agronomical important traits within controlled crosses, as a preliminary to a full QTL analysis. This population was evaluated in glasshouse and field conditions to expose the population to a wider range of genotype x environment effects.

Reasonable numbers of morphological traits were evaluated in this study for the vegetative growth stage, flowering and yield development stages. The distribution of

different traits was tested and a Box-Cox transformation used to convert non-normally distributed traits into normal distributions for QTL analysis.

4.3.1 Distribution of the traits

For all agro-morphological traits a distribution analysis was made using an Anderson-Darling test. Most traits were shown to be normally distributed and consistent over different environments for both F_2 and F_3 generations (flower no./plant, plant height, petiole length, terminal leaflet length and width, plant spread, stem no./plant, node no./plant, internode length, pod weight/plant, pod width, double seeded pods length and width, shelling% and 100-seed weight). Distribution of these characters normally indicates that they are more likely to be controlled by additive gene effect. Additive gene effects were also reported earlier for 100-seed weight and shelling percentage in bambara groundnut (Karikari, 2000). Other traits (days to emergence, days to flowering, growth habit and double seeded pods/plant) were non-normally distributed. However, some of these traits (leaf area, peduncle length, pod no./plant, double seeded pods/plant, pod length, seed length and biomass dry weight) did not show the same distribution pattern over different trials or across different generations, Box-Cox transformation pulled them back to a continuous and normally distribution. This might be due to differences in inter-planting distances in the glasshouse and field experiments or the effect of genotype x environment interaction. Jonah et al. (2012) also reported that seed length, pod length and pod width are largely under additive genetic control recording 100% broad sense heritability and predicted high genetic advance of these traits by evaluating twelve cultivars of bambara groundnut sourced from farmers collection in the north eastern Nigeria.

Days to emergence for both generations of F_3 and F_2 was characterized as a discontinuous trait, although it was not possible to determine a single gene effect as the trait did not match the expected pattern of Mendelian segregation ratio in both populations. In the glasshouse (F_3) data recorded for days to emergence only one individual line took longer to emerge than the DipC parent, however the difference in time to emergence between the parents of the cross did not exceed 4 days. In the F_2 population no difference was recorded for days to emergence of both parents. The result of this trait agrees with the findings of Basu et al. (2007c) studying segregation of this trait in an F_2 population derived from the cross of DipC x VSSP11.

Characterizing the number of flowers produced by the plant is laborious work, having to be done each 2-3 days for 3-4 months from the first day of flowering, this trait could be an important one in breeding terms. It was recorded for the F_3 population grown in glasshouse, and was found to follow a normal distribution, suggesting a multigenic trait.

Among the traits not distributed normally growth habit and eye pattern around the hilum were shown to follow a segregation pattern consistent with Mendelian inheritance. Incomplete dominance was hypothesised to control growth habit in the F_3 progenies in the glasshouse. Growth habit is one of the important morphological/agricultural traits and selection criteria derived in domestication and adaptation studies of bambara groundnut landraces have emphasized the study of this trait (Massawe et al., 2005). In the analysis of the wide (inter-sub-specific cross; DipC x VSSP11; Basu et al., 2007c), the F1 hybrid between the spreading spontenea type and the bunched subterranea type shows a morphology consistent with the wild spreading parent, suggesting that 'bunching' is a recessive mutation that has occurred during domestication.

The presence of eye pattern around the hilum was consistent with the hypothesis of control by complete dominance in the segregating F_3 population in the glasshouse. The monogenic inheritance of this trait was in agreement with what was detected previously in the segregation F_2 population of the wide cross (DipC x VSSP11) (Basu, 2005), however in the current study on the association of eye pattern with testa color was not confirmed as both parents of the current population possess creamy testa color, which made the following of the segregation for testa colour impossible.

4.3.2 Association of the traits

The characters studied here are most likely to have a complex relationship with each other. They mostly demonstrate substantial phenotypic variability and these characters could be used for breeding of this crop. A strong correlation was observed between yield and other traits studied here. The result was in agreement with previous reports (Ntundu et al., 2006; Ouedraogo et al., 2008). These correlations could serve as important criteria in breeding and selection of genotypes for this crop, using simpler or early traits which are correlated to later or more difficult to assess traits. Such correlations would need to be validated before use.

Days to emergence: Time required to initiate the first real leaf was positively correlated to the time taken for flowering in the F_2 and F_3 populations under study. In previous investigations it was also reported to show a positive but not significant correlation with days to 50% flowering (Nawab et al., 2008). A negligible negative correlation was observed for days to emergence with pod and seed yield. A non-significant correlation of days to emergence with seed weight was recorded in the early and late planting date of bambara groundnut by Benedict and Michael (2011). However days to emergence had no direct correlation with yield in bambara

groundnut in the current study, despite the significant and positive correlation of days to 50% emergence with pod yield reported by researchers in Zimbabwe (Makanda et al., 2009).

Days to flowering: This trait had negative and significant association with most of the vegetative growth traits and also with seed yield and biomass dry weight in both glasshouse and field trial for F_3 population. These relationships suggest that spending more time in the vegetative stage for this material will reduce time spent in the reproductive phase, resulting in lower yields. A similar result of lower yield in semi arid-conditions due to a longer vegetative stage was reported in the evaluation of 310 bambara groundnut accessions in the Northern area of Burkina Faso (Ouedraogo et al., 2008). It has been identified by Makanda et al. (2009) that poor yield in bambara groundnut is a function of delayed flowering. This association was not consistent in the F_2 population derived from the same cross as days to flowering was in a positive but not significant association with yield and its components.

Flower no./plant: Among vegetative growth traits, there was a significant association of flower number with plant spread accounted for 32.1% [R-Sq(adj) = 32.1 %] of the trait variation observed in plant spread. Furthermore, the negative and significant correlation detected between flower no./plant and days to flowering support a hypothesis; early flowering gives the plant time to deliver the stored energy into the seeds instead of partitioning to other organs. This possibility was also supported through the significant and positive correlation of flower no./plant with yield and its components. A high positive correlation of flower no./plant and seed weight was reported previously (Benedict and Michael, 2011).

Leaf dimensions: The measurements of terminal leaflet length and width were found to be correlated positively to seed yield and its components for the F_2 and F_3 generations, at different levels of significance, under glasshouse and field conditions. Increased photosynthetic area resulting from greater leaf size may have contributed to increase agronomical performance. Leaf length and width participate in determining the spread of the plant canopy, so selection for these traits could be used to control soil evaporation by optimal canopy spread. Another investigation also suggests longer leaved plants in bambara groundnut produce more pods and seed yield (Misangu et al., 2007).

Plant spread: Had significant and positive associations with other vegetative traits, especially with leaf dimensions and this was consistent for both segregating populations over all three experiments. A positive correlation of plant spread with biomass dry weight and seed weight was observed for all the experiments, although the association with seed weight did not reach significance in the field trial data. This combination of associations makes plant spread to be a good indicator for yield of bambara groundnut, in this material at least. Based on a strong positive correlation between plant spread and double seeded pods/plant in the glasshouse and TCRU experiments, increasing the yield of bambara groundnut in this material could be achieved by selection of plants having greater canopy spread, with double seeded pods providing a boost to yield. A positive correlation of canopy spread with pod yield was also reported in adaptation studies of bambara groundnut conducted in Owerri southeastern Nigeria (Onwubiko et al., 2011).

Internode length: This trait is a major component of plant architecture as it accounts for the major differences between bunch and spreading types. It is one of the important traits responsible for the variability of landraces (Siise and Massawe, 2012).

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Internode length was the main variable in an investigation assessing the level of diversity among 101 accessions to define collection and conservation strategies in bambara groundnut (Sévérin and Yao, 2011). In the current study correlations between internode length and other traits were consistent in both glasshouse and field trials for the F_3 population, internode length was also in a positive and significant association with other traits such as node no. and pod no./plant, pod weight, seed no. and seed weight. Internode length and days to flowering were negatively correlated. Thus higher yield could be obtained from plants having longer internode length, however selection efforts have worked to produce shorter internode length leading to a bunched habit (Smartt, 1985). It can be concluded that selection for this trait could result in better yield in this crop.

Biomass dry weight: Any improvement program for bambara groundnut should include high biomass production to emphasise bio-productivity and high harvest index to ensure partitioning biomass accumulation to the seed. Biomass dry weight had positive correlations with plant spread and the leaf measurements for the F_3 generation (glasshouse and the field) and the F_2 population grown in TCRU. The associations of other yield component traits with biomass dry weight were significant in the glasshouse and TCRU experiments, regression analysis suggested that nearly 90% of the variation in pod and seed weight could be explained on the basis of biomass dry weight for both experiments (glasshouse and TCRU).

Seed yield: In the current investigation the correlation of 100-seed weight to the seed yield was consistent for both segregating populations over the three experiments. Ofori (1996) in a correlation analysis in bambara groundnut also identified positive correlation of 100-seed weight with seed yield. Therefore, studying the trait of 100-seed weight was considered valuable in the breeding program as it was found to be

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among the important characters to be considered during selection of bambara groundnut (Karikari, 2000). Pod no./plant is an important component of seed yield in bambara groundnut, not surprisingly, and this has been noted through highly significant correlation between both traits in different environments. It has been concluded that the yield in bambara groundnut could be improved through selection for the pod no./plant (Adu-Dapaah and Sangwan, 2004).

Chapter 5: CONSTRUCTION OF A GENETIC LINKAGE MAP IN BAMBARA GROUNDNUT

5.1 Introduction

A genetic linkage map represents the relative order of genetic markers along a chromosome and the relative distance between them determined by recombination frequency. Based on this relationship, the markers in a genetic map are placed into linkage groupings which describe the gene order and high density maps can often assign individual linkage groups a chromosomal identity (Yeboah et al., 2007).

The construction of genetic linkage maps relies on the choice of parental lines to develop a controlled cross. The cross should be segregating for traits and reasonable levels of marker polymorphism are needed, which can be scored across the individuals of the population. It is possible to construct a genetic linkage map based on a number of different family structures; F_2, F_3, backcrosses, double haploid or Recombined Inbred Lines (RILs). Recombinational distances can be estimated through comparison of the number of recombinant genotypes for a pair of markers with the total number of observations (Wu et al., 2008).

5.2 Genetic mapping in related legume crops

Bambara groundnut belongs to the Phaseoloid-Millettioid clade, which contains most legume crops, such as pea, alfalfa (Medicago spp.), chickpea, soybean and common bean (Choi et al., 2004b). Investigating the genetic linkage map in related legume crops and emphasizing shared synteny among these species might help facilitate the identification of markers closely linked to traits of interest in bambara groundnut.

5.2.1 Soybean

The first soybean (Glycine max L. Merr.) (2n=2x=40) genetic linkage map of molecular markers was developed using 150 restriction fragment length polymorphism (RFLP) loci in an F_2 population derived from an inter-specific cross of G. max (A81-356022) x G. soja (PI468916) (Keim et al., 1990).

Five soybean maps constructed from the crosses of 'Minsoy' x 'Noir 1', 'Minsoy' x 'Archer', 'Archer' x 'Noir 1', 'Clark' x 'Harosoy', and A81-356022 x PI468916 were combined into an integrated genetic map spanning 2,523.6 cM (Kosambi) map distance across 20 linkage groups. A total of 1,849 markers were used, including 1,015 SSRs, 709 RFLPs, 73 RAPDs, 24 classical traits, six AFLPs, ten isozymes, and 12 others. On average, 51 SSR markers mapped per linkage group, ranging from 35 to 64. The average length of the interval between any two adjacent SSR markers was 2.5 cM. It was suggested that more SSRs than expected were closely linked and clustering of SSR markers on the soybean map was observed, suggesting an association of genes and SSRs. The primer sequences for all SSR loci, as well as the genetic maps of each of the 20 consensus linkage groups can be found on the SoyBase Web site of the USDA, ARS Soybean Genome Database (http://soybase.agron.iastate.edu/) (Song et al., 2004).

5.2.2 Cowpea

Omo-Ikerodah et al., (2008) reported construction of a linkage map to screen for QTL to resistance to flower bud thrips (Megalurothrips sjostedti) in cowpea (Vigna unguiculata (L.) Walp) using 145 RIL individuals (F_{10}). The population was developed from a cross between two cowpea lines, 'Sanzi' (resistant to FTh) and 'VITA7' (susceptible to FTh) and developed by the single seed descent method. A

total of 134 AFLP and the 5 SSR markers were scored in the developed mapping population to facilitate QTL analysis, covering 1620.1 cM of the cowpea genome.

A consensus genetic linkage map was constructed for cowpea [Vigna unguiculata (L.) Walp.] (2n=2x=22) using a high-throughput EST-derived SNP assay. The markers were applied to 741 recombinant inbred lines from six mapping populations. Approximately 90% of the SNPs were technically successful, providing 1,375 dependable markers. Of these, 928 were integrated into a consensus genetic map spanning 680 cM with 11 linkage groups and an average marker distance of 0.73 cM. Recently a new consensus map containing 1107 EST-derived SNP markers (856 bins) was reported by Lucas et al., 2011, which was developed through integrating 13 population-specific maps, segregating for the most important traits in this crop. Eleven of these populations were F_8 to F_{10} RIL populations developed by inbreeding and single seed descent while the remaining two were F_3–derived F_4 families. A total of 179 SNP markers were added to the previous mapping data set used by Muchero et al. (2009) and Xu et al. (2011). The improved methods of data analysis are realized in map characteristics when surveying synteny of cowpea with soybean, Medicago truncatula Gaertn, and Arabidopsis thaliana (L.) Heynh. Using HarvEST:Cowpea 1.27 (Wanamaker and Close, 2011) and Circos (Krzywinski et al., 2009).

In the soybean and M. truncatula genomes, homologous genes were identified for 85% and 80% of the SNPs mapped in cowpea, respectively Development of such a highly robust genetic map is of value to develop projects, including genome assembly, marker assisted breeding, quantitative trait loci (QTL) analysis, map-based cloning, and comparative genomics. These results support the evolutionary close relationship between cowpea and soybean and identify regions for synteny-based functional genomics studies in legumes (Lucas et al., 2011; Muchero et al., 2009).

Another investigation was conducted by Pottorff et al., 2012 to identify the candidate gene for leaf morphology in Cowpea [Vigna unguiculata (L.) Walp]. A RIL population of Sanzi (sub-globose leaf shape) x Vita 7 (hastate leaf shape) was used, showing considerable variation in leaf shape. A QTL for leaf shape, Hls (hastate leaf shape), was identified on the Sanzi x Vita 7 genetic map spanning from 56.54 cM to 67.54 cM on linkage group 15. The corresponding Hls locus was positioned on the cowpea consensus genetic map on linkage group 4, spanning from 25.57 to 35.96 cM. Synteny was examined using EST-derived SNP markers previously BLASTed and aligned to the soybean and Medicago genomes which are housed in the publicly available data set of HarvEST:Cowpea (http://harvest.ucr.edu). High co-linearity was observed for the syntenic Hls region in Medicago truncatula and Glycine max. One syntenic locus for Hls was identified on Medicago chromosome 7 Identifying 8 Medicago genes orthologous to cowpea SNP markers in that syntenic region of Medicago chromosome. While syntenic regions for Hls were identified on two soybean chromosomes, 3 and 19 (Figure 5-1). The Hls locus was identified on the cowpea physical map via SNP markers 1_0910, 1_1013 and 1_0992 which were identified in three BAC contigs; contig926, contig821 and contig25. The conserved gene order within the same legume family, cowpea, Medicago and soybean, enabled the identification of a candidate gene for the Hls locus (Pottorff et al., 2012).
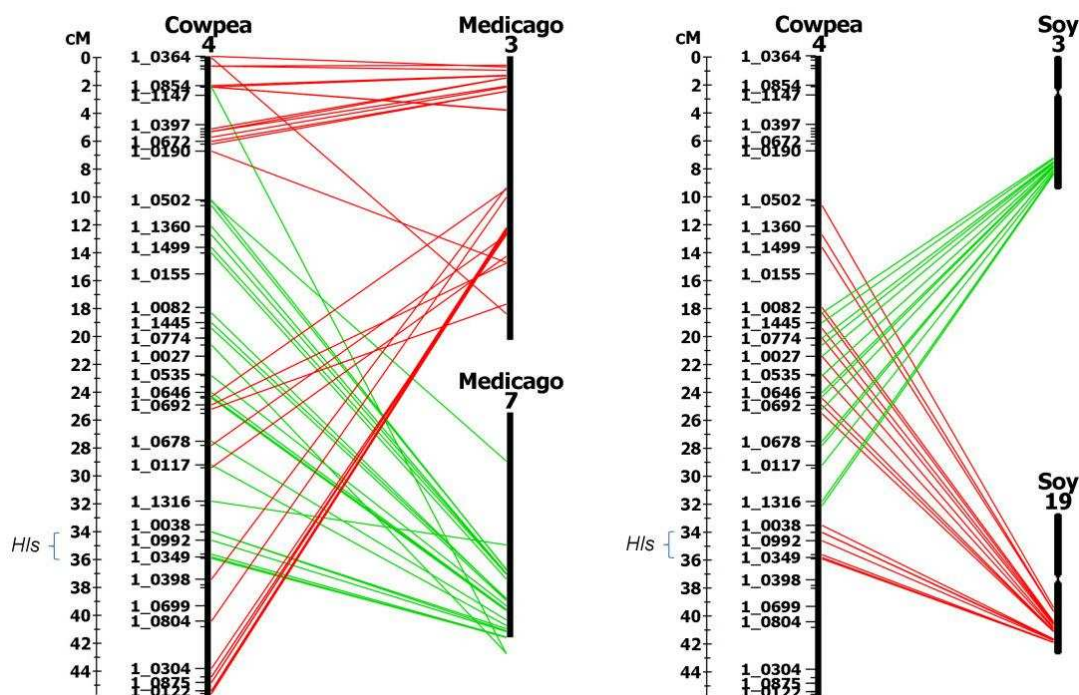
Figure 5-1: Synteny of the Hls locus for Cowpea with Medicago truncatula and Glycine max, using EST-derived SNP markers previously BLASTed and aligned to the sequenced genomes. The Hls locus which is on linkage group 4 of the cowpea consensus genetic map was syntenic with Medicago chromosomes 3 and 7. Two syntenic loci were identified for the Hls locus in soybean chromosomes 3 and 19. The syntenic map was constructed using the HarvEST: Cowpea database (http://harvest.ucr.edu) with a cut off e-score value of −10 and a minimum number of 10 lines drawn per linkage group. Colored lines indicate cowpea genes orthologous to genes on M. truncatula and G. max chromosomes (Pottorff et al., 2012).

5.2.3 Chickpea

Chickpea (Cicer arietinum L.) is a self-pollinated, diploid (2n=2x=16), grain legume crop with a genome size of 740 Mb (Arumuganathan and Earle, 1991). A comprehensive genetic map was constructed in chickpea using a recombinant inbred line (RIL) population of 131 individuals derived from the cross of ICC 4958 (C. arietinum)×PI 489777 (C. reticulatum). A total of 253 bacterial artificial chromosome BES-SSR and 675 DArT polymorphic markers between the parental genotypes were used along with genotyping data published recently (192 genic molecular markers

(GMMs) including 83 conserved orthologous sequences (COS)-based SNPs, 54 cleaved amplified polymorphic sequences (CAPS), 35 conserved intron spanning region (CISR) and 20 EST-derived SSR) (Gujaria et al., 2011). The map comprises 1,291 markers on eight linkage groups (LGs) spanning a total distance of 845.56 cM with an average inter-marker distance of 0.65 cM. This comprehensive genetic map with integrated BES-SSR markers will facilitate its anchoring to the physical map to accelerate map-based cloning of genes in chickpea and comparative genome evolution studies in legumes (Thudi et al., 2011).

5.2.4 Lentil

Lentil is self-pollinated, diploid (2n=2x=14) with a large genome size of approximately 4Gb (Arumuganathan and Earle, 1991). A linkage map was constructed to improve understanding of the genetic structure. The linkage map of lentil (Lens culinaris L.) was constructed for a 94 RIL population (developed from the parents Precoz x WA8949041) using 166 markers. The map consisted of 11 linkage groups spanning 1396.3 cM of the lentil genome at a minimum LOD score of 3. The length of LGs varied from 16 cM to 436.8 cM with average marker density of 8.4 cM. The number of marker loci per LG ranged from 6 to 41. LG1 was the group containing the most markers with an average marker density of 10.6 cM in length. There was great variability in these densities as most of the RAPD and ISSR markers were evenly distributed across the genome while the majority AFLP markers were clustered on LG1. The authors suggested that the map could be used to detect agronomically important genes and in marker assisted selection of this crop. (Tanyolac et al., 2010).

5.2.5 Alfalfa

The first genetic map was established for the model legume species of Medicago truncatula using an F_2 segregating population of 124 individuals. This map spanned 1225 cM with an average distance between adjacent markers of 470 kb/cM. It comprised 289 markers including RAPD, AFLP, known genes and isozymes arranged in 8 linkage groups. Markers were uniformly distributed throughout the map and segregation distortion was limited to only three linkage groups. Locating a number of common markers on the map, the eight linkage groups were shown to be homologous to those of diploid alfalfa (Medicago sativa), implying a good level of macrosynteny between the two genomes (Thoquet et al., 2002).

Comparative genome analysis has been performed between two closely related tribes (Trifoliae and Viciae) of the subfamily Papilionoideae with different basic chromosome numbers. The linkage map of diploid alfalfa (Medicago sativa) derived from F_2 segregating population was compared to those of homologous loci on the combined genetic map of pea (Pisum sativum) derived from RIL populations. The analysis aimed to analyze the degree of colinearity between their linkage groups. The linkage groups of Medicago and pea showed a high degree of colinearity as was also demonstrated by other researchers (Choi et al., 2004a). It was concluded that the difference in genome size between the two species (the pea genome is 5- to 10-fold larger than that of alfalfa) is not a consequence of genome duplication in the Pisum lineage. The high degree of synteny observed between pea and Medicago loci indicates that further map-based cloning of pea genes based on the available genome resources for Medicago truncatula and other related species will be productive which will enhance the strategy of transferring map information from this model legume to other related (Kalo et al., 2004).

5.2.6 Mungbean

A linkage map was constructed in mungbean (Vigna radiate L.) (2n=2x=22) using an F$_2$ population (186 individuals) derived from an intra-specific cross between the wild accession 'W021' (Vigna radiata var. sublobata) and cultivated line 'KUML29-1-3' (Vigna radiata var. radiata). JoinMap 3.0 program was used to develop the linkage map. A minimum LOD score of 3.0 was used as a threshold value for grouping the markers and mapping used the Kosambi map function. One hundred and fifty SSR markers were assigned into 11 linkage groups with 5 markers at least for each. The map covered 1,174.2 cM of the mungbean genome having an average marker density of 7.8 cM. Extensive genomic conservation has been revealed by comparing this map with azuki bean (Vigna angularis) and blackgram (Vigna mungo) linkage maps based on azuki common markers (Kajonphol et al., 2012).

5.2.7 Pigeon pea

The first genetic linkage map was developed for pigeon pea (Cajanus cajan spp. (L.) Mill sp.) (2n=2x=22) using a total of 554 diversity arrays technology (DArT) markers. The MAPMAKER/EXP version3 software was used to generate the map. The mapping population was an F$_2$ of 72 progenies derived from a cross between cultivated (C. cajan) genotype and its wild relative (C. scarabaeoides). Segregation distortion was 13%. Two groups of paternal and maternal genetic maps were generated with 172 and 122 unique marker loci, covering 451.6 cM and 270.0 cM, respectively. The authors state that those markers developed would be a useful genomic resource for inter-specific genetic analysis and the linkage map will allow map-based cloning of genes for biotic and abiotic stress from wild relatives of pigeon pea in the future (Yang et al., 2011).

The genetic linkage maps presented above were derived from different inter and intra-specific crosses using different kinds of molecular markers in legume crops to identify QTLs for the traits interested. Some of the maps constructed were proposed to be used in comparative genome analysis with the other non model legumes crops. The results also suggested close relationships between some of these legumes by identify a high degree of synteny. This could allows to identify markers and cloning genes and facilitate the strategy of transferring genomic information from this model legume such as soybean (Glycine max) and Medicago truncatula to other non-model related species such as bambara groundnut.

The aims of the research reported in this chapter were firstly to construct 'narrow' and 'wide' genetic linkage maps of bambara groundnut (Vigna subterranea (L.) Verdc.) to clarify the underlying genetic mechanisms controlling agronomic traits and facilitate MAS in future breeding programs, then combining these two maps to identify common markers in an attempt to build a consensus map. Two inter and intra-subspecific mapping populations were used. The first one was an F_3 segregating population derived from the cross of two domesticated landraces (DipC x Tiga necaru) based on SSR and DArT markers, while the second was a segregating F_2 population derived by crossing V. subterranea var. subterranea x V. subterranea var. spontanea. Three marker types of SSR, DArT and AFLP were used in constructing the second map.

5.3 Result

In this chapter the analysis of polymorphic markers, their segregation in the population under study and linkage map construction are presented for both 'narrow' and 'wide' maps. In addition, an attempt to combine the maps resulting from these two constructed maps is also presented here.

5.3.1 Polymorphism and markers for mapping

Polymorphic markers in a segregating F$_3$ *population of the 'narrow cross'*

Out of 124 SSR markers developed from the genome and transcriptome of bambara groundnut and from soybean (one marker) 45 primers were polymorphic (36.3%) and discriminated between the parental alleles of the cross. From this number 33 primers were used to screen all individuals in this population and in the construction of the genetic map. As there was not enough time or money, the other 12 polymorphic markers were not mapped in the population. Of the 7680 features present in the DArT slide array, 236 (3.1%) were identified as polymorphic markers in the cross and scored in the narrow cross population.

In total 269 polymorphic SSR and DArT markers were used to construct the initial map and showed linkage to at least one other marker using the segregating F$_3$ population derived from narrow cross of DipC x Tiga necaru landraces.

Polymorphic markers in the segregating F$_2$ *population of 'wide cross'*

The same primer sets of 124 SSR markers were tested for polymorphism in this population and 41 polymorphic primers were detected (33.1%). Parental alleles of this cross were scored for all the individuals using only 22 polymorphic SSR primers due

to shortage of time and money. These primers were combined with other sets previously developed for this population (Basu, 2005). Previously developed primers included 136 DArT, 141 AFLP and 2 SSR primers. In total, 301 polymorphic SSR, DArT and AFLP were used in linkage analysis and map construction using a segregating F_2 population derived from the cross of cultivated landrace (DipC) and wild accession (VSSP11).

5.3.2 Inheritance and the segregation distortion of markers

Marker segregation patterns and their potential distortion was detected automatically by JoinMap4, performing a Chi-square test against expected segregation patterns (p<0.05 for significance). In the narrow cross the segregation of markers was estimated from the second map run (RIL3 model) for phased markers (239). The locus genotype frequency table (Appendix 23) suggested 163 markers segregated in the expected Mendelian ratio of 3:2:3 and 3:5 for both marker types SSR and DArT, respectively, in the F_3 population. Seventy-six markers (32%) showed segregation distortion and did not segregate according to expected Mendelian inheritance. The segregation distortion in the DArT markers was found to be greater compared to SSRs (Table 5-1).

In the wide cross map 73% (218) of the markers evaluated segregated in the expected Mendelian ratio (Appendix 24). Eighty three markers from all marker types (SSRs, DArTs and AFLPs) were distorted (P≤ 0.05, chi-square test). The highest distortion ratio (36%) was among dominant AFLP markers for this map followed by SSR markers (29%) and the lowest distortion (19%) was observed for DArT markers (Table 5-1). The results of linkage analysis for both maps revealed that markers with distorted segregation were distributed throughout the genome.

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Table 5-1: Markers and their segregation patterns in both population types of narrow and wide cross of bambara groundnut.

Type of marker	F$_3$ population of narrow cross			F$_2$ population of wide cross		
	Mendelian Segregation ratio (x^2 at $p<0.05$)	Polymorphic marker	Distortion	Mendelian Segregation ratio (x^2 at $p<0.05$)	Polymorphic marker	Distortion
SSR	3:2:3	29	7 (24%)	1:2:1	24	7 (29%)
DArT	3:5	210	69 (33%)	1:3	136	26 (19%)
AFLP	-	-	-	1:3	139	50 (36%)
Co-dominant AFLP	-	-	-	1:2:1	2	0
Total markers	-	239	76 (32%)	-	301	83 (27%)

5.3.4 Linkage phase determination

With no information available on the parents' genotypes (DipC and Tiga necaru) in segregating F$_3$ population for DArT markers, phase was determined through initial analysis of the population as a Cross Pollinator (CP). Two hundred and thirty-six DArT markers were combined with 33 SSR markers, grouped and initial linkage maps determined. Out of 269 markers 239 grouped into 28 linkage groups, with their phases determined by JoinMap4. CP segregation type coding of <hkxhk> for all loci was converted into the RIL appropriate code, dependant on the marker type and the phase (a,h,b) = codominant markers , (a,c) = dominant coding where phase was determined as (0,0) and (b,d) = dominant coding where phase was determined to be (1,1). The .loc file recoded as a, b, c, d, h was used for linkage determination using an RIL3 model, producing 21 linkage groups of 238 markers. The other 31 unmapped markers were presented in Appendix 25.

5.3.5 Calculation of the map

The JoinMap4 program builds the map by adding loci one by one, starting with the pair for which there is the strongest linkage evidence. For each added locus the best

position is determined by comparing the goodness-of-fit of the resulting map for each tested position. When at the best position, the overall goodness-of-fit (measured as a cumulative chi-square score) should be lower when compared with other possible positions. This position is adopted, although the final position may still lead to an increase in overall group cumulative chi-sqr score which leads to an initial rejection of the new marker (the default 'jump' threshold is set at an increase in overall cumulative chi-sqr of 5). The locus is removed if the jump score increases above the default value of +5 (suggesting a conflict with the markers already mapped) or if strong negative distances are observed. The process is continued until all loci are tested once. This is the end of the first round resulting in Map1. Subsequently, a second attempt is made to add the previously removed loci to the map. This can be successful since the map will contain more loci than at the first attempt because of more pairwise data being used and testing local order may lead to changes in marker order. If high jump values or negative distances are still not resolved for the problematic loci in Map2, they will be removed again. This is the end of the second round resulting in Map 2. After that, all loci previously removed are added back to the map in the best possible positions, ignoring both jump and negative distance thresholds. This results in a final or third round of mapping giving rise to Map3. In this process each map is calculated using the pairwise data of loci present in the map, but only those pairs of loci that have a recombination frequency smaller than the REC (Recombination) threshold (0.4 default) and a LOD value larger than the LOD threshold (1.0 default) are used.

Constructing the linkage map using the narrow cross population resulted in performing more than one round of mapping (Map1) to build the map for the groups of 1, 5, 6 and 11. All of these groups except 11 had no significant conflicts and the

'jumped' markers from round 1 could be added in the second round (Map2) without a need to relax criteria. For group 11, because the third map for this group had shown a significant conflict between existing marker data from round two and the additional 'jumped' markers, the first round map was adopted. Note, the second round map did not lead to additional markers being added to Map2.

In the map constructed from the wide cross population the maps of linkage groups 1, 6, 7, 13, 14, 15, and 16 had three rounds of map, but the round one (Map1) was followed from these groups to assemble the linkage map in the segregating F_2 population. The large threshold value for jump in the second (Map2) and the third maps (Map3) of these groups indicated the poor fit of the added markers and all added markers (10) for these rounds were removed from the map (Table 5-2). These removed markers and those unmapped on the linkage maps of 'wide' map were present in Appendix 26.

Table 5-2: Map rounds for the linkage groups of 'narrow ' and 'wide' cross maps with the identified adopted rounds in the genetic maps.

Linkage groups	'Narrow' cross map		'Wide' cross map	
	No of round (Map)	Map used	No of round (Map)	Map used
1	2	2	3	1
2	1	1	1	1
3	1	1	1	1
4	1	1	1	1
5	2	2	1	1
6	2	2	3	1
7	1	1	3	1
8	1	1	1	1
9	1	1	1	1
10	1	1	1	1
11	3	1	1	1
12	1	1	1	1
13	1	1	3	1
14	1	1	3	1
15	1	1	3	1
16	1	1	3	1
17	1	1	1	1
18	1	1	1	1
19	1	1	1	1
20	1	1	1	1
21	1	1	-	-

5.3.6 Linkage map and marker distribution

Map construction was conducted with JoinMap4. It started with the calculation of LOD groupings (tree) and manually selection using a LOD score of 5.0 coming down to 3.0. Two hundred and sixty nine markers of SSR and DArT were employed in the linkage analysis of narrow cross map and the Haldane mapping function was used.

Initially map construction was setup to analyse the population under study as a Cross Pollinator (CP) to determine linkage phase for these markers, as the parental data was not available by the time of running slide-based DArT markers. Out of 269 markers (SSRs and DArTs) 239 were assigned to 28 linkage groups. The genotypes of DArT markers for the individuals were converted to different phases for the markers in linkage groups. Different classifications was arranged depends on their phases; a or c gave to {0,0} phase and b or d for {1,1} phase in an 'RIL3' population type.

All arranged markers (29 SSRs and 210 converted DArTs) were grouped again with JoinMap4 using 'independence LOD'. All markers except one were assigned to 21 linkage groups (Figure 5-2).

The 21 linkage groups spanned 608.6 cM of bambara groundnut genome in this map. The distance between two consecutive markers varied from 0-10.1 cM, with a mean of 3 cM. The number of markers per linkage group ranged from 2 to 46 markers with an average length of 28.98 cM. The 21 linkage groups spanned 608.6 cM of bambara groundnut genome in this map. The distance between two consecutive markers varied from 0-10.1 cM, with a mean of 3 cM. The number of markers per linkage group ranged from 2 to 46 markers with an average length of 28.98 cM. The longest group with 23 markers covered a distance of 76.4 cM.
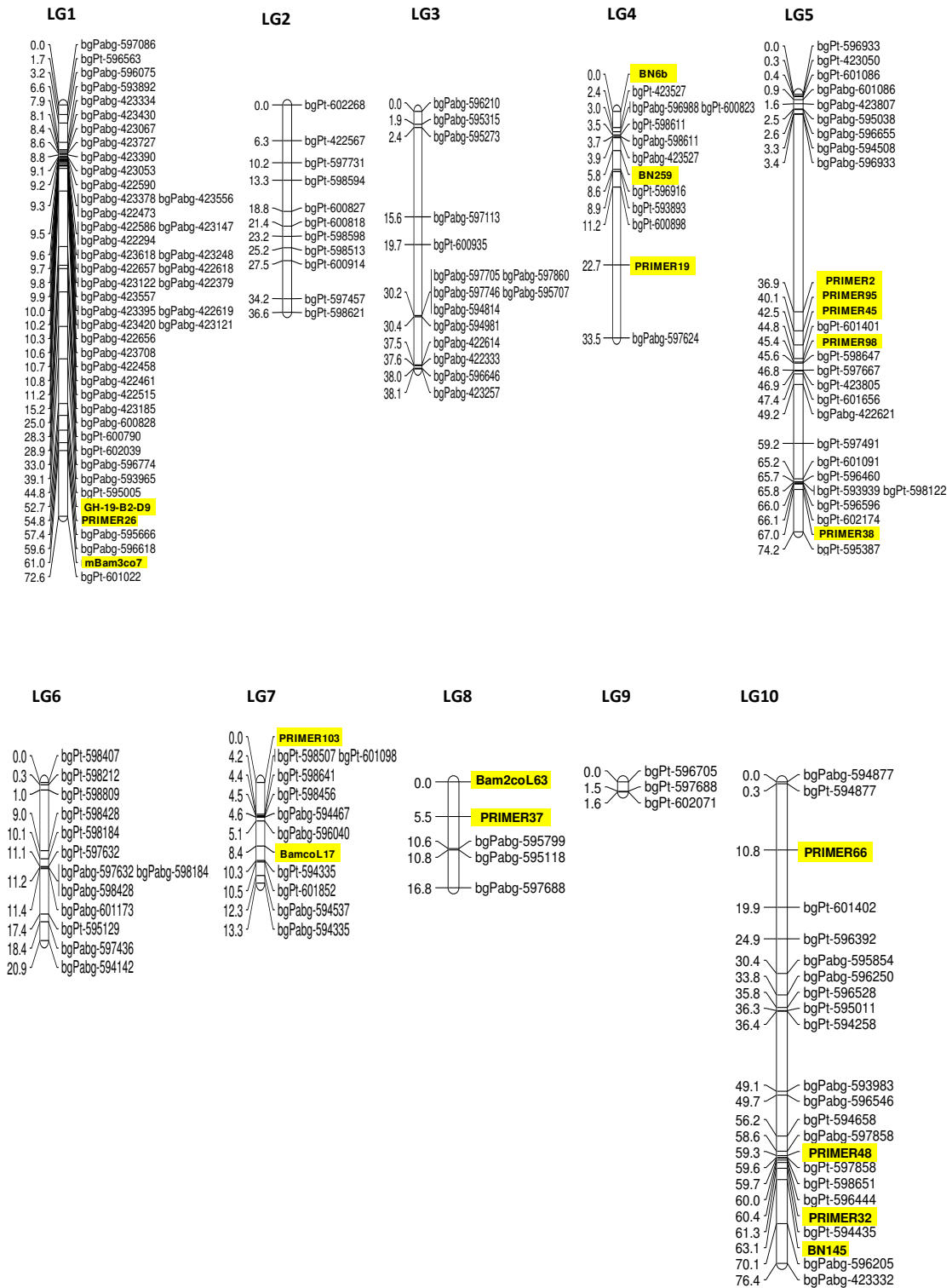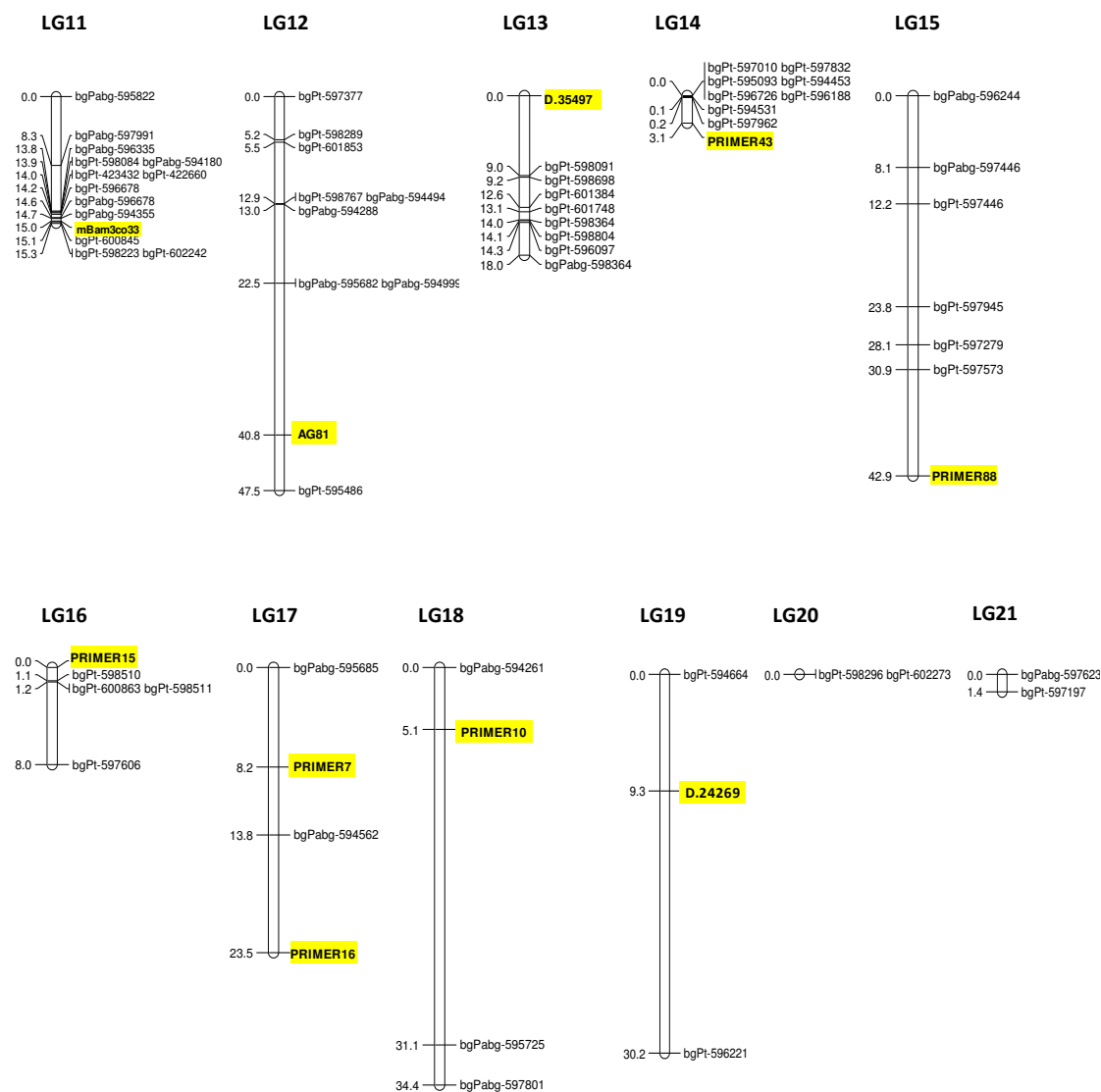
Figure 5-2: A genetic linkage map of 21 linkage groups. This was constructed in 73 F$_3$ individuals derived from the cross between individuals of the DipC and Tiga necaru landraces. The locations of 29 SSR and 209 DArT markers are given. Positions are given in centimorgan (Haldane units) to the right of the linkage groups and the name of markers to the left. Microsatellite markers were highlighted.

The final coverage of 608.6 cM was expected to equate to about 54 % of bambara groundnut genome, based on a default 100cM per chromosome and 11 chromosomes which the genome size (G) has been estimated from the partial linkage data according to the method of Hulbert et al. (1988):

$$G = \frac{n(n-1)}{2} \cdot \frac{2x}{yx}$$

Where n is the number of mapped markers, yx is the number of two-point linkages at a distance equal to x cM. This estimation applies directly when the markers are all informative in the same number of meioses in inbreeding species. Table 5-3 presents a summary of these results, with all groups adopting Map1 unless otherwise indicated.

Table 5-3: Distribution of the markers, linkage group size and marker density in the genetic map constructed in a F_3 population of DipC x Tiga necaru cross.

Linkage groups	Length (cM)	No. Of markers mapped in the groups			Average marker interval (cM)
		Total marker	SSR marker	DArT marker	
1 (Map2)	72.6	46	3	43	1.58
2	36.6	11	0	11	3.33
3	38.1	15	0	15	2.54
4	33.5	13	3	10	2.58
5 (map2)	74.2	28	5	23	2.65
6 (map2)	20.9	13	0	13	1.61
7	13.3	12	2	10	1.11
8	16.8	5	2	3	3.36
9	1.6	3	0	3	0.52
10	76.4	23	4	19	3.32
11	15.3	14	1	13	1.10
12	47.5	10	1	9	4.75
13	18.0	9	1	8	2.01
14	3.1	9	1	8	0.35
15	42.9	7	1	6	6.12
16	8.0	5	1	4	1.60
17	23.5	4	2	2	5.87
18	34.4	4	1	3	8.60
19	30.2	3	1	2	10.07
20	0.0	2	0	2	0.00
21	1.4	2	0	2	0.71
Total	608.61	238	29	209	-
Range	0- 77.43	2-46	1-5	2-45	0-10.1

The wide cross map was constructed using three marker types; SSR, DArT and AFLP. A total of 301 markers were subjected to linkage analysis, from which 194 markers

were assigned to 20 linkage groups (all groups adopting Map1). The mean marker-marker distance was 5.9cM. LG1 included the largest number of DArT markers (43). The greatest distance (109 cM) was observed for LG15 with 15 markers (Figure 5-3).
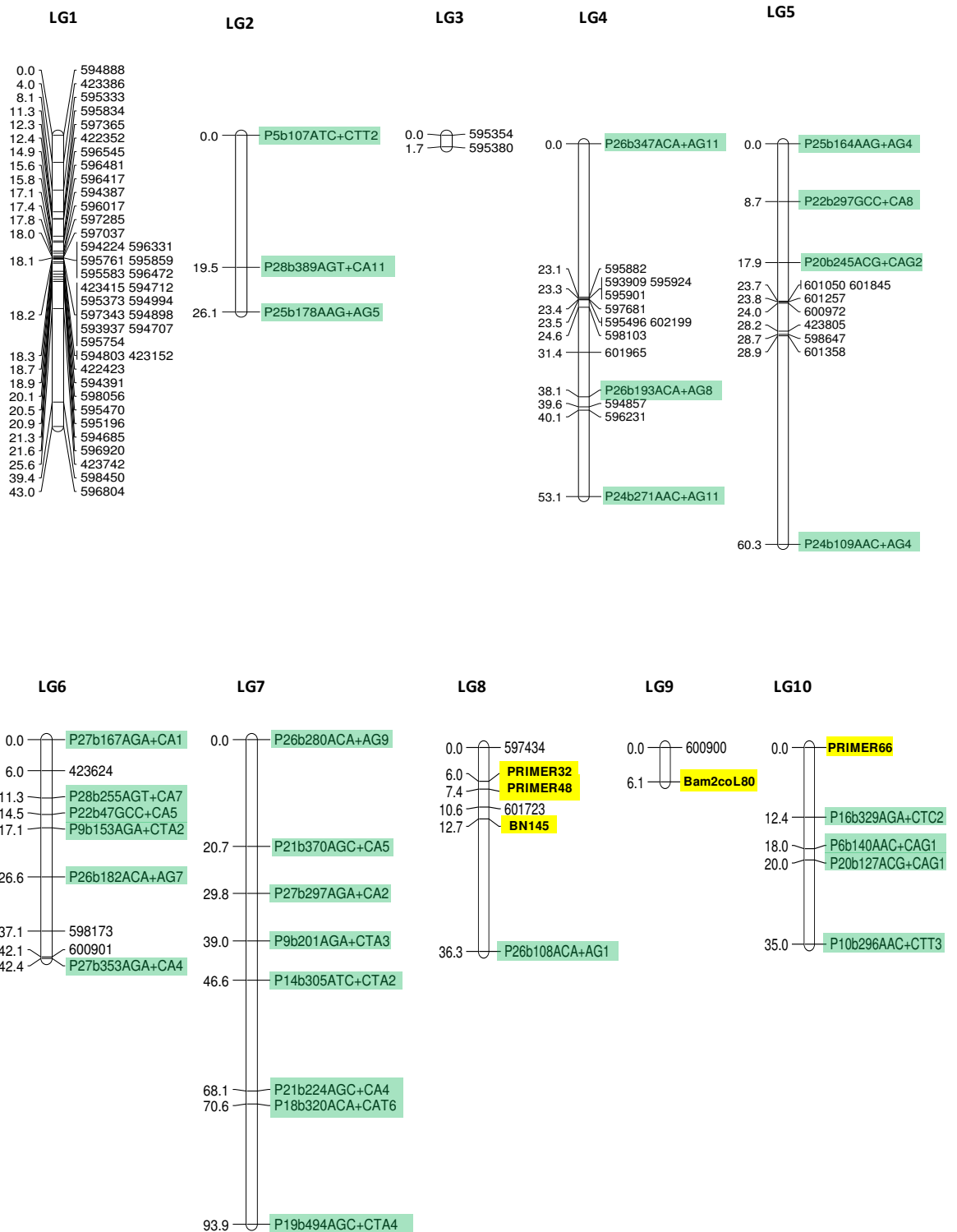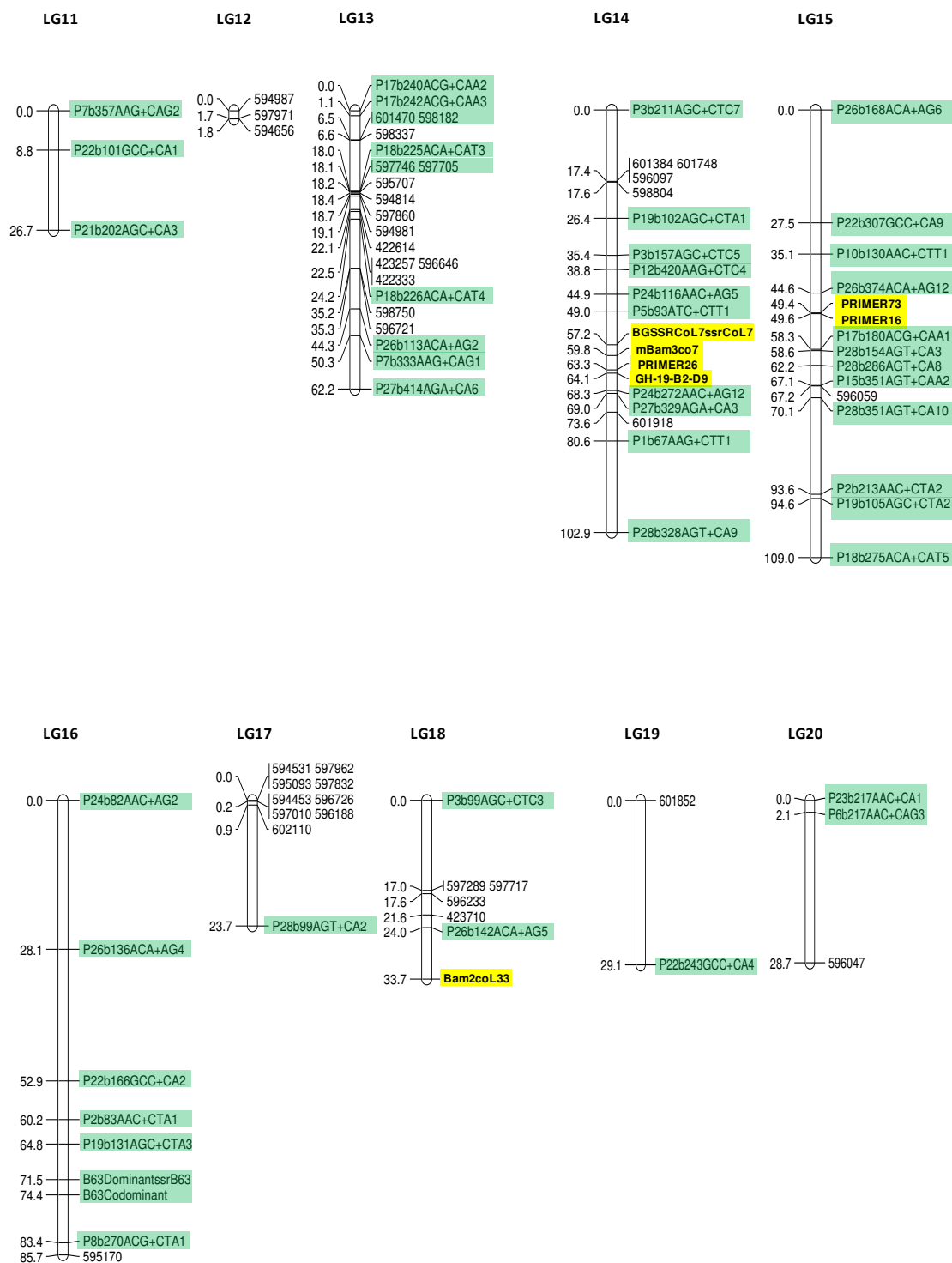
Figure 5-3: genetic linkage map of 20 linkage groups. This was constructed in 98 F₂ individuals derived from the cross between individuals of the DipC and VSSP11 landraces. The map represents the locations of 12 SSR, 110 DArT and 82 AFLP markers. Positions are given in centimorgan (Haldane units) to the right of the linkage groups and the name of markers to the left. Microsatellite and AFLP markers were highlighted on the linkage groups with yellow and green color, respectively.

The linkage map of the F_2 population derived from the wide cross spans a total of 901 cM, giving genome coverage of 79.6%, compared with the 67% of marker-marker linkage compared to the narrow cross map (Table 5-4).

Table 5-4: Distribution of the markers, linkage group size and marker density in the genetic map constructed in an F_2 population of DipC x VSSP11

Linkage groups	Length (cM)	No. of markers mapped in the groups				Average marker interval (cM)
		Total marker	SSR	DArT	AFLP	
1	43.0	41	-	41	-	1.05
2	26.1	3	-	-	3	8.69
3	1.7	2	-	2	-	0.83
4	53.1	14	-	11	3	3.79
5	60.3	11	-	7	4	5.48
6	42.4	9	-	3	6	4.71
7	93.9	8	-	-	8	11.74
8	36.3	6	3	2	1	6.05
9	6.1	2	1	1	0	3.05
10	35.0	5	1	-	4	7.00
11	26.7	3	-	-	3	8.91
12	1.8	3	-	3	-	0.58
13	62.2	22	-	15	7	2.83
14	102.9	19	4	4	11	5.41
15	109.0	15	2	1	12	7.27
16	85.7	9	-	1	8	9.52
17	23.7	10	-	9	1	2.37
18	33.7	7	1	4	2	4.81
19	29.1	2	-	1	1	14.55
20	28.7	3	-	1	2	9.57
Total	901.2	194	12	106	76	-
Range	1.7-105.7	2-45	1-4	1-45	1-12	0.83-14.55

5.3.7 Combining 'Narrow' and 'Wide' cross maps

JoinMap4 was used to combine linkage groups from both maps. ''Combine Maps'' command was used to align genetic maps obtained in different populations for a visual inspection of the marker order. The 'narrow' and 'wide' crosses carried a total of 8 common SSR and 24 DArT markers (Table 5-5). These were used to combine linkage groups from both crosses, where two or more common markers existed. The JoinMap4 function 'combine groups for map integration' was applied for the pairwise data, followed by regression mapping under default conditions. Two sets of linkage groups could only be linked through the existence of one common marker each and they were also shown in Figure 5-4. While it was possible to confirm common groups through one common marker on the same chromosome, their relative orientations were not determined.

Table 5-5: Number and the type of common markers in both 'narrow' and 'wide' cross population used to combine linkage groups

Linkage group	Linkage groups		Common marker	
	'narrow' cross map	'wide' cross map	number of markers	type of markers
combined group-1	1	14	3	SSR
combined group-2	10	8	3	SSR
combined group-3	3	13	10	DArT
combined group-4	5	5	2	DArT
combined group-5	14	17	8	DArT
combined group-6	13	14	4	DArT
non grouped a	17	15	1	SSR
non grouped b	10	10	1	SSR
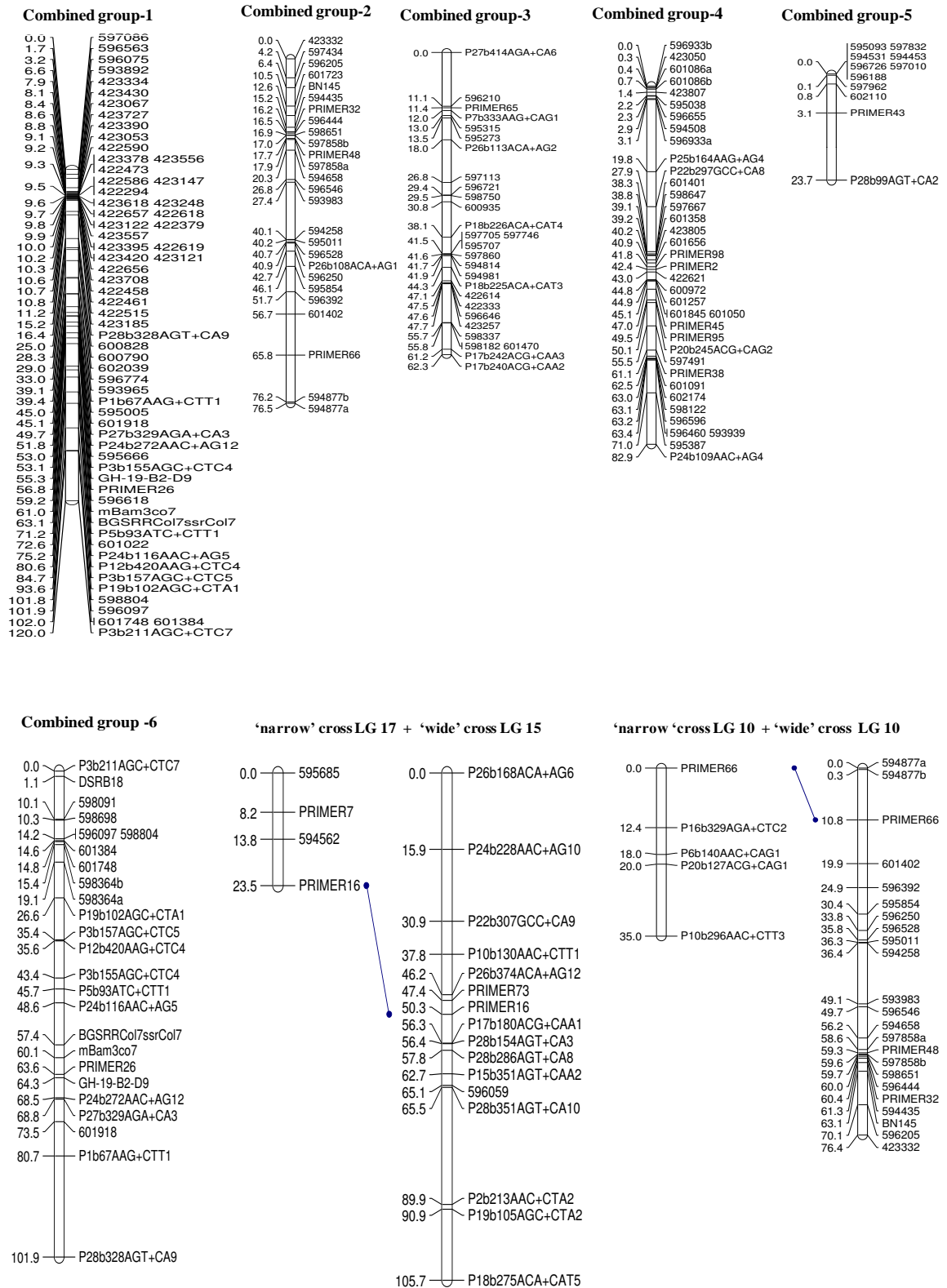
Figure 5-4: Combined linkage groups from the 'narrow' and 'wide' maps where two or more common markers exist; and the 'combined' linkage groups where a single common marker exists and relative orientation cannot be determined.

5.4 Discussion

5.4.1 Mapping populations

The same basic approaches were used to construct the first genetic linkage map of bambara groundnut, based on a population derived from a cross of individuals of a wild accession and of a cultivated landrace (Basu, 2005; Mayes et al., 2009) and an F_3 population derived from the cross between two individuals of domesticated landraces (DipC and Tiga necaru). Mapping in this cross aimed to further our understanding of the genetic basis of agronomically important traits in bambara groundnut. The genetic analysis of the previous cross had identified a number of genes important for the domestication process for bambara groundnut (Basu et al., 2007c). Construction of a comprehensive genetic linkage map relies upon polymorphism between the progenitors of the mapping population to study segregation patterns in the offspring and the existence of sufficient molecular markers. Although, a relatively low level of genetic polymorphism in the specific gene pool of bambara groundnut had been reported previously (Basu et al., 2007a), a polymorphism rate of 36.3% and 33.1% for SSRs was identified in narrow and wide cross populations of bambara groundnut, respectively. This level of polymorphism is higher than reports in a number of other legume crops. A polymorphism level of 16.1% was identified for the parental alleles using 945 SSR markers in 186 F_2 plants derived from a cross between an annual cultivated mungbean line 'KUML29-1-3' (Vigna radiata var. radiata) and an Australian wild perennial mungbean accession 'W021' (Vigna radiata var. sublobata) (Kajonphol et al., 2012). The polymorphism level remains reasonable even for transferred SSR markers from non-target legume species, for instance 109 transferable SSR markers from soybean were amplified successfully in peanut and

they detected 28% polymorphism among 4 cultivated peanut (Arachis hypogaea L)

lines (He et al., 2006).

5.4.2 Population size

Population sizes used in constructing genetic maps generally ranges from 50 to 250

individuals, however larger populations are required to determine the marker order for

high-resolution mapping (Mohan et al., 1997). The population size in most cases is

technically limited mainly by the amount of seed available from a single F1 cross.

This is clearly a species dependent issue. Population size is one of the factors that

affect the power of a QTL experiment to be able to identify the loci that underlie

phenotypic traits (Erickson et al., 2004). It has been demonstrated that the confidence

level generated for a QTL based on 100 individuals in genetic map drops from 90% to

60% when the population size is decreased to 50 individuals, affecting the power of

detecting the linkage as well as the estimate and accuracy of the recombination

frequency (Liu, 1998).

In order to construct the genetic map in the present study 73 F_3 progeny were derived

from a controlled cross between two single genotype accessions both with a

domesticated background. A population size of 75 recombinant inbred lines was used

to construct a linkage map in common bean (Freyre et al., 1998). Construction of the

wide cross genetic linkage map combining SSR, DArT and AFLP markers was based

on 98 F_2 progeny derived from a controlled cross between the domesticated landrace

(DipC) and the wild accession (VSSP11).

5.4.3 Phase issues with the dominant markers

Lack of available parental samples (DipC and Tiga necaru) for the segregating F_3 population at the time of the generation of the DArT array data resulted in a lack of phase information for these dominant markers. By the time the SSR data was generated, the parental genotypes had been recovered and confirmed by genetic fingerprinting. The linkage phases were determined prior to the final mapping. The genotype data was run in JoinMap4 as a cross pollinator (unknown linkage phase), after which the right phase could be determined for the initially grouped loci. Determining the correct linkage phase led to 30 markers which had weak association not being linked into the groups. Only markers showing linkage to other markers were used to develop the RIL3 .loc file. This decreased the number of markers to 239 for linkage analysis using the RIL3 model.

Linkage phase problems associated with some DArT and AFLP markers for the segregating F_2 population initially prevented map calculations in certain groups (Basu, 2005). Reversing the genotype codes for the population from (a, c) to (b, d) or vice versa, has solved the problem of suspect linkage for those markers and parental genotype data is also available for most markers, but not AFLPs.

5.4.4 Segregation distortion

Marker segregation distortion is commonly encountered in actual genetic mapping populations, leading to a skew in the frequency of genotypes from the expected Mendelian ratio (Lu et al., 2002) due to differential inheritance of alleles. The genotype frequencies for each locus were calculated by the JoinMap4 software to study segregation distortion. The segregation pattern was tested against normal

Mendelian expectation ratios through normal classification of genotypes using chi-square at p<0.05 based on a RIL3 model for the 'narrow' map and F_2 model for the 'wide' map.

Markers (SSR, DArT and AFLP) revealed high proportions of distorted loci (32% and 27%) from expected Mendelian ratios (P<0.05) in both narrow and wide cross maps, respectively. The distorted markers were found to be distributed across the linkage groups of both maps, rather than clustered on specific groups.

Skewed segregation ratios of the markers were reported to be highly variable in other studies. An investigation has reported 40.6% marker segregation distortion in an F_2 intra-specific population of Medicago tornata (Jenczewski et al., 1997). One of the highest frequencies of marker distortion of 73% was reported in an inter-specific recombinant inbred of tomato (Paran et al., 1995). In mapping and QTL analysis soybean (Glycine max) distortion of 19.93% of SSR markers on 14 linkage groups out of 20 was reported. The markers were distorted from the ratio of 1:1 in 106 RILs (F_9) derived from the cross of two soybean genotypes (BD2 and BX10) (Liang et al., 2010). Marker deviation from the expected ratio was thought to be a result of small population sizes, genotyping score errors, or the consequence of missing data (Millan et al., 2010). Other factors apart from the mapping population could affect the segregation of markers. (Zhang et al. (2006) reported that segregation distortion could be partially caused by gametophytic and sterility factors. Additionally, abortion of the parental gametes or zygotes, non-homologous recombination, transposable element and environmental agents would be counted among the factors involved (Knox and Ellis, 2002; Yamagishi et al., 2010). Among the 65 polymorphic SSR loci used in constructing a genetic map in barley , 22 loci (33.8%) showed genetic distortion (P<0.05), using 260 F_2 individuals derived from a male sterile line and elite cultivar.

It has been identified that all of the distorted markers were deviated toward male sterile parent (Liu et al., 2011).

5.4.5 Map construction and marker distribution

The genetic mapping of SSR and DArT markers was conducted in a population derived from the narrow cross. Twenty one linkage groups were identified, with a total 608.6 cM length to cover approximately 54% of bambara groundnut genome (based on 11 Morgans for 11 chromosomes as a crude assumption), although the high marker-marker linkage (238 out of 269 tested in the CP population) at 89% suggests more comprehensive coverage. Parental dissimilarity could be suppressing recombination, or potentially the developed markers could be clustered to particular regions of the bambara groundnut genome. The wide cross genetic linkage map constructed using an interspecific F_2 progeny had a total length of 901 cM with greater genome coverage (79.6%) compared to the narrow cross linkage map, however it had far lower marker-marker linkage of 67% (194 markers out of 301) resulted from 12 SSRs, 106 DArTs and 76 AFLPs. A possible explanation would be that the marker data for wide cross is less accurate than the data for the narrow cross, especially for AFLP markers (represented 141 out of 194 markers) which are largely responsible for the wider genome coverage of the map derived from the cross between DipC and VSSP11. The combination of three marker types in the wide cross map might also have a positive effect on coverage. It has been reported that different marker systems differ in the mechanism of detecting polymorphism, their genome coverage or location and the use of multiple marker types could be complemently and increase coverage overall (Adawy et al., 2005).

In the narrow cross map both kind of markers were evenly distributed across the linkage map, although the largest number of 43 and 41 DArT markers were located in linkage group 1 for both 'wide' and 'narrow' maps. The observed cluster of DArT markers is consistent with the earlier investigation of two major distinct DArT genotype groups (Stadler, 2009), including DipC in the smaller group containing the unusual repeat cluster As DipC was the maternal parent of both crosses here, this clustering effect is perhaps not unexpected. The persistency of DArT clustering seems to be reflected also in the wide cross map (LG1). Clustering of DArT markers in a particular genome region has been reported in chick pea, barley and wheat (Akbari et al., 2006; Semagn et al., 2006c; Thudi et al., 2011).

In the narrow cross, the markers in LG11 were only combined into a single map after three rounds of mapping. Because this included markers with negative distances, Map1, from the first round was used (and no further markers were added to Map2). While the second round (Map2) was used for the linkage groups 1, 5 and 6 as they allowed to map the optimum number of loci into the genetic map with no exceed of default jump (threshold value < 5) or negative map distances.

Three rounds were used to build the maps of 7 linkage groups for the wide cross map. A large jump in the goodness-of-fit of the second to third round indicated the poor fit of these additional loci. It was decided to remove 10 markers and adopt the map from round 1 (Map1) for the groups 1, 6, 7, 13, 14, 15, and 16.

Overall, the loss of 10 markers due to exceeding the default jump (5) or being associated with negative distances suggests that the map generated is relatively free from noisy data.

To our knowledge this is the first linkage map based on a combination of SSR and DArT markers in bambara groundnut. This map along with the improved wide cross map using a combination of SSR, DArT and AFLP markers, represents an important step toward genetic analysis of interesting agronomical traits in bambara groundnut.

The number of linkage groups in both narrow and wide cross map exceeded the expected number of 11 linkage groups for a comprehensive map in bambara groundnut (2n=2x=22). Since some of these groups had only a few markers, it can be concluded that the apparent excess of linkage groups might be due to incomplete coverage of the genome with the marker loci. We predict that the smaller groups will be brought together by adding more markers into the map and data has been generated from a sequence-based DArT approach which should allow this to be done. Unfortunately, time constraints prevented the use of this data in the current analysis

The SSR and DArT makers developed will be useful for comparative genomic analysis between the mapping populations of bambara groundnut and between this crop and other relative genomes. They are a pre-requisite for the application of more efficient breeding technologies such as marker-assistant selection and will give a better understanding of agronomical complex traits and phylogenetic analysis in bambara groundnut.

8.4.6 Attempt to combine the linkage maps

The 'narrow' and 'wide' linkage groups in bambara groundnut were combined where possible through a number of SSR and DArT markers. Combining linkage groups with at least two common markers was possible for 6 groups, however due to the lack

of a physical map for bambara groundnut the orientation of two other groups with single linkages was not.

The combined map will assist breeders to accurately select tightly-linked markers for agronomically important genomic regions for marker-assisted selection, will allow the positions of QTL for similar traits derived from the two crosses to be compared (where linkage exists) and will facilitate comparative mapping with other related legumes in the future (Millan et al., 2010).

Chapter 6: QUANTITATIVE TRAIT LOCI (QTL) MAPPING OF AGRONOMIC TRAITS

6.1 Introduction

QTL analysis is the study of genetic variation resulting from many genes with small effects to attempt to locate the genes responsible in complex traits and explore their effects and interactions (Kearsey, 1998). In order to identify molecular makers associated with QTLs, members of a random segregating population have to be scored first for a quantitative trait. The molecular genotype of each member of the population is then determined and a thorough search will be made for associations that might exist between the markers and the quantitative trait.

Since there has only been one attempt to date to identify molecular markers responsible for agronomic traits in bambara groundnut (Basu, 2005) a review of the QTL mapping for some agronomically important traits in related legume crops might enhance the understanding of these traits in bambara groundnut.

QTL mapping of agronomic traits in legume crops

In soybean (Glycine max L. Merr.) QTL mapping was conducted for agronomic traits across two years in a study by Wenxin et al. (2008) . A total of 136 SSR markers segregated in the population of 126 RIL (F_5) derived from a cross between two lines of late maturity (PI 171451) and early maturity (Hwaeomputkong). The markers were distributed over 20 linkage groups (LGs), covering 1073.9 cM of the soybean genome. A total of 15 QTLs were detected with LOD scores >3 for six major agronomic traits. QTLs of two traits (days to flowering and days to maturity) were

located at the same position in two linkage groups and reflected significant correlations among corresponding traits based on field data.

A SNP-based genetic linkage map was generated by Bobby et al. (2012) in soybean (Glycine max L. Merr.), using a RIL population derived from the cross between cultivars PI 438489B (resistant to sudden death syndrome) and Hamilton (high yield); n=50. A total of 18 QTLs were detected for days to germination, days to flowering, plant height, pod number, seed number, 100-seed weight, and total seed weight on 10 different chromosomes. The clusters of QTL for these traits were identified containing QTL controlling other important traits such as sudden death syndrome resistance and soybean cyst nematode resistance. The author emphasised the importance of these QTLs and identified that they may be introduced into breeding programs to develop soybean cultivars with high yield potential which have good resistance to these diseases.

In the model legume Lotus japonicus the first QTL analysis was performed by Gondo et al. (2007) for agronomic traits. A RIL population derived from the cross between the accessions Miyakojima MG-20 and Gifu B-129 was used in 2 year trial. The traits of yield and its components were studied. Some QTLs detected were co-located, especially those for pod length, pod width, seeds/pod, and seed mass. Seed mass QTLs were located at 5 locations that mapped to the corresponding genomic positions of equivalent QTLs in soybean, pea, chickpea, and mungbean. It was concluded that this study could provide key information on the traits studied for marker-assisted breeding of the important legume crops (Gondo et al., 2007).

Kajonphol et al., (2012) conducted a QTL analysis for mungbean (Vigna radiata L.) (2n=2x=22) to identify chromosome regions controlling agronomic traits, using an F_2 mapping population derived from an inter-specific cross between the wild and

cultivated mungbean. Twenty QTLs were identified controlling major agronomic characters for days to first flower, days to first pod maturity, days to harvest, 100 seed weight, number of seed per pod and pod length. Most of these QTLs were located on only two linkage groups. Extensive genome conservation between the mungbean map and maps for azuki bean (Vigna angularis) and blackgram (Vigna mungo) reveal the potential for cross-species genetic markers to detect marker-trait association among Vigna species.

In garden pea (Pisum sativum L.) Irzykowska and Wolko (2004) conducted interval mapping of QTLs for the agronomic traits of seed number, pod number, 1000-seed weight, seed yield, and seed protein content. Traits were measured in F_2 (114 plants) and in F_4 (104 RILs) plant populations derived from a cross of a large-seeded line (Wt10245) with a small-seeded line (Wt11238). The map consisted of a combination of 204 markers of different types (140 AFLPs, 24 RAPDs, 10 ISSRs, 5 CAPSs, 1 STS, 11 isozymes and 13 morphological markers). Thirty seven QTLs were detected across both populations on the seven pea chromosomes. A number of these QTLs for different traits were localized to the same map interval, possibly indicating pleiotropy or the actions of a common gene effecting an underlying trait. The authors postulate that these QTLs have an important role in controlling the yield and seed protein content in garden pea.

The aim of the current study was to carry out QTL analysis of bambara groundnut to identify regions of the genome that are contributing to variation in traits of agronomic importance. Detection, localization and estimating the effects of potential QTL could clarify the genetic mechanisms underlying agronomic traits to facilitate MAS in future breeding programs of bambara groundnut (Vigna subterranea (L.) Verdc.; 2n=2x=22). QTL analysis was performed for agronomic traits that were either quantitatively distributed or did not follow Mendelian segregation in the F_2 and F_3 progenies of the crosses. Where links between the 'wide' and 'narrow' maps could be identified, the potential presence of the same QTL in both maps was examined.

6.2 Result

QTL analysis was conducted for the two constructed linkage maps of 'narrow' and 'wide' crosses in bambara groundnut. The first analysis of the 'narrow' cross was carried out for agronomically important traits in an F_3 population derived from the cross between individual genotypes of the DipC and Tiga necaru landraces. The trait data came from the controlled environment tropical glasshouses based at Sutton Bonington (F_3; FutureCrop) and field experiments (F_3; Indonesia). Trait data for the segregating F_2 population from the same cross was also used to support the F_3 QTL analysis and was derived from single plants in a controlled environment glasshouse.

In the second analysis ('wide' cross), the QTL analysis was conducted for an updated map derived from the controlled cross between individuals of the DipC and VSSP11 landraces. Previous data from the segregating F_2 population of this cross was used in marker-trait analysis and was derived from controlled environment glasshouse measurements on single plants.

6.2.1 QTL analysis in 'narrow' cross map

6.2.1.1 Trait distribution

As a prerequisite for QTL analysis the distribution patterns of data were tested for the entire range of agronomic traits. Data came from the segregating F_3 population evaluated in FutureCrop glasshouse and Indonesian field and the F_2 population of the same cross evaluated in Tropical Crops Research Unit. The traits distribution was tested for different environments and generations using Anderson darling normality

tests. The description of the data distribution and the correlation between trait values was presented in Chapter 4. Non-normally distributed data were subjected to Box-Cox transformations prior to the QTL analysis. Transformation was used to improve the normality of traits for both F_2 and F_3 populations as shown in Table 6-1.

The initial tests suggested a non-normal distribution in these traits. Data from only two traits - growth habit and eye pattern around hilum - followed known inheritance patterns of Mendelian incomplete and complete dominance in the glasshouse, respectively. Traits that showed continuous distribution (in Chapter 4) and those transformed (Table 6-1) which tested as normally distributed after transformation were subjected to quantitative trait loci analysis using interval mapping. Non normal distributions were observed for days to emergence in both trials of F_3 progenies (glasshouse and field) and in the F_2 population. Growth habit (in glasshouse and field) and eye pattern around hilum (recorded in glasshouse only) were also tested to be non-normally distributed, despite attempts to transform the data to achieve normality. Detecting markers linked to QTLs for non-normally distributed data was performed only with Kruskal-Wallis analysis.

6.2.1.2 Marker and trait associations

Maximum LOD scores were determined by interval mapping of traits, with the most likely location for the QTL given at maximum LOD. LOD peaks ≥ 2 are presented as a potential/indicative QTL in the current investigation. Significant LOD thresholds for QTL determination were estimated for each trait after 10000 permutation tests. These ranged from LOD = 2.2 to LOD = 3.1 for different traits studied in the segregating F_2 and F_3 populations. A LOD score $\geq$ the significant threshold value was used to declare a QTL as significant.

Table 6-1: Statistical analysis of traits showing non-normal distributions, before and after transformation, for F$_2$ and F$_3$ progenies data derived from the narrow cross between DipC and Tiga necaru evaluated under different environments.

Trait	Data type	Min. value	Max. value	Median	Mean value	Variance	Skewness	Kurtosis	P value
				F$_3$ population evaluated in glasshouse					
Leaf no./plant	Non normal	29.50	148.25	60.75	62.49	325.58	2.07	7.89	0.00
	Transformed	5.43	12.18	7.79	7.83	1.12	1.22	4.16	0.01
Leaf area	Non normal	1402.34	9562.37	3200.75	3461.23	1925713.87	1.80	5.55	0.00
	Transformed	37.45	97.79	56.58	57.83	119.29	0.94	2.21	0.05
Double seeded pods/plant	Non normal	0.00	19.00	4.00	5.42	16.94	1.26	1.57	0.00
	Transformed	3.41	3.90	3.65	3.65	0.01	0.05	-0.50	0.64
Seed weight	Non normal	7.79	58.42	27.67	28.93	144.90	0.72	-0.11	0.00
	Transformed	2.79	7.64	5.26	5.27	1.22	0.29	-0.42	0.19
Biomass dry weight	Non normal	27.82	126.53	54.86	61.34	524.26	0.81	-0.02	0.00
	Transformed	5.27	11.25	7.41	7.70	2.01	0.49	-0.52	0.06
				F$_3$ population evaluated in the field					
Pod no./plant	Non normal	1.00	23.40	6.83	7.70	21.48	0.89	0.81	0.01
	Transformed	1.00	4.84	2.61	2.65	0.70	0.22	-0.51	0.25
Pod length/ plant	Non normal	8.00	20.00	13.51	13.61	3.14	0.66	3.75	0.01
	Transformed	2.83	4.47	3.68	3.68	0.06	0.16	3.66	0.02
				F$_2$ population evaluated in TRRU					
Double seeded pods/plant	Non normal	0.00	34.00	6.00	8.32	71.02	1.16	0.36	0.00
	Transformed	0.00	5.83	2.24	2.38	2.45	0.22	-0.72	0.01
Seed no./plant	Non normal	19.00	355.00	104.00	115.37	6049.15	1.03	0.99	0.00
	Transformed	4.36	18.84	10.20	10.13	12.83	0.30	-0.52	0.24
Biomass dry weight	Non normal	11.00	306.30	99.99	99.17	4482.23	0.64	-0.05	0.00
	Transformed	3.32	17.50	10.00	9.33	12.34	0.02	-0.96	0.02

The QTL results for individual traits here are presented separately and the trait data evaluated in all three experiments of FutureCrop glasshouse, Indonesian field and in TCRU (when presented) are introduced together for each trait.

Days to emergence: Kruskal-Wallis analysis revealed three potential QTL for days to emergence associated with DArT and SSR markers (bgPabg-601086, PRIMER66 and mBam3co33) on different linkage groups of 5, 10 and 11, respectively, valued K* >6 at p = 0.05 in the glasshouse (Table 6-2). Based on the field dataset, the marker bgPabg-423556 was identified on LG1 to be in association with this trait (K* = 7.3 at p = 0.01), in addition to two other associations with markers bgPt-598091 and bgPabg-601086 on LGs 13 and 5, respectively (Table 6-3). Data recorded for the F_2 progeny in the TCRU indicated a strong association of days to emergence with SSR marker PRIMER16 (K* = 12.3 at p = 0.005) on LG17 (Table 6-4).

Flower no./plant: A putative QTL was found for glasshouse data on LG8 at the 2.0 cM position mapped by interval mapping at a LOD score of 2.4, explaining 16% of the phenotypic variation. Marker bam2coL63 at 0.0 cM position (K* = 9.9 at p = 0.01) was the nearest to this locus.

Terminal leaflet length (cm): A significant QTL was detected on LG8 at the 2.0 cM position mapped by interval mapping at a LOD score of 3.2 (Figure 6-1). This genomic region explained 20.3% of the phenotypic variation and it is close to the marker bam2coL63 at 0.0 cM position (K* = 11.2 at p = 0.005). This marker also recorded a LOD score of 2, showing a just-putative QTL for this trait in the F_2 progenies grown in TCRU. Marker bam2coL63 was also found to be linked to flower no./plant.

Table 6-2: QTL mapping in bambara groundnut using interval mapping and Kruskal-Wallis analysis for the F$_3$ generation data of the 'narrow' cross in the FutureCrop glasshouses.

Traits	Linkage group	Position (cM)	Locus	Interval mapping				Kruskal-Wallis analysis		
				LOD	PT[a]	PVE[b]	Additive	K*	Df	Signifi. level
Days to emergence (DE)	11	15.0	mBam3co33					6.9	2	**
	5	0.9	bgPabg-601086					6.6	1	**
	10	10.8	PRIMER66					6.5	2	**
	12	12.9	bgPabg-594494					6.3	1	**
Flower no./plant (FN)	8	2.0		2.4	2.9	15.8	-16.7			
	8	0.0	Bam2coL63	2.3		15.3	-15.6	9.9	2	***
Terminal leaflet length (TLL)	8	2.0		3.2	2.6	20.3	-0.4			
	8	0.0	Bam2coL63	3.1		19.7	-0.4	11.2	2	****
Terminal leaflet width (TLW)	3	19.7	bgPt-600935	3.2	2.6	20.4	0.2	13.1	1	******
Leaf area (LA)	3	16.6		2.3	2.8	15.5	1.8			
	3	15.6	bgPabg-597113	2.3		15.3	1.4	6.5	1	**
Plant spread (PS)	4	0.0	BN6b	3.9	2.7	24.6	3.7	16.5	2	******
Stem no./plant (STN)	4	14.2		2.4	2.6	16.1	-0.9			
	4	11.2	bgPt-600898	2.3		15.4	-0.8	4.8	1	**
Node no./stem (NN)	1	33.0	bgPabg-596774	3.3	2.6	21.1	-1.1	8.8	1	****
	4	11.2	bgPt-600898	2.7		17.9	1.1	9.7	1	****
Internode length (IL)	4	3.0	bgPabg-596988	7.9	2.6	43.5	0.7	18.9	1	*******
Growth habit (GH)	4	0.0	BN6b					23.4	2	*******
	10	70.1	bgPabg-596205					8.3	1	****
	18	5.1	PRIMER10					7.8	2	**
Pod no./plant (PN)	1	33.0	bgPabg-596774	2.3	2.7	15.4	-7.0	8.8	1	****
Double seeded pods/plant (DPN)	4	1.0		3.4	2.9	22.0	0.5			
	4	0.0	BN6b	3.3		21.7	0.5			
Peduncle length (PEL)	4	1.0		9.7	2.7	50.3	0.9			
	4	2.4	bgPt-423527	9.6		49.9	0.9	8.1	1	****
Pod weight (PWE)	1	33.0	bgPabg-596774	2.6	2.5	17.0	-6.8	6.4	1	**
Pod length (PLE)	12	15.1		4.6	2.7	28.4	0.8			
	12	12.9	bgPt-598767	4.6		28.0	0.8	9.6	1	****
Pod width (PWD)	12	20.1		5.7	2.4	33.4	0.6			
	12	22.5	bgPabg-595682	5.5		32.7	0.5	17.7	1	*******
Pod length of double seeded (DPL)	1	0.0	bgPabg-597086	3.8	2.7	24.5	-1.5	14.7	1	******
	12	10.5		3.3		21.7	1.6			
Pod width of double seeded (DPW)	12	17.1		4.0	2.8	25.7	0.5			
	12	12.9	bgPt-598767	3.7		24.0	0.5	9.1	1	****
Seed length (SEL)	10	49.1	bgPabg-593983	2.5	2.7	16.4	0.4	9.6	1	****
Seed width (SEW)	12	15.1		2.0	2.7	13.6	0.3			
	12	12.9	bgPt-598767	2.0		13.5	0.3	4.6	1	**
Seed no./plant (SEN)	1	34.0		2.3	2.5	15.1	-7.9			
	1	33.0	bgPabg-596774	2.3		15.0	-7.6	7.6	1	***
Seed weight (SWT)	1	33.0	bgPabg-596774	2.7	2.6	17.8	-0.5	6.0	1	**
Biomass dry weight (BDW)	1	33.0	bgPabg-596774	3.5	3.0	22.4	-11.6	10.0	1	****
Shelling% (SH%)	7	13.3	bgPabg-594335	3.0	2.9	19.4	3.4	13.6	1	******
100-seed weight (HSW)	7	9.4		2.7	2.5	17.4	4.3			
	7	10.5	bgPt-601852	2.6		17.3	4.3	11.6	1	*****
Eye pattern around hilum (EP)	12	22.5	bgPabg-594999					29.68	1	*******
	18	0.0	bgPabg-594261					9.304	1	****

a : permutation-10000 times test

b : percentage of total phenotypic variation explained by the QTL

Significant level of K * values: *: 0.10, **: 0.05, ***: 0.01, ****: 0.005, *****: 0.001, ******: 0.0005, *******: 0.0001

Table 6-3: QTL mapping in bambara groundnut using interval mapping and Kruskal-Wallis analysis for F₃ generation data of 'narrow' cross in the field.

Traits	Linkage group	Position (cM)	Locus	Interval mapping				Kruskal-Wallis analysis		
				LOD	PT[a]	PVE[b]	Additive	K*	Df	Signifi. level
Days to emergence (DE)	1	9.3	bgPabg-423556					7.3	1	***
	13	9.0	bgPt-598091					6.9	1	***
	5	0.9	bgPabg-601086					5.8	1	**
Node no./stem (NN)	3	30.2	bgPabg-595707	2.8	2.7	18.4	1.0	10.8	1	****
Internode length (IL)	4	3.0	bgPabg-596988	7.1	2.7	40.9	0.3	20.9	1	*******
Growth habit (GH)	4	3.0	bgPabg-596988					18.1	1	*******
	4	0.0	BN6b					17.6	2	******
	18	5.1	PRIMER10					9.7	2	***
	14	0.0	bgPt-597832					7.4	1	***
Pod no./plant (PN)	18	3.0		2.5	3.1	16.3	-0.4			
	18	5.1	PRIMER10	2.4		15.8	-0.4	9.8	2	***
pod length (PLE)	11	3.0		3.2	2.5	20.9	0.1			
	11	0.0	bgPabg-595822	3.0		19.9	0.1	14.8	1	******
Biomass dry weight (BDW)	1	28.9	bgPt-602039	2.9	2.9	17.6	-1.8	17.9	1	*******

a : permutation-10000 times test

b : percentage of total phenotypic variation explained by the QTL

Significant level of K * values: *: 0.10, **: 0.05, ***: 0.01, ****: 0.005, *****: 0.001, ******: 0.0005, *******: 0.0001

Table 6-4: QTL mapping in bambara groundnut using interval mapping and Kruskal-Wallis analysis for F₂ generation data of narrow cross in TCRU

Traits	Linkage group	Position (cM)	Locus	Interval mapping				Kruskal-Wallis analysis		
				LOD	PT[a]	PVE[b]	Additive	K*	Df	Signifi. level
Days to emergence (DE)	17	23.5	PRIMER16					12.3	2	****
	5	74.2	bgPt-595387					7.8	1	***
	2	6.3	bgPt-422567					6.6	1	**
	4	3.7	bgPabg-598611					6.4	1	**
Terminal leaflet length (TLL)	8	0.0	Bam2coL63	2.0	2.6	11.6	-0.6	8.6	2	**
Terminal leaflet width (TLW)	5	74.2	bgPt-595387	2.6	2.5	15.3	0.3	10.4	1	****
Plant spread (PS)	4	33.5	bgPabg-597624	3.2	2.7	18.0	5.5	14.8	1	******
Pod no./plant (PN)	1	68.0		2.0	2.7	11.8	-35.3			
	1	72.7	bgPt-601022	1.9		11.0	-31.5	8.0	1	****
Double seeded pods/plant (DPN)	4	33.5	bgPabg-597624	3.2	2.8	19.2	0.7	13.1	1	******
Seed no./plant (SEN)	1	72.6	bgPt-601022	2.5	2.8	14.4	-30.7	7.65	1	***
Biomass dry weight (BDW)	1	67.0		2.0	2.9	11.5	-27.8			
	1	59.6	bgPabg-596618	2.0		11.3	-26.1	10.5	1	****
Shelling% (SH%)	12	47.5	bgPt-595486	4.8	2.6	26.3	-4.0	15.3	1	*******
100-seed weight (HSW)	11	0	bgPabg-595822	2.1	2.5	12.2	4.6	10.6	1	****
	12	47.5	bgPt-595486	2.1	2.5	12.1	-4.6	9.8	1	****

a : permutation-10000 times test

b : percentage of total phenotypic variation explained by the QTL

Significant level of K * values: *: 0.10, **: 0.05, ***: 0.01, ****: 0.005, *****: 0.001, ******: 0.0005, *******: 0.0001

Terminal leaflet width (cm): A single significant QTL was detected with LOD score 3.2 at 19.7 cM on LG 3. The locus bgPt-600935 was linked to this position and it was supported by Kruskal–Wallis output analysis at the same position with a high K* value (K* = 13.1 at p = 0.0005). Based on the data from the TCRU a significant QTL for this trait was detected on LG5 and linked with marker bgPt-595387 at 74.2 cM, having a LOD value of 2.6. Marker-trait linkages for terminal leaflet width were also revealed using Kruskal-Wallis analysis.
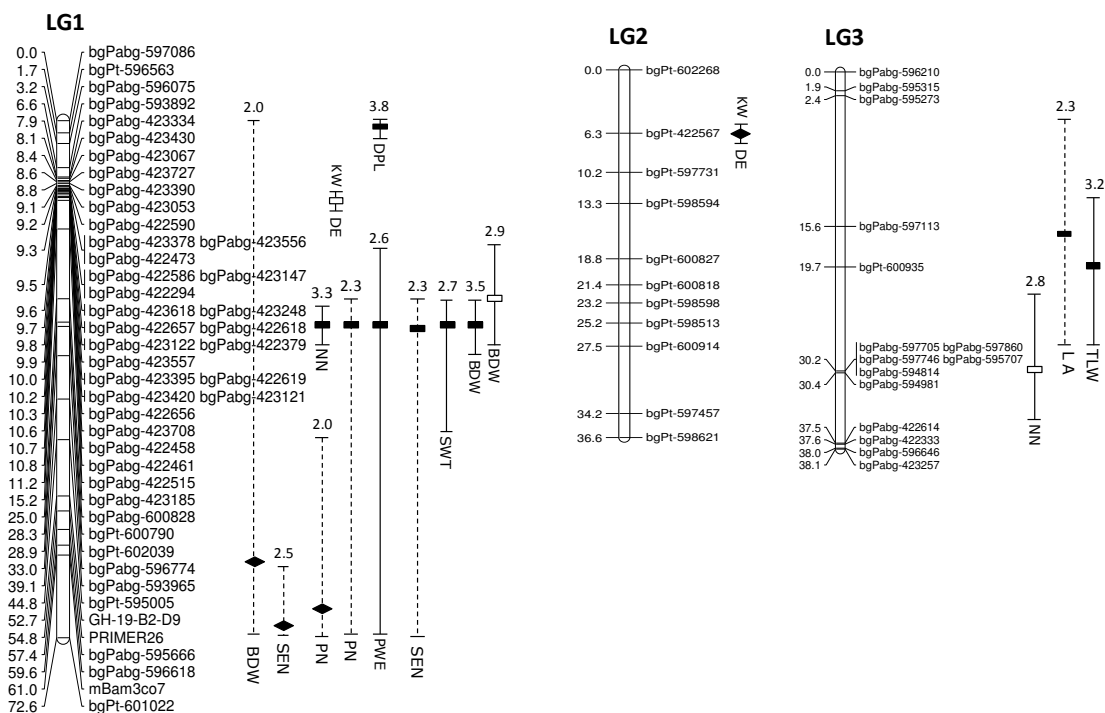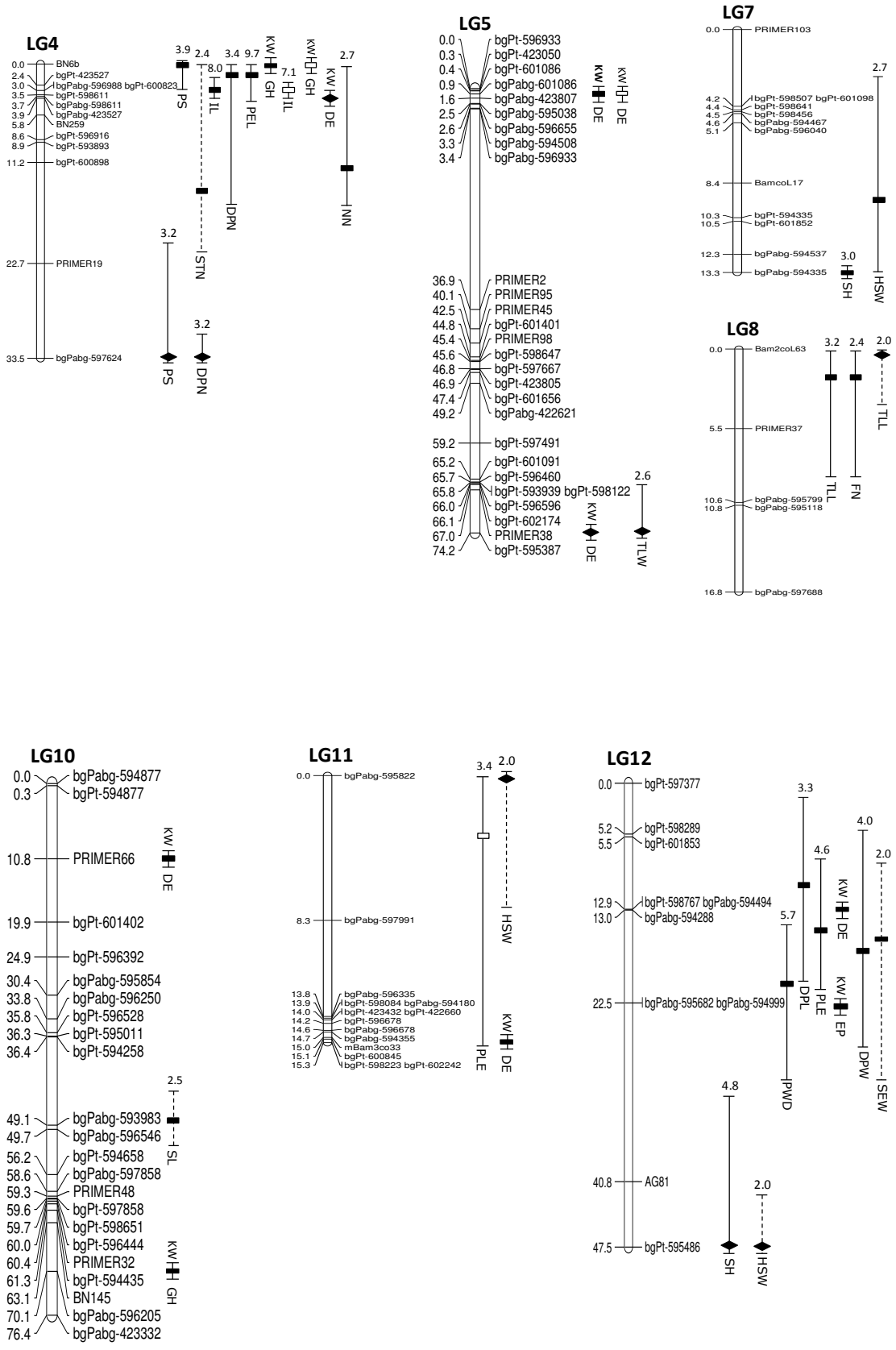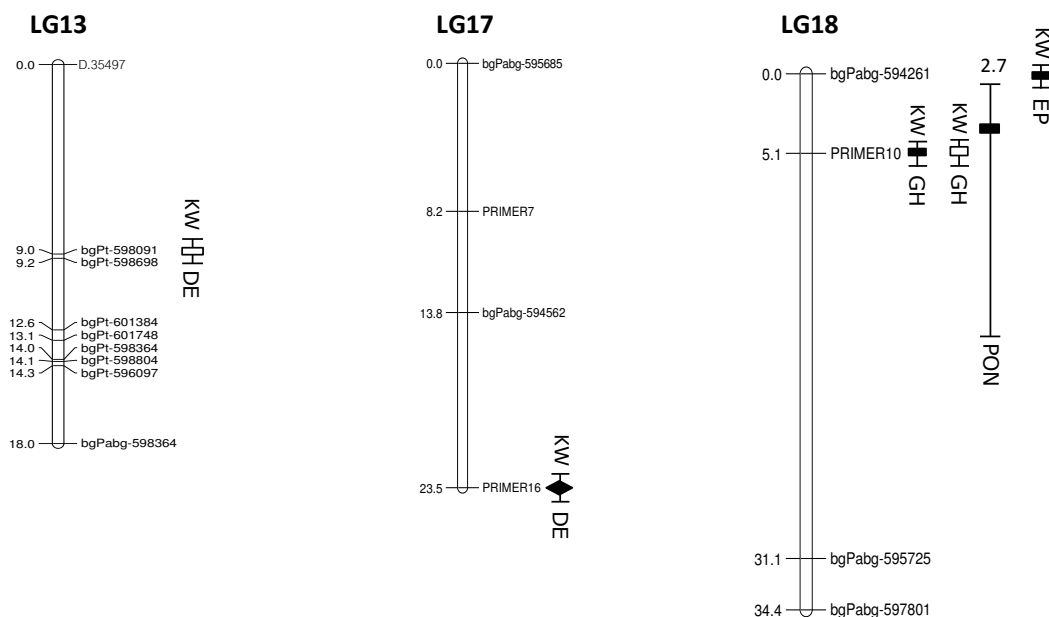
Figure 6-1: Map positions of the QTL on the 'narrow' cross (DipC x Tiga necaru) linkage map. Only linkage groups for which 'putative' or 'significant' QTL were found are shown. Positions are given in cM (Haldane units), to the right of the linkage groups. QTL terminology is described in the text. The position of the maximum LOD value of a particular QTL is written at the top of QTL pointer and indicated by a rectangle, in black for glasshouse experiment, white for field data and in black four pointed star for the F_2 progeny. QTL confidence intervals (1 LOD drop-off) are represented by plain lines with LOD score ≥ significant threshold value, determined by 10,000 permutation tests and by dotted lined for putative QTL below the significant threshold LOD value, but with LOD scores >2. QTLs detected with Kruskal-Wallis analysis are discontinuous with no confidence interval and the rectangular or four pointed star only represents the entire position of QTL.

Leaf area (cm^2): Based on glasshouse data a putative QTL for leaf area was identified on LG3 at 16.6 cM. This accounted for15.5% of the phenotypic variation of leaf area. The marker bgPabg-597113 at 15.6 cM is close to this QTL (K*= 6.5 at p =0.05).

Plant spread (cm): Interval mapping revealed a single QTL controlling plant spread on LG4 for the glasshouse data. This QTL was associated with the marker BN6b and had a LOD score of 3.9 and explained 24.6% of total phenotypic variation. A strong QTL-marker association was confirmed using non-parametric mapping (K* = 16.5 at

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p = 0.0005). In the F_2 progeny dataset the marker locus BN6b had a LOD score of 2.1 at the same position for plant spread, whereas the highest detected LOD value of 3.2 was at 33.5 cM, explaining 18% of the phenotypic variation and associated with marker bgPabg-597624 ($K^* = 14.8$ at p = 0.0005).

Stem no./plant: A putative QTL was mapped on the LG4 for stem no./plant, located at 14.2 cM for the glasshouse dataset. A peak LOD value of 2.4 was slightly lower than the genome-wide permutation test threshold (2.6; 10000 permutation). The closest marker to this genomic region was bgPt-600898 at 11.2 cM position ($K^* = 4.8$ at p = 0.05).

Node no./stem: Glasshouse data analysis detected two QTLs for this trait on LG1 and LG4. Both QTLs accounted for about 39% of total phenotypic variation. While F_3 progenies evaluated in the field suggested a major QTL on LG3 associated with node no./plant. This QTL was linked to marker bgPabg-595707 at a LOD value of 2.8 at 30.2 cM and accounted for 18.4% of the total phenotypic variance of node no./stem. The trait-marker association was also revealed through non-parametric mapping to be significant ($K^* = 10.8$ at p = 0.005).

Internode length (cm): Data analysis for the FutureCrop glasshouse and the field detected a major QTL for internode length mapped on LG4, with the highest LOD values of 7.9 and 7.1, respectively. This significant QTL was located at 3.0 cM and was associated with marker bgPabg-596988 and accounted for the greatest effects observed of 43.5% and 41% of total phenotypic variations in both datasets, respectively. KW analysis also identified the strongest association seen with internode length at this locus (at p = 0.0001).

Growth habit: Non-parametric mapping of the F_3 generation in FutureCrop glasshouses and the field showed a very strong association between this trait and marker BN6b on LG4. The association between this SSR marker and two other traits of plant spread and double seeded pods/plant was identified in the interval mapping analysis. Two other markers were linked to growth habit on LG10 and LG18 at different level of significant p value, whereas the field evaluation revealed that marker bgPabg-596988 was identified as the most strongly associated with this trait. This marker was potentially linked to internode length, being a major QTL in both glasshouse and the Indonesian field. Two other markers associated with growth habit were identified on LG18 (as detected also in the glasshouse) and LG14.

Pod no./plant: Two putative QTLs for pod no./plant were detected on LG1 at different locations for F_3 and F_2 progeny data analysed in FutureCrop glasshouse and TCRU, respectively. Interval mapping for the field dataset indicated another putative QTL for this trait on LG18 at 3.0 cM with a LOD value of 2.5. This locus explained 15.8% of phenotypic variation and was close to an SSR marker (primer10) ($K^* = 9.8$ at $p = 0.01$). The locus of primer10 was also associated with growth habit in nonparametric mapping of both glasshouse and field data.

Double seeded pods/plant: A QTL was detected for double seeded pods at 1.0 cM on the LG4, with a LOD score of 3.4 for the glasshouse F_3 dataset. This locus explains 22% of phenotypic variation. Marker BN6b was the nearest to this QTL and has been also associated with plant spread. Analysing F_2 progeny data detected a QTL for double seeded pods/plant on the same LG at a different location with a LOD score of 3.2 and an additive mode of action.

Peduncle length (cm): A major QTL was detected for peduncle length analysing the data of the F_3 progeny recorded in the glasshouse. It was located on LG4 with a maximum LOD score of 9.7 at 1.0 cM. This QTL explained 50.3% of the phenotypic variation in the glasshouse dataset. The marker bgPt-423527 at 2.4 cM was the nearest to this QTL ($K^* = 8.1$ at $p = 0.005$).

Pod weight (g/plant): Interval mapping located a single QTL for pod weight in the F_3 population grown in the FutureCrop glasshouse with LOD 2.6 at 33.0 cM on LG1. This genomic region explained 17% of the phenotypic variation and was associated with marker bgPabg-596774. This marker was also detected as associated with a putative QTL for pod no./plant.

Pod and seed dimensions: Analyzing the segregating F_3 population grown in the glasshouse detected four significant QTLs for pod length, pod width, pod length with double seeded and pod width with double seeded positioned close to each other. Marker bgPt-598767 was identified to be the nearest to the maximum LOD score of pod length, pod width of double seeded and seed width at 12.9 cM, whereas two other QTLs for pod length with double seeded and seed length were also mapped on LG1 and LG10, respectively. Based on the field dataset, another significant QTL for pod length was detected on LG11. The phenotypic variance explain by these QTL ranged from 13.5-33.4%.

Seed no. and seed weight (g/plant): On LG1 putative QTLs were detected for seed no./plant in the F_3 population of glasshouse and F_2 progenies of TCRU at 34.0 cM and 72.6 cM, respectively, although both had LOD scores under the significance threshold. Marker bgPabg-596774 at 33.0 cM was found to be the nearest to this locus in the F_3 generation and also detected as a significant QTL for seed weight.

Biomass dry weight (g/plant): Interval mapping analysis of the F_3 progeny grown in the glasshouse identified a major QTL for total biomass located on LG1. It was tightly linked with the marker bgPabg-596774 located at 33.0 cM with a LOD score of 3.5 explained 22.4% of the phenotypic variation. This genomic region was identified as containing 5 QTL for other traits (as a major QTL for node no./stem, pod weight and seed weight and as a putative QTL for pod no./plant and seed no./plant. Kruskal-Wallis analysis for non-parametric mapping also confirmed the significant association between this trait and the marker reported in the interval mapping.

A single QTL for biomass dry weight in the field trial was also detected on the same linkage group (LG1) at different positions; this region was linked to marker bgPt-602039, while analysis of data for the F_2 population detected a putative QTL for this trait at a different position on the same linkage group.

Shelling percentage: Interval mapping analysis of segregation in the F_3 and F_2 populations evaluated in glasshouse and TCRU indicated that shelling percent was effected by major QTLs. These two QTL mapped on LG7 and LG12 with the LOD score of 3.0 and 4.8, for both glasshouse and TCRU datasets, respectively. The high significant trait-marker association was confirmed through non-parametric mapping for both of QTLs.

100-seed weight (g): One significant QTL was detected for 100-seed weight in the F_3 generation in the glasshouse dataset. It was located on LG7 at 9.4 cM with a LOD score of 2.7. Analyzing the F_2 generation data suggested a putative QTL for this trait which mapped on LG11 and LG12 at a LOD peak recorded under the significant threshold value genome-wide (2.5).

Eye pattern around hilum: Non-parametric analysis revealed significant association between this trait and two DArT markers on LG12 and LG18. Marker bgPabg-594999 was found to be the most significantly linked (K* = 29.7 at p = 0.0001) on LG12 at 22.5 cM.

6.2.2. QTL analysis in the 'wide' cross map

A number of traits evaluated previously for the F_2 population of the inter-specific cross between DipC x VSSP11 (Basu, 2005) were analysed using the improved map which combined three marker types (SSR, DArT and AFLP). These traits included days to emergence, days to flowering from emergence, stems/plant, internode length, leaf area, specific leaf area (SLA), carbon isotope discrimination (CID), and 100-seed weight.

Days to emergence and days to flowering were both non-normally distributed and analysied with nonparametric mapping. Internode length data was used directly without transformation as the normality thresholds was P > 0.01 and A-square (0.89) was smaller than the 99% critical value (1.09). For stem no./plant transformed data was distributed normally and analyzed with parametric mapping, other traits were distributed normally and they were used directly for parametric analysis.

Marker and trait association

The LOD threshold for detection of putative QTLs was peak $\geq$ 2 for parametric results. Significance thresholds were determined by permutation testing of 10000 replications. Thresholds ranged from 3.1 to 3.6.

Days to emergence: An association between emergence and AFLP marker P19b494AGC+CTA4 was identified on LG7 (K* = 7.1 at p = 0.01), using non-parametric mapping. Other associations between marker P3b211AGC+CTC7 and this trait was detected on LG14, but scored a very low K* = 4; p = 0.05 (Table 6-5).

Table 6-5: QTL mapping in bambara groundnut using interval mapping and Kruskal-Wallis analysis for the F_2 generation data derived from the 'wide' cross of DipC x VSSP11

Trait	Linkage group	Position (cM)	Locus	Interval mapping				Kruskal-Wallis analysis		
				LOD	PT[a]	PVE[b]	Additive	K*	Df	Signifi. level
Days to emergence (DE)	7	93.9	P19b494AGC+CTA4					7.1	1	***
	14	0.0	P3b211AGC+CTC7					4.0	1	**
Days to flowering (DF)	10	18.0	P6b140AAC+CAG1					6.8	1	***
	7	70.6	P18b320ACA+CAT6					5.7	1	**
	15	67.1	P15b351AGT+CAA2					4.9	1	**
	14	64.1	GH-19-B2-D9					4.8	2	**
Leaf area (LA)	15	83.1		3.0	3.4	14.1	-17.6			
	15	94.6	P19b105AGC+CTA2	2.3		11.2	-22.5	5.27	1	**
Specific leaf area (SLA)	10	7.0		3.0	3.2	14.6	-9.3			
	10	12.4	P16b329AGA+CTC2	2.6		12.7	-7.8	9.9	1	****
Stem no./plant (STN)	13	1.1	P17b242ACG+CAA3	4.9	3.6	22.2	-2.6	15.7	1	*******
	1	20.9	595196	2.4		11.8	-2.1			
Internode length (IL)	9	0.0	600900	2.9	3.1	14	1.8			
	14	26.5	P19b102AGC+CTA1	2.5		12	1.6	10.1	1	****
	13	0.0	P17b240ACG+CAA2	2.4		11.8	1.5			
Carbon isotope discrimination CID	7	93.9	P19b494AGC+CTA4	4.7	3.3	22.1	0.0	17.4	1	*******
100-seed weight (HSW)	7	87.6		3.3	3.6	16.9	-2.6			
	7	93.9	P19b494AGC+CTA4	3.0		15.7	-4.0	12.0	1	*****

a : permutation-10000 times test

b : percentage of total phenotypic variation explained by the QTL

Significant level of K * values: *: 0.10, **: 0.05, ***: 0.01, ****: 0.005, *****: 0.001, ******: 0.0005, *******: 0.0001

Days to flowering: Kruskal-Wallis mapping indicated that days to flowering was associated with marker P6b140AAC+CAG1 on LG10 (K* = 6.8 at p = 0.01). Three other associations with both AFLP and SSR markers on three different linkage groups of 7, 14 and 15 were also identified (Figure 6-2).

Leaf area (cm^2): QTL analysis identified a putative QTL for leaf area on LG15 with a LOD score of 3.0 at 83.1 cM and this explained 14.1% of the total phenotypic

variance for this trait. Marker P19b105AGC+CTA2 was detected to be the nearest to this locus.

Specific leaf area (SLA): Interval mapping analysis identified a putative QTL for SLA located on LG10. The LOD score of 3.0 was under the genome-wide significance threshold value of 3.2, determined by permutation testing. The nearest marker to this genomic region was P16b329AGA+CTC2 which showed an association with the trait studied in nonparametric mapping ($K^* = 9.9$; $p = 0.005$).
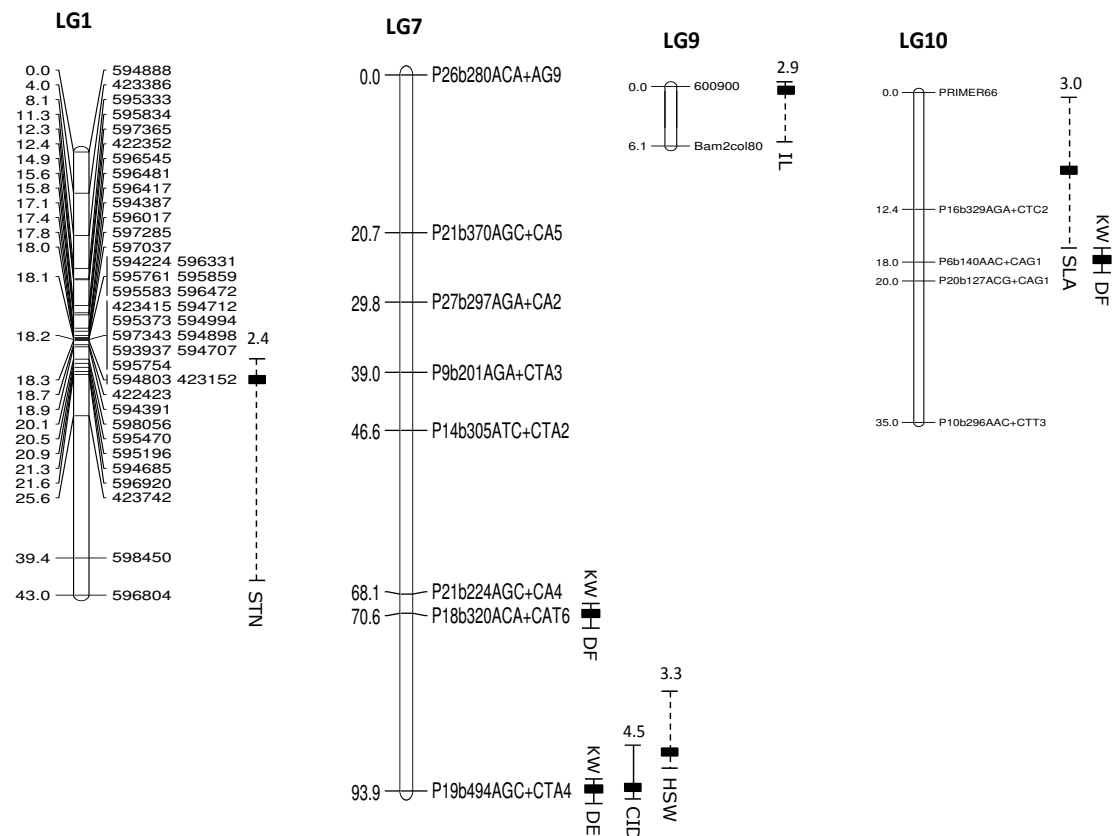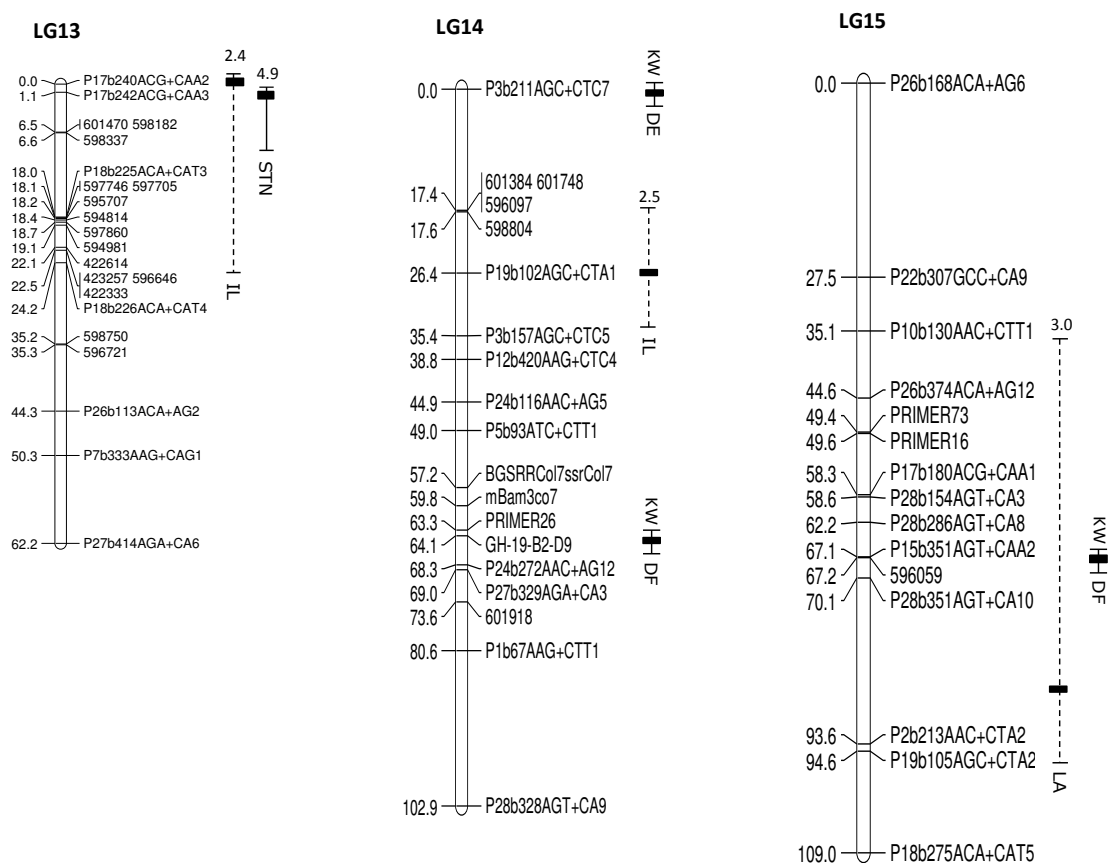
Figure 6-2: Map positions of the QTL on the wide cross (DipC x VSSP11) linkage map. Only linkage groups for which QTL were found are shown. Positions are given in cM (Haldane units) to the right of the linkage groups. QTL terminology is described in the text. The position of the maximum LOD value is written at the top of the QTL pointer and indicated by a rectangle in black. The maximum confidence LOD values of a particular QTL (1 LOD drop-off) are represented by plain lines with LOD score ≥ significant threshold value, as determined by 10,000 permutation tests and by dotted lined for putative QTL below the significant threshold LOD value but ≥ 2 LOD score. QTLs detected with Kruskal-Wallis analysis are discontinuous with no confidence interval and the rectangular only represents the entire position of QTL.

Stem no./plant: A significant QTL on LG13 was mapped using interval mapping at a LOD score of 4.9. The marker P17b242ACG+CAA3 was found to be the most significantly linked ($K^* = 15.7$; $p = 0.0001$) locus to this QTL for stem no./plant which was mapped at 1.7 cM and explained 22.2% of total phenotypic variance.

Another putative QTL was detected for this trait on LG1 at LOD = 2.4. DArT marker 595196 was linked to this trait at 20.9 cM.

Internode length (cm): Interval mapping for the inter-specific wide cross population derived from DipC and VSSP11 detected a putative QTL for internode length on LG9 at 0.0 cM with a LOD score of 2.9. Marker 600900 was linked to this locus and the phenotypic variance explained was 14%. Two other putative QTLs for internode length were located on LG13 and LG14 and were in association with AFLP markers P17b240ACG+CAA2 and P19b102AGC+CTA1, respectively. These three loci together explained about 38% of the phenotypic variation of internode length observed in this population.

Carbon isotope discrimination (CID): A single QTL for CID was identified on LG7 at 93.9 cM with the LOD sore of 4.7. It was found to be associated strongly with marker P19b494AGC+CTA4 ($K^* = 17.4$ at $p = 0.0001$). This genomic region explained 22.1% of phenotypic variance observed for CID in the F_2 progeny of the wide cross (DipC x VSSP11).

100-seed weight (g): Interval mapping also revealed a putative QTL for 100-seed weight on LG7 at 87.6 cM, having a LOD score of 3.3 (significant threshold value = 3.6; by permutation, 10,000 replications). The marker P19b494AGC+CTA4 was the nearest to this QTL ($K^* = 15.7$; $p = 0.001$) and the interval mapping suggested that this explained 16.9% of total phenotypic variance. This marker was also found to be associated with the QTL of CID

6.3 Discussion

The majority of biological traits are genetically complex. Mapping quantitative trait loci (QTL) is a powerful means for estimating many parameters of the genetic architecture for a trait and potentially identifying the genes responsible for the expressed phenotypic variation. QTL mapping is a key tool for studying the genetic architecture of complex traits in plants, facilitating estimation of the minimum number of genome regions that affect a trait, the distribution of gene effects, and the relative importance of additive and non-additive gene action (Laurie et al., 2004; Mackay, 2004).

The populations used for the map construction of both 'narrow' and 'wide' cross were segregating for a number of agronomic traits, which either showed a continuous or discrete distribution.

6.3.1 QTL detected

In order to obtain more reliable QTLs for the agronomically important traits a single QTL-model analysis of Interval Mapping was used to try to identify QTL for all the trait data that followed a normal distribution. The Kruskal-Wallis test of Marker-QTL associations was implemented for non-normally distributed traits at a significance threshold $p < 0.05$ (Van Ooijen and Maliepaard, 2001).

6.3.2 LOD significance threshold for QTL detection

It was possible in the current study to carry out a permutation test to determine the empirical significance thresholds (Churchill and Doerge, 1994). MapQTL6 offers this

test for interval mapping to determine the significance threshold of the LOD score. The significance threshold in this study varied from 2.4 to 3.6 for different trait in different mapping populations. They were determined based on the actual data rather than an assumed normally distributed data, being freedom from constraints on probability distribution and size of samples (Senko and Kuznetsova, 2006). Calculation of this threshold was dependent on the genome-wide and population type under study. LOD scores greater than the genome-wide significance threshold value was considered as 'significant QTL' for traits studied and those above LOD = 2, but below the calculated thresholds as 'putative QTL'.

6.3.3 QTLs detected in the 'narrow' cross map

A stable QTL was detected for internode length in the F_3 population of the narrow cross for both glasshouse and field trials dataset. The phenotypic variation explained in association with the marker bgPabg-596988 was >40%. The significant association of this trait with both seed no./plant and seed weight/plant could be of importance, as selection for internode length should help to improve seed yield in bambara groundnut by selection for this early trait. Internode length is considered as one of the most variable traits between different landraces and could be important in selection of genotypes in a breeding program (Siise and Massawe, 2012). Internode length also had a strong negative correlation with growth habit in both environments (r = -0.8) and this association might be used to structure the plant architecture according to the target environment. Bunch types appear easier to manage in mixed cropping systems in low input subsistence farming. While for controlled crossing spreading types could be easier to deal with because the flowers of bambara groundnut are very small and are more compact in the bunch type, while genotypes with longer internode lengths

offer easier access to flower buds for emasculation and pollination. Furthermore, QTL detected for internode length has indicated a positive additive effect for the alleles contributed from the parents to increase the internode length (Table 6-2 and Table 6-3). The finding of residual internode variation in a domesticated x domesticated cross is interesting, as it is one of the major domestication traits detected (Basu et al., 2007c) and suggests that variation for this trait has not been completely lost.

Both position and the magnitude of the QTLs for internode length on the map were stable across FutureCrop glasshouse and Indonesian field for the F_3 population derived from 'narrow' cross. This QTL could be a good candidate for MAS in the yield enhancement programs to help to suit different morphotypes to different environmental conditions. It could also be used to develop material for better management of this crop in the field.

Interval mapping analysis of the F_3 generation in the FutureCrop glasshouse for the 'narrow' map recorded the highest QTL LOD peak (9.7) for peduncle length on LG4. A significant QTL for double seeded pods/plant was also found in this genomic region and its association with peduncle length was identified previously through their significant trait correlation (r= +0.53 at p= 0.000). Higher additive effects compared to the dominance effects associated with the QTL of these two traits were detected and found to be in the same direction of effect, which is consistent with their associations (Appendix 27). Marker bgPt-423527 was the nearest marker to this locus and only 0.5 cM from the centre of the main QTL for internode length. The level of pods produced under the soil surface could be predicted through measurement of peduncle length in the population grown in FutureCrop glasshouse. Those lines having longer peduncles located the pods under the soil surface. Burying pods under the soil is considered to be an advantage of groundnut compared to other legumes,

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making harvesting time less critical, as the pods can be left in the soil for considerable time without serious loses (Whitmore, 2000). This trait is also associated in this cross with biomass dry weight and seed weight/plant, through an increase in pods containing double seeds for those lines having a longer peduncle. All of these trait relationships were previously suggested through Pearson's correlation coefficient analysis. Thus, this QTL could be one of the candidates for marker assisted selection for the yield in bambara groundnut.

An emphasis on biomass and distribution from the source into different sinks is a key focus of breeding programmes. QTL analysis can assist in understanding the genetic basis for this and how the environment may effect this accumulation and distribution of carbon. The marker bgPabg-596774 on LG1 is a target marker for a number of QTL, being a major QTL for biomass dry weight, node no./plant, pod weight and seed weight/plant and as a putative QTL for pod no. and seed no./plant. Their relationships have been confirmed through strong trait associations in FutureCrop glasshouse data for the narrow cross population. These associations are reflected in the direction of additive effects observed for the coincident QTLs of these traits. This situation of multiple traits affected by a single marker bgPabg-596774 might come from the linkage of multiple QTLs or it could be pleitropy; a single gene affecting several biochemical pathways leading to many different phenotypes at different levels of organization. This has been identified in a wide range of species (Prokop, 2004). Previous studies on soybean also identified more than one QTL for different traits mapped on the same loci (Wenxin et al., 2008; Zhang et al., 2004).

Microsatellite marker Bam2coL63 is found to be a candidate marker for the QTL of leaflet length analysed in both F_2 and F_3 populations in the controlled environment glasshouses for the 'narrow' cross population. This marker could potentially be used

to predict biomass dry weight as terminal leaf length and biomass dry weight are associated traits (r= +0.273; p=0.019 and +0.441; p= 0.000, respectively).

In total 18 significant QTL and 7 putative QTL were detected using interval mapping in the 'narrow' cross map, in addition to loci for days to emergence, growth habit and eye pattern around hilum detected with nonparametric mapping. Most of the QTLs detected were clustered on linkage groups 1, 4 and 12. A major QTL for internode length was detected on LG4 of the F_3 generation grown in glasshouse and in the field. The highest LOD value of 9.7 for peduncle length was located close to the internode length locus. A QTL at marker locus bgPabg-596774 was also detected for seed yield and some other yield-related traits located on LG1.

6.3.4 QTL analysis in the 'wide' cross map

QTL detection was based on LOD thresholds estimated by permutation tests (10000 permutations, P = 0.05), to account for the effects of known major QTL of multiple tests through their association with genetic markers for the entire experiment (Doerge and Churchill, 1996). The thresholds adopted here ranged from LOD = 2.9-3.6 for different traits studied in this cross. Two major QTLs were detected for the traits of stem no./plant and CID on LG13 and LG7 (LOD= 4.9 and 4.7), respectively. However the QTLs detected for leaf area, specific leaf area and 100-seed weight had LOD scores ≥ 3, they were considered as putative because they were detected with LOD score lower than significant threshold value of permutation tests. Two and three QTLs for the traits stem no./plant and internode length were located on different linkage groups, respectively. Only two traits (days to emergence and days to flowering) were not normally distributed and their association with markers on different linkage groups was determined based on Kruskal-Wallis analysis with a

significance level of 0.05- 0.01. Insufficient parental data on days to emergence and days to flowering hindered the identification of discrete classes of parental values to analyse the segregation of F_2 data for these two traits (Basu, 2005). The AFLP marker P19b494AGC+CTA4 located at 93.9 cM was detected as the reference marker for the QTL effect on days to emergence, CID and 100-seed weight. The significant association between these three traits was detected previously by Basu (2005) using Pearson's correlation coefficient analysis.

Due to the difficulty in characterizing phenotype of traits based on single plants the wide cross linkage groups probably harboured fewer QTLs for agronomic traits. The lack of replication is considered one of the major disadvantages of using F_2 population in QTL analysis, because the phenotypic evaluation on single plants is usually not considered reliable for some quantitative traits (Rakshit et al., 2012; Semagn et al., 2010). It has been suggested that studies conducted in a single environment are likely to underestimate the number of QTLs that can influence a trait (Paterson et al., 1991). For such environment-specific QTL, one would only be able to know that the QTL acts at locations where the environmental conditions are the same (Bolek et al., 2005). Moreover, the limited number of common markers between 'wide' and 'narrow' maps for both SSR and DArT markers has also limited the attempt to combine linkage groups of these two maps, which has affected the proper identification of links between the QTLs detected on both maps. The only link detected between the QTLs of the combined maps of both crosses was for days to emergence on the linkage groups 13 and 14 of 'narrow' and 'wide' cross maps, respectively. The QTL for this trait in both linkage groups was in close association with the common DArT markers 601384 and 601748 and these two linkage groups were joined together in the combined map of group 6 (Figure 6-3).
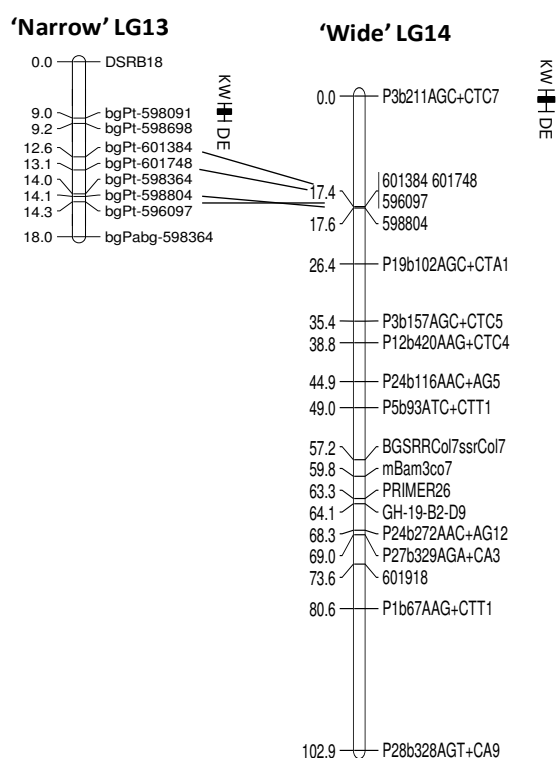
Figure 6-3: Related linkage groups identified through comparison of markers in the populations derived from both wide and narrow crosses in bambara groundnut.

One hundred and seven markers were not mapped to any linkage groups in the 'wide' map. The largest number of unmapped markers were AFLP markers (64 out of 141 markers) followed by DArTs (30 out of 106), although the highest proportion of unmapped markers (54%) was for SSR markers from the total number of 24 markers involved. It is expected that increasing the number of markers and the addition of new marker types to this population such as conserved ortholog set markers from other related legumes (soybean and Medicago) could help to identify and locate other QTLs, by increasing the coverage.

6.3.5 Summary

The results of QTLs analysis are presented and several genomic regions containing QTLs were found to explain variation for agronomic traits. The aim of this work has been to provide an understanding of biological mechanisms controlling these traits. The 'wide' map constructed between a wild and cultivated bambara groundnut accession has been used to determine the basis for domestication traits (Basu et al., 2007c; Basu, 2005). While the 'narrow' map constructed in a domesticated (DipC) x domesticated (Tiga necaru) cross was expected to segregate for agronomic traits, rather than domestication or major morphological traits.

Microsatellite markers involved in constructing the linkage map were derived from both genomic and transcriptomic regions. No marker-trait association was identified for transcritome SSR markers in the current study for both 'narrow' and 'wide' map. The possible reason is that both maps contain a limited number of transcriptome SSRs (2 and 3) for 'narrow' and 'wide' linkage map, respectively, and they might be associated with other traits not investigated here.

However, while the populations under study here were not big enough to construct a high resolution map for QTL study (Collard et al., 2005), the loci in the framework maps of 'narrow' and 'wide' maps were within 10 cM (3.04 cM and 5.91 cM, respectively) generally recommended for QTL analysis. The QTL identified could provide a resource for identifying the regions of bambara genome which contain genes for agronomically important traits and developing molecular markers for MAS in the breeding program of this crop. It has been declared that uniformly distributed loci every 10–20 cM within the entire genome could give a significant increase in the relative effectiveness of MAS and QTL identification (Stuber et al., 1999).

Chapter 7. GENERAL DISCUSSION

7.1 Introduction

Investigating the genetic architecture of crop plants has paid significant dividends in the case of many crops and especially cereals, such as wheat and rice, to meet the continually growing needs of Humankind in terms of increased yield and improved quality (Cholin, 2009). Plant breeding efforts are yet to make such an impact in the case of legumes as here yields have not kept pace with those of cereals. Despite the reported drought tolerance in bambara groundnut coupled with reasonable resistance to diseases, pests and adaption to poor soils, crop production is at relatively low levels, possibly due to low and unpredictable yields (Mayes et al., 2009). Bambara groundnut is cultivated throughout tropical regions of Africa and considered as an important food security crop in sub-Saharan Africa. The farmers there still rely on local landraces which can be inherently low yielding due to poor physical and genetic quality of seeds and poor crop management. Development of high yielding and adapted genotypes of bambara groundnut is an important strategic approach to try to increase food security through use of bambara groundnut.

Most of the agronomically important traits in bambara groundnut are quantitatively inherited (Olukolu et al., 2012). Due to the genotype-by-environment interaction in these traits most yield components have low heritable variation. Hence, phenotypic selection based on conventional breeding techniques alone will have some limitations in breeding programs for yield improvement in this crop. Employing new biotechnological tools such as DNA markers for mapping and identifying genes for desirable traits could solve some aspects of this problem. Construction of molecular

linkage maps is now a routine way to trace valuable alleles in a segregating population.

In this study we aimed to generate a framework map of an F_3 segregating population derived from the intra-subspecific cross (DipC x Tiga necaru) and to improve the partial genetic map developed previously (Basu, 2005) from an inter-subspecific cross between a domesticated landrace and a wild ancestor. A number of microsatellites (derived from different microsatellite-enriched libraries) and Diversity Arrays Technology (DArT) markers were used to carry out map construction and allow a QTL analysis of agronomically important traits. An attempt to combine the 'wide' cross and the 'narrow' cross was made to identify common markers to QTL effects for bambara groundnut and to improve our understanding of domestication. This could help breeders to effectively pyramid genes for agronomically important traits into single cultivars in a much shorter time than would be possible by conventional breeding.

7.2 Molecular markers development, characterization and validating the F_3 population under study

An overview of molecular marker (SSR and DArT) characterization and the polymorphic markers used in both 'narrow' and 'wide' cross populations under study is described in Figure 7-1.
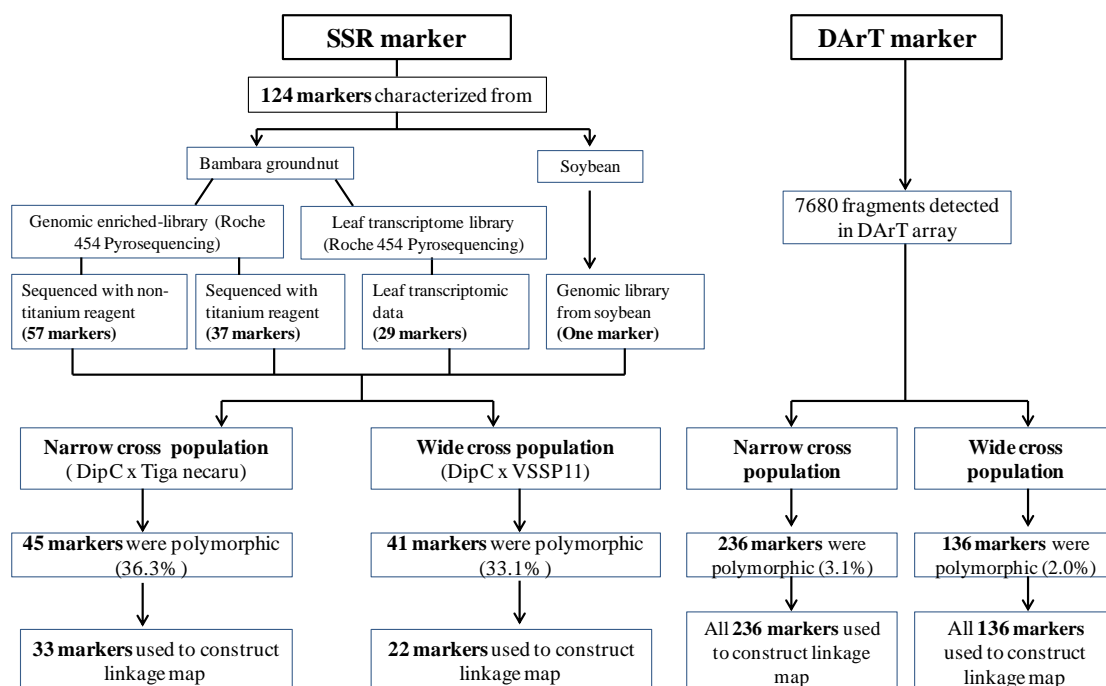
Figure 7-1: Diagram of SSR and DArT markers, their characterization and the number of polymorphic markers used in both 'narrow' and 'wide' cross populations of this study.

7.2.1 SSR markers

Microsatellite markers can be found in both coding and non-coding regions in all prokaryotic and eukaryotic genomes (Kalia et al., 2011; Trivedi, 2004). The markers used in this study were derived from different microsatellite-enriched libraries of both regions. Out of a total 124 SSRs screened 94 markers were developed from a genomic-enriched repeat library sequenced with non-titanium and titanium reagents (Roche 454 Pyrosequencing), representing the first two sets. The other set of 29 SSR markers were developed from a bambara groundnut leaf transcriptome library (Roche 454 Pyrosequencing). In addition, one SSR marker from soybean was also used in this investigation. Transferring SSR markers between the legume crops has become more common recently through linkage mapping and assessing genetic diversity (Chandra, 2011). A high rate of amplification of SSR markers from other legume species (azuki

bean, cowpea, and mungbean) in bambara groundnut was also reported by Somta et al. (2011b). Wang et al. (2004a) suggested that transferring SSRs across species and genera could be an efficient approach to develop DNA markers, especially for minor crops.

Commercial application and quick detection of SSR allele using agarose or simple polyacrylamide gel systems can be achieved by any laboratory with minimum infrastructure, meaning that this technique has been widely used in studies of genetic diversity (Senior et al., 1998; Stewart et al., 2011). However, such systems cannot resolve small difference in allele size (Jones et al., 1997).

Agarose gel (2.5%) was used in our investigation to detect the parental alleles in the populations under study for 4 primer pairs (D.35497, Primer10, Primer82 and D.48339) which had 34bp, 31bp, 28bp and 36bpbp allelic differences, respectively. A 3% agarose gel has been used by a number of researchers to screen for polymorphism of SSR markers with more than 20bp differences in allelic size (Ashkani et al., 2012; Beyene et al., 2005; Legesse et al., 2007; Wietholter et al., 2008).

Allelic size differences for other primers in both 'narrow' and 'wide' cross populations which could not be clearly scored with an agarose gel were determined using the CEQ^{TM} 8000 (Genetic Analysis System, Beckman Coulter, USA). The product sizes were scored manually from the electrophoretograms, taking into account the entire microsatellite profile as the automated calling system is unable to cope simply with changes in relative peak heights between alleles and the shift in overall microsatellite 'shape' is more informative. To reduce the costs and facilitate screening of large numbers of potential microsatellites with the Beckman CEQ^{TM} 8000, a three primer system was used (Schuelke, 2000). Further reduction in the cost of screening

the microsatellite markers was achieved by multiplexing PCR products which showed a wide product size differences between microsatellites in their individual pre-screens for size and polymorphism. Up to three SSRs were pooled. All the primers were labeled only with fluorescent blue dye D4. This was to avoid the problem of colour bleed through (e.g. as reported by Molosiwa, 2012) and the comparatively week D2 dye (Black).

Polymorphism of SSRs

Out of a total of 124 SSR markers, 45 were detected as polymorphic (36.3%) for the parental alleles in the F_3 population derived from the cross between DipC and Tiga necaru, while 41 markers were polymorphic (33.1%) in the F_2 population from the cross between DipC and VSSP11. Low levels of genetic polymorphism in the specific gene pool of bambara groundnut had been reported previously (Basu et al., 2007a). However, as all 124 markers had been prescreened against 24 genotypes believed to reflect existing variation within bambara groundnut (Stadler, 2009), there is a presumption that they are functional microsatellites. In an assessment of genetic diversity in bambara groundnut by Somta et al. (2011b) 35 primers out of 188 amplified SSR markers (19%) were polymorphic in eight accessions collected from the major growing regions of the world. Among these polymorphic markers, four came from those previously developed in bambara groundnut (10 SSR in total) by Basu et al. (2007a). The others were from related species: azuki bean, cowpea and mungbean. Variable levels of polymorphism in SSR markers were detected in different legume seed crops. Kajonphol et al. (2012) identified 16.1% polymorphic SSR markers when screened in the parental genotypes of wild and cultivated mungbean in an F_2 population using 945 markers. In a QTL study screening for

Fusarium wilt resistance gene in chickpea, Qadir et al. (2007) identified a high polymorphism rate (70.3%) for SSR markers between the parents of the mapping population (BG 256 and WR 315). The highest polymorphism rate (81.3%) was detected in pigeon pea by screening 40 genotypes (representing different Cajanus species including eight wild accessions) with 16 SSRs (Saxena et al., 2010).

Individuals genotypes from the DipC landrace (V. subterranea subterranea) were involved in both 'wide' and 'narrow' crosses, being the female parent crossed with landraces of VSSP11 (V. subterranea spontenea) and Tiga necaru (V. subterranea subterranea), respectively. Higher polymorphism was expected in the inter-subspecific cross (DipC x VSSP11) than in the intra-subspecific cross (DipC x Tiga necaru) but a contrasting result was initially obtained with more polymorphic markers for narrow cross. Overall, similar levels of SSR polymorphism was observed in both crosses despite one segregating for domestication traits and the other for agronomic traits and diversity analysis (Stadler, 2009; Molosiwa, 2012) suggested that the DipC parent used in the 'wide' cross is actually closely related to the wild relative used in the same cross. Stadler (2009) also revealed considerable genetic distance between the DipC and Tiga necaru landraces when analyzing the genetic relationships of 87 bambara groundnut genotypes using 296 polymorphic DArT markers.

However, there was a small sample size of the common SSR markers (26 polymorphic SSRs in common from 45 and 41 polymorphic SSRs in the 'narrow' and 'wide' crosses, respectively) (Table 7-1). The authenticity of the DipC female parental genotype of both crosses was confirmed through amplification of the same allelic size with 17 common markers for both populations. Different allele sizes for the DipC parent in both populations with other common markers (9) is likely to come from the fact that different individuals of the DipC landrace were used as the maternal

parent. Massawe et al. (2005) reported genotype variations within bambara groundnut

landraces and DipC appears to be a particularly variable landrace (Mayes et al, 2009).

Table 7-1: Common microsatellite markers and the alleles of the DipC parent used in both 'wide' and 'narrow' crosses

Primers	DipC parent used in narrow cross	DipC parent used in wide cross	allele	Source of primer
PRIMER10	260	268	different	
PRIMER15	238	240	different	
PRIMER19	273	270	different	
PRIMER26	183	183	match	
PRIMER32	247	220	different	
PRIMER38	194	194	match	
PRIMER48	238	244	different	
PRIMER66	225	219	different	
PRIMER98	264	274	different	Genomic DNA library of bambara groudnut
GH-19-B2-D9	236	236	match	
Bam2coL80	220	220	match	
Bam2coL33	253	239	different	
mBam3co7	267	267	match	
BN145	150	143	different	
PRIMER65	172	172	match	
PRIMER16	189	189	match	
PRIMER85	248	248	match	
AG81	202	202	match	Genomic DNA library of Soybean
D.42026	238	238	match	
D.8148	244	244	match	
D.8999	203	203	match	
D.37053	181	181	match	Leaf transcriptome library of bambara groundnut
D.12522	328	328	match	
D.24269	246	246	match	
D.51646	185	185	match	
D.2094	224	224	match	

7.2.2 Validation of the F$_3$ populations under study

The segregating F$_3$ population was tested for residual heterozygosity before

construction of the genetic map. All 73 individual lines were screened with 33

polymorphic SSR markers. Residual heterozygosity of the population was found to be

24.9% which matches the predicted Mendelian ratios expected from the population

history. In addition, none of the lines was identified as being out-crossed. The tests

made confirmed that the population under study is an F_3 which can be used confidently in construction of a genetic map.

7.2.3 DArT assay

DArT as a high-throughput and hybridized-based microarray platform was developed to overcome some of the limitations of other molecular marker technologies, such as RFLP, AFLP and SSR (Akbari et al., 2006). Since no DNA sequence information is required in developing DArT this technique allows for the typing of tens of thousands of loci in parallel with reduced cost per data point after the initial platform development (Kilian et al., 2005; Wenzl et al., 2004). It has proved to be the most cost-and time-efficient approach for under-utilized crops such as bambara groundnut (Mayes et al., 2009; Olukolu et al., 2012; Stadler, 2009). The procedure of generating DArT markers, screening for polymorphisms and genotyping was conducted by Diversity Arrays Pty. Ltd., Yarralumla, Australia. Due to the high locus specificity of these markers, they can be easily arranged into genetic linkage maps (Akbari et al., 2006; Wenzl et al., 2006). In the current study a total of 236 polymorphic markers (3.1%) were detected for the individuals of the F_3 population derived from the cross between DipC and Tiga necaru. They were integrated with polymorphic SSR markers to construct a genetic linkage map of bambara groundnut in this population. DArT was also used to generate additional markers for the existing initial map derived from the inter-subspecific cross in bambara groundnut with approximately 2% of DArT array elements revealing polymorphism. Higher levels of polymorphism at 5.5% were detected by Stadler (2009) in the construction of initial DArT marker discovery array from 38 landrace individuals using the restriction endonucleases combination PstI/AluI. However, this would be expected to reflect the overall levels of

polymorphism within the species, rather than in specific controlled crosses. In other crops such as barley, 2.9 to 10.4% polymorphism levels were found in discovery arrays prepared from nine 768-clone PstI libraries from two genetically distant cultivars (Clipper and Sahara) (Wenzl et al., 2004).

7.3 Rapid verification of presumed hybrids

Although a convenient hybridization system has been developed to obtain a large number of hybrid seeds in bambara groundnut (Suwanprasert et al., 2006), the cleistogamous nature of V. subterranea flower causes a high rate of self-pollination in this crop in nature (Uguru and Agwatu, 2006). Due to the maternal control of seed coat colour, distinguishing between genuine F_1 hybrid seed and self-pollinated seed is not possible in the F_1 and the segregation of seed colour can only be observed in the F_3 seeds produced (Basu et al, 2007c). To shortcut this process PCR-based molecular marker fingerprinting systems have been developed (e.g. Sundaram et al. 2008). For further acceleration of this process we have investigated the possibility of extracting DNA directly from the seed endosperm instead of growing the presumed hybrids for DNA extraction, aiming to rapidly verify the F_1 seeds in a way that does not reduce their viability. Genomic DNA was extracted successfully using the GenElute Plant Genomic DNA kit (Sigma Aldrich) giving the greatest DNA yields with 30 minutes incubation. The viability of the seeds was checked by a germination test and statistical analysis revealed that germination frequency of the seeds was not affected by this treatment. A similar method has been used by Kamiya and Kiguchi (2003) to extract DNA from soybean seeds. This simple and rapid method could be a useful tool even in the next filial generations to perform marker-assisted selection, even before sowing of the seeds.

7.4 Normality tests and association between the traits

The distribution of trait data was tested using Anderson Darling Normality test for the F_3 populations derived from 'narrow' cross in both Future Crop glasshouse and the field trial, and for the F_2 population from the same cross evaluated in the TCRU. Normal distribution of traits was confirmed in most of the trait values over different environments for both F_2 and F_3 generations (flower no./plant, plant height, petiole length, terminal leaflet length and width, leaf area, plant spread, stem no./plant, node no./plant, internode length, pod no./plant, double seeded pods/plant, peduncle length, pod weight/plant, pod length, pod width, double seeded pods length and width, seed length, biomass dry weight, shelling% and 100-seed weight), which indicates their control is more likely to be under multiple gene effects. Although some of these traits did not exhibit the same distribution pattern over all trials for both generations, Box-Cox transformation pulled many of them back to a continuous and normal distribution. Our results are in agreement with what was reported by other researchers on the effect of multiple additive genes for a number of these agronomic traits in bambara groundnut (Jonah et al., 2012; Karikari, 2000).

The other traits of days to emergence, days to flowering, growth habit and eye pattern around hilum were characterized as discrete traits even after transformation. The trait distribution of both growth habit and eye pattern around hilum was found to follow a segregation pattern consistent with Mendelian inheritance. Incomplete and complete dominance were hypothesized to control both traits, respectively. The monogenic inheritance of eye pattern around hilum is in agreement with previous work by Basu (2005) in the segregating F_2 population derived from the 'wide' cross which has DipC as the maternal parent of the cross.

7.5 Phenotypic correlation

The low heritability of most quantitative traits of economic interest complicates the selection process, while understanding of their relationship with those of high heritability could aid indirect selection (Shimelis and Shiringani, 2010).

The characters studied here are most likely to have complex inter-relationships. In order to determine the phenotypic relationships that exist among traits, Pearson's correlation coefficient analysis was calculated for the population derived from intra-subspecific cross between DipC and Tiga necaru. Pearson's correlation coefficient describes the direction and degree to which one variable is linearly related to another (Bolboaca and Jantschi, 2006). This test was performed for the F_3 population evaluated in the FutureCrop glasshouse and the Indonesian field trial and also for the F_2 population derived from the same cross in the TCRU.

Most of the vegetative growth traits and yield component traits were in a strong association with yield in bambara groundnut for the different trials. These results are in accordance with earlier published association between these traits (Makanda et al., 2009; Ntundu et al., 2006; Ouedraogo et al., 2008).

The negative association of days to flowering with the other traits, especially seed yield may lead to the hypothesis that the genotypes with early flowering may have a longer reproductive phase to deliver the stored and newly captured carbon into the seeds, leading to higher yields. Increasing the leaf number, leaf area, internode length and the optimal canopy spread should contribute positively to agronomical performance and increase the accumulation of biomass that goes into the seed.

A strong positive correlation was observed between yield and other traits studied here. Pod no./plant is a component of seed yield in bambara groundnut and considered as the most important yield component traits (Adu-Dapaah and Sangwan, 2004; Chijioke

et al., 2010; Makanda et al., 2009; Ofori, 1996). This has been confirmed in this study by the detection of a highly significant positive correlation (r=> 0.8 at p= 000) between both traits across different environments. It can been concluded that yield in bambara groundnut could be effectively improved through selection of these yield contributing traits. Given that bambara groundnut has a photoperiod requirement in some landraces for pod set/filling, The apparently self-evident correlation may not hold in non 12-hour photoperiod environments (Harris and Azam-Ali, 1993; Linnemann and Craufurd, 1994).

The association results between the traits studied was in agreement to the previously reported in the present of a strong correlation between the yield and other related traits in bambara groundnut. However these results needs further investigation to be validated, they may support the hypothesis that using early or simple traits associated with the later or more difficult will serve the assessment of the traits in selection of desirable genotypes for bambara groundnut.

7.6 Construction of genetic linkage map

The genetic studies reported earlier by Basu (2005) were based on an inter-subspecific cross. The genetic analysis of this cross has identified a number of genes important for the domestication process in Bambara groundnut (Basu et al., 2007c). The second genetic map based on an intra-subspecific cross exploits variation within the domesticated landraces gene pool, using an F_3 population derived from the cross between two domesticated landraces (DipC and Tiga necaru). This cross was expected to show variation for traits of breeding interest (agronomic traits rather than domestication traits). An overview of both map construction and the attempt to combine the maps is described in Figure 7-2.
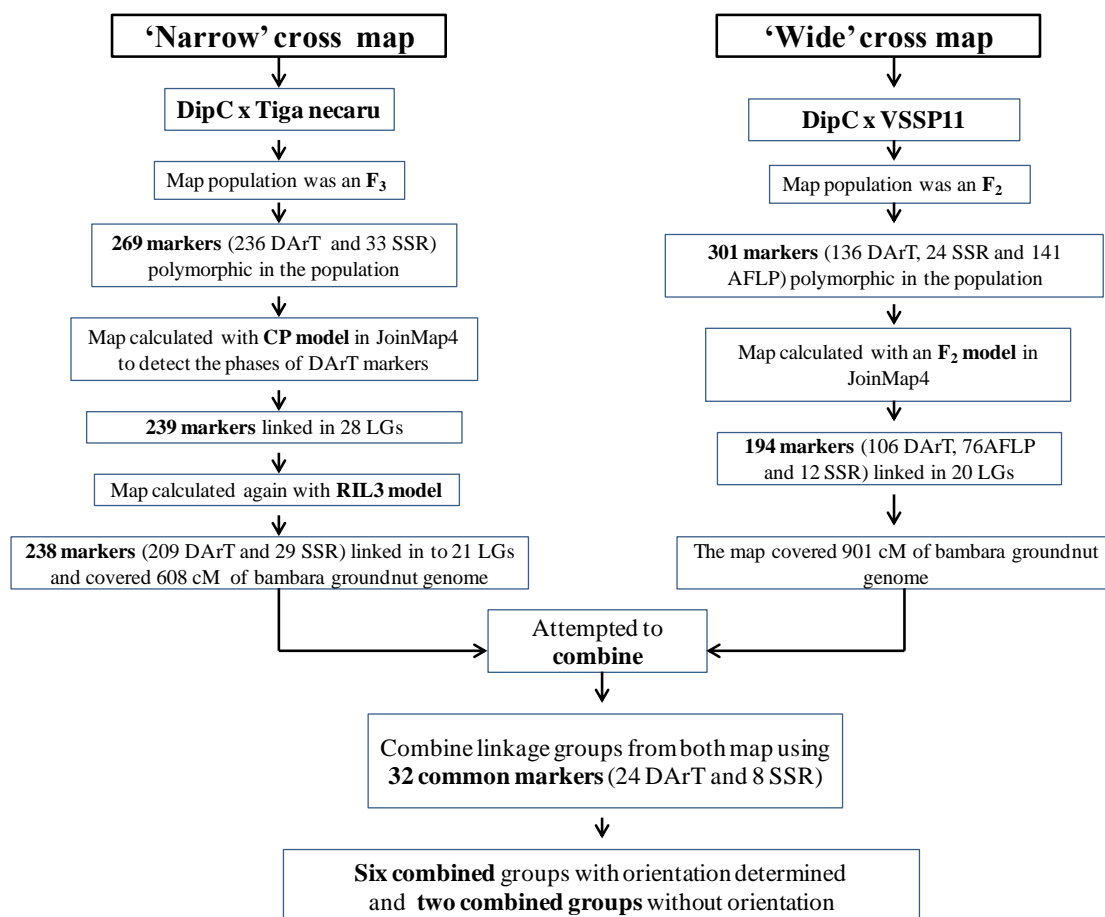
Figure 7-2: A flow diagram of the process of construction of the genetic linkage maps in the 'narrow' and 'wide' cross population and their combination.

7.6.1 Population size to construct the maps

A total number of 73 F_3 progeny were used to construct the 'narrow' cross genetic map. They were derived from a controlled cross between two single genotype accessions of domesticated background. Although, a similar population size of 75 recombinant inbred lines was used to construct a linkage map in common bean (Freyre et al., 1998), larger populations are required to determine the marker order for high-resolution mapping (Mohan et al., 1997). The size of population is considered the limiting factor for segregating populations and is determined by the number of seed from a single F_1 cross. As landraces are known to be composed of numerous

inbred lines in bambara groundnut (Massawe et al., 2005), it is not possible to use multiple genotype accessions from a landrace to generate multiple F_1 seed whose self-pollinated F_2 can be combined into a single large F_2 population. In an F_2 population replications over time or space also cannot be carried out as each single plant represents a different genotype (Semagn et al., 2010), while in the F_3 population single plant assessment can be replaced by the assessment of F_3 families.

The population of 98 F_2 progeny maintained as dried leaf in silica was re-extracted to be used in the improvement of the previous partial genetic map for an inter-subspecific cross between a domesticated landrace and a wild ancestor (Basu et al., 2007c).

7.6.2 Marker distortion

With the development of molecular linkage maps, numerous examples of segregation distortion have been reported on many plant species (He et al., 2001). In the current study the segregation patterns of the markers and detection of any distortion was tested by JoinMap4 through performance of a Chi-square test ($p < 0.05$). Thirty two percent of the markers (SSR and DArT) were found to be distorted in the narrow cross, based on a RIL3 model. While in the wide cross population 27% of all three marker types (SSR, DArT and AFLP) were distorted, based on an F_2 model of segregation. The results of linkage analysis for both maps revealed that markers with distorted segregation were distributed throughout the genome. Our markers for both maps showed lower distortion than the 41% distortion reported by Basu (2005) in the linkage map constructed in bambara groundnut using 115 AFLPs and one SSR marker. However, lower distortion (19.93%) of SSR markers was reported in 106

RILs (F_9) derived from the cross of two soybean (Glycine max) genotypes (BD2 and BX10) (Liang et al., 2010).

Segregation distortion is influenced by many factors, some of these are: mapping population, genetic transmission, gametic and zygotic selection, non-homologous recombination, gene transfer, transposable elements and environmental agents (Knox and Ellis, 2002; Yamagishi et al., 2010). Wu et al. (2001) demonstrated that 21.7% segregation distortion was caused by a genetic transmission effect using an RIL population from a cross between two cultivated soybean Kefeng1 and Nannong1138-2. It was also reported that segregation distortion could be partially caused by gametophytic and sterility factors (Zhang et al., 2006). In constructing a genetic map in barley a total of 22 loci among 65 polymorphic SSR loci (33.8%) were distorted in 260 F_2 individuals derived from a male sterile line and an elite cultivar. Distorted loci were found to be skewed toward the male sterile parent (Liu et al., 2011). These results suggested that the phenomenon of segregation distortion occurs commonly in hybrid populations (Konishi et al., 1990). In progeny derived from inter- and intra-specific crosses the distortion is thought to be caused by competition among gametes for preferential fertilization or from abortion of the gamete or zygote (Faris et al., 1998).

7.6.3 Linkage map and marker distribution

Map construction was conducted with JoinMap4. Out of 269 markers involved in construction the 'narrow' map 238 marker (29 SSRs and 209 converted DArTs) were assigned in to 21 linkage groups which cover 608.6 cM of the bambara groundnut genome. Map construction was undertaken by running the population under the RIL3

model after the initial analysis of the population as a Cross Pollinator (CP) to determine the phases of DArT markers, by comparison with SSRs.

In the 'wide' cross map three marker types of SSR, DArT and AFLP were involved with a total number of 301 markers. One hundred and ninety four markers were assigned to 20 linkage groups spanning a total of 901 cM.

Some of the linkage groups in both maps had only a few markers and incomplete coverage of the genome led to both maps having more than the expected 11 linkage groups corresponding to chromosomes (2n=2x=22) for the bambara genome. Adding more markers to these two maps should join these small groups together, although undetected genetic reasons for fragmented groups may also exist.

The map of 'narrow' cross covered approximately 54% of bambara groundnut genome, however more comprehensive coverage was expected based on the marker-marker linkage (89%). Clustering of the developed markers to particular regions of the genome or inhibition of recombination by parental dissimilarity could be one reason of the lower than expected map length. The linkage map of the F_2 population derived from the 'wide' cross had greater genome coverage of 79.6% compared to 'narrow' map, however it had lower marker-marker linkage of 67% than the 'narrow' map. The combination of three marker types could be one of the reasons for greater coverage of the bambara genome by the 'wide' map. Adawy et al. (2005) reported that different marker systems differ in detecting polymorphism and their genome coverage.

Unusual clustering of DArT markers was observed in both 'wide' and 'narrow' maps, which seems to confirm earlier observations of two major clusters of accessions, based on of DArT markers including DipC in the smaller group containing the

unusual repeat cluster (Stadler, 2009). However, the source of this clustering is not quite clear an introgression from an alien species might be the reason or it could represent a repetitive sequence which has proliferated locally within the chromosome from a specific progenitor sequence within specific lineages. In the genomes of chick pea, barley and wheat clustering of DArT markers in a particular genome region has also been reported (Akbari et al., 2006; Semagn et al., 2006c; Thudi et al., 2011).

We have developed the first linkage maps based on a combination of SSR and DArT markers in bambara groundnut. This map will be useful for comparative genomic analysis between the mapping populations in this crop and also between bambara groundnut and other related legume crops. This map along with the improved wide cross map using a combination of SSR, DArT and AFLP markers, represents an important step toward genetic analysis of interesting agronomic traits in bambara groundnut. These markers developed could be used in marker-assisted selection for complex agronomic traits and phylogenetic analysis in bambara groundnut.

7.6.4 Combined maps

Both 'narrow' and 'wide' maps were initially combined using the JoinMap4 software through 32 common markers (SSRs and DArTs). Thirty common markers were combined in 6 linkage groups with at least 2 common markers each. However, two other groups were linked with only one SSR marker each on the same chromosome, so resolving their relative orientation was not possible. The combined map will assist breeders to localize QTLs to genomic regions for marker-assisted selection. Addition of more common markers will assist in map integration. It will also make comparative mapping with other related legumes easier in the future (Millan et al., 2010), which

ultimately will give valuable information about QTL regions in related crops which may also be important for bambara groundnut.

7.7 QTL analysis

The genetic architecture of complex traits in plants can be investigated by QTL mapping which can identify the genomic regions responsible, elucidate the effect of the gene(s) and (eventually) the structure of gene itself (Laurie et al., 2004; Mackay, 2004). QTL analysis was carried out in this study to identify regions of bambara groundnut genome that are responsible of genetic mechanisms for the agronomic traits.

The first step in QTL mapping is to have a linkage map with good coverage of markers. Interval mapping (IM) was used to detect loci accounting for traits data variation in the populations derived from the intra-subspecific and inter-subspecific crosses of bambara groundnut. The IM model is considered more powerful than the analysis of variance at individual marker loci being more flexible for missing genotype data and is more feasible when the markers are widely spaced (Broman, 2001). LOD scores are used to measure the strength of evidence for the presence of a QTL effecting a trait. Where the LOD score is higher than the permutation test threshold, a QTL for that trait can be declared. Generally, the most likely position of the QTL is at the maximum LOD, with a 1 LOD drop in probability on either side of the maximal point defining the confidence interval. The detection of QTL for different generations in different environments for both 'narrow' and 'wide' cross populations are summarised in Figure 7-3.
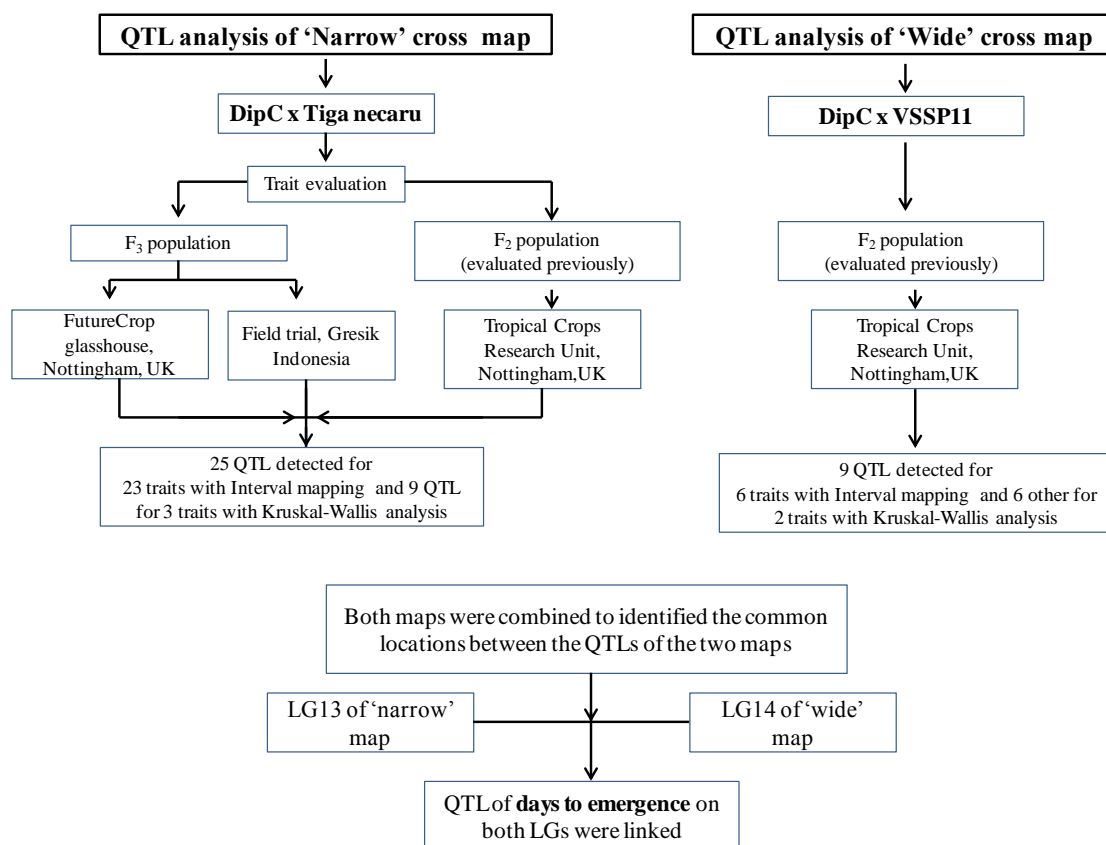
Figure 7-3: Diagram of the QTL detection scheme for traits in the 'narrow' and 'wide' cross populations, evaluated in different generations and for different environments.

7.7.1 QTL analysis of 'narrow' cross map

We have found several genomic regions detecting QTLs for agronomic traits in both FutureCrop glasshouse and the Indonesian field. These results were also supported by data collected from the same intra-subspecific cross for the F_2 generation grown in TCRU.

Eighteen significant QTL and 7 putative QTL were detected for 23 agronomic traits using an interval mapping in addition to nine others detected for days to emergence, growth habit and eye pattern around helium using non-parametric mapping for the F_3 population evaluated in FutureCrop glasshouse. Individual QTLs detected with interval mapping explained 13.5 to 50.3% of the total phenotypic variation. Two

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QTLs were detected for each of the traits node no./stem and pod length of double seeded, explaining 39% and 36.2% of phenotypic variance observed for both traits in the F_3 progeny evaluated in FutureCrop glasshouse. A smaller number of QTL for these traits were detected and recorded in the Indonesian field trial and the TCRU for the F_3 and F_2 generations, respectively. The possible reason is that the typical tropical growth condition of this crop in the FutureCrop glasshouse allowed the genes responsible to these traits to be expressed properly more than the field condition, as most of the agronomic traits are quantitative with multi-gene response having minor phenotypic effect and they are sensitive to the environmental changes (Chenu et al., 2011; Choudhary et al., 2008). Furthermore, the technical measurement issue has caused the number of traits involved in QTL analysis to be less in both field and TCRU trials compared to the FutureCrop glasshouse. The QTL for internode length and biomass dry weight were considered stable across the FutureCrop glasshouse and the Indonesian field trial for the F_3 population derived from the cross of DipC x Tiga necaru. These QTLs could be a good candidates for MAS in a yield enhancement program under different environment conditions. Association of the trait values with the same marker in the FutureCrop glasshouse and the field was also recorded for the traits of days to emergence and growth habit detected by Kruskal-Wallis analysis (due to the non-normal distribution of the trait). Other QTLs for plant spread, leaflet length and pod no./plant were detected on the same linkage groups for the F_3 population evaluated in the FutureCrop glasshouse and TCRU.

Potentially pleiotropic effects of single loci on multiple phenotypic traits have been identified in current study; the marker bgPabg-596774 on LG1 was associated with six QTL related to yield. Three other QTL for the pod and seed dimensions were also positioned on LG12 and associated with marker bgPt-598767. The causal relationship

between these traits was confirmed through their strong association in the data analysis of the FutureCrop glasshouse population, as described in Chapter 4, and the allelic effects of coincident QTLs in the same direction as traits correlated supported the hypothesis that traits variation attributed to this coincident QTLs (Appendix 28). Pleiotropy effects have been identified in a wide range of species, including legumes (Bobby et al., 2012; Irzykowska and Wolko, 2004; Prokop, 2004; Wenxin et al., 2008; Zhang et al., 2004).

The QTLs detected for both peduncle length and internode length were located close to each other within 1cM distance on the same linkage group (LG4). These two traits gave the highest LOD scores in the analysis, with values of 9.7, and 7.9, respectively. Most of the QTLs detected here could be considered valuable and of potential use in MAS of this crop, aiming to develop the yield and the performance of bambara groundnut.

The QTL of traits Internode length, growth habit, plant spread and peduncle length located on LG4 were within the combined confidence interval, indicating that they could be affected by the same gene (s). Using conserved synteny of the marker sequences at this location with the other related legumes might accelerate comparisons of the gene order and identifying candidate gene (s) responsible to these traits. In Cowpea, homologous genes were identified for 85 and 80% of the SNPs markers when soybean and M. truncatula genome sequences were compared (Lucas et al., 2011; Muchero et al., 2009).

Both dominance and additive effects were evaluated for the QTL detected with the interval mapping. Most of QTLs detected for the F_3 population evaluated in the FutureCrop glasshouse were more likely to be under the effect of additive effect with

the presence of a few dominance effects such as flower no./plant, plant spread, internode length, seed no./plant and biomass dry weight, while the QTL of leaf area appeared to have more dominance effect. Additive effect was also predominant for QTLs of the field trial except for the traits of node no./plant and biomass dry weight showing dominant gene action for their QTLs. While the F_2 population evaluated for the same 'narrow' cross the effect of dominance effects for the QTL of different traits seem to be more than those detected for the segregating F_3 population (Appendix 27, Appendix 28 and Appendix 29). QTLs with large additive effects are valuable for breeders as they might result in larger difference in the traits of interest (Bradshaw et al., 2008). The coincidence of seed yield QTL with that of yield component such as (biomass dry weight and flower no./plant) with high additive effect offers a means for selecting for seed yield by efficient selection for its component.

7.7.2 QTL analysis in the 'wide' cross map

The eight traits evaluated previously in the F_2 population derived from the wide cross (DipC x VSSP11) (Basu, 2005) were included in the QTL analysis of the updated 'wide' map. QTLs detected for stem no./plant and CID were significant, a putative QTL was detected for other traits of leaf area, specific leaf area and 100-seed weight. Two and three QTLs for the traits stem no./plant and internode length were located on different linkage groups, and the loci for each traits together explained about 34% and 38% of the phenotypic variation, respectively. Days to emergence and days to flowering were non-normally distributed and they were found in significant association with AFLPs and SSRs marker loci on different linkage groups (7, 10, 14 and 15). Insufficient parental data on days to emergence and days to flowering and the possibility of transgressive segregation for these traits might hindered the

identification of discrete classes of parental value to analyse the segregation patterns in the F_2 for these two traits. The three traits of days to emergence, CID and 100-seed weight as found in significant association with each other, QTLs being located at the same position of marker P19b494AGC+CTA4 on LG7.

The QTL identified in any one mapping experiment are likely to describe only a small part of the global genetic architecture of the trait within the species as a whole. For a better understanding of the genetic architecture of natural variation for the trait, the use of multiple populations connected through common markers could help to understand the species level variation for the trait of interest (Symonds et al., 2005).

In the combined linkage groups of 'wide' and 'narrow' maps a common location was determined between the QTLs for days to emergence. This trait in both maps was in close association with the common DArT markers 601384 and 601748. The limited QTL linkage between the two maps is likely to be a function of a limited number of integrated linkage groups and also due to some limitation of phenotypic assessment in an F_2 population based on single plant assessment which led to fewer QTLs for agronomic traits (Paterson et al., 1991; Rakshit et al., 2012; Semagn et al., 2010). Increasing the number of common marker from other related legumes (soybean and Medicago) might help to link more QTLs through combining more linkage groups from both maps or additional marker systems in bambara groundnut (potentially DArT Seq scored in both populations) could significantly improve integration of the maps.

In the QTL analysis with the interval mapping those detected for leaf area, Carbon isotope discrimination and 100-seed weight had dominance effect, while the others either had only additive effect or both additive and dominance effects (Appendix 30).

7.7.3 An overview of QTL analysis

To determine the basis for domestication traits and the recruitment of the wild ancestor into a domesticated 'landrace', the 'wide' map was constructed between a wild and cultivated accession (Basu et al., 2007c; Basu, 2005). The subsequent development of domesticated landraces and trait variation within domesticated material was investigated through the 'narrow' map constructed here between domesticated landraces. The latter cross was developed to provide a better understanding of the genetic control of biological mechanisms controlling agronomic traits. The results of the QTL analysis have been presented and several genomic regions containing QTLs for agronomic traits identified, with a number of these stable across different environments. The QTLs detected here are specific to the populations' analyses for the given study; it is quite possible that future studied will discover additional QTL regions contributing to agronomic traits phenotypes because of genetic variance across and within landraces. Although the number of markers integrated to construct the genetic linkage maps, the loci in both framework maps were generally within recommended distance for QTL analysis (<10 cM) (Doerge, 2002). The QTL identified could provide a resource for identifying the regions of the bambara genome which contains genes for agronomically important traits and to develop molecular markers for MAS in the breeding of bambara groundnut.

7.8 Summary of progress achieved in the study

This project has investigated various aspects of developing molecular markers, phenotypic evaluation of the populations, construction of genetic maps and QTL analysis. The results of these investigations can be summarised into a number of main points.

1- Development of polymorphic SSR markers for both 'wide' and 'narrow' cross populations, generated from a genomic-enriched repeat library sequenced with non-titanium and titanium reagents (Roche 454 Pyrosequencing), a leaf transcriptome library (Roche 454 Pyrosequencing) and a single cross-species SSR from the soybean genome (Chapter 3).

2- Developing polymorphic DArT marker data for the F_3 population, derived from 'narrow' cross of domesticated bambara groundnut (Chapter 3).

3- Confirmation of the residual heterozygosity present in the 'narrow' cross. A set of 33 polymorphic SSR was used and the individuals of F_3 population were found to match with the predicted Mendelian ratios expected from the population history (Chapter 3).

4- Rapid extraction of genomic DNA from the seed endosperm without affecting seed viability, using the GenElute Plant Genomic DNA kit (Sigma Aldrich) (Chapter 3).

5- Associations between important agronomic traits in the segregating F_2 and F_3 populations derived from the 'narrow' cross evaluated in controlled environment glasshouse and Indonesian field experiments (Chapter 4).

6- Construction of a genetic linkage map in bambara groundnut using a total of 238 SSR and DArT polymorphic markers in the F_3 population (Chapter 5).

7- Improving the wide cross map using a combination of SSR, DArT and AFLP markers in the F_2 population (Chapter 5).

8- Attempting to combine both 'narrow' and 'wide' maps using JoinMap4 with 30 common markers (SSRs and DArTs) (Chapter 5).

9- QTL mapping of polygenic agronomic traits, identify 18 QTLs for the traits evaluated in the population derived from the 'narrow' cross and 6 QTLs for traits of 'wide' cross population evaluated previously (Chapter 6).

10- Co-location of a QTL for days to emergence in both 'wide' and 'narrow' (Chapter 6).

7.9 Future work

Based on the results obtained from the present study, the following future lines of work can be proposed briefly.

- Already validated candidate marker (bgPabg-596774) could be used for MAS. The QTL region could be fine mapped for use in map-based cloning or subjected to a functional genomics approach. Development of the population into full RILs (it is now at F_6) or the development of Near Isogenic Lines (NILs) could allow the evaluation of the effects of this QTL alone and also the development of heterozygous plants for the QTL region, allowing a large-scale fine mapping programme.

- Further validation of prominent candidate markers/QTLs for various traits is required in different populations, across locations and/seasons, before using them in the breeding program of bamabara groundnut.

- Saturate linkage maps with more markers so that the extensive phenotypic data, including drought and heat tolerance and photoperiodic control of pod filling, could be efficiently used for further QTL detection. DArT Seq data was generated on the narrow cross, but time did not allow a full analysis of this for genetic mapping.

- Increasing the number of common markers using COS primers (screened previously with the pooled DNA from bambara groundnut (Basu, 2005) along with other COS primers designed from related legume crops, Medicago truncatula Gaertn and soybean (Glycine max) http://www.phytozome.net/soybean (Kaur et al., 2011; Varshney et al., 2009b), to screen the polymorphism in the populations under study. This might help to link more QTLs through combining more linkage groups from both 'wide' and 'narrow' maps and would also allow alignment of

plant genomes between of bambara groundnut and other legume species to predict the gene content and gene order in bambara groundnut through comparative genetic maps.

- Further development of selected lines in future generation to identify superior RILs for various traits which can be used in the future crop improvement program of bambara groundnut before release of new cultivars.

- More sequencing data, especially transcriptomic sequence, can be generated for this crop at relatively low cost using Next Generation Sequencing technology. The sequences developed could be used directly to develop more markers (SSR) markers or to develop synteny of bambara groundnut to close relative legume sequences to analyse gene expression and potentially allow tracking of candidate gene from other relatives to bambara groundnut.

- Developing this segregating population through repeat crossing and development of more lines. This would allow greater QTL analysis power and allow the detection of further QTLs for MAS. It would also allow better localization of effects (due to increased meiotic crossing over). Single seed descent could be used to develop fixed lines for further analysis.

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Appendices

Appendix 1: First set of microsatellite makers developed from Roche 454 pyrosequencing technology used in bambara groundnut.

No.	Primer name	Sequence F	Length	Sequence R	Length
1	PRIMER1	AACTTGCCATACGTGGAAGG	20	ACACGCTGCATAATTCACCA	20
2	PRIMER2	CGTGGATACCCATACCGTCT	20	TAAGTCCATTTTGTCCGATTGA	22
3	PRIMER4	CATTGTCTCTGCCACCATTTT	21	CAGACTGGGATTTGCATGTG	20
4	PRIMER5	CTGCTGTGGTGAGCTTTTGT	24	CTCCTTGCAGCTAAGCGTCT	21
5	PRIMER6	TACGGTCCTACACGGGAAAC	20	ACCTGTCCAGCCGCAATTA	19
6	PRIMER7	GTAGGCCCAACACCACAGTT	26	GGAGGTTGATCGATGGAAAA	20
7	PRIMER8	GGAAGAGTGCGTTTTGGTGT	22	CTGTGTGGACCCCAGAAAAT	20
8	PRIMER10	TCAGTGCTTCAACCATCAGC	24	GACCAAACCATTGCCAAACT	24
9	PRIMER15	AGGAGCAGAAGCTGAAGCAG	21	CCAATGCTTTTGAACCAACA	21
10	PRIMER16	CCGGAACAGAAAACAACAAC	21	CGTCGATGACAAAGAGCTTG	21
11	PRIMER19	AGGCAAAAACGTTTCAGTTC	20	TTCATGAAGGTTGAGTTTGTCA	22
12	G11-9-B2-D9	ATCAAAATCAAGCAAATGAGA	21	ACCTTTTACGCTCATTTTAACCAG	24
13	PRIMER23	CAGTAGCCATAATTTGCTATGAACA	27	CGAATCACCATTCAATACGC	22
14	PRIMER26	CGCTCATTTTAACCAGACCTC	24	CAAACAAACCAACGGAATGA	22
15	PRIMER27	ACACCGCCATCATGAGATTT	26	CATTTCAGGATTTGGGAGGA	25
16	PRIMER30	AATGCAAGATTTTGGCTTGG	24	CCCACTCAAACCATACACCA	23
17	PRIMER31	GCTAAGGTGGAGTGGTGGAA	26	CAATCATCTTTTGCGCTTCA	25
18	PRIMER32	TTCACCTGAACCCCTTAACC	21	AGGCTTCACTCACGGGTATG	20
19	PRIMER37	CCGATGGACGGGTAGATATG	23	GCAACCCTCTTTTTCTGCAC	22
20	PRIMER38	TCACACTTGCAATGGTGCTT	24	TCGTTGTTTCTCTTTTCATTGC	24
21	PRIMER42	TCGTACCGAATCACCATTCA	23	CAGTAGCCATAATCTGCTATGAACA	27
22	PRIMER43	CTTGATGCTACCGAGAGAGAG	27	AGGCTCCAACAATGCGATAG	25
23	PRIMER44	TGTGGGCGAAAATACACAAA	27	TCGTCGAATACCTGACTCATTG	27
24	PRIMER45	CGTGGATACCCATACCGTCT	22	AAGTCCATTTTGTCCGATTGA	23
25	PRIMER47	ACCCATTGCACGTCATAGAA	21	GGGTGAACTACACCACCTTCA	21
26	PRIMER48	TACCTGCATTCGGGCACAGTT	20	TTCACTCTTTCTTGATCACATGC	23
27	PRIMER52	TGCTTACCGCTTGAAGGACT	20	GCACCATCATCGACTTCCTT	20
28	PRIMER56(Z)	TGGCATGAGCATCATTCATT	20	CAAGGCTATGTCACGGGTTT	20
29	PRIMER63	TTTAGGAATCAAGAAAGGCAAG	22	GCGAGGCAAAAGAAAATGAC	20
30	PRIMER65	GGACGTGAATCGATGGAGAT	20	TCCTTCCCCCTTCTCTGATT	20
31	PRIMER66	CGTTAGATCTGAGACGCCATT	21	CATCCATCACCTGTCACCAG	20
32	PRIMER72	AACTTGCCATACGTGGAAGG	20	ACACGCTGCATAATTCACCA	20
33	PRIMER73	CACCCTCCAAGACCATCTTC	20	CCGAATCCTGATTCTTCCAA	20
34	PRIMER74	GAGCAGGTGGTGGTGGTT	18	GTGAGCTCGTCGTGTTTCTG	20
35	PRIMER81	CCGGAACAGAAAACAACAAC	20	CGTCGATGACAAAGAGCTTG	20
36	PRIMER82	AATCGTGGTCTCCCATTGAG	20	AGGAGCAGAAGCTGAAGCAG	20
37	PRIMER84(Z)	AGAGGAAACCACCATTGCAC	20	GCATCACACGAATTTCTCACA	21
38	PRIMER85	TTTCCAGATTGGATCGTTGA	20	TGTCTTCACACCGGAATTTG	20
39	PRIMER88	TGTGGTTGTGCTCCTTCTCA	20	GGGAAGAAGAGTGAAGTTGGAA	22
40	PRIMER91	ATGGCTGTTAGTGGCGTGAG	20	AGACCTGGGAACTTCCACCT	20
41	PRIMER95	AAGTCCATTTTGTCCGATTGA	21	CGTGGATACCCATACCGTCT	20
42	PRIMER96	TCTTATGCCCTCACCCATTC	20	ATTCAGACCAGTGCCCAAAA	20
43	PRIMER98	TTTTGTCACTGTTTGCCACAA	21	AGATTTATATCTGGATGAGAGAGAGAG	27
44	PRIMER103	AAAATTCAAAGGCCTGGAAAAA	21	TTTTTGAGTTCTGCGAGCAA	20
45	PRIMER104	ACGAGTGTGCGTGTGTGTTT	20	GGATCAGGCTCCACATAACC	20
46	PRIMER105	CAGTGGCAATGAATATCTCAGG	22	TGGCTGTTCTCTTGTGAATCC	21
47	Bamcol17	AACCTGAGAGAAGCGCGTAGAGAA	24	GGCTCCCTTCTAAGCAGCAGAACT	24
48	Bamcol24	ATTGTGCTCAGCTAGGATGTCACG	24	GAACTTGGCGAGACTATGCCTCAT	24
49	Bam2col58	GCGGATGGAAGTTTTGGAAGTGTA	24	TTGCTTCCTCCCCCAACATTACTA	24
50	Bam2col63	AAAATCTCACTCGGATGGCATGTG	24	TGGAATCACCTGATAGTAGTGTATTGG	27
51	Bam2col80	GAGTCCAATAACTGCTCCCGTTTG	24	ACGGCAAGCCCTAACTCTTCATTT	24
52	Bam2col13	CCATCATGAGATTTGCCTTTCCTT	24	TAAGCATTTCAGGATTTGGGAGGA	24
53	Bam2col33	ATGTTCCTTCGTCCTTTTCTCAGC	24	AAAACAATCTCTGCCCCAAAAAGA	24
54	mBam3co7	GGGTTAGTGATAATAAATGGGTGTG	25	GTCATAGGAAAGGACCAGTTTCTC	24
55	mBam3co18	TAGGTTATGAGGTAAGCATTTCAGG	25	TGGATATGTGTCTCTATGTTTCCAC	25
56	mBam3co33	TGTGTCTGTTTGTGGGGATATGTA	24	TTATCCCGGTCCTAATTCATCTTA	24
57	mBam3co39	CAGTAGCCATAATTTGCTATGAACA	25	CACATCAATCAAAAATCTCGGTAG	24
58	AG81*	ATTTTCCAACTCGAATTGACC	21	TCATCAATCTCGACAAAGAATG	22

*primer from soybean

Appendix 2: Second set of microsatellite makers developed from Roche 454 pyrosequencing technology used in bambara groundnut.

No.	Primer name	Sequence F	Length	Sequence R	Length
1	BN1	CACTTCCGACTCGCTCTGTT	20	ggcagtctcagCTCTTGCTT	20
2	BN4	AGCACCAACTACAGCAGCAA	20	GTAATCCCAGGAACTACCACCTC	23
3	BN6a	GAATGTTCCAGACACTACCCTGTT	24	GGTTACTCTGTTTGGCTTTGTTAG	24
4	BN6b	CACTACCCTGTTCTTCATCCGT	22	CATTGCACGTCATAGAATTTGG	22
5	BN10	GTTACGATGACCAATATGCTTCC	23	CTTGGACTTCGCATAACTTCCTT	23
6	BN11	aatacctaccgaccaacgacac	22	gtccgttcggctagttctca	20
7	BN19	ATGTCGCACTCTCCTTCACC	20	CTTACGCGTGGACTAACCAA	20
8	BN21	AGTTGCAATGCAGCAACAACAC	22	ACGCGTGGACTAACATGAGGA	21
9	BN24	ATCCCTAGCCAAATGACCTACC	22	GCTTTAGGTGTGGCGGATT	19
10	BN28	ACCTCCTTGGTAATACCTCTCTC	23	ggcagtctcagCTCTTGCTTAC	22
11	BN30	CAAACCAACGGAATGAGTTATG	22	ggcagtctcagCTCTTGCTTAC	22
12	BN37	GGAACAGAAACAGCAACAACC	21	acgcgtcgatgacaagaga	19
13	BN39	CAATGTTTTCACTCAGCAGCA	21	tcgttggtaGGAACCGAAAC	20
14	BN41	GGTTTGCAACCCTCGAATAA	20	acggtcgtcgactgacaagag	21
15	BN44	CATTCACAGCAGCAACACTACTT	23	CGTACGTCTACGCGTCTTCTC	21
16	BN53	CAGGCAGAGTATCAGCAGCA	20	ACTAACCCGACCTAGCCCATT	21
17	BN57	GTTCCAGACACTACCCTGTTCTTC	24	ggcagtctcagCTCTTGCTTAC	22
18	BN63	AGCGAGACGCAAGTTTCATTAC	22	ggcagtctcagCTCTTGCTTAC	22
19	BN64a	AGCGAGACGCAAGTTTCATTAC	22	GTGGACGTACGCGAGGAG	18
20	BN64b	AGCGAGACGCAAGTTTCATTAC	22	gtcgagtctcagCTCTTGTCTTAC	24
21	BN67a	AACAACCTTCACAACTACAACCAC	24	CCAGGCCCTGTTACATCAA	19
22	BN67b	CACACCCTTAATGATACAACCA	22	TGGACTAACAGATGGAAGTGAA	22
23	BN145	GGCACTGGTAGCAACGAAA	19	CGTGGACGTAACAACACAACAC	22
24	BN151a	GGCACTGGTAGCAACGAAA	19	CGTCGGACGTAACAACAACA	20
25	BN151b	ATCGCTTCCACCGTAGAAAGTAG	23	TCGGACGTAACAACAACAACAC	22
26	BN164	ACCGGGAATTCTGGCACT	18	cctaccctcccttcggtgt	19
27	BN167	CTGGTAGCAACGAAAAGGGTAA	23	TCTACACTCTCTCTTCTGTCGTCTTC	26
28	BN168a	ACATTCACAGCAGCAACACTACTT	24	CTCTACACACTTACCTTCCGGTTC	24
29	BN168b	CACTACTTTGCCACATTTTCCA	23	TCTACACACTTACCTTCCGGTTC	23
30	BN169	ATCGCTTCCACCGTAGAAAGTAG	23	GTCTCTCGCTCTTCGTCTCGT	21
31	BN206	GGTTTCCATTCATACAAGCCAT	22	GGCAGTCTCAGCTCTTGCTTAC	22
32	BN207	GATTGGAGGGAATGAATGTGAT	22	GGACTACAGTGCTGGTTGCAT	21
33	BN209	GTCATTGTGAGATCCTTTTCCA	22	GTACGCTCGTCTCGTCGTC	20
34	BN249	CCTCACTTGAACGAAGCTAGGA	22	GAAACGAACGGACGAAGAAG	20
35	BN259	CGATTGCACGTCATAGAATTTG	22	GTTCCAGACACTACCCTCGTTC	22
36	BN260	AGGGAGCGTGGCAATGTAT	19	ACGCCTTCGTTCGTTTACC	19
37	BN275	CTTGCATTTCTATGAGCAGTGG	23	CTATCCCCTCGTGTGCCTT	19

Appendix 3: Microsatellite makers developed from deep sequencing of bambara groundnut.

No.	Primer name	Sequence F	Length	Sequence R	Length
1	D.5953	TCAATTCAAGACTTCCTGCTTTC	23	TAGGGTTTTTATTAGGCACTCTGC	24
2	D.15508	AACTGTATGTAGCAGGGCTTCG	22	GGAGATAGAGAGCTACAGAGAATCG	25
3	D.25551	GCACACAACATAACATAAGGGACAC	25	GTTGAAAACCGAACTCATCATTCTC	25
4	D.36186	GGCGTTTCGATTTCTTCGACTAT	23	GAATATGACCTCAGAGCCATTCCT	24
5	D.42026	TCGTGAAGAATGATTGTCTTTGAT	24	TCTATTCAGCTGTTGCTACTCTGG	24
6	D.8148	CAAACAGTTGGTGTACTTGAATGG	24	CAATTAACAAGTTCCATGATGTGC	24
7	D.11587	TTGAACATTGGAGTTTCTCTTGGT	24	AGAAAGGTCCAACACCTTCTCTTTC	25
8	D.8999	GTGTTATCCGGTATTCGTCAAAAC	24	GTCCCTTCAACCTCTCATCTTCC	23
9	D.21950	ACAGCAGAAACCCTAATTTGTGAG	24	AACCAAATTTCACTCTCCTTTTCAC	25
10	D.37053	AAGCTTCTTAGCCCAGAACAGCTA	24	GTCATCCTATTTCTCCCACATCGT	24
11	D.12522	AGAGGTTGAAGAGGCATAGAGTTG	24	ACCACTAACCCAATACAATAGATGG	25
12	D.14265	GGCGAAGTGATTATTATATAAGATTAGG	28	ATCAGAATATCTTCGTTCGAAACTC	25
13	D.15619	GTGTTCAGGCAAAATCCATGTG	22	ACAAAATTTCTTTGGTGGGTCTCT	24
14	D.16501	TCATAGAGGAAGTTGAGGAACTGG	24	TCATCATCATTCATCCAATAACAG	24
15	D. 21310	GTTTTGATTTCAAAGGGATTATCAC	25	ACAACTGAGATTGCAGAAACGC	22
16	D.24269	AGGTTCATGATCGTAGATGTGGAT	24	ACGATATCATACTGACATGTTTCATAC	27
17	D.32937	GGTGAAAATCAGCAGTACCGAAT	23	GTTTCGTCACCATTACCCTCTC	22
18	D.35497	ACTTTTAGCTCTTGTCAGGAAACG	24	TCTTTCTACTTTTCTCTGGCTGGT	24
19	D.48339	CTGACCCATACATATCCATCATCA	24	AGATAGTTCTTTCCACTGCCATGC	24
20	D.51646	CGGCAATATAATTCAAAATCACTATG	26	GTGGAGTTTGAAGAAAACGGATCT	24
21	D.655	CTTCAATCCCAAAGTCAATTTCTG	24	CACATTTTCAACTCTGTTTCGTCA	24
22	D.1006	CTCAGTTTCACCACTCTCCAAAAC	24	CATCAGCATTACCAAGAATGACC	23
23	D.7215	ATCAAGGGTTTGAGGAGTTGAAAT	24	GGTGAAGTGAGTGTGGAAAAAGTT	24
24	D.8387	CAATGTTGTTGTTGTTATGGTGGT	24	CTAGTCCATTGTTGTCCCAAAATC	24
25	D.11860	GAAATCAACATCCAAACACATGAA	24	ACAAAATCACACACAAAACACTCG	24
26	D.125	CCACAAGTACCTAACTCCCAGCTA	24	TCTGACTCTACCGTTTTAGGTTGC	24
27	D.1050	ACAGATGCTTAGAAGATGGAGCTG	24	TTTTCAGGCTCTATTTTTGTCCTG	24
28	D.1591	TTGCAGAAAAATTACAGAAACCAG	24	TTATTTACTCGTTTCCCCAATCAA	24
29	D.2094	ACTCCTGGAAGACGAGTCTGTG	22	GTTCACACATGGTGCTAAATTGAA	24

Appendix 4: Normality plot for leaf area in the F$_3$ population of the field data.

Field trial

Average: 672.182
StDev: 329.697
N: 70

Anderson-Darling Normality Test
A-Squared: 0.326
P-Value: 0.514
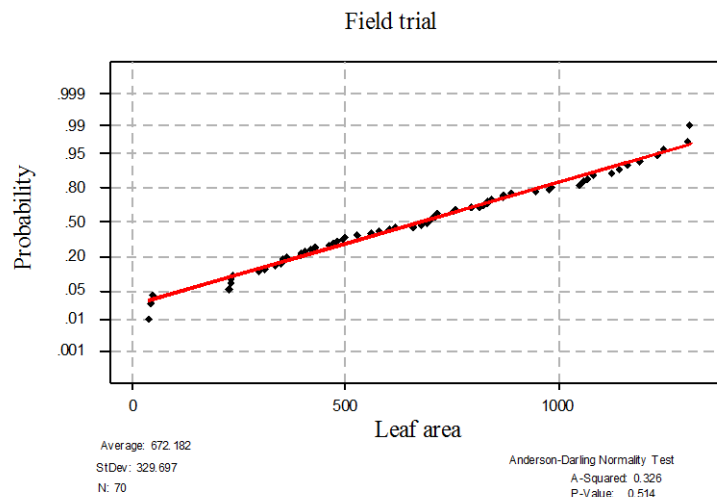
Appendix 5: Histogram of normal distribution for internode length in the glasshouse.
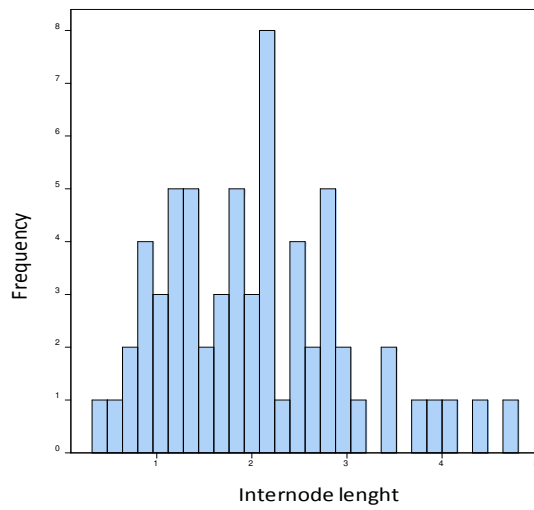
Appendix 6: Histogram of normal distribution for internode length in the field.
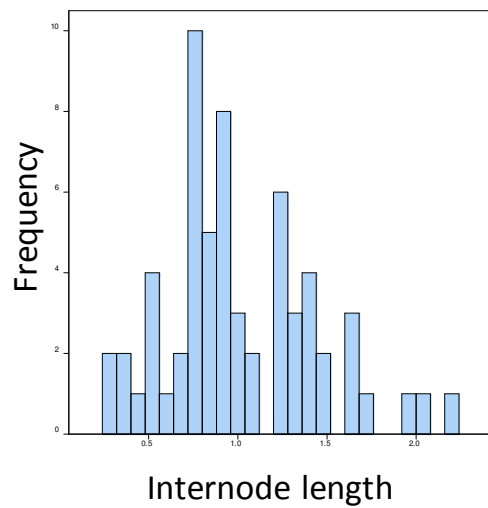
Appendix 7: Histogram of non-normal distribution for growth habit in the glasshouse.
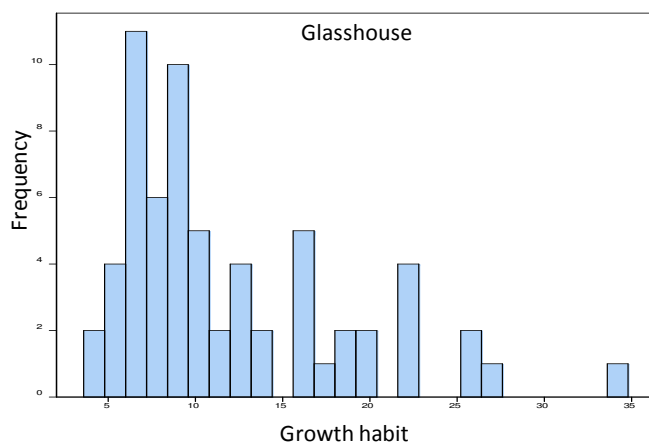
Appendix 8: Histogram of non-normal distribution of growth habit in the Field.
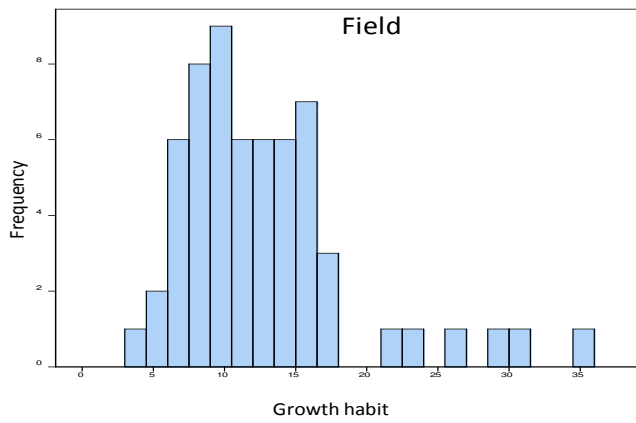
Appendix 9: Normality plot for pod no./plant in the F$_3$ population of the glasshouse.
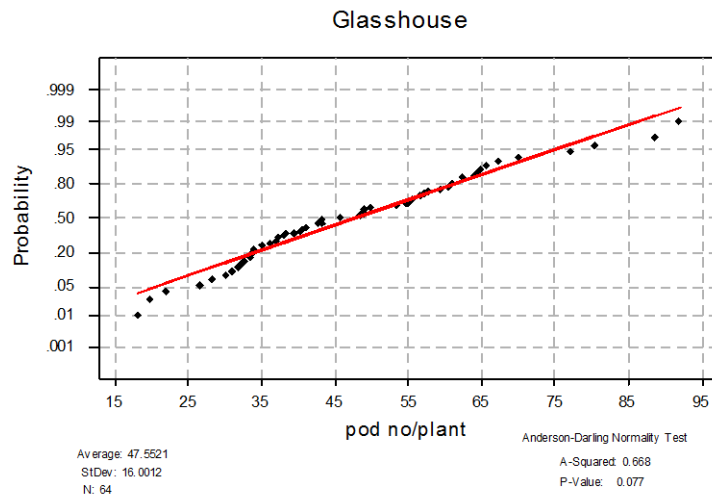
Appendix 10: Normality plot for pod no./plant (transformed) in the F$_3$ population of the field.
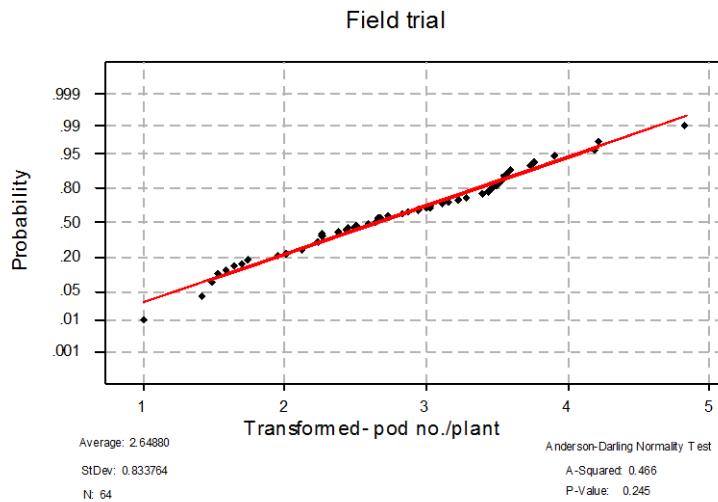
Appendix 11: Normality plot for double seeded pods/plant (transformed) in the F_3 population of the glasshouse.
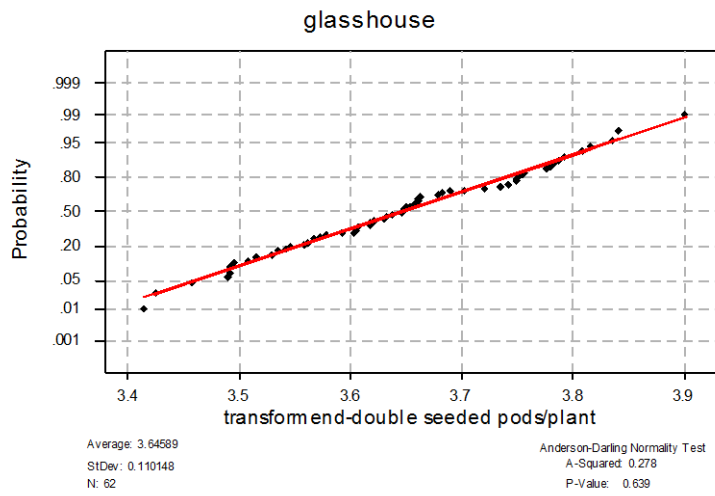
Appendix 12: Normality plot for seed weight (transformed) in the F_3 population of the glasshouse.
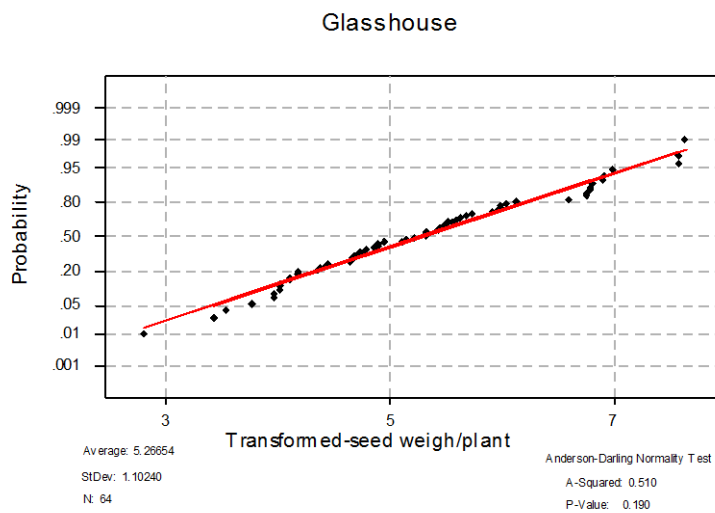
Appendix 13: Histogram of normal distribution of the length and width of terminal leaflet for F_2 progenies data in TCRU.
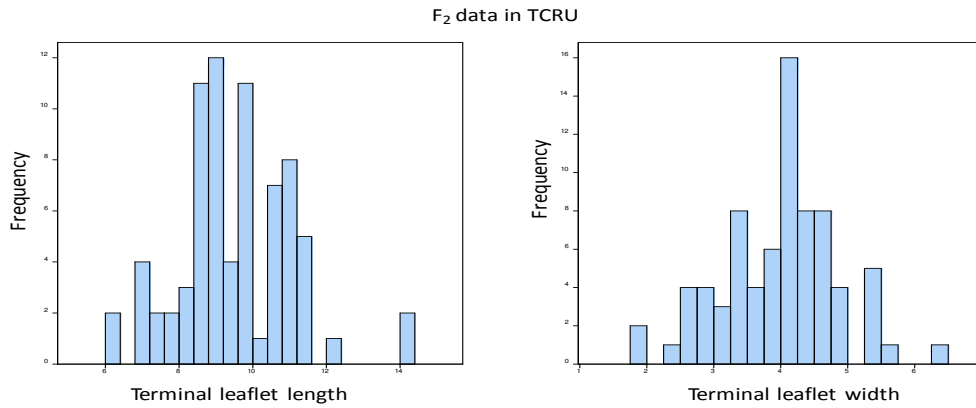
Appendix 14: Normality plot for seed no./plant (transformed) in the F_2 population grown in TCRU.
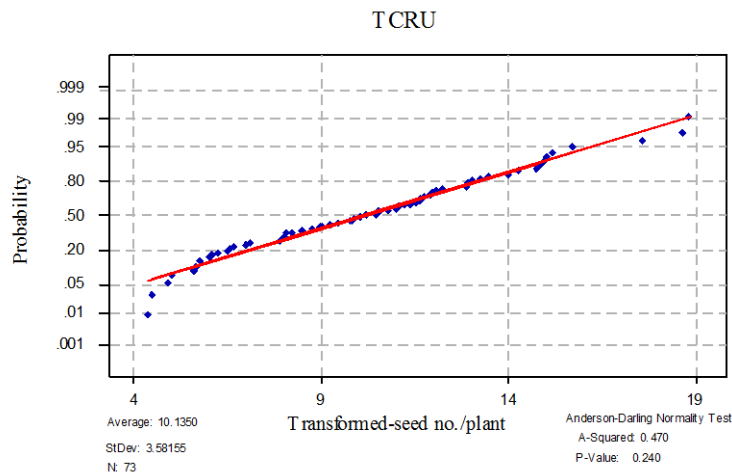
Appendix 15: A fitted line regression plot of flower no./plat with both plant spread and biomass dry weight in F_3 population of glasshouse.

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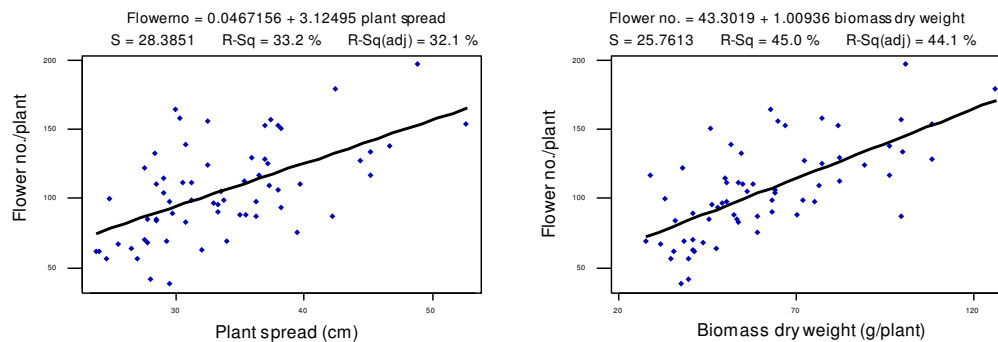
Appendix 16: A fitted line regression plot of plant spread with both growth habit and biomass dry weight in F₃ population of glasshouse.

plant spread = 40.8135 - 0.595520 growth habit

S = 5.05183   R-Sq = 37.8 %   R-Sq(adj) = 36.8 %

plant spread = 20.8257 + 0.209137 biomass dry weight

S = 4.21055   R-Sq = 56.8 %   R-Sq(adj) = 56.1 %
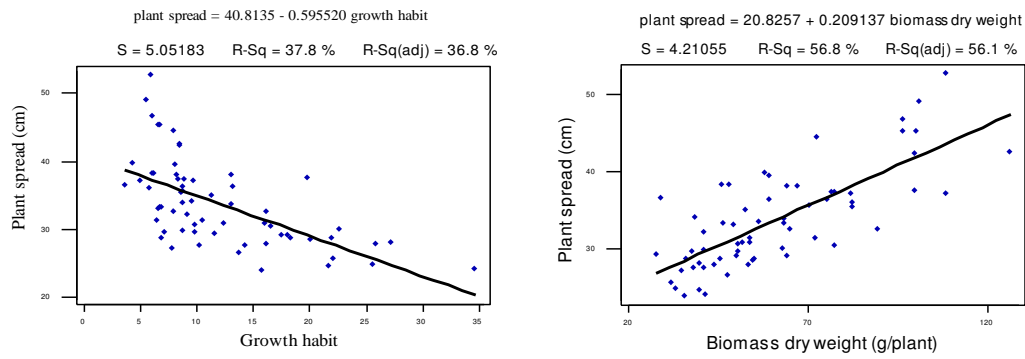
Appendix 17: A fitted line regression plot of pod no./plant with both of node no./stem and seed weight in F₃ population of glasshouse.

Podno./plant = 1.75814 + 5.28875 node no./stem

S = 11.1867   R-Sq = 51.9 %   R-Sq(adj) = 51.1 %

Podno./plant = 13.6861 + 1.17051 seed weight/plant

S = 7.64473   R-Sq = 77.5 %   R-Sq(adj) = 77.2 %
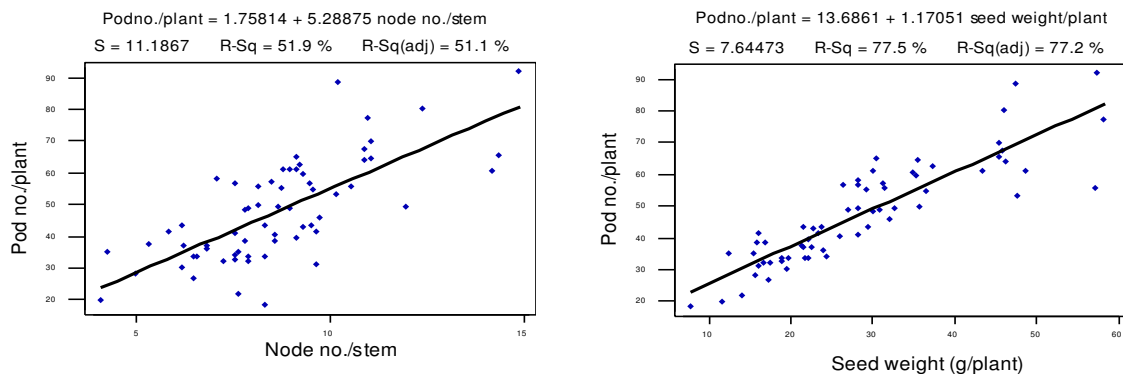
Appendix 18: A fitted line regression plot for plant height with biomass dry weight in the F₃ population of the Field.

Plant height = 8.55713 + 0.753757 biomass dry weight

S = 2.18371   R-Sq = 57.4 %   R-Sq(adj) = 56.7 %
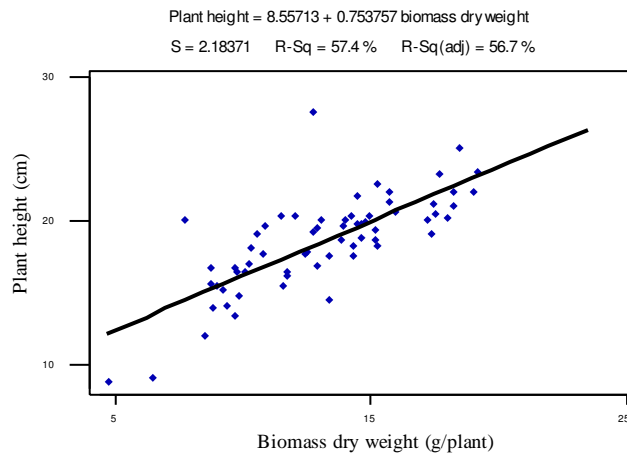
Appendix 19: A fitted line regression plot for internode length with growth habit and seed weight in the F$_3$ population of the field.

Internode length = 1.70899 - 0.0531969 growth habit

S = 0.257562   R-Sq = 63.5 %   R-Sq(adj) = 62.9 %

Internode length = 0.756156 + 0.0867348 seed weight

S = 0.379230   R-Sq = 15.4 %   R-Sq(adj) = 13.8 %
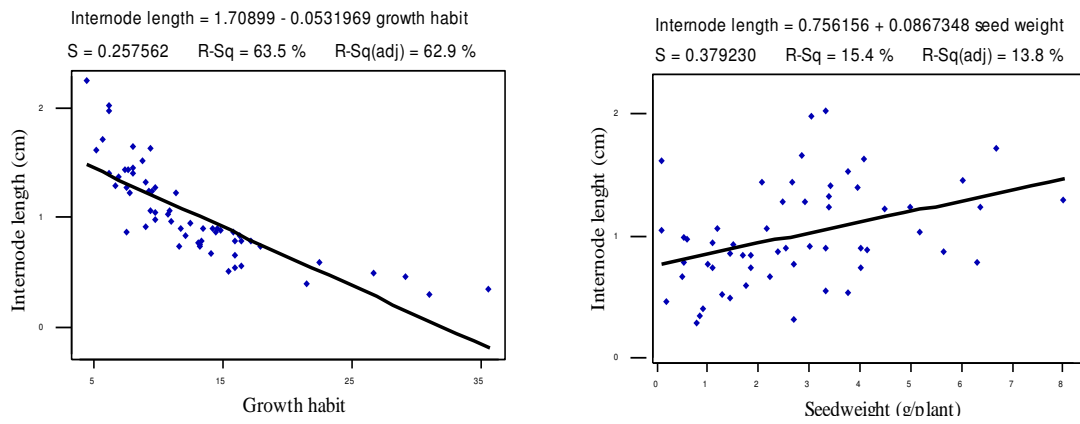
Appendix 20: A fitted line regression plot for seed weight with pod weight in the F$_3$ population of the Field.

Regression Plot

Seed weight = 0.0063412 + 0.650766 pod weight

S = 0.837295   R-Sq = 79.9 %   R-Sq(adj) = 79.6 %

Appendix 21: A fitted line regression plot for plant spread with biomass dry weight in the F_2 population of narrow cross.

Biomass dry weight = -140.088 + 4.26199 plant spread

S = 39.8138   R-Sq = 65.1 %   R-Sq(adj) = 64.6 %
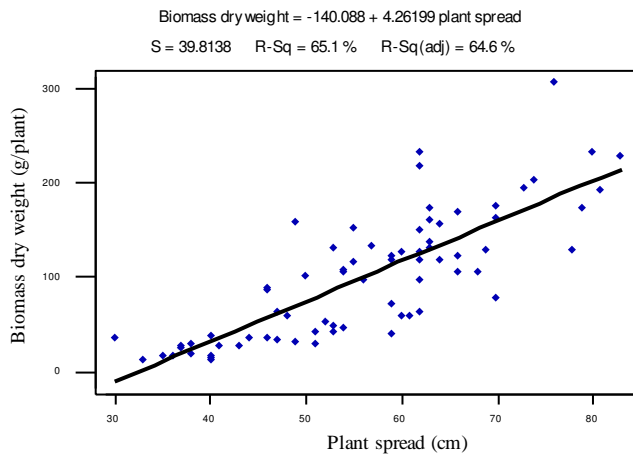
Appendix 22: A fitted line regression plot for growth habit with plant spread in the F_2 population of narrow cross.

Seed weight = -58.0990 + 1.74037 Plant spread

S = 17.3631   R-Sq = 62.1 %   R-Sq(adj) = 61.5 %
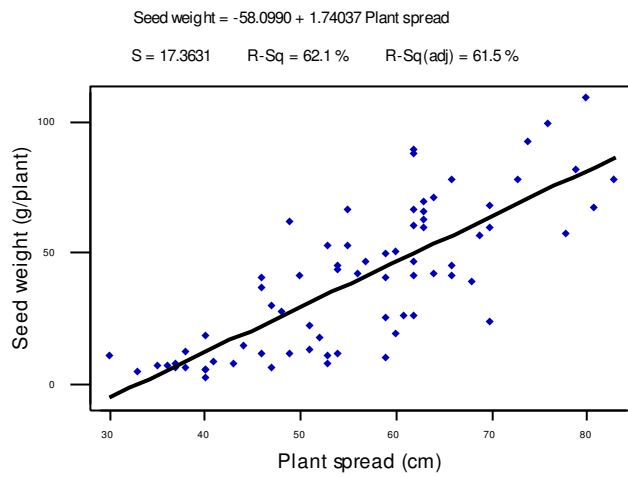
Appendix 23: The locus genotype frequency for narrow cross map after phase detection.

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
bgPabg-594562	25	0	0	42	0	6	0	1	-	[a:h+b+c]
bgPabg-595666	25	0	0	42	0	6	0	1	-	[a:h+b+c]
bgPt-594335	25	0	0	41	0	7	0	1	-	[a:h+b+c]
bgPt-597197	22	0	0	37	0	14	0	1	-	[a:h+b+c]
bgPt-602174	22	0	0	37	0	14	0	1	-	[a:h+b+c]
bgPt-598456	24	0	0	41	0	8	0.01	1	-	[a:h+b+c]
bgPt-598510	21	0	0	36	0	16	0.01	1	-	[a:h+b+c]
bgPt-598594	21	0	0	36	0	16	0.01	1	-	[a:h+b+c]
bgPt-600863	21	0	0	36	0	16	0.01	1	-	[a:h+b+c]
bgPabg-595315	25	0	0	43	0	5	0.02	1	-	[a:h+b+c]
bgPabg-597801	26	0	0	42	0	5	0.02	1	-	[a:h+b+c]
bgPabg-594305	23	0	0	40	0	10	0.03	1	-	[a:h+b+c]
bgPabg-596075	27	0	0	43	0	3	0.03	1	-	[a:h+b+c]
bgPt-596460	23	0	0	40	0	10	0.03	1	-	[a:h+b+c]
bgPt-601402	22	0	0	35	0	16	0.03	1	-	[a:h+b+c]
bgPabg-423257	24	0	0	42	0	7	0.04	1	-	[a:h+b+c]
bgPt-601656	0	0	21	0	33	19	0.04	1	-	[a+h+d:b]
bgPabg-593892	25	0	0	44	0	4	0.05	1	-	[a:h+b+c]
bgPabg-595273	25	0	0	44	0	4	0.05	1	-	[a:h+b+c]
bgPt-594531	20	0	0	31	0	2	0.06	1	-	[a:h+b+c]
bgPabg-597632	23	0	0	41	0	9	0.07	1	-	[a:h+b+c]
bgPabg-601173	23	0	0	41	0	9	0.07	1	-	[a:h+b+c]
bgPt-593893	23	0	0	41	0	9	0.07	1	-	[a:h+b+c]
bgPt-598647	0	0	25	0	39	9	0.07	1	-	[a+h+d:b]
bgPabg-422614	27	0	0	42	0	4	0.08	1	-	[a:h+b+c]
DSRB18	28	17	27	0	0	1	0.09	2	-	[a:h:b]
bgPt-598428	24	0	0	37	0	12	0.09	1	-	[a:h+b+c]
bgPabg-422333	26	0	0	40	0	7	0.1	1	-	[a:h+b+c]
bgPt-596097	22	0	0	40	0	11	0.11	1	-	[a:h+b+c]
bgPt-596596	25	0	0	38	0	10	0.13	1	-	[a:h+b+c]
bgPabg-594981	27	0	0	41	0	5	0.14	1	-	[a:h+b+c]
bgPabg-596210	27	0	0	41	0	5	0.14	1	-	[a:h+b+c]
bgPabg-596646	27	0	0	41	0	5	0.14	1	-	[a:h+b+c]
bgPabg-597746	27	0	0	41	0	5	0.14	1	-	[a:h+b+c]
bgPt-600898	0	0	20	0	37	16	0.14	1	-	[a+h+d:b]
bgPabg-594814	28	0	0	42	0	3	0.19	1	-	[a:h+b+c]
bgPabg-595707	28	0	0	42	0	3	0.19	1	-	[a:h+b+c]
bgPt-594664	23	0	0	43	0	7	0.2	1	-	[a:h+b+c]
bgPt-601022	23	0	0	34	0	16	0.2	1	-	[a:h+b+c]
bgPt-601091	19	0	0	36	0	1	0.2	1	-	[a:h+b+c]
bgPabg-595725	24	0	0	45	0	4	0.22	1	-	[a:h+b+c]
bgPabg-597705	27	0	0	40	0	6	0.22	1	-	[a:h+b+c]
bgPabg-597860	27	0	0	40	0	6	0.22	1	-	[a:h+b+c]
bgPt-593939	27	0	0	40	0	6	0.22	1	-	[a:h+b+c]
bgPabg-594999	0	0	24	0	45	4	0.22	1	-	[a+h+d:b]
bgPabg-594261	0	0	24	0	35	14	0.25	1	-	[a+h+d:b]
bgPt-596392	26	0	0	38	0	9	0.27	1	-	[a:h+b+c]
bgPt-600935	26	0	0	38	0	9	0.27	1	-	[a:h+b+c]
bgPabg-595685	0	0	22	0	42	9	0.27	1	-	[a+h+d:b]
bgPabg-597086	28	0	0	41	0	4	0.28	1	-	[a:h+b+c]
bgPt-598511	18	0	0	35	0	2	0.28	1	-	[a:h+b+c]
bgPt-597279	0	0	19	0	37	17	0.3	1	-	[a+h+d:b]
bgPabg-597623	20	0	0	39	0	14	0.33	1	-	[a:h+b+c]
bgPt-598641	20	0	0	39	0	14	0.33	1	-	[a:h+b+c]
bgPt-600823	0	0	20	0	39	14	0.33	1	-	[a+h+d:b]
bgPabg-598428	22	0	0	43	0	8	0.37	1	-	[a:h+b+c]
PRIMER10	25	20	28	0	0	0	0.39	2	-	[a:h:b]
bgPabg-595682	0	0	24	0	47	2	0.41	1	-	[a+h+d:b]

Appendix 23 (continued).

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
bgPt-601748	19	0	0	38	0	16	0.42	1	-	[a:h+b+c]
bgPt-602039	0	0	19	0	38	16	0.42	1	-	[a+h+d:b]
bgPt-596705	21	0	0	29	0	23	0.43	1	-	[a:h+b+c]
bgPabg-594467	27	0	0	38	0	8	0.45	1	-	[a:h+b+c]
bgPabg-596244	0	0	27	0	38	8	0.45	1	-	[a+h+d:b]
bgPabg-597436	22	0	0	44	0	7	0.49	1	-	[a:h+b+c]
bgPabg-597991	26	0	0	36	0	11	0.52	1	-	[a:h+b+c]
bgPt-597945	0	0	24	0	33	16	0.52	1	-	[a+h+d:b]
bgPt-598364	18	0	0	37	0	18	0.53	1	-	[a:h+b+c]
bgPabg-595854	29	0	0	40	0	4	0.6	1	-	[a:h+b+c]
bgPabg-596250	0	0	23	0	47	3	0.64	1	-	[a+h+d:b]
bgPt-423805	0	0	17	0	36	20	0.67	1	-	[a+h+d:b]
PRIMER66	24	20	29	0	0	0	0.68	2	-	[a:h:b]
bgPt-598651	26	0	0	35	0	12	0.68	1	-	[a:h+b+c]
bgPt-602268	26	0	0	35	0	12	0.68	1	-	[a:h+b+c]
bgPt-597632	18	0	0	38	0	17	0.69	1	-	[a:h+b+c]
bgPt-602071	22	0	0	29	0	22	0.69	1	-	[a:h+b+c]
bgPabg-423248	0	0	24	0	32	17	0.69	1	-	[a+h+d:b]
bgPt-596678	0	0	24	0	32	17	0.69	1	-	[a+h+d:b]
bgPt-601384	20	0	0	42	0	11	0.73	1	-	[a:h+b+c]
bgPabg-598184	21	0	0	44	0	8	0.75	1	-	[a:h+b+c]
Bam2coL63	25	17	31	0	0	0	0.77	2	-	[a:h:b]
PRIMER103	29	19	23	0	0	2	0.79	2	-	[a:h:b]
bgPt-598122	17	0	0	37	0	19	0.83	1	-	[a:h+b+c]
PRIMER88	28	21	24	0	0	0	0.84	2	-	[a:h:b]
bgPt-597446	18	0	0	39	0	16	0.85	1	-	[a:h+b+c]
bgPt-422660	0	0	24	0	31	18	0.88	1	-	[a+h+d:b]
bgPabg-422621	20	0	0	43	0	10	0.89	1	-	[a:h+b+c]
bgPabg-594142	21	0	0	45	0	7	0.91	1	-	[a:h+b+c]
bgPt-423432	0	0	25	0	32	16	0.98	1	-	[a+h+d:b]
bgPt-598804	17	0	0	38	0	18	1.02	1	-	[a:h+b+c]
bgPabg-596040	30	0	0	39	0	4	1.05	1	-	[a:h+b+c]
bgPt-595486	28	0	0	36	0	9	1.07	1	-	[a:h+b+c]
bgPt-597731	20	0	0	44	0	9	1.07	1	-	[a:h+b+c]
bgPabg-423332	0	0	20	0	44	9	1.07	1	-	[a+h+d:b]
bgPabg-596546	0	0	28	0	36	9	1.07	1	-	[a+h+d:b]
bgPabg-596618	21	0	0	46	0	6	1.08	1	-	[a:h+b+c]
bgPabg-598364	26	0	0	33	0	14	1.09	1	-	[a:h+b+c]
bgPt-598084	0	0	26	0	33	14	1.09	1	-	[a+h+d:b]
bgPt-597858	29	0	0	37	0	7	1.17	1	-	[a:h+b+c]
bgPabg-422590	0	0	29	0	37	7	1.17	1	-	[a+h+d:b]
bgPt-422567	27	0	0	34	0	12	1.19	1	-	[a:h+b+c]
GH-19-B2-D9	23	19	31	0	0	0	1.21	2	-	[a:h:b]
bgPt-598767	25	0	0	31	0	17	1.22	1	-	[a:h+b+c]
bgPabg-597446	19	0	0	43	0	11	1.24	1	-	[a:h+b+c]
bgPabg-593983	0	0	30	0	38	5	1.27	1	-	[a+h+d:b]
bgPabg-596774	0	0	30	0	38	5	1.27	1	-	[a+h+d:b]
bgPabg-597624	0	0	30	0	38	5	1.27	1	-	[a+h+d:b]
bgPabg-423067	0	0	28	0	35	10	1.3	1	-	[a+h+d:b]
bgPabg-422473	0	0	29	0	36	8	1.4	1	-	[a+h+d:b]
PRIMER48	32	15	26	0	0	0	1.43	2	-	[a:h:b]
bgPt-596563	17	0	0	40	0	16	1.43	1	-	[a:h+b+c]
bgPt-597606	16	0	0	38	0	19	1.43	1	-	[a:h+b+c]
bgPt-601098	16	0	0	38	0	19	1.43	1	-	[a:h+b+c]
bgPt-596916	19	0	0	44	0	10	1.45	1	-	[a:h+b+c]
bgPt-598621	19	0	0	44	0	10	1.45	1	-	[a:h+b+c]
bgPt-594453	20	0	0	46	0	7	1.46	1	-	[a:h+b+c]
bgPt-596188	20	0	0	46	0	7	1.46	1	-	[a:h+b+c]
bgPt-596726	20	0	0	46	0	7	1.46	1	-	[a:h+b+c]
PRIMER15	28	22	23	0	0	0	1.48	2	-	[a:h:b]
bgPabg-595822	30	0	0	37	0	6	1.51	1	-	[a:h+b+c]

Appendix 23 (continued).

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
bgPabg-594335	31	0	0	38	0	4	1.62	1	-	[a:h+b+c]
bgPabg-597858	31	0	0	38	0	4	1.62	1	-	[a:h+b+c]
bgPabg-598611	20	0	0	47	0	6	1.67	1	-	[a:h+b+c]
bgPt-595093	19	0	0	45	0	9	1.67	1	-	[a:h+b+c]
bgPt-597667	19	0	0	45	0	9	1.67	1	-	[a:h+b+c]
bgPt-598184	14	0	0	35	0	24	1.67	1	-	[a:h+b+c]
bgPt-600818	19	0	0	45	0	9	1.67	1	-	[a:h+b+c]
bgPabg-596988	21	0	0	49	0	3	1.68	1	-	[a:h+b+c]
mBam3co7	22	21	30	0	0	0	1.72	2	-	[a:h:b]
PRIMER32	32	14	27	0	0	0	1.78	2	-	[a:h:b]
bgPt-595129	28	0	0	33	0	12	1.84	1	-	[a:h+b+c]
BN6b	22	19	32	0	0	0	1.87	2	-	[a:h:b]
bgPabg-594355	20	0	0	48	0	5	1.9	1	-	[a:h+b+c]
bgPt-600827	18	0	0	44	0	11	1.9	1	-	[a:h+b+c]
bgPabg-596335	0	0	31	0	37	5	1.9	1	-	[a+h+d:b]
bgPt-595387	0	0	26	0	30	17	1.9	1	-	[a+h+d:b]
bgPt-597573	0	0	18	0	44	11	1.9	1	-	[a+h+d:b]
PRIMER43	25	15	33	0	0	0	1.94	2	-	[a:h:b]
bgPabg-423420	0	0	29	0	34	10	1.96	1	-	[a+h+d:b]
bgPabg-422461	0	0	30	0	35	8	2.08	1	-	[a+h+d:b]
bgPabg-423053	0	0	30	0	35	8	2.08	1	-	[a+h+d:b]
bgPt-601853	0	0	17	0	43	13	2.15	1	-	[a+h+d:b]
PRIMER26	24	15	33	0	0	1	2.17	2	-	[a:h:b]
bgPt-598507	15	0	0	39	0	19	2.18	1	-	[a:h+b+c]
bgPabg-423334	0	0	31	0	36	6	2.2	1	-	[a+h+d:b]
bgPt-597491	26	0	0	29	0	18	2.24	1	-	[a:h+b+c]
bgPabg-422379	0	0	29	0	33	11	2.28	1	-	[a+h+d:b]
bgPabg-422586	0	0	29	0	33	11	2.28	1	-	[a+h+d:b]
bgPabg-596678	0	0	29	0	33	11	2.28	1	-	[a+h+d:b]
BamcoL17	31	21	21	0	0	0	2.38	2	-	[a:h:b]
bgPt-596444	30	0	0	34	0	9	2.4	1	-	[a:h+b+c]
bgPt-597962	17	0	0	44	0	12	2.41	1	-	[a:h+b+c]
bgPt-598611	17	0	0	44	0	12	2.41	1	-	[a:h+b+c]
PRIMER7	24	24	25	0	0	0	2.43	2	-	[a:h:b]
bgPabg-593965	0	0	33	0	38	2	2.44	1	-	[a+h+d:b]
BN259	23	16	34	0	0	0	2.58	2	-	[a:h:b]
bgPabg-594537	29	0	0	32	0	12	2.62	1	-	[a:h+b+c]
bgPabg-594877	19	0	0	49	0	5	2.65	1	-	[a:h+b+c]
bgPt-594877	18	0	0	47	0	8	2.67	1	-	[a:h+b+c]
PRIMER16	27	12	32	0	0	2	2.95	2	-	[a:h:b]
PRIMER38	30	23	20	0	0	0	3.47	2	-	[a:h:b]
PRIMER19	19	21	33	0	0	0	4.13	2	-	[a:h:b]
PRIMER95	19	23	31	0	0	0	4.28	2	-	[a:h:b]
mBam3co33	21	16	36	0	0	0	4.48	2	-	[a:h:b]
bgPabg-423185	0	0	40	0	24	9	17.07	1	*******	[a+h+d:b]
bgPt-596933	0	0	48	0	10	15	50.69	1	*******	[a+h+d:b]
bgPabg-596933	0	0	54	0	12	7	55.31	1	*******	[a+h+d:b]
bgPt-423050	0	0	49	0	8	16	57.12	1	*******	[a+h+d:b]
bgPt-601086	0	0	44	0	4	25	60.09	1	*******	[a+h+d:b]
bgPabg-594508	0	0	58	0	12	3	61.44	1	*******	[a+h+d:b]
bgPabg-423807	0	0	54	0	9	10	62.49	1	*******	[a+h+d:b]
bgPabg-596655	0	0	53	0	8	12	63.48	1	*******	[a+h+d:b]
bgPabg-601086	0	0	53	0	7	13	66.15	1	*******	[a+h+d:b]
bgPabg-595038	0	0	60	0	10	3	69.43	1	*******	[a+h+d:b]
bgPt-596221	39	0	0	27	0	7	13.13	1	******	[a:h+b+c]
bgPt-423527	7	0	0	42	0	24	11.27	1	*****	[a:h+b+c]
bgPt-597457	11	0	0	53	0	9	11.27	1	*****	[a:h+b+c]
bgPabg-422515	0	0	35	0	29	9	8.07	1	****	[a+h+d:b]
bgPt-598223	12	0	0	49	0	12	8.27	1	****	[a:h+b+c]
bgPt-596528	0	0	11	0	47	15	8.5	1	****	[a+h+d:b]
bgPt-602242	10	0	0	47	0	16	9.69	1	****	[a:h+b+c]

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Appendix 23 (continued).

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
AG81	39	8	26	0	0	0	10.76	2	****	[a:h:b]
bgPabg-423727	0	0	35	0	31	7	6.79	1	***	[a+h+d:b]
bgPabg-597688	13	0	0	48	0	12	6.82	1	***	[a:h+b+c]
bgPt-598698	11	0	0	44	0	18	7.19	1	***	[a:h+b+c]
bgPabg-423557	0	0	34	0	29	10	7.29	1	***	[a+h+d:b]
bgPt-594258	0	0	16	0	47	10	3.94	1	**	[a+h+d:b]
bgPabg-594494	0	0	18	0	52	3	4.15	1	**	[a+h+d:b]
bgPabg-423556	0	0	32	0	32	9	4.27	1	**	[a+h+d:b]
bgPt-594658	0	0	16	0	48	9	4.27	1	**	[a+h+d:b]
bgPt-595005	0	0	32	0	32	9	4.27	1	**	[a+h+d:b]
bgPabg-596205	33	0	0	33	0	7	4.4	1	**	[a:h+b+c]
bgPabg-423121	0	0	33	0	33	7	4.4	1	**	[a+h+d:b]
bgPt-598296	14	0	0	44	0	15	4.42	1	**	[a:h+b+c]
bgPabg-594288	0	0	18	0	53	2	4.47	1	**	[a+h+d:b]
bgPt-598407	13	0	0	42	0	18	4.51	1	**	[a:h+b+c]
bgPt-598598	15	0	0	47	0	11	4.68	1	**	[a:h+b+c]
bgPt-597688	32	0	0	31	0	10	4.75	1	**	[a:h+b+c]
bgPabg-423378	0	0	32	0	31	10	4.75	1	**	[a+h+d:b]
bgPabg-423708	0	0	32	0	31	10	4.75	1	**	[a+h+d:b]
bgPt-598212	14	0	0	45	0	14	4.77	1	**	[a:h+b+c]
bgPt-600914	16	0	0	50	0	7	4.95	1	**	[a:h+b+c]
bgPabg-597113	34	0	0	33	0	6	5.02	1	**	[a:h+b+c]
bgPt-600845	15	0	0	48	0	10	5.04	1	**	[a:h+b+c]
bgPt-602273	14	0	0	46	0	13	5.14	1	**	[a:h+b+c]
bgPabg-422619	0	0	31	0	29	13	5.14	1	**	[a+h+d:b]
bgPt-598513	15	0	0	49	0	9	5.4	1	**	[a:h+b+c]
bgPabg-423430	0	0	34	0	32	7	5.53	1	**	[a+h+d:b]
bgPt-594435	31	0	0	28	0	14	5.7	1	**	[a:h+b+c]
bgPabg-594180	0	0	33	0	30	10	5.95	1	**	[a+h+d:b]
bgPabg-595799	15	0	0	51	0	7	6.15	1	**	[a:h+b+c]
bgPt-597377	30	0	0	26	0	17	6.17	1	**	[a:h+b+c]
bgPt-601401	12	0	0	44	0	17	6.17	1	**	[a:h+b+c]
bgPt-600790	32	0	0	28	0	13	6.42	1	**	[a:h+b+c]
bgPabg-595118	15	0	0	52	0	6	6.53	1	**	[a:h+b+c]
bgPabg-600828	0	0	33	0	29	11	6.54	1	**	[a+h+d:b]
PRIMER2	17	25	31	0	0	0	6.91	2	**	[a:h:b]
PRIMER45	18	27	28	0	0	0	7.42	2	**	[a:h:b]
bgPabg-423527	16	0	0	43	0	14	2.71	1	*	[a:h+b+c]
bgPabg-422458	0	0	30	0	33	10	2.75	1	*	[a+h+d:b]
bgPt-601852	14	0	0	39	0	20	2.78	1	*	[a:h+b+c]
bgPabg-423147	0	0	31	0	34	8	2.88	1	*	[a+h+d:b]
bgPt-597010	17	0	0	46	0	10	2.97	1	*	[a:h+b+c]
bgPt-597832	17	0	0	46	0	10	2.97	1	*	[a:h+b+c]
bgPabg-423618	0	0	29	0	31	13	3	1	*	[a+h+d:b]
bgPabg-422294	0	0	30	0	32	11	3.14	1	*	[a+h+d:b]
bgPabg-422618	0	0	30	0	32	11	3.14	1	*	[a+h+d:b]
bgPabg-422656	0	0	30	0	32	11	3.14	1	*	[a+h+d:b]
bgPabg-422657	0	0	30	0	32	11	3.14	1	*	[a+h+d:b]
bgPabg-423395	0	0	31	0	33	9	3.27	1	*	[a+h+d:b]
bgPt-595011	0	0	17	0	47	9	3.27	1	*	[a+h+d:b]
bgPt-598091	16	0	0	45	0	12	3.31	1	*	[a:h+b+c]
bgPabg-423122	0	0	30	0	31	12	3.55	1	*	[a+h+d:b]
bgPt-598289	0	0	17	0	48	8	3.57	1	*	[a+h+d:b]
bgPt-598809	15	0	0	44	0	14	3.67	1	*	[a:h+b+c]
bgPabg-423390	0	0	32	0	33	8	3.82	1	*	[a+h+d:b]
DSRB16	24	13	36	0	0	0	4.64	2	*	[a:h:b]
PRIMER37	21	26	26	0	0	0	4.84	2	*	[a:h:b]
PRIMER98	36	11	26	0	0	0	5.67	2	*	[a:h:b]
BN145	35	10	28	0	0	0	5.87	2	*	[a:h:b]

Appendix 24: The locus genotype frequency for wide cross map of phase determination

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
P1b251AAG+CTT4	25	0	0	73	0	0	0.01	1	-	[a:h+b+c]
P2b83AAC+CTA1	26	0	0	72	0	0	0.12	1	-	[a:h+b+c]
P2b213AAC+CTA2	26	0	0	72	0	0	0.12	1	-	[a:h+b+c]
P3b87AGC+CTC2	0	0.00	23	0	75.00	0	0.12	1	-	[a+h+d:b]
P3b99AGC+CTC3	25	0.00	0	73	0.00	0	0.01	1	-	[a:h+b+c]
P3b155AGC+CTC4	18	0.00	0	80	0.00	0	2.3	1	-	[a:h+b+c]
P3b157AGC+CTC5	0	0	25	0	73.00	0	0.01	1	-	[a+h+d:b]
P3b264AGC+CTC8	0	0	26	0	72	0	0.12	1	-	[a+h+d:b]
P4b224AGC+CAG	0	0	23	0	75	0	0.12	1	-	[a+h+d:b]
P5b93ATC+CTT1	0	0	27	0	71	0	0.34	1	-	[a+h+d:b]
P5b107ATC+CTT2	24	0	0	74	0	0	0.01	1	-	[a:h+b+c]
P5b191ATC+CTT3	0	0	19	0	79	0	1.65	1	-	[a+h+d:b]
P6b140AAC+CAG1	22	0	0	76	0	0	0.34	1	-	[a:h+b+c]
P6b187AAC+CAG2	26	0	0	72	0	0	0.12	1	-	[a:h+b+c]
P6b217AAC+CAG3	27	0	0	71	0	0	0.34	1	-	[a:h+b+c]
P7b333AAG+CAG1	21	0	0	77	0	0	0.67	1	-	[a:h+b+c]
P7b357AAG+CAG2	0	0	29	0	69	0	1.1	1	-	[a+h+d:b]
P8b270ACG+CTA1	0	0	30	0	68	0	1.65	1	-	[a+h+d:b]
P9b153AGA+CTA2	0	0	22	0	76	0	0.34	1	-	[a+h+d:b]
P9b201AGA+CTA3	0	0	20	0	78	0	1.1	1	-	[a+h+d:b]
P9b205AGA+CTA4	22	0	0	76	0	0	0.34	1	-	[a:h+b+c]
P9b221AGA+CTA5	0	0	21	0	77	0	0.67	1	-	[a+h+d:b]
P9b331AGA+CTA6	27	0	0	71	0	0	0.34	1	-	[a:h+b+c]
P10b130AAC+CTT1	0	0	23	0	75	0	0.12	1	-	[a+h+d:b]
P10b296AAC+CTT3	23	0	0	75	0	0	0.12	1	-	[a:h+b+c]
P12b374AAG+CTC2	19	0	0	79	0	0	1.65	1	-	[a:h+b+c]
P12b420AAG+CTC4	22	0	0	76	0	0	0.34	1	-	[a:h+b+c]
P14b305ATC+CTA2	0	0	22	0	76	0	0.34	1	-	[a+h+d:b]
P15b351AGT+CAA2	28	0	0	70	0	0	0.67	1	-	[a:h+b+c]
P15b646AGT+CAA3	0	0	21	0	77	0	0.67	1	-	[a+h+d:b]
P16b121AGA+CTC1	22	0	0	76	0	0	0.34	1	-	[a:h+b+c]
P16b329AGA+CTC2	19	0	0	79	0	0	1.65	1	-	[a:h+b+c]
P17b180ACG+CAA1	0	0	19	0	79	0	1.65	1	-	[a+h+d:b]
P17b240ACG+CAA2	20	0	0	78	0	0	1.1	1	-	[a:h+b+c]
P17b242ACG+CAA3	0	0	28	0	70	0	0.67	1	-	[a+h+d:b]
P18b186ACA+CAT1	0	0	28	0	70	0	0.67	1	-	[a+h+d:b]
P18b221ACA+CAT2	0	0	25	0	73	0	0.01	1	-	[a+h+d:b]
P18b225ACA+CAT3	0	0	20	0	78	0	1.1	1	-	[a+h+d:b]
P18b226ACA+CAT4	26	0	0	72	0	0	0.12	1	-	[a:h+b+c]
P19b102AGC+CTA1	0	0	27	0	71	0	0.34	1	-	[a+h+d:b]
P19b105AGC+CTA2	27	0	0	71	0	0	0.34	1	-	[a:h+b+c]
P20b127ACG+CAG1	22	0	0	76	0	0	0.34	1	-	[a:h+b+c]
P20b245ACG+CAG2	23	0	0	75	0	0	0.12	1	-	[a:h+b+c]
P21b132AGC+CA1	0	0	27	0	71	0	0.34	1	-	[a+h+d:b]
P21b154AGC+CA2	0	0	26	0	72	0	0.12	1	-	[a+h+d:b]
P21b224AGC+CA4	0	0	30	0	68	0	1.65	1	-	[a+h+d:b]
P21b370AGC+CA5	0	0	18	0	80	0	2.3	1	-	[a+h+d:b]
P22b101GCC+CA1	0	0	29	0	69	0	1.1	1	-	[a+h+d:b]
P22b166GCC+CA2	0	0	25	0	73	0	0.01	1	-	[a+h+d:b]
P22b243GCC+CA4	23	0	0	75	0	0	0.12	1	-	[a:h+b+c]
P22b47GCC+CA5	0	0	26	0	72	0	0.12	1	-	[a+h+d:b]
P22b252GCC+CA6	0	0	30	0	68	0	1.65	1	-	[a+h+d:b]
P22b285GCC+CA7	21	0	0	77	0	0	0.67	1	-	[a:h+b+c]
P22b297GCC+CA8	29	0	0	69	0	0	1.1	1	-	[a:h+b+c]
P22b307GCC+CA9	0	0	25	0	73	0	0.01	1	-	[a+h+d:b]
P23b217AAC+CA1	27	0	0	71	0	0	0.34	1	-	[a:h+b+c]
P23b308AAC+CA3	0	0	29	0	69	0	1.1	1	-	[a+h+d:b]
P24b79AAC+AG1	27	0	0	71	0	0	0.34	1	-	[a:h+b+c]
P24b159AAC+AG8	31	0	0	67	0	0	2.3	1	-	[a:h+b+c]
P24b221AAC+AG9	31	0	0	67	0	0	2.3	1	-	[a:h+b+c]
P24b289AAC+AG13	0	0	29	0	69	0	1.1	1	-	[a+h+d:b]
P24b291AAC+AG14	18	0	0	80	0	0	2.3	1	-	[a:h+b+c]
P25b93AAG+AG1	0	0	25	0	73	0	0.01	1	-	[a+h+d:b]
P25b115AAG+AG3	25	0	0	73	0	0	0.01	1	-	[a:h+b+c]
P25b199AAG+AG6	21	0	0	77	0	0	0.67	1	-	[a:h+b+c]
P25b270AAG+AG7	0	0	20	0	78	0	1.1	1	-	[a+h+d:b]
P25b320AAG+AG9	0	0	29	0	69	0	1.1	1	-	[a+h+d:b]
597705	17	0	0	43	0	38	0.36	1	-	[a:h+b+c]

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Appendix 24 (continued).

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
P25b482AAG+AG12	30	0	0	68	0	0	1.65	1	-	[a:h+b+c]
P26b113ACA+AG2	26	0	0	72	0	0	0.12	1	-	[a:h+b+c]
P26b116ACA+AG3	21	0	0	77	0	0	0.67	1	-	[a:h+b+c]
P26b136ACA+AG4	0	0	31	0	67	0	2.3	1	-	[a+h+d:b]
P26b142ACA+AG5	0	0	25	0	73	0	0.01	1	-	[a+h+d:b]
P26b193ACA+AG8	0	0	29	0	69	0	1.1	1	-	[a+h+d:b]
P26b280ACA+AG9	0	0	29	0	69	0	1.1	1	-	[a+h+d:b]
P26b282ACA+AG10	0	0	24	0	74	0	0.01	1	-	[a+h+d:b]
P26b374ACA+AG12	0	0	23	0	75	0	0.12	1	-	[a+h+d:b]
P27b167AGA+CA1	0	0	27	0	71	0	0.34	1	-	[a+h+d:b]
P27b329AGA+CA3	18	0	0	80	0	0	2.3	1	-	[a:h+b+c]
P27b353AGA+CA4	0	0	31	0	67	0	2.3	1	-	[a+h+d:b]
P27b383AGA+CA5	0	0	23	0	75	0	0.12	1	-	[a+h+d:b]
P27b414AGA+CA6	30	0	0	68	0	0	1.65	1	-	[a:h+b+c]
P28b70AGT+CA1	0	0	21	0	77	0	0.67	1	-	[a+h+d:b]
P28b99AGT+CA2	21	0	0	77	0	0	0.67	1	-	[a:h+b+c]
P28b154AGT+CA3	25	0	0	73	0	0	0.01	1	-	[a:h+b+c]
P28235+237AGT+CA4+5	23	46	29	0	0	0	1.1	2	-	[a:h:b]
P28b241AGT+CA6	18	0	0	80	0	0	2.3	1	-	[a:h+b+c]
P28b255AGT+CA7	0	0	24	0	74	0	0.01	1	-	[a+h+d:b]
P28b286AGT+CA8	31	0	0	67	0	0	2.3	1	-	[a:h+b+c]
P28b351AGT+CA10	31	0	0	67	0	0	2.3	1	-	[a:h+b+c]
AG81	17	59	22	0	0	0	4.59	2	-	[a:h:b]
BGSRRCol7ssrCol7	21	46	28	0	0	3	1.13	2	-	[a:h:b]
B63DominantssrB63	0	0	28	0	64	6	1.45	1	-	[a+h+d:b]
B63Codominant	23	38	31	0	0	6	4.17	2	-	[a:h:b]
PRIMER32	20	51	15	0	0	12	3.56	2	-	[a:h:b]
Bm2coL33	22	46	20	0	0	10	0.27	2	-	[a:h:b]
BN145	21	52	15	0	0	10	3.73	2	-	[a:h:b]
PRIMER98	18	40	24	0	0	16	0.93	2	-	[a:h:b]
mBam3co7	16	45	27	0	0	10	2.8	2	-	[a:h:b]
PRIMER15	26	44	19	0	0	9	1.11	2	-	[a:h:b]
Bam2col80	20	26	15	0	0	37	2.15	2	-	[a:h:b]
GH-19-B2-D9	14	41	27	0	0	16	4.12	2	-	[a:h:b]
PRIMER65	27	40	19	0	0	12	1.91	2	-	[a:h:b]
PRIMER16	23	46	16	0	0	13	1.73	2	-	[a:h:b]
PRIMER38	24	41	24	0	0	9	0.55	2	-	[a:h:b]
PRIMER73	19	49	20	0	0	10	1.16	2	-	[a:h:b]
PRIMER82	22	41	25	0	0	10	0.61	2	-	[a:h:b]
PRIMER10	17	48	23	0	0	10	1.55	2	-	[a:h:b]
PRIMER19	27	37	19	0	0	15	2.52	2	-	[a:h:b]
423624	0	0	19	0	50	29	0.24	1	-	[a+h+d:b]
594856	17	0	0	52	0	29	0	1	-	[a:h+b+c]
594857	11	0	0	56	0	31	2.63	1	-	[a:h+b+c]
595170	17	0	0	50	0	31	0	1	-	[a:h+b+c]
596903	0	0	20	0	46	32	0.99	1	-	[a+h+d:b]
594400	0	0	19	0	43	36	1.05	1	-	[a+h+d:b]
596605	0	0	13	0	53	32	0.99	1	-	[a+h+d:b]
596233	12	0	0	47	0	39	0.68	1	-	[a:h+b+c]
597717	13	0	0	45	0	40	0.21	1	-	[a:h+b+c]
596311	0	0	14	0	43	41	0.01	1	-	[a+h+d:b]
596059	16	0	0	52	0	30	0.08	1	-	[a:h+b+c]
422614	14	0	0	46	0	38	0.09	1	-	[a:h+b+c]
594987	0	0	16	0	47	35	0.01	1	-	[a+h+d:b]
594981	14	0	0	44	0	40	0.02	1	-	[a:h+b+c]
601441	0	0	15	0	43	40	0.02	1	-	[a+h+d:b]
597860	15	0	0	45	0	38	0	1	-	[a:h+b+c]
423415	0	0	16	0	44	38	0.09	1	-	[a+h+d:b]
597289	15	0	0	43	0	40	0.02	1	-	[a:h+b+c]
594656	0	0	16	0	42	40	0.21	1	-	[a+h+d:b]
423049	0	0	16	0	42	40	0.21	1	-	[a+h+d:b]
595013	0	0	15	0	41	42	0.1	1	-	[a+h+d:b]
596646	15	0	0	42	0	41	0.05	1	-	[a:h+b+c]
423257	15	0	0	43	0	40	0.02	1	-	[a:h+b+c]
422333	15	0	0	41	0	42	0.1	1	-	[a:h+b+c]
597343	0	0	16	0	39	43	0.49	1	-	[a+h+d:b]
595707	17	0	0	44	0	37	0.27	1	-	[a:h+b+c]
423152	0	0	18	0	43	37	0.66	1	-	[a+h+d:b]

Appendix 24 (continued).

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
594814	17	0	0	43	0	38	0.36	1	-	[a:h+b+c]
597279	0	0	18	0	45	35	0.43	1	-	[a+h+d:b]
597746	17	0	0	41	0	40	0.57	1	-	[a:h+b+c]
597971	0	0	18	0	41	39	0.95	1	-	[a+h+d:b]
423386	16	0	0	42	0	40	0.21	1	-	[a:h+b+c]
595948	0	0	18	0	40	40	1.13	1	-	[a+h+d:b]
597945	0	0	18	0	42	38	0.8	1	-	[a+h+d:b]
597087	0	0	16	0	39	43	0.49	1	-	[a+h+d:b]
595380	0	0	18	0	40	40	1.13	1	-	[a+h+d:b]
595797	0	0	19	0	38	41	2.11	1	-	[a+h+d:b]
423742	17	0	0	41	0	40	0.57	1	-	[a:h+b+c]
596776	0	0	18	0	35	45	2.27	1	-	[a+h+d:b]
594685	0	0	20	0	39	39	2.49	1	-	[a+h+d:b]
595006	0	0	20	0	42	36	1.74	1	-	[a+h+d:b]
598173	0	0	19	0	41	38	1.42	1	-	[a+h+d:b]
423258	18	0	0	37	0	43	1.75	1	-	[a:h+b+c]
601723	0	0	10	0	52	36	2.6	1	-	[a+h+d:b]
594707	9	0	0	47	0	42	2.38	1	-	[a:h+b+c]
593909	11	0	0	54	0	33	2.26	1	-	[a:h+b+c]
596231	12	0	0	52	0	34	1.33	1	-	[a:h+b+c]
595901	12	0	0	55	0	31	1.8	1	-	[a:h+b+c]
602199	12	0	0	54	0	32	1.64	1	-	[a:h+b+c]
600900	0	0	13	0	48	37	0.44	1	-	[a+h+d:b]
597681	12	0	0	54	0	32	1.64	1	-	[a:h+b+c]
595924	12	0	0	53	0	33	1.48	1	-	[a:h+b+c]
601918	12	0	0	48	0	38	0.8	1	-	[a:h+b+c]
595496	12	0	0	52	0	34	1.33	1	-	[a:h+b+c]
423805	14	0	0	52	0	32	0.51	1	-	[a:h+b+c]
422423	11	0	0	45	0	42	0.86	1	-	[a:h+b+c]
598804	13	0	0	47	0	38	0.36	1	-	[a:h+b+c]
598647	14	0	0	50	0	34	0.33	1	-	[a:h+b+c]
601358	14	0	0	50	0	34	0.33	1	-	[a:h+b+c]
595882	13	0	0	52	0	33	0.87	1	-	[a:h+b+c]
600972	14	0	0	49	0	35	0.26	1	-	[a:h+b+c]
598750	14	0	0	49	0	35	0.26	1	-	[a:h+b+c]
596721	14	0	0	48	0	36	0.19	1	-	[a:h+b+c]
601257	14	0	0	47	0	37	0.14	1	-	[a:h+b+c]
601845	15	0	0	49	0	34	0.08	1	-	[a:h+b+c]
601050	15	0	0	49	0	34	0.08	1	-	[a:h+b+c]
597434	0	0	15	0	49	34	0.08	1	-	[a+h+d:b]
598337	14	0	0	45	0	39	0.05	1	-	[a:h+b+c]
596097	15	0	0	46	0	37	0.01	1	-	[a:h+b+c]
595754	13	0	0	45	0	40	0.21	1	-	[a:h+b+c]
596331	14	0	0	45	0	39	0.05	1	-	[a:h+b+c]
594898	14	0	0	45	0	39	0.05	1	-	[a:h+b+c]
601965	15	0	0	44	0	39	0.01	1	-	[a:h+b+c]
423710	15	0	0	43	0	40	0.02	1	-	[a:h+b+c]
601384	16	0	0	46	0	36	0.02	1	-	[a:h+b+c]
601470	17	0	0	47	0	34	0.08	1	-	[a:h+b+c]
598182	17	0	0	45	0	36	0.19	1	-	[a:h+b+c]
594803	15	0	0	43	0	40	0.02	1	-	[a:h+b+c]
594994	15	0	0	42	0	41	0.05	1	-	[a:h+b+c]
596047	19	0	0	47	0	32	0.51	1	-	[a:h+b+c]
601748	17	0	0	43	0	38	0.36	1	-	[a:h+b+c]
594224	16	0	0	44	0	38	0.09	1	-	[a:h+b+c]
593937	15	0	0	41	0	42	0.1	1	-	[a:h+b+c]
595761	16	0	0	44	0	38	0.09	1	-	[a:h+b+c]
594391	17	0	0	44	0	37	0.27	1	-	[a:h+b+c]
597175	0	0	20	0	46	32	0.99	1	-	[a+h+d:b]
596042	0	0	19	0	43	36	1.05	1	-	[a+h+d:b]
597962	16	0	0	42	0	40	0.21	1	-	[a:h+b+c]
597832	16	0	0	41	0	41	0.29	1	-	[a:h+b+c]
595583	17	0	0	41	0	40	0.57	1	-	[a:h+b+c]
594712	18	0	0	43	0	37	0.66	1	-	[a:h+b+c]
597285	18	0	0	43	0	37	0.66	1	-	[a:h+b+c]
597010	17	0	0	42	0	39	0.46	1	-	[a:h+b+c]
602110	17	0	0	42	0	39	0.46	1	-	[a:h+b+c]
595093	17	0	0	42	0	39	0.46	1	-	[a:h+b+c]

Appendix 24 (continued).

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
595373	17	0	0	40	0	41	0.71	1	-	[a:h+b+c]
596726	17	0	0	38	0	43	1.02	1	-	[a:h+b+c]
594531	18	0	0	39	0	41	1.32	1	-	[a:h+b+c]
596188	18	0	0	39	0	41	1.32	1	-	[a:h+b+c]
422352	19	0	0	39	0	40	1.86	1	-	[a:h+b+c]
596017	20	0	0	41	0	37	1.97	1	-	[a:h+b+c]
596545	18	0	0	38	0	42	1.52	1	-	[a:h+b+c]
594387	20	0	0	39	0	39	2.49	1	-	[a:h+b+c]
597365	19	0	0	37	0	42	2.38	1	-	[a:h+b+c]
597037	19	0	0	37	0	42	2.38	1	-	[a:h+b+c]
596417	20	0	0	40	0	38	2.22	1	-	[a:h+b+c]
598056	19	0	0	38	0	41	2.11	1	-	[a:h+b+c]
595470	19	0	0	36	0	43	2.67	1	-	[a:h+b+c]
595859	19	0	0	38	0	41	2.11	1	-	[a:h+b+c]
P1b67AAG+CTT1	0	0	42	0	56	0	16.67	1	*******	[a+h+d:b]
P8b315ACG+CTA2	0	0	46	0	52	0	25.16	1	*******	[a+h+d:b]
P12b105AAG+CTC1	0	0	43	0	55	0	18.63	1	*******	[a+h+d:b]
P23b287AAC+CA2	44	0	0	54	0	0	20.69	1	*******	[a:h+b+c]
P24b271AAC+AG11	0	0	43	0	55	0	18.63	1	*******	[a+h+d:b]
P24b392AAC+AG15	0	0	50	0	48	0	35.39	1	*******	[a+h+d:b]
P24b499AAC+AG16	0	0	48	0	50	0	30.05	1	*******	[a+h+d:b]
P25b94AAG+AG2	0	0	48	0	50	0	30.05	1	*******	[a+h+d:b]
P25b299AAG+AG8	0	0	44	0	54	0	20.69	1	*******	[a+h+d:b]
P25b444AAG+AG10	0	0	45	0	53	0	22.87	1	*******	[a+h+d:b]
P26b108ACA+AG1	42	0	0	56	0	0	16.67	1	*******	[a:h+b+c]
PRIMER66	12	31	43	0	0	12	29.05	2	*******	[a:h:b]
598450	33	0	0	32	0	33	23.02	1	*******	[a:h+b+c]
595671	0	0	0	68	0	30	22.67	1	*******	[a:h+b+c]
P14b264ATC+CTA1	0	0	41	0	57	0	14.82	1	******	[a+h+d:b]
P19b494AGC+CTA4	0	0	40	0	58	0	13.07	1	******	[a+h+d:b]
P25b178AAG+AG5	0	0	41	0	57	0	14.82	1	******	[a+h+d:b]
P28b328AGT+CA9	0	0	41	0	57	0	14.82	1	******	[a+h+d:b]
P1b161AAG+CTT3	39	0	0	59	0	0	11.44	1	*****	[a:h+b+c]
P24b109AAC+AG4	39	0	0	59	0	0	11.44	1	*****	[a:h+b+c]
P24b151AAC+AG7	0	0	39	0	59	0	11.44	1	*****	[a+h+d:b]
DSRB20	37	40	12	0	0	9	14.96	2	*****	[a:h:b]
P1b88AAG+CTT2	0	0	12	0	86	0	8.5	1	****	[a+h+d:b]
P11b80AAG+CAA1	37	0	0	61	0	0	8.5	1	****	[a:h+b+c]
P12b403AAG+CTC3	37	0	0	61	0	0	8.5	1	****	[a:h+b+c]
P22b207GCC+CA3	38	0	0	60	0	0	9.92	1	****	[a:h+b+c]
P24b82AAC+AG2	0	0	12	0	86	0	8.5	1	****	[a+h+d:b]
P26b168ACA+AG6	0	0	38	0	60	0	9.92	1	****	[a+h+d:b]
P26b534ACA+AG13	11	0	0	87	0	0	9.92	1	****	[a:h+b+c]
P27b297AGA+CA2	0	0	11	0	87	0	9.92	1	****	[a+h+d:b]
P28b570AGT+CA12	38	0	0	60	0	0	9.92	1	****	[a:h+b+c]
DSRB17	21	32	36	0	0	9	12.08	2	****	[a:h:b]
DSRB19	34	31	21	0	0	12	10.63	2	****	[a:h:b]
P5b204ATC+CTT4	0	0	13	0	85	0	7.2	1	***	[a+h+d:b]
P28b389AGT+CA11	0	0	36	0	62	0	7.2	1	***	[a+h+d:b]
596162	0	0	23	0	35	40	6.64	1	***	[a+h+d:b]
597026	0	0	24	0	37	37	6.69	1	***	[a+h+d:b]
598161	25	0	0	39	0	34	6.75	1	***	[a:h+b+c]
P3b83AGC+CTC1	0	0	15	0	83	0	4.91	1	**	[a+h+d:b]
P3b211AGC+CTC7	0	0	16	0	82	0	3.93	1	**	[a+h+d:b]
P9b84AGA+CTA1	34	0	0	64	0	0	4.91	1	**	[a:h+b+c]
P10b203AAC+CTT2	0	0	33	0	65	0	3.93	1	**	[a+h+d:b]
P15b319AGT+CAA1	0	0	16	0	82	0	3.93	1	**	[a+h+d:b]
P18b275ACA+CAT5	16	0	0	82	0	0	3.93	1	**	[a:h+b+c]
P18b320ACA+CAT6	0	0	34	0	64	0	4.91	1	**	[a+h+d:b]
P19b131AGC+CTA3	0	0	34	0	64	0	4.91	1	**	[a+h+d:b]
P21b202AGC+CA3	0	0	34	0	64	0	4.91	1	**	[a+h+d:b]
P24b116AAC+AG5	0	0	34	0	64	0	4.91	1	**	[a+h+d:b]
P24b126AAC+AG6	15	0	0	83	0	0	4.91	1	**	[a:h+b+c]
P24b228AAC+AG10	0	0	33	0	65	0	3.93	1	**	[a+h+d:b]
P24b272AAC+AG12	0	0	34	0	64	0	4.91	1	**	[a+h+d:b]
P24b510AAC+AG17	34	0	0	64	0	0	4.91	1	**	[a:h+b+c]
P25b164AAG+AG4	33	0	0	65	0	0	3.93	1	**	[a:h+b+c]
P26b182ACA+AG7	0	0	15	0	83	0	4.91	1	**	[a+h+d:b]

Appendix 24 (continued).

Locus	a	h	b	c	d	-	X2	Df	Signif.	Classification
P26b347ACA+AG11	34	0	0	64	0	0	4.91	1	**	[a:h+b+c]
PRIMER48	21	53	12	0	0	12	6.53	2	**	[a:h:b]
594941	0	0	21	0	35	42	4.67	1	**	[a+h+d:b]
595018	0	0	22	0	34	42	6.1	1	**	[a+h+d:b]
596804	21	0	0	33	0	44	5.56	1	**	[a:h+b+c]
422577	21	0	0	34	0	43	5.1	1	**	[a:h+b+c]
601017	0	0	8	0	56	34	5.33	1	**	[a+h+d:b]
601131	0	0	8	0	56	34	5.33	1	**	[a+h+d:b]
598669	0	0	8	0	53	37	4.6	1	**	[a+h+d:b]
595196	21	0	0	37	0	40	3.89	1	**	[a:h+b+c]
595834	21	0	0	36	0	41	4.26	1	**	[a:h+b+c]
P3b185AGC+CTC6	0	0	32	0	66	0	3.06	1	*	[a+h+d:b]
P21b394AGC+CA6	0	0	32	0	66	0	3.06	1	*	[a+h+d:b]
P24b88AAC+AG3	0	0	17	0	81	0	3.06	1	*	[a+h+d:b]
P25b459AAG+AG11	0	0	17	0	81	0	3.06	1	*	[a+h+d:b]
PRIMER85	31	37	19	0	0	11	5.25	2	*	[a:h:b]
PRIMER26	13	47	28	0	0	10	5.52	2	*	[a:h:b]
595354	0	0	21	0	40	37	2.89	1	*	[a+h+d:b]
594888	0	0	21	0	39	38	3.2	1	*	[a+h+d:b]
595675	20	0	0	37	0	41	3.09	1	*	[a:h+b+c]
594513	19	0	0	34	0	45	3.33	1	*	[a:h+b+c]
601852	22	0	0	41	0	35	3.31	1	*	[a:h+b+c]
598103	0	0	22	0	42	34	3	1	*	[a+h+d:b]
600901	0	0	21	0	38	39	3.53	1	*	[a+h+d:b]
596920	21	0	0	38	0	39	3.53	1	*	[a:h+b+c]
595333	20	0	0	35	0	43	3.79	1	*	[a:h+b+c]
596472	20	0	0	36	0	42	3.43	1	*	[a:h+b+c]
596481	20	0	0	36	0	42	3.43	1	*	[a:h+b+c]
594453	20	0	0	36	0	42	3.43	1	*	[a:h+b+c]

Appendix 25: Unmapped DNA markers of the 'narrow' map in bambara groundnut derived from the cross of DipC and Tiga necaru.

No	DNA markers	Type of marker	No	DNA markers	Type of marker
1	Bam2coL33	SSR	17	bgPt-596650	DArT
2	Bam2coL80		18	bgPt-596869	
3	PRIMER65		19	bgPt-596950	
4	PRIMER85		20	bgPt-597130	
5	bgPabg-593922	DArT	21	bgPt-597585	
6	bgPabg-595641		22	bgPt-598164	
7	bgPabg-596877		23	bgPt-598235	
8	bgPabg-597557		24	bgPt-598385	
9	bgPabg-598400		25	bgPt-598669	
10	bgPabg-601035		26	bgPt-598683	
11	bgPt-594663		27	bgPt-601017	
12	bgPt-594957		28	bgPt-601027	
13	bgPt-595456		29	bgPt-601131	
14	bgPt-595565		30	bgPt-601486	
15	bgPt-596047		31	bgPabg-594305	
16	bgPt-596476				

Appendix 26: Unmapped DNA markers of the 'wide' map in bambara groundnut derived from the cross of DipC and VSSP11.

No	DNA markers	Type of marker	No	DNA markers	Type of marker
1	PRIMER10	SSR	55	P24b221AAC+AG9	AFLP
2	PRIMER15		56	P24b228AAC+AG10	
3	PRIMER19		57	P24b289AAC+AG13	
4	PRIMER38		58	P24b291AAC+AG14	
5	PRIMER65		59	P24b392AAC+AG15	
6	PRIMER82		60	P24b499AAC+AG16	
7	PRIMER85		61	P24b510AAC+AG17	
8	PRIMER98		62	P24b79AAC+AG1	
9	D.32937		63	P24b88AAC+AG3	
10	D.48339		64	P25b115AAG+AG3	
11	D.51646		65	P25b199AAG+AG6	
12	AG81ssrAG81		66	P25b270AAG+AG7	
13	422577	DArT	67	P25b299AAG+AG8	
14	423049		68	P25b320AAG+AG9	
15	423258		69	P25b444AAG+AG10	
16	423386		70	P25b459AAG+AG11	
17	594400		71	P25b482AAG+AG12	
18	594513		72	P25b93AAG+AG1	
19	594856		73	P25b94AAG+AG2	
20	594941		74	P26b116ACA+AG3	
21	595006		75	P26b282ACA+AG10	
22	595013		76	P26b534ACA+AG13	
23	595018		77	P27b383AGA+CA5	
24	595671		78	P28235+237AGT+CA4+5	
25	595675		79	P28b241AGT+CA6	
26	595797		80	P28b570AGT+CA12	
27	595948		81	P28b70AGT+CA1	
28	596042		82	P3b155AGC+CTC4	
29	596162		83	P10b203AAC+CTT2	
30	596311		84	P12b105AAG+CTC1	
31	596605		85	P12b403AAG+CTC3	
32	596776		86	P12b420AAG+CTC4	
33	596903		87	P16b329AGA+CTC2	
34	597026		88	P18b221ACA+CAT2	
35	597087		89	P1b251AAG+CTT4	
36	597175		90	P1b88AAG+CTT2	
37	597279		91	P21b132AGC+CA1	
38	597945		92	P21b154AGC+CA2	
39	598161		93	P21b202AGC+CA3	
40	598669		94	P21b224AGC+CA4	
41	601017		95	P21b370AGC+CA5	
42	601131		96	P21b394AGC+CA6	
43	601441		97	P22b243GCC+CA4	
44	P12b105AAG+CTC1	AFLP	98	P22b47GCC+CA5	
45	P18b186ACA+CAT1		99	P3b264AGC+CTC8	
46	P21b394AGC+CA6		100	P3b83AGC+CTC1	
47	P22b207GCC+CA3		101	P3b87AGC+CTC2	
48	P22b252GCC+CA6		102	P3b99AGC+CTC3	
49	P22b285GCC+CA7		103	P4b224AGC+CAG	
50	P23b287AAC+CA2		104	P5b191ATC+CTT3	
51	P23b308AAC+CA3		105	P5b204ATC+CTT4	
52	P24b126AAC+AG6		106	P8b270ACG+CTA1	
53	P24b151AAC+AG7		107	P8b315ACG+CTA2	
54	P24b159AAC+AG8				

Appendix 27: Additive, dominance effect and the residual variance after fitting the QTL for interval mapping analysis, using the F₃ generation data of the 'narrow' cross in the FutureCrop glasshouses

Traits	Linkage group	Position (cM)	Locus	mu_A	mu_H	mu_B	Variance	Additive	Dominance
Flower no./plant (FN)	8	2	-	86.1	108.1	119.5	978.6	-16.7	5.3
	8	0	Bam2coL63	87.6	107.3	118.8	986.3	-15.6	4.0
Terminal leaflet length (TLL)	8	2	-	7.8	8.2	8.6	0.5	-0.4	0.0
	8	0	Bam2coL63	7.8	8.2	8.6	0.5	-0.4	0.0
Terminal leaflet width (TLW)	3	19.7	bgPt-600935	3.8	3.3	3.4	0.2	0.2	-0.3
Leaf area (LA)	3	16.6	-	62.8	44.9	59.1	96.8	1.8	-16.1
	3	15.6	bgPabg-597113	62.3	44.2	59.5	96.9	1.4	-16.7
Plant spread (PS)	4	0	BN6b	37.5	34.3	30.1	29.9	3.7	0.5
Stem no./plant (STN)	4	14.2	-	9.4	10.0	11.2	2.5	-0.9	-0.3
	4	11.2	bgPt-600898	9.5	10.0	11.1	2.5	-0.8	-0.3
Node no./stem (NN)	1	33	bgPabg-596774	7.6	8.3	9.7	3.7	-1.1	-0.4
	4	11.2	bgPt-600898	9.8	8.7	7.6	3.8	1.1	0.0
Internode length (IL)	4	3	bgPabg-596988	2.9	2.0	1.4	0.5	0.7	-0.1
Pod no./plant (PN)	1	33	bgPabg-596774	39.8	49.9	53.8	212.7	-7.0	3.1
Double seeded pods/plant (DPN)	4	1	-	2.8	2.1	1.8	0.6	0.5	-0.2
	4	0	BN6b	2.8	2.1	1.8	0.6	0.5	-0.2
Peduncle length (PEL)	4	1	-	3.8	3.0	2.1	0.5	0.9	0.1
	4	2.4	bgPt-423527	3.8	3.0	2.1	0.5	0.9	0.0
Pod weight (PWE)	1	33	bgPabg-596774	29.1	40.0	42.8	175.8	-6.8	4.0
Pod length (PLE)	12	15.1	-	16.7	16.1	15.2	0.9	0.8	0.1
	12	12. 9	bgPt-598767	16.7	16.0	15.2	1.0	0.8	0.0
Pod width (PWD)	12	20.1	-	13.5	12.9	12.3	0.4	0.6	0.0
	12	22.5	bgPabg-595682	13.5	12.9	12.4	0.4	0.5	0.0
Pod length of double seeded (DPL)	1	0	bgPabg-597086	23.5	24.8	26.5	5.0	-1.5	-0.2
	12	10.5	-	25.9	25.0	22.6	5.1	1.6	0.7
Pod width of double seeded (DPW)	12	17.1	-	13.7	13.3	12.7	0.5	0.5	0.1
	12	12.9	bgPt-598767	13.7	13.2	12.7	0.5	0.5	0.0
Seed length (SEL)	10	49.1	bgPabg-593983	11.4	11.3	10.7	0.6	0.4	0.3
Seed width (SEW)	12	15.1	-	9.4	9.2	8.9	0.3	0.3	0.0
	12	12.9	bgPt-598767	9.4	9.2	8.9	0.3	0.3	0.0
Seed no./plant (SEN)	1	34	-	42.9	52.1	58.6	265.5	-7.9	1.4
	1	33	bgPabg-596774	43.3	52.1	58.4	265.8	-7.6	1.2
Seed weight (SWT)	1	33	bgPabg-596774	4.7	5.5	5.7	1.0	-0.5	0.4
Biomass dry weight (BDW)	1	33	bgPabg-596774	48.9	61.5	72.2	404.6	-11.6	1.0
Shelling% (SH%)	7	13.3	bgPabg-594335	82.1	78.8	75.3	32.4	3.4	0.1
100-seed weight (HSW)	7	9.4	-	60.2	53.3	51.5	60.3	4.3	-2.6
	7	10.5	bgPt-601852	60.2	53.4	51.6	60.5	4.3	-2.5

Appendix 28: Additive, dominance effect and the residual variance after fitting the QTL for interval mapping analysis, using the F₃ generation data of 'narrow' cross in the field

Traits	Linkage group	Position (cM)	Locus	mu_A	mu_H	mu_B	Variance	Additive	Dominance
Node no./stem (NN)	3	30.2	bgPabg-595707	16.7	11.2	14.6	12.1	1.0	-4.5
Internode length (IL)	4	3	bgPabg-596988	1.4	1.0	0.7	0.1	0.3	-0.1
Pod no./plant (PN)	18	3	-	2.2	2.5	3.0	0.6	-0.4	-0.1
	18	5.1	PRIMER10	2.2	2.5	3.0	0.6	-0.4	-0.1
pod length (PLE)	11	3		3.8	3.6	3.6	0.0	0.1	-0.1
	11	0	bgPabg-595822	3.8	3.7	3.6	0.0	0.1	0.0
Biomass dry weight (BDW)	1	28.9	bgPt-602039	12.4	11.2	16.0	11.1	-1.8	-3.0

Appendix 29: Additive, dominance effect and the residual variance after fitting the QTL for interval mapping analysis, using the F₂ generation data of narrow cross in TCRU

Traits	Linkage group	Position (cM)	Locus	mu_A	mu_H	mu_B	Variance	Additive	Dominance
Terminal leaflet length (TLL)	8	0	Bam2coL63	9.0	9.7	10.2	2.0	-0.6	0.1
Terminal leaflet width (TLW)	5	74.2	bgPt-595387	4.4	4.5	3.7	0.6	0.3	0.5
Plant spread (PS)	4	33.5	bgPabg-597624	60.6	62.3	49.6	125.4	5.5	7.2
Pod no./plant (PN)	1	68	-	77.2	141.6	147.7	5440.3	-35.3	29.1
	1	72.7	bgPt-601022	86.5	134.3	149.5	5556.3	-31.5	16.4
Double seeded pods/plant (DPN)	4	33.5	bgPabg-597624	3.1	3.0	1.6	1.8	0.7	0.7
Seed no./plant (SEN)	1	72.6	bgPt-601022	83.8	122.1	145.3	5340.0	-30.7	7.6
Biomass dry weight (BDW)	1	67	-	63.5	117.5	119.0	3889.4	-27.8	26.2
	1	59.6	bgPabg-596618	62.5	119.0	114.8	3796.6	-26.1	30.3
Shelling% (SH%)	12	47.5	bgPt-595486	69.4	73.3	77.4	33.4	-4.0	-0.1
100-seed weight (HSW)	11	0	bgPabg-595822	37.5	31.7	28.3	115.4	4.6	-1.2
	12	47.5	bgPt-595486	28.4	33.5	37.6	115.6	-4.6	0.5

Appendix 30: Additive, dominance effect and the residual variance after fitting the QTL for interval mapping analysis, using the F₂ generation data derived from the 'wide' cross of DipC x VSSP11

Trait	Linkage group	Position (cM)	Locus	mu_A	mu_H	mu_B	Variance	Additive	Dominance
Leaf area (LA)	15	83.1	-	396.3	317.1	434.5	3586.1	-19.1	-98.3
	15	94.6	P19b105AGC+CTA2	384.6	318.9	429.6	3714.9	-22.5	-88.3
Specific leaf area (SLA)	10	7	-	133.0	149.5	151.6	199.4	-9.3	7.2
	10	12.4	P16b329AGA+CTC2	136.5	148.4	152.1	203.8	-7.8	4.1
Stem no./plant (STN)	13	1.1	P17b242ACG+CAA3	9.5	10.6	14.8	14.7	-2.6	-1.5
	1	20.9	595196	9.8	10.9	14.0	16.7	-2.1	-1.0
Internode length (IL)	9	0	600900	11.7	8.5	8.2	8.2	1.8	-1.4
	14	26.5	P19b102AGC+CTA1	11.2	9.5	8.0	8.4	1.6	-0.1
	13	0	P17b240ACG+CAA2	10.8	9.7	7.8	8.4	1.5	0.4
Carbon isotope discrimination CID	7	93.9	P19b494AGC+CTA4	21.0	19.6	21.0	0.9	0.0	-1.4
100-seed weight (HSW)	7	87.6	-	49.4	37.5	54.7	120.7	-2.6	-14.5
	7	93.9	P19b494AGC+CTA4	43.6	41.1	51.7	122.5	-4.0	-6.6